Systematic Mutagenesis of the Murine Gammaherpesvirus 68 M2 Protein Identifies Domains Important for Chronic Infection[▽]

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Murine gammaherpesvirus 68 (MHV68) infection of inbred mice represents a genetically tractable small-animal model for assessing the requirements for the establishment of latency, as well as reactivation from latency, within the lymphoid compartment. By day 16 postinfection, MHV68 latency in the spleen is found in B cells, dendritic cells, and macrophages. However, as with Epstein-Barr virus, by 3 months postinfection MHV68 latency is predominantly found in isotype-switched memory B cells. The MHV68 M2 gene product is a latency-associated antigen with no discernible homology to any known cellular or viral proteins. However, depending on experimental conditions, the M2 protein has been shown to play a critical role in both the efficient establishment of latency in splenic B cells and reactivation from latently infected splenic B cells. Inspection of the sequence of the M2 protein reveals several hallmarks of a signaling molecule, including multiple PXXP motifs and two potential tyrosine phosphorylation sites. Here, we report the generation of a panel of recombinant MHV68 viruses harboring mutations in the M2 gene that disrupt putative functional motifs. Subsequent analyses of the panel of M2 mutant viruses revealed a functionally important cluster of PXXP motifs in the C-terminal region of M2, which have previously been implicated in binding Vav proteins (P. A. Madureira, P. Matos, I. Soeiro, L. K. Dixon, J. P. Simas, and E. W. Lam, *J. Biol. Chem.* 280:37310–37318, 2005; L. Rodrigues, M. Pires de Miranda, M. J. Caloca, X. R. Bustelo, and J. P. Simas, *J. Virol.* 80:6123–6135, 2006). Further characterization of two adjacent PXXP motifs in the C terminus of the M2 protein revealed differences in the functions of these domains in M2-driven expansion of primary murine B cells in culture. Finally, we show that tyrosine residues 120 and 129 play a critical role in both the establishment of splenic latency and reactivation from latency upon explant of splenocytes into tissue culture. Taken together, these analyses will aid future studies for identifying M2 interacting partners and B-cell signaling pathways that are manipulated by the M2 protein.

The latent stage of herpesvirus infections is an essential aspect of persistent infection of the host. The gammaherpesviruses are characterized by their abilities to establish latency in B or T lymphocytes as well as the association of this family of viruses with the development of lymphomas and lymphoproliferative diseases. Research on the human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) has been largely restricted to in vitro analyses due to the limited host ranges of these pathogens. Murine gammaherpesvirus 68 (MHV68) is a type 2 gammaherpesvirus that naturally infects wild murid rodents (6, 32, 40, 50). As such, one of the major advantages of the MHV68 model system is the opportunity to address the genetic requirements for gammaherpesvirus pathogenesis, host control, and lymphomagenesis in the context of infection of a natural host (12, 13, 35, 36, 45, 48, 57).

Following intranasal inoculation of inbred strains of mice, MHV68 undergoes acute-phase replication in a variety of anatomical sites, including the lungs and the spleen. A subsequent latent infection is established and is marked by a CD4⁺ T-cell-dependent splenomegaly which peaks at 14 to 21 days postinfection (dpi) (14, 49–51, 56, 60). The majority of MHV68

latency at early times after infection is found in a variety of B-cell subsets in the spleens of mice as well as in macrophages and dendritic cells (53, 54). However, as with EBV, long-term latency is predominantly restricted to germinal center and memory B cells (53, 54). With respect to EBV infection, there is mounting evidence to support the hypothesis that upon infection of naïve B cells, a subset of EBV genes that facilitate the proliferation and subsequent activation of these cells are expressed, driving these cells to form germinal centers and subsequently differentiate into memory B cells (4, 53, 55). During the germinal center reaction, specific EBV latency-associated gene products (e.g., LMP1 and LMP2a) are proposed to mimic the requisite signals normally provided by antigen and antigen-specific T-helper cells to allow differentiation into memory B cells (1, 8, 22, 64). Following establishment of latency in the memory compartment, EBV shuts down viral gene expression and leaves the site of primary infection to seed additional latency reservoirs in secondary lymphoid organs. We have hypothesized that MHV68, like EBV, encodes antigens with the potential to manipulate normal B-cell signaling pathways to establish a latent infection in memory B cells (17, 34, 63).

The M2 open reading frame (ORF) is located in a cluster of unique genes at the left end of the MHV68 genome and shares positional homology with latency and transformation-associated genes present in other characterized gammaherpesviruses (48, 57, 58). The M2 gene was shown to be transcribed in

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latently infected tissue *in vivo* and to encode a latency-associated antigen in the MHV68 latently infected murine B-lymphoma cell line S11 (18, 43, 59). Through the use of a viral recombinant harboring a translational stop codon in the M2 ORF, the contribution of the M2 gene product to MHV68 replication and latency was analyzed following intranasal and intraperitoneal inoculation of mice. Regardless of dose of infection, loss of M2 resulted in no defect in acute-phase replication in the lungs (17, 19, 29). However, M2 was shown to be required for the establishment of MHV68 latency in the spleen at 16 dpi following low-dose inoculation with 100 PFU of virus administered intranasally. Notably, upon inoculation with 100 PFU of M2 mutant virus administered intraperitoneally or intranasal inoculation with 4×10^5 PFU, the M2 mutant virus was capable of establishing latency in splenic B cells but under these infection conditions exhibited a profound reactivation defect (17). Interestingly, we previously observed that at 6 weeks postinfection following an intraperitoneal inoculation of the M2 mutant virus, there was a >10-fold-higher frequency of viral genome-positive naïve B cells than for the control marker rescue virus (17). This accumulation of virus in the naïve B-cell compartment may reflect a role for the M2 protein in driving differentiation of latently infected naïve B cells.

M2 is a unique protein that lacks discernible homology to any known cellular or pathogen protein; however, inspection of the amino acid sequence reveals many hallmarks of a bona fide signaling molecule, including multiple PXXP motifs. Previous studies have shown that M2 is capable of interacting with a number of SH3 domain-containing proteins, including Vav1, -2, and -3; Fyn; the tyrosine kinases TXK and Tec; Nck2; Grb2; endophilins II and III; Ras GTPase-activating protein 1; and Rho GTPase-activating protein 4 (30, 43). Furthermore, Madureira et al. (30) and Rodrigues et al. (43) demonstrated that three PXXP motifs located in the C-terminal half of M2 are required to bind Vav1 and induce phosphorylation of Vav1 leading to downstream Rac1 stimulation. Additionally, the M2 protein harbors three tyrosine residues, two of which are predicted to be potential phosphorylation sites (Fig. 1) and have been shown to be essential for a trimolecular complex with Vav1 and the Src family kinase Fyn (43). However, the use of an MHV68 M2 mutant virus was not employed to investigate the importance of the proline domains required for Vav binding or the tyrosine phosphorylation sites within M2 (30, 43). Recently, M2 was demonstrated to be phosphorylated on multiple threonine and serine residues as well (25). Other biochemical analyses of M2 revealed a role for M2 in down-regulating STAT1/2 levels, resulting in inhibition of interferon-mediated transcriptional activation and inhibition of DNA damage-induced apoptosis via interaction with the DDB1/COP9/cullin repair complex and the ATM DNA damage signal transducer (26). Again, these findings were not supported by genetics using MHV68 M2 mutant viruses (26, 27).

Here, we present the results of a systematic mutagenesis of the M2 protein in the context of the viral genome. Pathogenesis of recombinant viruses harboring various mutations in the M2 gene were analyzed in mice to identify functionally important interaction motifs as well as to assess the importance of previously characterized domains implicated in specific interactions of the M2 protein with cellular proteins. In addition, the data gathered from the mutagenesis screen were used to

FIG. 1. Deduced amino acid sequence of the M2 protein, with PXXP motifs and candidate tyrosine phosphorylation sites indicated in bold. Each PXXP motif is numbered, and the mutations introduced into each motif are indicated above the M2 sequence. The mutations of proline residues 70, 71, and 73 disrupt both the P3 and P4 PXXP motifs. Also shown are the mutations introduced into the tyrosine residues at positions 120 and 129, which were mutated to either phenylalanine or aspartic acid. The P7 PXXP motif is the only consensus class I SH3 binding motif (K/RxxPxxP) present in the M2 protein.

identify motifs required for M2-dependent expansion of primary B cells in tissue culture.

MATERIALS AND METHODS

Viruses and tissue culture. The MHV68 genome, cloned as a bacterial artificial chromosome (BAC), was a kind gift from Ulrich Koszinowski (2) and was the wild-type (wt) virus used to derive all the mutant viruses in this paper. Virus passage and titer determination were performed as previously described (10, 33). NIH 3T12 cells, Cos-1 cells, and mouse embryonic fibroblast (MEF) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U of penicillin per ml, 100 mg of streptomycin per ml, 10% fetal calf serum (FCS), and 2 mM l-glutamine (cMEM). Vero-Cre cells were maintained in cMEM supplemented with 300 µg of hygromycin B/ml. All cells were maintained at 37°C in a 5% CO₂ environment. Vero-Cre cells were a kind gift from David Leib, Washington University, St. Louis, MO. MEF cells were obtained as previously described (38).

Generation of virus mutants. An MHV68 genomic fragment containing the region from bp 2403 to bp 6262 (WUMS sequence) (58) was cloned into the Litmus-38 plasmid (Lit38-M2) as previously described (19). All point mutations within the M2 ORF were generated by site-specific PCR mutagenesis, using Lit38-M2 as a template. Individual point mutations were introduced by overlapping PCR including inside primers (listed below for each mutant) and outside primers approximately 1,500 bp from the mutagenesis site within the M2 ORF. Each M2 mutant PCR product was purified and ligated to the pCR Blunt vector (Invitrogen, United States) and designated as described here. The naming of the potential functional motifs is summarized in Fig. 1. The primers were as follows: for M2.Stop(2), Oligo1 (5' CCA CCA GGC CGA AGC TTA CGG ATT GGG AAT C) and Oligo2 (5' CCA ATC CGT AAG CTT CGG CCT GGT GGA TG), generating a translational stop codon at bp 4566 and introducing a HindIII restriction site; for P1, M2.P3A/P5A Oligo1 (5' CTT GTG GAG CTG TTG CGG CCA TTA CCT GAA AAC) and Oligo2 (5' GGT AAT GGC CGC AAC AGC TCC ACA AGG AAA GAT), mutating amino acids 3 and 5 from prolines to alanines, introducing a silent mutation at bp 4592 yielding an AluI restriction site; for P2, M2.P35A/P38A Oligo1 (5' ATC CAG GCC TCG CTG GCA CGG TAG TTC CCG TCT G) and Oligo2 (5' CTA CCG TGC CAG CGA GGC CTG GAT CTT AGG CC), mutating amino acids 35 and 38 from prolines to alanines, introducing a BglI restriction site; for P3/4, M2.P70A/P71A/P73A Oligo1 (5' AGT CCT TGC CAA AGC CGC GCG CTG AGG TCT GC) and Oligo2 (5' TCA GCG CGC GGC TTT GGC AAG GAC TCG CTT TCC), mutating amino acids 70, 71, and 73 from prolines to alanines, introducing a BssHII restriction site; for P5, M2.P95A/P98A Oligo1 (5' GCC GCG CCT TCC

