The MHV-68 latent protein, M2, does not have homology to any known viral or cellular proteins, and its function is unclear. To define the role played by M2 during MHV-68 latency as well as the molecular mechanism involved, we used M2 as bait to screen a yeast two-hybrid mouse B-cell cDNA library. Vav1 was identified as an M2-interacting protein in two independent screenings. Subsequent yeast two-hybrid interaction studies showed that M2 also binds to Vav2, but not Vav3, and that three "PXXP" motifs located at the C terminus of M2 are important for this interaction. The interactions between M2 and Vav proteins were also confirmed in vivo in 293T and WEHI-231 B-cells by co-immunoprecipitation assays. Rac1/GST-PAK "pull-down" experiments and Western blot analysis using a phospho-Vav antibody demonstrated that expression of M2 in WEHI-231 cells enhances Vav activity. We further showed in WEHI-231 cells that M2 expression promotes proliferation and survival and is associated with enhanced cyclin D2 and repressed p27Kip1, p130, and Bim expression. Taken together, these experiments suggest that M2 might have an important role in disseminating the latent virus during the establishment and maintenance of latency by modulating B-cell receptor-mediated signaling events through Vav to promote B-cell activation, proliferation, and survival.

γ-Herpesviruses comprise a subfamily of animal double-stranded DNA viruses widespread in nature. There are two known human γ-herpesviruses, namely Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8) also known as Kaposi’s sarcoma herpesvirus, which are largely disseminated in the population. Infections by the γ-herpesviruses have been implicated in the development of lymphoproliferative diseases and in some cases, tumorigenesis (1, 2). One of the characteristic biological features of the γ-herpesviruses is their ability to establish a life-long latent infection in lymphocytes, during which only a few viral genes are expressed. One hypothesis is that these viral genes play a major role in the establishment and maintenance of viral latency. Understanding the molecular mechanisms whereby latency-associated proteins alter the regulation of the cellular pathways of the infected host cell, as well as evade immune surveillance will provide essential information for devising therapeutic strategies against these viruses. γ-Herpesviruses employ similar strategies to subvert the infected host cell, encoding for viral proteins, which often interfere with cellular processes in the host cell, in particular targeting the transcriptional machinery and signaling pathways. In EBV, the EBNA-1, EBNA-2, EBNA-3A, -B, -C, and EBNA-LP latency-associated proteins are involved in the altered transcriptional regulation of the host’s genes (3, 4), whereas the LMP-1 and LMP-2A proteins are involved in the altered regulation of endogenous cellular signaling pathways. In the case of LMP-1, it functions as a constitutively active CD40 receptor. This viral protein binds and activates TRAF-2 and -3 proteins leading to NF-κB activation and consequently cell proliferation (5, 6). It has been demonstrated that LMP-2A associates with the cellular tyrosine kinases Fyn, Lyn, and Syk, modulating their interactions with the B-cell receptor (7, 8), thereby acting in the same manner as a constitutively active B-cell receptor. In B-cell receptor-negative B-cells, LMP-2A activates signals that are usually generated by the B-cell receptor to inhibit apoptosis of infected cells (9). LMP-2A has been shown to activate the phosphatidylinositols 3-kinase/Akt (also called PKB) signaling cascade, which has a major role in cell proliferation and survival (10, 11). During latency, HHV-8 expresses viral proteins, such as the D-type cyclin homologue (v-cyclin) (12, 13), the transcription regulators LANA-1 and LANA-2 (vIRF-3) (14, 15), kaposin A (16), and vFLIP (17) to manipulate cellular signaling pathways. Another HHV-8 latent protein, LAMP, has been shown to inhibit B-cell receptor-dependent signal transduction and to associate with the cellular proteins TRAF-1, -2, and -3, suggesting that it might also mimic the function of the CD40 receptor (18, 19).