ATG CGG ACT TTA ACG TCG ACC TAA G) and Oligo2 (5' GTT AAA GTC CGC ATG GAA GGC GCG GCC GAG TCC TGT AC), mutating amino acids 95 and 98 from prolines to alanines, resulting in the loss of an NcoI restriction site; for Y120/129F, M2.Y120F/Y129F Oligo1 (5' GGC TGG ATA AAG ACT GGT TCA CTG TTA GCT GTT TCA AAG A) and Oligo2 (5' CAT CTT TGA AAC AGC TAA CAG TGA ACC AGT CTT TAT CC), followed by Oligo3 (5' CCT GAA GAA AAC ATC TTT GAA ACA GCT AAC AGT GAA CC) and Oligo4 (5' CTG TTA GCT GTT TCA AAG ATG TTT TCT TCA GGA CTT CC), mutating amino acids 120 and 129 from tyrosines to phenylalanines and introducing a silent mutation at bp 4241 yielding an AluI restriction site; for Y120/129D, M2.Y120D/Y129D Oligo1 (5' GGC TGG ATA TCG ACT GGT TCA CTG TTA GCT GTT TCA TCG A) and Oligo2 (5' CAT CGA TGA AAC AGC TAA CAG TGA ACC AGT CGA TAT CC), followed by Oligo3 (5' CCT GAA GAA AAC ATC GAT GAA ACA GCT AAC AGT GAA CC) and Oligo4 (5' CTG TTA GCT GTT TCA TCG ATG TTT TCT TCA GGA CTT CC), mutating amino acids 120 and 129 from tyrosines to aspartic acids and introducing a silent mutation at bp 4241 yielding an AluI restriction site; for P6, M2.P155A/P158A Oligo1 (5' CTA GGC CCA GCT ACA AGA GCT TTG CCT AAA) and Oligo2 (5' GGC AAA GCT CTT GTA GCT GGG CCT AGA TTT), mutating amino acids 155 and 158 from prolines to alanines, introducing two AluI restriction sites; for P7, M2.P160A/P163A Oligo1 (5' GTT GGT TCG CGA GTT TAG CCA AAG GTC TTG TAG G) and Oligo2 (5' GAC CTT TGG CTA AAC TCG CGA ACC AAC ACC), mutating amino acids 160 and 163 from prolines to alanines, introducing a BstUI restriction site; for P8, M2.P167A/P170A Oligo1 (5' CGT ATC TCC GCG TTC ATG GCG TGT TGG TTC G) and Oligo2 (5' CAA CAC GCC ATG AAC GCG GAG ATA CGT CTT CC), mutating amino acids 167 and 170 from prolines to alanines and introducing a silent mutation at bp 4097 yielding a DdeI restriction site; for P9, M2.P175A/P178A Oligo1 (5' CCC TGA GAT ACG TCT AGC TAT TAT TGC ACC ATC C) and Oligo2 (5' GGT TCA TCT GAT GTT AAT GTT GAA GGG CAG GCC), mutating amino acids 175 and 178 from prolines to alanines, introducing an AluI restriction site; for P8/9, M2.P167A/P170A/P175A/P178A, constructed using M2.P175A/P178A as a template with Oligo1 (5' CGC GGA GAT ACG TCT AGC TAT TAT TGC ACC ATC C) and Oligo2 (5' GGA TGG TGC AAT AAT AGC TAG ACG TAT CTC CGC G), mutating amino acids 167 and 170 from prolines to alanines and introducing a silent mutation at bp 4097 yielding a DdeI restriction site; for P3/4/7, M2.P70A/P71A/P73A/P160A/P163A, constructed using M2.P160A/P163A as a template and the M2.P70A/P71A/P73A oligonucleotides as described above; and for Y129F/P7, M2.Y129F/P160A/P163A, constructed using M2.P160A/P163A as a template and M2.Y120F/Y129F Oligo1 and Oligo2 as described above.

An M2 marker rescue pCR Blunt plasmid was generated by PCR using Lit38-M2 as a template and designated M2.MR. All M2 mutant pCR Blunt plasmids and M2.MR were sequenced to verify the introduction of the site-directed point mutations and the absence of unwanted mutations. All recombinant viruses were generated by allelic exchange in *Escherichia coli*, as described by Smith and Enquist (33, 46). Briefly, the NotI and BamHI restriction sites within pCR Blunt were used to liberate the MHV68 genomic region contained within the plasmid. This fragment was cloned into the suicide vector pGS284, which harbors an ampicillin gene and a levansucrase cassette for positive and negative selection, respectively. The resulting plasmid was transformed into S17 λ pir *E. coli* cells and mated to GS500 *E. coli* (RecA⁺) containing wt MHV68 BAC. Cointegrants were selected on Luria-Bertani (LB) agar plates containing chloramphenicol (Cam) and ampicillin and were resolved following overnight growth in LB medium with Cam. Next, bacteria were plated on LB agar plates containing Cam and 7% sucrose to select for loss of pGS284 vector sequence. Individual colonies harboring site-specific point mutations within M2 were identified by colony PCR followed by restriction endonuclease digestion. Positive clones were grown in LB medium with Cam, and BAC DNA was purified with a midi-prep kit (Qiagen, Hilden, Germany) as described in the manufacturer's modified protocol. The presence of site-specific point mutations and the absence of unwanted mutations within the region of homologous recombination were confirmed by sequencing and Southern blotting. Virus stocks were generated by Superfect (Qiagen, Hilden, Germany) transfection of recombinant MHV68 BAC DNA into Vero-Cre cells as previously described (33). In wells showing a cytopathic effect (CPE), virus was harvested, cleared of cell debris, and used to infect Vero-Cre cells in order to generate high-titer stocks. Following the presence of CPE in Vero-Cre cells, samples were harvested, homogenized, clarified, and aliquoted for storage at -80°C. Virus stock titers were determined by a plaque assay as previously described (10, 19). Virus stocks were generated by transfection of recombinant MHV68.BAC DNA into a Vero cell line constitutively expressing Cre-recombinase (Vero-Cre). LoxP sites flanking the BAC vector allowed for Cre recombinase-mediated excision of the BAC vector from the viral

genome during virus growth in this cell line. Removal of the BAC vector was assessed by a diagnostic PCR (data not shown), and all viral stocks employed in these studies were negative for the presence of any BAC vector sequences.

The structure of recombinant viruses was verified by Southern blot analyses (Fig. 2) and sequence analysis. To sequence the M2 region of the resulting mutant viruses, DNA was amplified using primers annealing outside the region of homologous recombination. In addition, diagnostic restriction endonuclease digestions were analyzed by Southern blot analysis, using a probe from the M2 ORF (genomic coordinates, bp 4031 to 4627). To generate MHV68M2.P1, amino acids 3 and 5 were mutated from prolines to alanines and a silent mutation at bp 4592 yielding an AluI restriction site was introduced. MHV68M2.P1 digestion with AluI resulted in a unique band of 792 bp, compared to digestion of MHV68M2.P1.MR, yielding a band of 1,051 bp (Fig. 2B, lanes 1 and 2). To generate MHV68M2.P2, prolines 35 and 38 were replaced with alanines, introducing a BglII restriction site. MHV68M2.P2 digestion with BglII resulted in two bands (6,390 bp and 2,066 bp), compared to the single band (8,456 bp) obtained following BglII digestion of wt MHV68 (Fig. 2B, lanes 3 and 4). MHV68M2.P3/4 was generated by mutation of amino acids 70, 71, and 73 from prolines to alanines, introducing a BssHII restriction site. MHV68M2.P3/4 digestion with BssHII yielded two bands (4,396 bp and 927 bp), compared to the single band (5,321 bp) obtained following BssHII digestion of the wt virus (Fig. 2B, lanes 5 and 6). To generate MHV68M2.P5, prolines 95 and 98 were mutated to alanine residues, resulting in the loss of an NcoI restriction site. MHV68M2.P5 digestion with NcoI yielded a single band (2,789 bp), compared to two bands (2,058 bp and 731 bp) for the wt virus (Fig. 2B, lanes 7 and 8). The extraneous, submolar bands in lanes 7 and 8 are attributed to nonspecific hybridization of the ³²P-labeled molecular size standard probe. MHV68M2.P6 was generated by mutation of amino acids 155 and 158 from prolines to alanines, introducing two AluI restriction sites. MHV68M2.P6 digestion with AluI resulted in a unique band of 708 bp, compared to the band of 1,051 bp obtained following AluI digestion of the wt virus (Fig. 2C, lanes 1 and 2). To construct MHV68M2.P7, prolines 160 and 163 were mutated to alanines, introducing a BstUI restriction site. MHV68M2.P7 digestion with BstUI resulted in a single band (727 bp), compared to the two bands (727 bp and 540 bp) obtained following BstUI digestion of wt MHV68 (Fig. 2C, lanes 3 and 4). MHV68M2.P8 was generated by mutation of amino acids 167 and 170 from prolines to alanines, introducing a silent mutation at bp 4097 yielding a DdeI restriction site. MHV68M2.P8 digestion with DdeI resulted in two bands (857 bp and 597 bp), compared to DdeI digestion of MHV68M2.P8.MR, yielding a single band (857 bp) (Fig. 2C, lanes 5 and 6). To generate MHV68M2.P9, prolines 175 and 178 were mutated to alanines, introducing an AluI restriction site. MHV68M2.P9 digestion with AluI resulted in a unique band of 770 bp, compared to AluI digestion of MHV68M2.P9.MR, yielding a band of 1,051 bp (Fig. 2C, lanes 7 and 8). Subsequently, MHV68M2.P9 was used as a template to generate MHV68M2.P8/9. Thus, prolines 167 and 170 were replaced with alanines and a silent mutation at bp 4097 yielding a DdeI restriction site was introduced. MHV68M2.P8/9 digestion with DdeI resulted in two bands (857 bp and 597 bp), compared to DdeI digestion of MHV68M2.P8/9.MR, yielding a single band (857 bp) (Fig. 2C, lanes 9 and 10). MHV68M2.P7 was used as a template to generate MHV68M2.P3/4/7. Hence, mutation of amino acids 70, 71, and 73 from prolines to alanines introduced a BssHII restriction site. MHV68M2.P3/4/7 digestion with BssHII resulted in two bands (4,396 bp and 927 bp), compared to the single band (5,321 bp) obtained following BssHII digestion of the wt virus (Fig. 2C, lanes 11 and 12). To generate MHV68M2.Y120/129F, tyrosines 120 and 129 were mutated to phenylalanines and a silent mutation at bp 4241 yielding an AluI restriction site was introduced. MHV68M2.Y120/129F digestion with AluI resulted in unique bands of 610 bp and 441 bp, compared to AluI digestion of MHV68M2.Y120/129F.MR, yielding a single band (1,051 bp) (Fig. 2D, lanes 1 and 2). To generate MHV68M2.Y120/129D, tyrosines 120 and 129 were replaced with aspartic acids and a silent mutation at bp 4241 yielding an AluI restriction site was introduced. MHV68M2.Y120/129D digestion with AluI resulted in unique bands of 610 bp and 441 bp, compared to AluI digestion of MHV68M2.Y120/129D.MR, yielding a single band (1,051 bp) (Fig. 2D, lanes 3 and 4). MHV68M2.P7 was used as a template to generate MHV68M2.Y129F/P7. Tyrosine residue 129 was mutated to phenylalanine, and a silent mutation at bp 4241 yielding an AluI restriction site was introduced. MHV68M2.Y129F/P7 digestion with AluI resulted in unique bands of 610 bp and 441 bp, compared to AluI digestion of MHV68M2.Y129F/P7.MR, yielding a single band (1,051 bp) (Fig. 2D, lanes 5 and 6). The observation of a 1,051-bp band in Fig. 2D, lanes 2, 4, and 6, is the result of incomplete digestion of this DNA fragment. Submolar, extraneous bands are the result of nonspecific hybridization of the ³²P-labeled molecular size standard probe.

In addition, because the original M2.Stop virus contained a translation stop codon inserted at residue 108 and thus could produce a truncated M2 protein, we

FIG. 2. Construction of M2 mutant viruses and corresponding marker rescue viruses. (A) The MHV68 genome cloned as a BAC (2) was used to generate all M2 recombinant viruses by RecA-mediated recombination. For M2 mutant viruses exhibiting robust establishment-of-latency and/or reactivation-from-latency phenotypes, a marker rescue virus in which the wt M2 sequences were targeted to the M2 ORF by allelic exchange to restore the wt phenotype was generated. The DNA probe used for the Southern blot analyses contained sequence from the M2 ORF (genomic coordinates, bp 4031 to 4627). Relevant restriction sites are indicated. A, AluI; Bg, BglI; D, DdeI; N, NcoI; BU, BstUI; Bs, BssHII. (B and C) Southern blot analyses of recombinant viruses harboring mutations in the indicated PXXP motifs of M2, along with corresponding marker rescue viruses. (D) Southern blot analyses of recombinant viruses harboring mutations in the indicated tyrosine residues of M2 and their corresponding marker rescue viruses. (E) Southern blot analysis of M2.Stop(2). ³²P-labeled molecular size standards (Lambda DNA-BstEII digest; New England Biolabs, Beverly, MA) were included in each Southern blot analysis. WT, wt MHV68; MR, marker rescue virus.

generated a second M2.Stop virus [M2.Stop(2)]. The M2.Stop(2) mutant contains a translation stop codon (and a diagnostic HindIII restriction site) inserted at bp 4566, 13 amino acids downstream of the translation initiation codon, in the viral genome. MHV68M2.Stop(2) digestion with HindIII resulted in two bands (4,459 bp and 1,696 bp), compared to the single band (6,155 bp) obtained following HindIII digestion of the wt virus (Fig. 2E, lanes 1 and 2). This virus was constructed for the purpose of serving as an M2 null virus control and was used as a reference point to compare all other M2 mutant viruses discussed in this

paper. Notably, the phenotype of the M2.Stop(2) virus was indistinguishable from that of the original M2.Stop virus (see Fig. 4C and D).

Mammalian cell transfections. A 2-kb region of the MHV68 genome (bp 3954 to 5955) including M2 exon 1 and the M2 protein-coding region of exon 2, as well as part of the predicted 3' untranslated region (18), was cloned into the mammalian expression vector pIRES2-EGFP (Clontech, Mountain View, CA), generating the plasmid M2.MEX. The following primers were utilized: Oligo1 (5' GAA TTC TGT CTG GTC TGT CAC GCT TC) and Oligo2 (5' AGA TCT

ACC ATG AGG TCT GCA CCA CG). All relevant M2 mutants were cloned into M2.MEX as follows: the BamHI and XhoI restriction sites within M2.MEX were used to liberate a portion of the wt M2 sequence from the backbone vector; XhoI and BglII restriction sites were employed to cut the replacement M2 sequence containing the mutation of interest from the relevant M2 mutant pCR Blunt plasmid. For all transfections, 1×10^6 Cos-1 cells were plated in 100-mm culture dishes and allowed to grow overnight. Ten micrograms of plasmid DNA was used per transfection with the *TransIT-LT1* transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. At 48 hours posttransfection, cells were washed twice with phosphate-buffered saline (PBS) and lysed in 0.5 ml ELB buffer (50 mM HEPES [pH 7.2], 250 mM NaCl, 2 mM EDTA, 0.1% NP-40 substitute, 1 mM NaF, 1 mM Na_3VO_4 , and 0.01% protease inhibitors [Sigma, St. Louis, MO]) or 0.5 ml CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer (50 mM HEPES [pH 7.2], 250 mM NaCl, 2 mM EDTA, 0.1% CHAPS, 1 mM NaF, 1 mM Na_3VO_4 , and 0.01% protease inhibitors) for 20 min at 4°C. Following lyses, cells were scraped into 1.5-ml tubes and cleared of cellular debris by centrifugation. M2 immunoprecipitations were performed as described below.

Antibodies and immunoprecipitation and CHX assays. For M2 immunoprecipitations, affinity-purified chicken polyclonal anti-M2 antibodies (Gallus Immunotech, Ontario, Canada) were generated against the peptides GGRPQRPP LPRTRFC and CSTRSLMMLDSG, corresponding to amino acids 82 to 94 and 150 to 161, respectively. Preimmune sera from these animals did not recognize M2 in any experiments described in this paper. In addition, rabbit antiserum was generated against the M2 protein. The full-length M2 ORF (bp 4031 to 4627) was PCR amplified from purified MHV68 DNA to incorporate a six-histidine tag at the 5' end (CAC CAT CAT CAT CAC), cloned into the bacterial expression vector pET30(a)+ (Novagen, Madison, WI), and transformed into BL21(DE3) competent *E. coli* (Novagen). Following transformation, expression of the six-His-tagged M2 ORF was induced by 0.8 mM IPTG (isopropyl- β -D-thiogalactopyranoside). The M2 protein was purified by continuous gradient elution from a Ni^{2+} column (Qiagen, Germany) per the manufacturer's instructions. The M2 antisera were obtained from rabbits boosted 12 times with the M2 protein (Cocalico Biochemicals, Reamstown, PA). Serum was heat inactivated at 56°C for 30 min, sterile filtered, and aliquoted for storage at -80°C. Preimmune sera from these rabbits did not recognize M2 in any experiments described in this paper. For M2 immunoprecipitations, the cell lysates were precleared with 200 μ l of PrecipHen (Gallus Immunotech, Ontario, Canada) and 1 μ l of 1 mg/ml of preimmune sera at 4°C for 30 min. Following centrifugation, the anti-M2 185R chicken antibody was added to precleared lysates at a dilution of 1:100 by volume. After rotating an hour at 4°C, 20 μ l of PrecipHen was added to the lysates and allowed to rotate at 4°C overnight. The following day, immunoprecipitations were washed four times with ELB lysis buffer, resuspended in 25 μ l of fresh ELB buffer and 25 μ l of 2% sodium dodecyl sulfate (SDS) loading buffer, boiled for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% nonfat milk in PBS-Tween, immunoblotted with the indicated antibody, and visualized by chemiluminescence using the ECL western blotting detection system (Amersham Biosciences). For cycloheximide (CHX) experiments, 2×10^6 Cos-1 cells were plated in two 100-mm culture dishes and allowed to grow overnight. Ten micrograms of plasmid DNA was used per transfection per 100-mm dish. At 6 hours posttransfection, cells were trypsinized and pooled, and equivalent cell numbers were dispensed to nine 10-mm culture dishes. At 24 hours posttransfection, 50 μ g/ml CHX was added to each 10-mm dish. Cells were washed twice with PBS and lysed in ELB buffer at the indicated time points. Immunoblots were probed with the M2 antiserum, treated with sodium azide, and then probed with antibody against green fluorescent protein (GFP). An affinity-purified immunoglobulin G goat anti-GFP antibody (Rockland) was used to detect GFP. Protein half-life was determined by calculating an M2-to-GFP immunoblot band intensity ratio at each time point and then determining the time at which the ratio is 50% of that at the zero time point. Protein band intensities were measured using ImageQuant TL (Amersham Biosciences).

Mice, infections, and organ harvests. Female C57BL/6J mice (catalog no. 000664; The Jackson Laboratory, Bar Harbor, ME) were housed at the Yerkes vivarium or Whitehead vivarium in accordance with university and federal guidelines. Mice between 8 and 12 weeks of age were placed under isoflurane anesthesia prior to intranasal inoculation with 100 PFU of virus in 20 μ l of cMEM. Splenocytes were harvested into cMEM, homogenized, and filtered through a 100- μ m-pore-size nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Red blood cell lysis buffer (Sigma, St. Louis, MO) was used to eliminate erythrocytes. Pooled splenocytes from five mice were used in all relative experiments. Lungs were harvested into cMEM and stored at -80°C.

Plaque assay. Plaque assays were performed as previously described (10), with the following modifications. NIH 3T12 cells were plated in six-well plates 24 h prior to infection at 2.5×10^5 cells per well. Harvested lungs were subjected to three rounds of mechanical disruption of 1 min each, using 1.0-mm zirconia-silica beads (Biospec Products, Bartlesville, OK) in a Mini-Beadbeater-8 (Biospec Products). Serial 10-fold dilutions of lung homogenate were plated on NIH 3T12 monolayers in a 200- μ l volume. Infections were performed for 1 h at 37°C. Immediately following infection, the plates were overlaid with 5 ml of cMEM containing 2% methylcellulose. On day 7 postinfection, the plates were stained with a neutral red overlay and plaques were scored 24 h later. The titers of all samples were determined in parallel with a known laboratory standard. The limit of detection for this assay is 50 PFU.

Limiting-dilution ex vivo reactivation analyses. Limiting-dilution analysis for determining the frequency of cells containing virus capable of reactivating from latency was performed as previously described (60, 61). Briefly, splenocytes were resuspended in cMEM as described above and plated in serial twofold dilutions (starting with 10^5 cells) onto MEF monolayers in 96-well tissue culture plates. Twelve dilutions were plated per sample, and 24 wells were plated per dilution. Wells were scored for CPE at 21 days postplating. The presence of preformed infectious virus was determined by plating parallel samples of mechanically disrupted cells onto MEF monolayers. This process kills >99% of live cells, which allows preformed infectious virus to be discerned from virus reactivating from latently infected cells (60–62). The level of sensitivity of this assay is 0.2 PFU (60).

Limiting-dilution nested-PCR detection of MHV68 genome-positive cells. Limiting-dilution analysis for determining the frequency of cells harboring the viral genome was performed using a single-copy-sensitive nested PCR assay as previously described (61, 62). Briefly, splenocyte samples were counted, washed, and resuspended in isotonic buffer and plated in serial threefold dilutions in a background of 10^4 uninfected NIH 3T12 cells in 96-well plates (MWG Biotech, High Point, NC). Plates were covered with PCR foil (Eppendorf Scientific, Westbury, NY), and cells were lysed with proteinase K for 6 h at 56°C. Ten microliters of round 1 PCR mix was added to each well by foil puncture. Upon completion of first-round PCR, 10 μ l of round 2 PCR mix was added to each well by foil puncture. Following second-round PCR, products were resolved by ethidium bromide staining on 2% agarose gels. Cell lyses and PCR were performed with a PrimusHT thermal cycler (MWG Biotech, High Point, NC). Twelve PCRs were performed for each sample dilution, and a total of six dilutions were performed per sample. Every PCR plate contained control reaction mixtures (uninfected cells and 10 copies, 1 copy, or 0.1 copy of plasmid DNA in a background of 10^4 cells) as previously described (61, 62). All of the assays demonstrated approximately single-copy sensitivity with no false positives.

Retrovirus production and transduction. Using the M2.MR or indicated M2 mutant pCR Blunt plasmid as a template, the M2 ORF was flanked with BglII restriction sites by using Oligo1 (5' CAG CTC AGA TCT ATG GCC CCA ACA CCC) and Oligo2 (5' CAG CTC AGA TCT TTA CTC CTC GCC CCA) and cloned into the pMSCV-IRES-Thy1.1 vector (a kind gift from Phillipa Marrack). Positive clones were sequence verified. Retroviruses were generated using the BOSC23 producer cell line. BOSC23 cells (2×10^6) were plated on 60 mM collagen II-coated plates. Twenty-four hours later, 10 μ g of the pMSCV vector was transfected into the BOSC23 cells by using the LT-293T reagent (Mirus, Madison, WI) according to the manufacturer's protocol. At 48 hours posttransfection, retroviral supernatants were harvested and centrifuged at 2,000 rpm for 10 min to clear cell debris. Primary murine B cells were transduced by removing 700 μ l of cRPMI and replacing it with 1 ml of retroviral supernatant and 5 μ g/ml polybrene. Cells were centrifuged at 2,500 rpm for 1 hour at 30°C. Seven hundred fifty microliters of retroviral supernatant was removed and replaced with fresh cRPMI.

B-cell isolation and flow cytometry. Spleens were homogenized and erythrocytes were removed with red blood cell lysis buffer (Sigma, St. Louis, MO). B cells were resuspended in PBS containing 0.5% FCS and stained with rat anti-mouse CD16/32 (Fc block) on ice for 15 min in the dark. B cells were isolated by depletion of non-B cells, using a B-cell isolation kit and autoMACS (Miltenyi Biotec, Cologne, Germany) as previously described (17). B-cell purity was examined by staining for CD19; all B cells isolated were 93 to 97% pure. Cells were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10 mM nonessential amino acids, and 25 μ g/ml of lipopolysaccharide (LPS; Sigma). Following transduction, cells were stained with Thy1.1-APC (eBiosciences) for 20 min. B cells were analyzed on an LSR II flow cytometer. Data were analyzed using FlowJo software (TreeStar, Inc., San Carlos, CA).

Statistical analyses. Data were analyzed with GraphPad Prism software (GraphPad Software, San Diego, CA). Data were subjected to nonlinear regres-

sion analysis to determine the single-cell frequency for each limiting-dilution analysis. From the Poisson distribution, the frequencies of reactivation and viral genome-positive cells were obtained from the nonlinear regression fit of the data, where the regression line intersected 63.2%.

RESULTS

Generation of a panel of recombinant viruses harboring point mutations in the M2 gene. Based on the presence of multiple PXXP motifs in the M2 protein (Fig. 1), we predicted that M2 might function as an adaptor protein, interacting with multiple SH3 domain-containing proteins. The plethora of potential SH3 binding partners suggested that several of the PXXP motifs in M2 could contribute to the function of the protein in the establishment of and/or reactivation from MHV68 latency. In addition, if the role of M2 in a latent infection relies on its ability to manipulate lymphocyte signaling pathways, the tyrosine residues within M2 (upon phosphorylation) might mediate interactions with SH2 domain-containing proteins. Therefore, to systematically identify which of these motifs plays a role in the function of M2 in the establishment of and/or reactivation from MHV68 latency in the spleen, a panel of 13 recombinant viruses containing mutations in the M2 gene targeted to either individual PXXP motifs or tyrosine residues was constructed. In the case of mutating PXXP motifs, we mutated specific proline residues to alanines, while mutation of tyrosine residues involved mutation to either phenylalanine or aspartic acid (Fig. 1). Mutagenesis of the viral genome was carried out with *E. coli* by using a previously characterized BAC clone of the MHV68 genome (2), with specific point mutations being introduced into the M2 ORF by allelic exchange as described by Smith and Enquist (33, 46). The details of the generation and initial characterization of these viral mutants is described in detailed in Materials and Methods. Notably, for each mutant virus the presence of site-specific point mutations, and the absence of unwanted mutations within the region of homologous recombination, was confirmed by DNA sequencing of BAC DNA, coupled with Southern blot analyses (Fig. 2). In addition, for those mutant viruses that exhibited a significant phenotype, a marker rescue virus was generated to ensure that the phenotype mapped to the M2 gene.

To verify that the point mutations introduced into the M2 protein did not give rise to a misfolded protein, resulting in an unstable molecule, we attempted to detect the M2 protein following infection of a variety of cell lines with the recombinant viruses. Analysis of M2 expression was assessed by immunoprecipitation and subsequent immunoblotting following MHV68 infection of a variety of cell lines (NIH 3T12 fibroblasts, the A20 and WEHI-231 B-cell lines, the murine macrophage cell line RAW, and the murine myeloma cell line NSO). In none of these infected cultures could we detect expression of the M2 protein (data not shown). This result was not surprising in the case of cell lines productively infected by MHV68, as M2 transcripts are found at very low abundance during the virus replication cycle (3, 31, 52). However, these analyses also indicated that infection of B-cell lines or a macrophage cell line, which might be expected to allow establishment of a latent infection, also did not result in detectable M2 expression. As such, it appears that M2 gene expression is

tightly regulated during virus infection, and to date, we have failed to identify an appropriate cell line where M2 expression can be detected following virus infection.

To circumvent the inability to detect the M2 protein upon infection of cultured cell lines, each M2 mutant was cloned into a mammalian expression vector (pIRES2-EGFP) harboring a GFP expression cassette and transiently transfected into Cos-1 cells. The presence of an internal ribosome entry site-GFP cassette provided an internal control for transfection efficiency and experimental variation. At 48 hours posttransfection, cells were lysed and M2 was immunoprecipitated with an affinity-purified chicken polyclonal anti-M2 antibody (anti-M2 185R) generated by immunizing chickens with two peptides from the M2 protein (a peptide from the N-terminal domain of M2 and a peptide from the C-terminal domain of M2). Following SDS-PAGE, M2 was identified by immunoblot analysis with a rabbit polyclonal M2 antiserum generated against recombinant M2 purified from *E. coli* (Fig. 3B). Importantly, the appropriately sized M2 species was detected for each mutant (approximately 29 kDa, consistent with the observations of others [29, 30, 43]) (Fig. 3B and data not shown). To verify that the M2 mutants exhibiting significant latency phenotypes (see below) express M2 protein with stability similar to that of the wt protein, specific M2 mutants were transiently transfected into Cos-1 cells, followed by treatment with CHX to halt protein synthesis. Samples were harvested for Western blot analyses of M2 and GFP expression at the indicated times posttreatment (Fig. 3C). Protein half-lives were calculated for each M2 mutant, which demonstrated that none of these mutations significantly altered M2 protein stability (Fig. 3D).

PXXP motifs in the C terminus of M2 play an important role in the establishment of MHV68 latency in the spleen. To directly assess the contribution of each PXXP motif to the function of M2 in the context of a latent MHV68 infection in vivo, 10 M2 mutant viruses were characterized. Notably, for those M2 mutant viruses exhibiting robust establishment-of-latency and/or reactivation-from-latency phenotypes, a marker rescue virus in which wt M2 sequences were targeted by allelic exchange to replace the mutant M2 ORF was constructed. Thus, the marker rescue viruses served to rule out the potential contribution to the observed phenotype of distal mutations that might have been incorporated during the recombination process.

Previously, we have shown that the M2 gene is required for the efficient establishment of MHV68 latency in the spleen at 16 dpi following a 100-PFU intranasal inoculation with MHV68M2.Stop (17). Therefore, these conditions were chosen to evaluate the abilities of recombinant viruses harboring point mutations in the M2 gene to establish and reactivate from latency. C57BL/6J mice were infected via intranasal inoculation with 100 PFU of either wt MHV68, recombinant M2 mutant virus, or the corresponding marker rescue virus. At 16 dpi, spleens were harvested and single-cell suspensions were prepared for analysis of latency in bulk splenocytes.

To investigate the requirement of these putative interaction domains in M2 for the establishment of latency in the spleen, the frequencies of viral genome-positive cells were determined using a previously described limiting-dilution PCR assay (61, 62). To examine the abilities of these M2 mutant viruses to reactivate from latency, total splenocytes were subjected to a

FIG. 3. Expression of mutant M2 proteins in mammalian cells. (A) Schematic illustration of the locations of PXXP motifs and tyrosine residues in the M2 protein which have been targeted for mutation in this report. (B) Cos-1 cells were transiently transfected with plasmids expressing the indicated forms of mutant M2 protein. At 48 hours posttransfection, cells were harvested and M2 immunoprecipitations were performed as described in Materials and Methods. (C) Cos-1 cells were transiently transfected with plasmids expressing the indicated forms of mutant M2 protein and GFP. At 24 hours posttransfection, 50 μ g/ml CHX (+) or a vehicle control (–) was added to each sample, and cell lysates were harvested at the indicated times, in hours, posttreatment. Protein half-life was calculated by determining the time point at which the band intensity ratio of M2 to GFP was 50% of this ratio at time zero. WT, wt M2; Cntl, vector control; α , anti; IP, immunoprecipitation; $t_{1/2}$, half-life.

limiting-dilution ex vivo reactivation analysis as previously described (60, 62). Furthermore, to verify that the observed virus-induced CPE in the reactivation analyses was the result of reactivation from latency and not the presence of preformed infectious virus at the time of harvest, splenocytes were mechanically disrupted and plated in parallel. Importantly, little or no preformed infectious virus was observed in any of the analyses in this study (data not shown).

Examination of recombinant viruses harboring mutations in either the P1 or the P2 PXXP motif revealed that neither mutation had a significant impact on the establishment of latency (Fig. 4A and Table 1). However, in mice infected with

MHV68M2.P1 there was a significant defect in virus reactivation compared to what was found for the wt virus, with only 1 in 60,042 splenocytes reactivating from latency (Fig. 4B and Table 1). Infection with the P1 marker rescue virus (MHV68M2.P1.MR) rescued the observed reactivation defect. In contrast to the P1 mutant, the MHV68M2.P2 mutant exhibited only a mild defect in virus reactivation (1 in 10,070 splenocytes) compared to wt MHV68 (Fig. 4B and Table 1). These results identify the P1 domain as playing a role in virus reactivation from latency but indicate that this domain is largely dispensable for establishment of latency.

Splenocytes harvested from mice infected with wt MHV68

TABLE 1. Frequencies of viral genome-positive and reactivating splenocytes at day 16 postinfection

Virus	Total no. of splenocytes recovered	Reciprocal frequency of viral genome-positive cells ^a	Genome frequency relative to wt virus frequency	Estimated no. of viral genome-positive cells ^b	Reciprocal frequency of reactivating cells ^a	Reactivation frequency relative to wt virus frequency	Estimated no. of reactivating cells ^b	Reactivation efficiency (%) ^c
wt MHV68	4.12×10^7	169	1.0	243,787	3,115	1.0	13,226	5.4
M2.Stop(2)	2.64×10^7	6,423	0.03^d	4,110^d	$>1 \times 10^6$	<0.01	<25^d	~0.5
M2.P1	8.97×10^7	420	0.40	213,571	60,042	0.05	1,494^d	0.7
M2.P1.MR	1.12×10^8	215	0.79	520,930	4,046	0.77	27,682	5.3
M2.P2	5.24×10^7	183	0.92	286,339	10,070	0.31	5,203	1.8
M2.P3/4	6.82×10^7	223	0.75	305,829	5,745	0.54	11,871	3.9
M2.P5	6.30×10^7	172	0.98	366,279	6,046	0.52	10,420	2.8
M2.P6	8.37×10^7	130	1.30	643,846	1,777	1.75	47,102	7.3
M2.P7	3.66×10^7	390	0.43	93,846	15,145	0.21	2,417	2.6
M2.P8	4.18×10^7	2,123	0.08^d	19,689^d	85,725	0.04	488^d	2.5
M2.P8.MR	6.08×10^7	178	0.95	341,573	7,876	0.40	7,720	2.3
M2.P9	5.75×10^7	1,898	0.09^d	30,295^d	56,125	0.06	1,024^d	3.4
M2.P9.MR	6.80×10^7	160	1.06	425,000	3,126	1.0	21,529	5.1
M2.P8/9	6.03×10^7	1,901	0.09^d	31,720^d	149,416	0.02	404^d	1.3
M2.P8/9.MR	1.04×10^8	161	1.05	645,963	4,420	0.70	23,529	3.6
M2.P3/4/7	4.33×10^7	361	0.47	119,944	9,569	0.33	4,525	3.8
M2.Y120/129F	8.97×10^7	492	0.34	182,317	15,700	0.20	5,716	3.1
M2.Y120/129F.MR	5.62×10^7	212	0.80	265,094	4,421	0.70	12,712	4.8
M2.Y120/129D	5.24×10^7	2,203	0.08^d	23,786^d	150,377	0.02	348^d	1.5
M2.Y120/129D.MR	7.95×10^7	145	1.17	548,276	2,785	1.11	28,546	5.2
M2.Y129F/P7	2.03×10^7	3,306	0.05^d	6,140^d	231,489	0.01	88^d	1.4
M2.Y129F/P7.MR	6.72×10^7	190	0.89	353,684	2,297	1.36	29,255	8.4

^a Determined from the mean of at least two independent experiments, with splenocytes pooled from five mice per experimental group.

^b Estimated number per spleen, derived from the experimental frequency data and the total number of splenocytes recovered.

^c Percentage of viral genome-positive splenocytes that reactivate virus.

^d The data of significance are indicated in bold.

or the M2.P3/4 mutant exhibited nearly identical frequencies of viral genome-positive cells (1 in 169 and 1 in 223 cells, respectively), while mice infected with the M2.P3/4/7 mutant exhibited a slightly lower frequency of viral genome-positive cells (1 in 361) (Fig. 4C and Table 1). As expected, a 40-fold reduction in the establishment of latency was observed following infection with the M2 null virus MHV68M2.Stop(2) (Fig. 4C and Table 1). The frequencies of splenocytes reactivating virus were very similar for wt MHV68 and the M2.P3/4 mutant (1 in 3,115 and 1 in 5,745 splenocytes, respectively), while the M2.P3/4/7 mutant exhibited a slightly greater defect in virus reactivation (1 in 9,569 splenocytes) (Fig. 4D and Table 1). The lower frequency of splenocytes reactivating MHV68 following M2.P3/4/7 infection appears to be largely accounted for by the slight defect in establishment of latency described above (Fig. 4C and Table 1). Splenocytes harvested from mice infected

with MHV68M2.Stop(2) displayed a severe decrease in reactivation frequency (<1 in 1×10^6 cells reactivating virus) (Fig. 4D and Table 1), confirming our previously described data (employing the original M2.Stop mutant virus) indicating that in the absence of M2, reactivation from MHV68 latency is severely impaired (17, 19, 29). As described in Materials and Methods, the MHV68.Stop(2) mutant is a newly generated M2 null virus in which a translation stop codon was inserted just downstream of the M2 translation initiation codon. This mutant was generated to address the possible impact that a truncated M2 protein being generated from the previously characterized M2.Stop virus (17, 19) could have on the observed phenotype as well as to ensure that all viruses analyzed in this study share a common genetic background (from the cloned MHV68 BAC).

Both the P5 and P6 domains were dispensable for establish-

FIG. 4. Identification of PXXP motifs and tyrosine residues in the M2 protein that are involved in establishment of and/or reactivation from MHV68 latency in the spleen. C57BL/6J mice were infected intranasally with 100 PFU of wt MHV68, a recombinant M2 mutant virus, or its corresponding marker rescue virus, and spleens were harvested at 16 dpi. Bulk splenocytes were analyzed by limiting-dilution ex vivo reactivation assays and limiting-dilution viral genome PCR assays, as described in Materials and Methods. (A, C, E, G, I, K and M) Frequency of splenocytes harboring viral genome. A limiting-dilution PCR assay was used to determine the frequencies of splenocytes harboring the viral genome. Samples were serially diluted into a background of 10^4 uninfected cells, lysed, and subjected to nested PCR to detect the viral genome (see Materials and Methods). (B, D, F, H, J, L, and N) Frequency of splenocytes reactivating virus. Results are shown for a limiting-dilution ex vivo reactivation assay in which intact splenocytes were serially diluted on MEF indicator monolayers in parallel with mechanically disrupted samples (to distinguish between virus reactivating from latency and preformed infectious virus). In this report, little or no preformed infectious virus was detected for any of the viruses analyzed (data not shown). For each sample dilution, 24 wells were scored for the presence of CPE (see Materials and Methods). Data are expressed as the mean percentages of wells positive for virus (CPE or viral DNA) $\pm$ the standard errors of the means. Curve fit lines for both assays were derived through nonlinear regression analyses. The dashed line indicates 63.2%, from which the frequency of cells reactivating virus or the frequency of cells harboring the viral genome was determined based on Poisson distribution. The data shown represent the means for at least two independent experiments. The recombinant virus nomenclature is described in the legends to Fig. 1 and 3 and Materials and Methods.

ment of latency, while mutation of the P7 motif modestly reduced the frequency of latently infected splenocytes (Fig. 4E). Analysis of reactivation also indicated that mutation of the P5 and P6 domains had little effect on virus reactivation (Fig. 4F and Table 1). However, consistent with the observed decrease in establishment of latency, mutation of the P7 domain modestly decreased the frequency of splenocytes reactivating virus compared to that for the wt virus (1 in 15,145 versus 1 in 3,115, respectively) (Fig. 4F and Table 1). The data on the P7 mutant virus indicate that the phenotype of the triple PXXP domain mutant virus (MHV68M2.P3/4/7) discussed above is due to the mutation of prolines 160 and 163. As discussed below (see Discussion), the studies investigating the interaction of the guanine nucleotide exchange factor proteins Vav1 and Vav2 with M2 demonstrate that the P7 motif is largely responsible for this interaction (30). Based on the analysis of the P7 domain presented here, this raises some question as to the functional significance of the Vav-M2 interaction in the establishment of virus latency.

Mutation of either the P8 or the P9 motif resulted in a significant defect in the establishment of latency compared to what was found for wt M2. The frequencies of viral genome-positive cells were 1 in 2,123 for the P8 mutant and 1 in 1,898 for the P9 mutant, compared to 1 in 169 for the wt virus (Fig. 4G and Table 1). Consistent with the defect in the establishment of latency, when reactivation from latency was assessed the P8 mutant was found to exhibit a ca. 30-fold defect (1 in 85,725 splenocytes) and the P9 mutant a ca. 20-fold defect (1 in 56,125 splenocytes) compared to the wt virus (1 in 3,115 splenocytes) (Fig. 4H and Table 1). Notably, both the P8 and P9 marker rescue viruses exhibited wt levels for establishment of and reactivation from latency (Fig. 4G and H and Table 1). Thus, we conclude that the P8 and P9 PXXP motifs in M2 play an important role in the establishment of splenic latency. Based on the similarity between the phenotypes obtained by mutating either the P8 or the P9 motif, we considered the possibility that these two PXXP motifs participate in the same pathway with respect to M2 function *in vivo*. To investigate this hypothesis, a recombinant M2 mutant virus harboring mutations of both the P8 and P9 motifs (MHV68M2.P8/9) was constructed. Notably, the M2.P8/9 double mutant exhibited an establishment of latency (1 in 1,901) nearly identical to that for the individual P8 and P9 domain mutations (Fig. 4I and Table 1). However, mutation of both the P8 and P9 motifs resulted in a slightly greater defect in virus reactivation, with ca. 50-fold-less virus reactivation from latency in the spleen (only 1 in 149,416 cells) than in a wt MHV68 infection (Fig. 4J and Table 1). The reactivation phenotypes observed following inoculation with MHV68M2.P8 and MHV68M2.P9 can, in large part, be attributed to a failure to efficiently establish latency in the spleen. Although the combinatorial mutant virus (MHV68M2.P8/9) was severely compromised in its ability to establish latency, this M2 mutant also revealed a defect in reactivation from latency (Fig. 4J and Table 1). These results demonstrate the importance of the C-terminal proline-rich motifs in M2 in the context of a latent MHV68 infection *in vivo*. Notably, as discussed below, an *ex vivo* assay that we have recently developed to examine M2 function in primary B cells demonstrated distinct roles for the P8 and P9 PXXP motifs (see Discussion and Fig. 6).

Tyrosine residues 120 and 129 of M2 are required for efficient MHV68 reactivation from latency in the spleen. A number of unique gammaherpesvirus antigens contain tyrosine residues that are involved in binding cellular proteins containing SH2 domains and have been shown to modulate lymphocyte signaling pathways during viral infection. Specifically, LMP2A utilizes an immuno-tyrosine activation motif (ITAM) to usurp signaling pathways normally associated with the cytoplasmic tail of the B-cell receptor (BCR) (5, 7, 11, 15, 27, 44, 53). The KSHV K1 and K15 proteins also contain tyrosine motifs capable of interacting with SH2 domains (11, 23, 24). Although M2 contains three tyrosines, none of which comprise a consensus ITAM, only residues 120 and 129 were scored as potential phosphorylation sites by the NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos/>). Recently, these residues have been shown to be capable of being phosphorylated in Cos-1 cells (43). Based on the hypothesis that M2 manipulates B-cell signaling pathways to promote the establishment of and/or reactivation from latency, two recombinant M2 mutant viruses were constructed to evaluate the importance of tyrosine residues 120 and 129 in the context of a latent infection. Site-specific PCR mutagenesis was employed to substitute either phenylalanines or aspartic acids for the indicated amino acids (Fig. 1).

C57BL/6J mice were infected intranasally with 100 PFU of MHV68M2.Y120/129F or MHV68M2.Y120/129F.MR. At 16 dpi, approximately fivefold less virus reactivation from latency was observed in the spleen after infection with MHV68M2.Y120/129F (1 in 15,700 cells) than in a wt virus infection. Infection with MHV68M2.Y120/129F.MR revealed normal levels of reactivation (1 in 4,421 cells) (Fig. 4L and Table 1). Upon evaluation of establishment of latency, 1 in 492 splenocytes harbored the viral genome from mice infected with the M2.Y120/129F mutant. Following inoculation with MHV68M2.Y120/129F.MR, 1 in 212 splenocytes contained the viral genome (Fig. 4K and Table 1). From mice infected with MHV68M2.Y120/129D, the frequency of reactivation was only 1 in 150,377 splenocytes, whereas MHV68M2.Y120/129D.MR infection led to a frequency of 1 in 2,785 splenocytes (Fig. 4L and Table 1). To determine if the 50-fold decrease in reactivation was the result of inefficient establishment of latency, the frequency of viral genome-positive cells was investigated. Following inoculation with MHV68M2.Y120/129D, 1 in 2,203 splenocytes harbored the viral genome and MHV68M2.Y120/129D.MR infection revealed wt levels of splenic latency (1 in 145 cells) (Fig. 4K and Table 1). Although the M2.Y120/129D mutant was severely compromised in its ability to reactivate from latency, these analyses reveal an additional defect in the establishment of latency. Notably, no defect in acute virus replication was observed in the lungs at 9 dpi following MHV68M2.Y120/129D inoculation (Fig. 5B). Examination of these M2 mutant viruses demonstrates the importance of these tyrosine residues in virus reactivation and the establishment of splenic latency. Furthermore, these results suggest that during the course of a latent infection, the phosphorylation status of the residue(s) may be tightly regulated. By substituting aspartic acids for these tyrosines, the residues assume the charge of being constitutively phosphorylated and are hence incapable of being dephosphorylated. Therefore, an uncontrolled activation status may be disadvantageous to M2 function in a latent

FIG. 5. M2 recombinant viruses display normal acute-phase replication in vivo following intranasal inoculation. C57BL/6J mice were infected intranasally with 100 PFU of a recombinant M2 mutant virus or its corresponding marker rescue virus, and the left lung was extracted at 9 dpi (d9). (A) M2 recombinant viruses with site-directed mutations in PXXP motifs and the corresponding marker rescue viruses. (B) M2 recombinant viruses with site-directed mutations in tyrosine residues and the corresponding marker rescue viruses. The results shown were compiled from two independent experiments with three mice per experiment (each symbol represents data from an individual mouse). Virus titers in lungs were determined by a plaque assay on NIH 3T12 monolayers as described in Materials and Methods. The dashed line indicates the level of detection of the plaque assay (50 PFU), and the solid line indicates the mean virus titer of each group. The recombinant virus nomenclature is described in the legends to Fig. 1 and 3 and Materials and Methods.

infection. Alternatively, a constitutively active protein in the midst of proper signaling cascades within a latently infected cell may lead to aberrant cell proliferation, differentiation, and/or gene transcription, subsequently altering the natural course of MHV68 pathogenesis.

An obvious extension of these mutagenesis studies would be the construction of recombinant viruses harboring mutations in multiple functional motifs of M2 (e.g., the C-terminal PXXP motifs in conjunction with tyrosine residues 120 and 129). Along this line, it has been shown in vitro that M2 can form a trimolecular complex with Src kinases and Vav1 and via this interaction M2 facilitates phosphorylation of Vav family members (43). We considered the possibility that a number of proteins involved in lymphocyte signaling contain both SH2 and SH3 domains; therefore, disruption of specific PXXP motifs and tyrosine residues in M2 might augment the phenotype observed. To this end, an M2 mutant virus (MHV68M2.Y129F/P7) in which alanine substitutions for prolines 160 and 163 in the P7 motif were introduced (implicated in Vav binding), in conjunction with a mutation of tyrosine 129 to phenylalanine, was engineered. Infection of mice with MHV68M2.Y129F/P7 revealed a ca. 20-fold defect in the establishment of latency and an estimated 75-fold defect in reactivation from latency (Fig. 4M and N and Table 1). Notably, this double mutant is much more impaired for both establishment of and reactivation from latency than either the Y120/129F mutant or the P7 mutant; indeed, the Y129F/P7 mutant is nearly as impaired as the M2 null mutant [compare M2.Stop(2) data in Fig. 4C and D to results for the M2.Y129F/P7 mutant in Fig. 4M and N]. Importantly, the M2.Y129F/P7 marker

rescue virus exhibited wt levels for establishment of and reactivation from latency, demonstrating that the observed defects map to the tyrosine and P7 mutations. Thus, these data suggest that disruption of any single functional domain in the M2 protein may not completely reveal the importance of the domain in M2 function in vivo, perhaps because mutating a single domain does not completely disrupt the formation of multiple protein complexes involving M2 (i.e., these may be recruited to the complex via other protein-protein interactions).

M2 mutant viruses display normal acute-phase replication in the lungs following intranasal inoculation. The M2 gene product has previously been shown to be dispensable for acute-phase virus replication in the lung following intranasal inoculation (17, 19, 29). However, to rule out the possibility of defects in acute virus replication contributing to the observed latency phenotypes, we assessed virus replication in the lungs of mice following intranasal inoculation. C57BL/6J mice were infected intranasally with 100 PFU of either specific M2 mutants or their corresponding marker rescue viruses. The left lung was harvested at 9 dpi, and virus titers were determined by a plaque assay on NIH 3T12 fibroblasts. We limited these analyses to those M2 mutant viruses that exhibited significant latency phenotypes. The M2 PXXP mutant viruses that exhibited significant latency phenotypes all replicated to levels similar to those of their corresponding marker rescue viruses (Fig. 5A). In addition, M2 recombinant viruses with site-directed mutations in tyrosine residues also demonstrated acute-phase replication at levels comparable to those of their corresponding marker rescue viruses (Fig. 5B). Thus, we conclude that the mutations introduced into the M2 gene do not appear to grossly alter acute virus replication in vivo.

M2 can interact with endophilin II and Grb2 in mammalian cells in a glutathione S-transferase pulldown assay. To begin to assess the interaction of cellular proteins with M2, we carried out a yeast two-hybrid screen using a murine spleen cell cDNA library and a murine day 17.5 embryo library. The SH3 domain-containing proteins endophilin II, endophilin III, and Grb2 were among the putative M2-interacting partners identified in the murine day 17.5 embryo library screen. Furthermore, we determined that the SH3 domain of endophilin II (and presumably that of endophilin III) is both necessary and sufficient to mediate interaction with the M2 antigen in the yeast *Saccharomyces cerevisiae*, and the region of M2 required for its interaction with endophilin II in yeast is located in the amino terminus (data not shown). Next, we demonstrated an interaction between M2 and endophilin II in mammalian cells and verified that P3/4 is required for M2 to bind endophilin II (data not shown). From our mutagenesis of M2, the P3/P4 domains did not score as playing an important role in MHV68 latency. As discussed in the previous section, it is possible that endophilin interacts with M2 in a large protein complex, being recruited by other proteins as well as M2. As such, it is possible that mutation of the endophilin interaction domain on M2 does not abrogate a functional interaction with M2 in vivo.

To further investigate the interaction of Grb2 with M2, Cos-1 cells were transiently transfected with wt or mutant M2 expression vectors. At 48 hours posttransfection, recombinant Grb2 protein fused to glutathione S-transferase was used to pull down the various forms of M2 (see Materials and Methods), followed by immunoblotting for the presence of M2.

FIG. 6. M2 expression in primary B cells results in expansion of transduced population. (A) Representative flow cytometry data from 3 days posttransduction. (B) M2-expressing B cells expand in culture over time. Triplicate B-cell cultures were transduced with wt M2 or the indicated M2 mutant retrovirus and allowed to rest for 48 h before analysis. Cells were stained with anti-Thy1.1 and analyzed daily for Thy1.1-positive cells. Trypan blue exclusion was used to count absolute numbers of live and dead cells in triplicate wells every day; notably, absolute numbers of cells per well were unchanged over time. Data are representative of two independent experiments.

Individual M2 mutants harboring proline-to-alanine mutations at residues 70, 71, and 73 (M2.P3/4) or residues 160 and 163 (M2.P7) were capable of binding Grb2 (data not shown). However, an M2 mutant containing alanine substitutions at residues 70, 71, 73, 160, and 163 (M2.P3/4/7) was unable to interact with Grb2 (data not shown). These results are consistent with the observations in yeast that both the amino-terminal and carboxy-terminal domains of M2 harboring PXXP motifs are capable of binding Grb2 (data not shown). In addition, consistent with this mapping data, we assessed binding of Grb2 to other M2 PXXP mutants and determined that each of these M2 mutants was capable of binding Grb2 (data not shown). As with the endophilin interaction with M2, Grb2 binding mapped to domains that do not appear to be critical for M2 function in vivo. However, it is worth noting that mutation of the P7 domain, along with mutation of tyrosine 129, resulted in a large defect in MHV68 latency. This is particularly interesting since the Grb2 protein, in addition to containing 2 SH3 domains, also contains an SH2 domain. The latter domain might be involved in recruiting Grb2 to a phosphorylated form of M2, a possibility that future studies will address.

Mutation of the P8 motif or the Y129F/P7 motifs, but not the P9 motif, abrogates M2-driven B-cell proliferation and cellular IL-10 expression. We have recently shown that M2 expression in primary murine B cells enhances B-cell proliferation and survival, which is dependent in part on the induction of cellular interleukin-10 (IL-10) expression by M2 (A. M. Siegel,

J. H. Herskowitz, and S. H. Speck, submitted for publication). To determine whether any of the functional domains that we have identified here are required for M2-driven IL-10 expression and B-cell proliferation, we engineered recombinant mouse stem cell virus (MSCV) retroviral vectors harboring either wt M2, M2.Stop, M2.P8, M2.P9, or M2.Y129F/P7 (see Materials and Methods). These vectors also harbor a linked expression cassette composed of a truncated Thy1.1 gene cloned downstream of an internal ribosome entry site, which allows retroviral transduction efficiency to be monitored by surface staining for Thy1.1 expression. B cells purified from spleens of naïve C57Bl/6 mice were stimulated overnight with LPS, followed by removal of the LPS and infection with the recombinant MSCV retroviruses (Fig. 6). At 48 hours posttransduction, there were similar frequencies of Thy1.1⁺ B cells in all cultures (Fig. 6B). Over time, expression of M2 drives a rapid expansion of transduced B cells whereas P9 exhibits a more gradual effect. At 6 days posttransduction, nearly 100% of the B-cell culture transduced with M2 or P9 was Thy1.1⁺, compared to 20% of the culture transduced with M2Stop(2), P8, or Y129F/P7 (Fig. 6B).

Notably, the increase in the percentage of Thy1.1⁺ B cells did not coincide with an increase in cell number in the primary B-cell cultures transduced with M2 compared to that in the negative-control MSCV vector (M2.Stop)-transduced B-cell cultures (data not shown). The latter observation is surprising and suggests the possibility that M2-expressing B cells come to

dominate the culture by out-competing the nontransduced B cells for some limiting reagent required for survival. Alternatively, the M2-expressing B cells may secrete a factor(s) that promotes the death of the nontransduced B cells. In addition, we have shown in other experiments that the M2-expressing B cells do proliferate slightly more and survive slightly better than the control-transduced B cells (Siegel et al., submitted). Regardless, it is important to emphasize that this is a robust phenotype that has been observed in every analysis of M2 function in primary B cells over multiple independent experiments carried out with independent preparations of the recombinant MSCV vectors. We are actively pursuing the mechanism(s) underlying this phenomenon.

Although the P8, P9, and Y129F/P7 mutations all revealed robust latency phenotypes *in vivo*, only the P8 and Y129F/P7 mutations failed to exhibit the dominance of M2-expressing B cells in the transduced primary B-cell cultures (Fig. 6). In contrast, the P9 domain was not required for M2-dependent expansion of primary B cells, although mutation of this domain did result in slower kinetics of the dominance phenotype than in wt M2 (Fig. 6B). Thus, while the latency phenotypes of P8 and P9 raised the possibility that they might function in the same pathway, the analysis of M2 function in primary B cells demonstrates that these domains have distinct functions. This demonstrates the importance of individual functional motifs in M2 and supports the hypothesis that M2 is a multifunctional protein that may interact with a number of different cellular or viral proteins. Furthermore, the dominance of M2-expressing B cells in this culture system is consistent with the hypothesis that M2 may facilitate the establishment of MHV68 latency or virus reactivation by enhancing B-cell proliferation and/or survival.

DISCUSSION

During the early phases of MHV68 infection, the virus can be found in both B cells and non-B cells in the spleens of mice. However, like EBV infection, MHV68 infection ultimately is found predominantly in GC and memory B cells. We have hypothesized that MHV68, like EBV, encodes antigens with the potential to alter normal B-cell signaling pathways to access the memory B-cell reservoir. Consistent with this hypothesis, we have determined that the M2 gene product of MHV68 is a latency-associated antigen that is required for the efficient establishment of latency, as well as reactivation from latency, in splenic B cells (17). The M2 protein is a unique protein that lacks discernible homology to any known viral or cellular proteins but does contain multiple PXXP motifs that have been shown to be involved in the interaction of M2 with several cellular SH3 domain-containing proteins. In addition, the M2 protein harbors tyrosine, threonine, and serine residues that can be phosphorylated (25, 43). In this report, the contributions of each PXXP motif and tyrosine residues 120 and 129 were evaluated with respect to establishment of latency in the spleen as well as reactivation of MHV68 from latency. A systematic mutagenesis of these domains revealed two PXXP motifs, the P8 and P9 domains, located at the C terminus of M2, that are necessary for efficient establishment of latency in the spleen. In addition, a third PXXP motif, P7, when mutated in the context of mutation of tyrosine 129 to phenylalanine,

also scored as functionally important. Notably, the P7 PXXP motif has been implicated in binding Vav (30, 43).

Evaluation of M2 PXXP motifs with respect to establishment of MHV68 latency in the spleen and reactivation from latency. Since the M2 protein is unique to MHV68, various databases (PROSITE, PATTINPRO, PSORT, DGPI, and NetPhos2.0) were searched to determine if M2 harbored any obvious localization or functional motifs. The database algorithms recognized no predicted signal peptide, hydrophobic region consistent with a transmembrane domain, prenylation motif, nuclear localization sequence, endoplasmic reticulum retention signal, or coiled-coil region. M2 has been shown to localize to the cytoplasmic face of the plasma membrane in B cells (29) and Cos-1 cells (43) (also confirmed by our unpublished results). The intracellular localization of M2, along with the presence of multiple PXXP motifs and tyrosine residues, led to the hypothesis that M2 might function to modulate B-cell signaling pathways. Using a yeast two-hybrid screen, we found that M2 is capable of binding the SH3 domain-containing endophilin II/III proteins as well as Grb2 (our unpublished results). Endophilin family members have been shown to be regulatory components of clathrin-coated-pit formation (41, 42) and mediate ligand-dependent down-regulation of the growth factor receptors Met and EGF (37, 47). Grb2, which harbors two SH3 domains flanking an SH2 domain, is an adaptor molecule responsible for assembling signaling complexes at receptor internalization sites to facilitate downstream signaling cascades. The requirements for these protein-protein interactions in mammalian cells were verified, and the physiological relevance of these interactions was tested by analyzing the pathogenesis of recombinant viruses harboring mutations that ablate the interaction of M2 with these cellular proteins. Unfortunately, MHV68M2.P3/4 (which harbors mutations in the endophilin II binding site) lacked a substantial latency phenotype, and MHV68M2.P3/4/7 (which harbors mutations in the Grb2 binding sites) displayed only a modest (threefold) decrease in the reactivation from latency compared to that for wt MHV68 infection (Fig. 4D). These analyses could reflect that (i) these protein-protein interactions do not play a significant role in the function of M2 during a latent MHV68 infection, (ii) the M2-endophilin II interaction or M2-Grb2 interactions *in vivo* have alternative requirements that are not revealed by studies *in vitro*, or (iii) these protein-protein interactions are important but are facilitated by other interactions (e.g., other M2 interacting proteins or other interaction domains in the M2 protein, such as a phosphorylated tyrosine residue); as such, mutation of the implicated PXXP motifs does not result in a profound defect in M2 function. Importantly, these results demonstrate the importance of evaluating the contribution of amino acid binding motifs in the context of a viral infection *in vivo*, in addition to characterizing such interactions in overexpression systems in culture.

Previously, Madureira et al. (30) identified the SH3 domain-containing adaptor proteins Vav1 and Vav2 as M2 interacting partners, again using a yeast two-hybrid screen of a mouse B-cell cDNA library. It was determined that mutating prolines 155, 158, 160, 163, 167, and 170 ablated the M2 interaction with the C terminus SH3 domains of Vav1 and Vav2 (30). Furthermore, it was demonstrated that this interaction is largely mediated through the interaction of the C-terminal

SH3 domains of Vav1 and Vav2 with prolines 160 and 163 of M2, which comprise the M2 P7 domain (Fig. 1). These authors suggest that the adjacent P6 and P8 domains may also play a role in Vav binding; however, no difference in Vav1 or Vav2 binding was observed when a P7 mutant was compared to a P6/P7/P8 triple mutant in a coimmunoprecipitation from transiently transfected 293T cells (30). Subsequent studies demonstrated that Vav binding to M2 is required to induce phosphorylation of Vav1 leading to downstream Rac1 stimulation (43). However, the use of an MHV68 M2 mutant virus was not employed to investigate the importance of the proline domains required for Vav binding (30, 43). These analyses demonstrate that mutation of P7 alone has little impact on M2 function *in vivo*, raising the question of the functional significance of this interaction. As discussed above, it is certainly possible that other interacting proteins or domains within M2 serve to compensate for the loss of the M2 P7 PXXP motif, but this remains to be investigated.

Our mutational analysis also revealed the importance of three PXXP motifs in M2 in the context of MHV68 infection *in vivo*. A severe decrease in the ability of the virus to reactivate from latency was observed following infection with the MHV68M2.P1 mutant. In addition, the MHV68M2.P8 and MHV68M2.P9 mutants each exhibited a ca. 10-fold decrease in the establishment of splenic latency. That the P1 mutation appears to selectively affect virus reactivation, while the P9 mutation (as well as the P8 mutation) predominantly affects establishment of latency, argues that there are distinct functions of M2 involved in the establishment of latency and reactivation from latency. Furthermore, the dominance of M2-expressing primary B cells in culture suggests that M2 may promote the establishment of MHV68 latency or virus reactivation by enhancing B-cell proliferation and/or survival. Interestingly, the P8 motif, but not P9, is required for M2-dependent expansion of primary B cells. However, the P9 mutation displayed slower kinetics of expansion than wt M2 (Fig. 6B). Could the slower expansion of B cells observed in P9-transduced cultures indicate a requirement for P9 in a specific subset of B cells in the culture? The importance of these analyses is that they clearly distinguish between the functions of the P8 and P9 motifs, providing evidence that these motifs likely interact with different cellular proteins. To date, none of the observed interactions of cellular proteins with M2 have mapped to either the P1 or the P9 motif, and the data suggesting that Vav may interact with the P8 motif (in addition to the P7 motif) are currently unconvincing.

Several unique gammaherpesvirus antigens with signaling functions harbor the PXXP core consensus motif required for interacting with SH3 domains. The herpesvirus saimiri Tip protein utilizes its SH3 binding domain to interact with the major T-cell tyrosine kinase Lck, leading to inhibition of T-cell receptor-regulated signal transduction (20, 21). The KSHV latency-associated membrane protein, or K15, contains an SH3 binding motif in its cytoplasmic tail and is capable of inhibiting BCR-mediated signal pathways (9, 11, 16, 39). The EBV LMP2a antigen, famous for its ability to usurp BCR signal transduction, harbors three PXXP cores within its amino-terminal domain; however, these motifs have yet to be shown to mediate interaction(s) with a cellular protein(s) (28). A striking functional commonality among these unique gammaher-

pesvirus antigens is the down-regulation of lymphocyte signaling pathways. In the case of LMP2a, it has been hypothesized that this function may help to suppress spontaneous virus reactivation evoked by host cell activation or proliferation.

Targeted mutagenesis of M2 tyrosine residues 120 and 129 in the context of a latent MHV68 infection. Another hallmark of many signaling molecules is phosphorylated tyrosine residues, which mediate interactions with SH2 domain-containing proteins. Several unique gammaherpesvirus antigens harbor SH2 binding motifs as well as consensus ITAMs, including EBV LMP2a, KSHV K1 and K15, and rhesus rhadinovirus R1. In the cases of LMP2a and K1, the ITAMs can be utilized as surrogate BCR signal transducers (5, 7, 11, 15, 27, 44, 53); however, testing the relevance of these amino acid motifs in the context of a natural host infection has not been done. The M2 protein harbors three tyrosine residues, two of which are predicted to be potential phosphorylation sites and shown to be essential for a trimolecular complex with Vav1 and the Src family kinase Fyn following overexpression of all three proteins in Cos-1 cells (43). Furthermore, using *in vitro* kinase assays, Fyn-mediated phosphorylation of M2 has been demonstrated (43). However, to date, M2 has not been shown to be tyrosine phosphorylated in B cells. We have examined M2 expression in A20 and WeHi B-cell lines, as well as endogenous M2 in the MHV68-positive S11 B-lymphoma cell line, for tyrosine-phosphorylated species of M2 without success. Our inability to detect tyrosine-phosphorylated M2 could be due to either rapid dephosphorylation in B cells or rapid turnover of tyrosine-phosphorylated M2. If so, then it may be very difficult to detect tyrosine-phosphorylated M2 protein. Thus, the phenotype of the Y120/129F mutant may reflect the loss of a critical M2 function mediated through interaction of an SH2 domain-containing protein with tyrosine-phosphorylated M2. In contrast, the severe phenotype of the Y120/129D mutant (which should mimic a constitutively phosphorylated form of M2) may reflect the need to tightly regulate the half-life of this M2 species. As such, if the Y120/129D mutant is unable to be targeted for degradation (or dephosphorylation), an overabundance of a constitutively active form of M2 may be detrimental to the ability of the virus to transition to quiescence in a germinal center or memory B cell, leading to the observed negative phenotype rather than the anticipated positive phenotype.

To test the significance of these amino acids in the context of an MHV68 infection, two separate M2 mutant viruses were constructed by replacing tyrosine residues 120 and 129 with phenylalanine (MHV68M2.Y120/129F) or aspartic acid (MHV68M2.Y120/129D). A fivefold reduction in reactivation from latency was observed after inoculation with MHV68M2.Y120F/Y129F in comparison to that for a wt infection (Fig. 4L). However, upon infection of mice with MHV68M2.Y120D/Y129D, there was a 50-fold reduction in reactivation from latency. These results suggest that if one or both of these tyrosine residues are phosphorylated during the course of a latent infection, the phosphorylation statuses of these residues are tightly regulated. This hypothesis is driven by the historical logic that substituting phenylalanine for tyrosine renders the amino acid unable to be phosphorylated; however, by mutating a tyrosine to aspartic acid or glutamic acid, the residue assumes the charge of being constitutively phosphorylated and is thus incapa-

ble of being dephosphorylated. Therefore, it is formally possible that rendering M2 constitutively active during a latent MHV68 infection actually leads to defects in the establishment of latency. Alternatively, introduction of an unregulated, constitutively active protein in the context of ongoing signaling cascades within a B cell may deregulate a number of pathways leading to aberrant cell proliferation, differentiation, or immune function, ultimately affecting the course of MHV68 infection.

Striking phenotypes observed following infection with M2 recombinant viruses harboring point mutations in PXXP motifs or tyrosine residues demonstrate the importance of individual motifs and support the hypothesis that M2 is a multifunctional protein interacting with a number of different cellular proteins. For example, M2 may utilize one or more of its PXXP motifs to mediate an interaction with an SH3 domain to promote the establishment of latency in a B cell, whereas regulated phosphorylation of its tyrosine residues may facilitate an SH2 domain interaction responsible for virus reactivation. Presumably, these functions could be executed via interaction with a single protein, such as Grb2 or Vav, both of which have SH3 and SH2 domains. Alternatively, multiple interactions could occur with any number of signaling molecules. An obvious next step would be the investigation of combinatorial M2 recombinant viruses harboring mutations in C terminus PXXP motifs along with amino acid substitutions for tyrosine residues 120 and 129. To this end, an M2 mutant virus containing alanine substitutions for prolines 160 and 163 (comprising the consensus class I SH3 binding domain) as well as a phenylalanine substitution for tyrosine 129 was constructed. Following infection of mice, MHV68M2.Y129F/P7 revealed a >70-fold defect in virus reactivation from latency in the spleen. This is the first M2 mutant virus to display a reactivation defect similar to that observed with M2 null mutants.

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REFERENCES

- Adler, B., E. Schaadt, B. Kempkes, U. Zimmer-Strobl, B. Baier, and G. W. Bornkamm. 2002. Control of Epstein-Barr virus reactivation by activated CD40 and viral latent membrane protein 1. *Proc. Natl. Acad. Sci. USA* **99**:437–442.
- Adler, H., M. Messerle, M. Wagner, and U. H. Koszinowski. 2000. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. *J. Virol.* **74**:6964–6974.
- Ahn, J. W., K. L. Powell, P. Kellam, and D. G. Alber. 2002. Gammaherpesvirus lytic gene expression as characterized by DNA array. *J. Virol.* **76**:6244–6256.
- Babcock, G. J., D. Hochberg, and A. D. Thorley-Lawson. 2000. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity* **13**:497–506.
- Beaufils, P., D. Choquet, R. Z. Mamoun, and B. Malissen. 1993. The (YXXL/I)2 signalling motif found in the cytoplasmic segments of the bovine leukaemia virus envelope protein and Epstein-Barr virus latent membrane protein 2A can elicit early and late lymphocyte activation events. *EMBO J.* **12**:5105–5112.
- Blaskovic, D., M. Stancekova, J. Svobodova, and J. Mistrikova. 1980. Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virol.* **24**:468.
- Burkhardt, A. L., J. B. Bolen, E. Kieff, and R. Longnecker. 1992. An Epstein-Barr virus transformation-associated membrane protein interacts with *src* family tyrosine kinases. *J. Virol.* **66**:5161–5167.
- Caldwell, R. G., J. B. Wilson, S. J. Anderson, and R. Longnecker. 1998. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9**:405–411.
- Choi, J. K., B. S. Lee, S. N. Shim, M. Li, and J. U. Jung. 2000. Identification of the novel K15 gene at the rightmost end of the Kaposi's sarcoma-associated herpesvirus genome. *J. Virol.* **74**:436–446.
- Clamby, E. T., H. W. Virgin IV, and S. H. Speck. 2002. Characterization of a spontaneous 9.5-kilobase-deletion mutant of murine gammaherpesvirus 68 reveals tissue-specific genetic requirements for latency. *J. Virol.* **76**:6532–6544.
- Damania, B., J. K. Choi, and J. U. Jung. 2000. Signaling activities of gammaherpesvirus membrane proteins. *J. Virol.* **74**:1593–1601.
- Doherty, P. C., J. P. Christensen, G. T. Belz, P. G. Stevenson, and M. Y. Sangster. 2001. Dissecting the host response to a gamma-herpesvirus. *Philos. Trans. R. Soc. Lond. B* **356**:581–593.
- Doherty, P. C., R. A. Tripp, A. M. Hamilton-Easton, R. D. Cardin, D. L. Woodland, and M. A. Blackman. 1997. Tuning into immunological dissonance: an experimental model for infectious mononucleosis. *Curr. Opin. Immunol.* **9**:477–483.
- Efstathiou, S., Y. M. Ho, S. Hall, C. J. Styles, S. D. Scott, and U. A. Gompels. 1990. Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. *J. Gen. Virol.* **71**:1365–1372.
- Fruehling, S., and R. Longnecker. 1997. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction. *Virology* **235**:241–251.
- Glenn, M., L. Rainbow, F. Aurade, A. Davison, and T. F. Schulz. 1999. Identification of a spliced gene from Kaposi's sarcoma-associated herpesvirus encoding a protein with similarities to latent membrane proteins 1 and 2A of Epstein-Barr virus. *J. Virol.* **73**:6953–6963.
- Herskowitz, J., M. A. Jacoby, and S. H. Speck. 2005. The murine gammaherpesvirus 68 M2 gene is required for efficient reactivation from latently infected B cells. *J. Virol.* **79**:2261–2273.
- Husain, S. M., E. J. Usherwood, H. Dyson, C. Coleclough, M. A. Coppola, D. L. Woodland, M. A. Blackman, J. P. Stewart, and J. T. Sample. 1999. Murine gammaherpesvirus M2 gene is latency-associated and its protein a target for CD8(+) T lymphocytes. *Proc. Natl. Acad. Sci. USA* **96**:7508–7513.
- Jacoby, M. A., H. W. Virgin IV, and S. H. Speck. 2002. Disruption of the M2 gene of murine gammaherpesvirus 68 alters splenic latency following intranasal, but not intraperitoneal, inoculation. *J. Virol.* **76**:1790–1801.
- Jung, J. U., S. M. Lang, U. Friedrich, T. Jun, T. M. Roberts, R. C. Desrosiers, and B. Biesinger. 1995. Identification of Lck-binding elements in tip of herpesvirus saimiri. *J. Biol. Chem.* **270**:20660–20667.
- Jung, J. U., S. M. Lang, T. Jun, T. M. Roberts, A. Veillette, and R. C. Desrosiers. 1995. Downregulation of Lck-mediated signal transduction by tip of herpesvirus saimiri. *J. Virol.* **69**:7814–7822.
- Kilger, E., A. Kieser, M. Baumann, and W. Hammerschmidt. 1998. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* **17**:1700–1709.
- Lagunoff, M., R. Majeti, A. Weiss, and D. Ganem. 1999. Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. USA* **96**:5704–5709.
- Lee, H., J. Guo, M. Li, J. K. Choi, M. DeMaria, M. Rosenzweig, and J. U. Jung. 1998. Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. *Mol. Cell. Biol.* **18**:5219–5228.
- Liang, X., M. T. Pickering, N. H. Cho, H. Chang, M. R. Volkert, T. F. Kowalik, and J. U. Jung. 2006. Deregulation of DNA damage signal transduction by herpesvirus latency-associated M2. *J. Virol.* **80**:5862–5874.
- Liang, X., Y. C. Shin, R. E. Means, and J. U. Jung. 2004. Inhibition of interferon-mediated antiviral activity by murine gammaherpesvirus 68 latency-associated M2 protein. *J. Virol.* **78**:12416–12427.
- Longnecker, R., B. Druker, T. M. Roberts, and E. Kieff. 1991. An Epstein-Barr virus protein associated with cell growth transformation interacts with a tyrosine kinase. *J. Virol.* **65**:3681–3692.
- Longnecker, R., M. Merchant, M. E. Brown, S. Fruehling, J. O. Bickford, M. Ikeda, and R. N. Harty. 2000. WW- and SH3-domain interactions with Epstein-Barr virus LMP2A. *Exp. Cell Res.* **257**:332–340.
- Macrae, A. I., E. J. Usherwood, S. M. Husain, E. Flano, I. J. Kim, D. L. Woodland, A. A. Nash, M. A. Blackman, J. T. Sample, and J. P. Stewart. 2003. Murid herpesvirus 4 strain 68 M2 protein is a B-cell-associated antigen important for latency but not lymphocytosis. *J. Virol.* **77**:9700–9709.
- Madureira, P. A., P. Matos, I. Soeiro, L. K. Dixon, J. P. Simas, and E. W. Lam. 2005. Murine gamma-herpesvirus 68 latency protein M2 binds to Vav signaling proteins and inhibits B-cell receptor-induced cell cycle arrest and apoptosis in WEHI-231 B cells. *J. Biol. Chem.* **280**:37310–37318.
- Mistrikova, J., and D. Blaskovic. 1985. Ecology of the murine alphaherpesvirus and its isolation from lungs of rodents in cell culture. *Acta Virol.* **29**:312–317.
- Moorman, N. J., D. O. Willer, and S. H. Speck. 2003. The Gammaherpes-

- virus 68 Latency-Associated Nuclear Antigen Homolog Is Critical for the Establishment of Splenic Latency. *J. Virol.* **77**:10295–10303.
34. Moser, J. M., J. W. Upton, R. D. Allen III, C. B. Wilson, and S. H. Speck. 2005. Role of B-cell proliferation in the establishment of gammaherpesvirus latency. *J. Virol.* **79**:9480–9491.
 35. Nash, A. A., B. M. Dutia, J. P. Stewart, and A. J. Davison. 2001. Natural history of murine gamma-herpesvirus infection. *Philos. Trans. R. Soc. Lond. B* **356**:569–579.
 36. Nash, A. A., and N. P. Sunil-Chandra. 1994. Interactions of the murine gammaherpesvirus with the immune system. *Curr. Opin. Immunol.* **6**:560–563.
 37. Petrelli, A., G. F. Gilestro, S. Lanzardo, P. M. Comoglio, N. Migone, and S. Giordano. 2002. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. *Nature* **416**:187–190.
 38. Pollock, J. L., and H. W. Virgin IV. 1995. Latency, without persistence, of murine cytomegalovirus in the spleen and kidney. *J. Virol.* **69**:1762–1768.
 39. Poole, L. J., J. C. Zong, D. M. Ciuffo, D. J. Alcendor, J. S. Cannon, R. Ambinder, J. M. Orenstein, M. S. Reitz, and G. S. Hayward. 1999. Comparison of genetic variability at multiple loci across the genomes of the major subtypes of Kaposi's sarcoma-associated herpesvirus reveals evidence for recombination and for two distinct types of open reading frame K15 alleles at the right-hand end. *J. Virol.* **73**:6646–6660.
 40. Rajcani, J., D. Blaskovic, J. Svobodova, F. Ciampor, D. Huckova, and D. Stanekova. 1985. Pathogenesis of acute and persistent murine herpesvirus infection in mice. *Acta Virol.* **29**:51–60.
 41. Reutens, A. T., and C. G. Begley. 2002. Endophilin-1: a multifunctional protein. *Int. J. Biochem. Cell Biol.* **34**:1173–1177.
 42. Ringstad, N., H. Gad, P. Low, G. Di Paolo, L. Brodin, O. Shupliakov, and P. De Camilli. 1999. Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron* **24**:143–154.
 43. Rodrigues, L., M. Pires de Miranda, M. J. Caloca, X. R. Bustelo, and J. P. Simas. 2006. Activation of Vav by the gammaherpesvirus M2 protein contributes to the establishment of viral latency in B lymphocytes. *J. Virol.* **80**:6123–6135.
 44. Scholle, F., R. Longnecker, and N. Raab-Traub. 1999. Epithelial cell adhesion to extracellular matrix proteins induces tyrosine phosphorylation of the Epstein-Barr virus latent membrane protein 2: a role for C-terminal Src kinase. *J. Virol.* **73**:4767–4775.
 45. Simas, J. P., and S. Efstathiou. 1998. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. *Trends Microbiol.* **6**:276–282.
 46. Smith, G. A., and L. W. Enquist. 1999. Construction and transposon mutagenesis in *Escherichia coli* of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. *J. Virol.* **73**:6405–6414.
 47. Soubeyran, P., K. Kowanetz, I. Szymkiewicz, W. Y. Langdon, and I. Dikic. 2002. Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* **416**:183–187.
 48. Speck, S. H., and H. W. Virgin. 1999. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. *Curr. Opin. Microbiol.* **2**:403–409.
 49. Stevenson, P. G., and P. C. Doherty. 1999. Non-antigen-specific B-cell activation following murine gammaherpesvirus infection is CD4 independent in vitro but CD4 dependent in vivo. *J. Virol.* **73**:1075–1079.
 50. Sunil-Chandra, N. P., S. Efstathiou, J. Arno, and A. A. Nash. 1992. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. *J. Gen. Virol.* **73**:2347–2356.
 51. Sunil-Chandra, N. P., S. Efstathiou, and A. A. Nash. 1992. Murine gamma-herpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. *J. Gen. Virol.* **73**:3275–3279.
 52. Symensma, T. L., D. Martinez-Guzman, Q. Jia, E. Bortz, T. T. Wu, N. Rudra-Ganguly, S. Cole, H. Herschman, and R. Sun. 2003. COX-2 induction during murine gammaherpesvirus 68 infection leads to enhancement of viral gene expression. *J. Virol.* **77**:12753–12763.
 53. Thorley-Lawson, D. A. 2001. Epstein-Barr virus: exploiting the immune system. *Nat. Rev. Immunol.* **1**:75–82.
 54. Thorley-Lawson, D. A., and G. J. Babcock. 1999. A model for persistent infection with Epstein-Barr virus: the stealth virus of human B cells. *Life Sci.* **65**:1433–1453.
 55. Thorley-Lawson, D. A., and K. P. Mann. 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. *J. Exp. Med.* **162**:45–59.
 56. Usherwood, E. J., A. J. Ross, D. J. Allen, and A. A. Nash. 1996. Murine gammaherpesvirus-induced splenomegaly: a critical role for CD4 T cells. *J. Gen. Virol.* **77**:627–630.
 57. Virgin, H. W., and S. H. Speck. 1999. Unraveling immunity to gamma-herpesviruses: a new model for understanding the role of immunity in chronic virus infection. *Curr. Opin. Immunol.* **11**:371–379.
 58. Virgin, H. W., IV, P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck. 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J. Virol.* **71**:5894–5904.
 59. Virgin, H. W., IV, R. M. Presti, X. Y. Li, C. Liu, and S. H. Speck. 1999. Three distinct regions of the murine gammaherpesvirus 68 genome are transcriptionally active in latently infected mice. *J. Virol.* **73**:2321–2332.
 60. Weck, K. E., M. L. Barkon, L. I. Yoo, S. H. Speck, and H. I. Virgin. 1996. Mature B cells are required for acute splenic infection, but not for establishment of latency, by murine gammaherpesvirus 68. *J. Virol.* **70**:6775–6780.
 61. Weck, K. E., S. S. Kim, H. W. Virgin IV, and S. H. Speck. 1999. B cells regulate murine gammaherpesvirus 68 latency. *J. Virol.* **73**:4651–4661.
 62. Weck, K. E., S. S. Kim, H. W. Virgin IV, and S. H. Speck. 1999. Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. *J. Virol.* **73**:3273–3283.
 63. Willer, D. O., and S. H. Speck. 2003. Long-term latent murine gammaherpesvirus 68 infection is preferentially found within the surface immunoglobulin D-negative subset of splenic B cells in vivo. *J. Virol.* **77**:8310–8321.
 64. Zimmer-Strobl, U., B. Kempkes, G. Marschall, R. Zeidler, C. Van Kooten, J. Bancheau, G. W. Bornkamm, and W. Hammerschmidt. 1996. Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. *EMBO J.* **15**:7070–7078.