It is evident that the biological functions of some of these γ-herpesvirus latent proteins are to promote lymphocyte proliferation and survival to preserve and propagate the viral genomes during latent infection. Mammalian cell proliferation requires successful transition through cell cycle phases (G1, S, G2, and M) (20, 21). The expression of D-type cyclins (cyclins D1, D2, and D3) is predominantly regulated by extracellular mitogenic (or inhibitory) signals and are rate-limiting for transition through G1. Once cells have reached late G1, E-type
cycins (E1 and E2) accumulate and allow S phase initiation, followed by induction of cyclin A in S phase. The D-type cycins preferentially associated with and activate CDK-4 and -6, whereas cyclin E/A with CDK2. The primary physiological substrate for the CDKs is the retinoblastoma (pRb) family of pocket proteins (pRb, p107, and p130). When hypophosphorylated, the pocket proteins bind to and inactivate the E2F family of transcription factors that are required for the transcription of genes that are necessary for entry into S phase. The activities of the CDKs are negatively regulated by two families of CDK inhibitors, namely the Cip/Kip family (comprising p21Cip1, p27Kip1, and p57Kip2) and the INK4 family. The Cip/Kip family inhibits a broad range of cyclin-CDK complexes, whereas the INK4 family members specifically inhibit cyclin D-CDK4/6 (21). The HHV-8 v-cyclin D has been shown to be refractory to the inhibitory effects of the Cip/Kip family of CKIs, and has been shown to increase the rate of proliferation of the infected cells, thereby propagating the viral genome (22). Another mechanism whereby the viral genome is propagated during latency is via inhibition of apoptosis.

The Bcl-2 family of proteins is central to regulating the commitment of cells to apoptosis (programmed cell death) (23, 24). The Bcl-2 family of proteins contains both anti- and pro-apoptotic members and can be divided into three distinct classes: the anti-apoptotic sub-group (e.g. Bcl-2 and Bcl-XL); the pro-apoptotic, multi-Bcl-2 homology (BH) domain sub-group (e.g. Bak and Bax); and the pro-apoptotic, BH3 domain-only sub-group (e.g. Bad, Bik, Bid, Bim, PUMA, and Bmf). The pro-apoptotic BH3-only member of the Bcl-2 family, Bim, plays a crucial role in regulating apoptosis in lymphocytes during negative selection. Bim, like other BH3-only Bcl-2 proteins, mediates apoptosis through inducing mitochondrial cytochrome c release, which in turn activates caspase-9, caspase-3, and eventually the downstream cell death machinery (23, 25). Bim is believed to promote apoptosis by neutralizing pro-survival Bcl-2 proteins in a mechanism that requires members of the multi-BH-domain pro-apoptotic subgroup.

The murine γ-herpesvirus 68 (MHV-68) is closely related to the human HHV-8 and EBV. Because γ-herpesviruses often share similar mechanisms to manipulate the host cellular signaling cascades, MHV-68 is often used as a rodent model of γ-herpesviral infection. M2 is a unique latency-associated protein of MHV-68. It has no known functions, yet is expressed from the viral genome, and cloned into the EcoRI site of pCMV-Myc (Clontech) (48). The murine vav2 gene was PCR-amplified from Mus musculus vav2 cDNA, using the primers 5’CGG AAT TCA GCA GTG GCC GCA A and 3’TCT AGA ATT CAG CAG AAA AGA CAG GCA and cloned into the EcoRI site of pACT2 MCS in-frame with Gal4AD, this plasmid was designated pACT2-Vav2. Restriction sites are highlighted in bold, and nucleotide modifications are underlined. vav2SH gene encoding for amino acids 536–869 of Vav2 was PCR-amplified from M. musculus vav2 cDNA using the primers 5’GAG AAT TCT CAG GGG TAC TTG TGT TT and 3’CAG GAA TTC TGT CCA GGT CT and cloned into the EcoRI site of pACT2 MCS in-frame with Gal4AD, and this plasmid was designated pACT2-Vav2SH. vavSH gene encoding for amino acids 531–860 of Vav3 was PCR-amplified from M. musculus vav3 cDNA using the primers 5’TAT CCC GGC TTC TTA TGT TTT and 3’GTC TCT CAG GAG ATT TTA GTG CA and cloned into the EcoRI site of pACT2 MCS in-frame with Gal4AD, and this plasmid was designated pACT2-Vav3SH.

Plasmids for Protein Expression in Bacterial Cells—The M2 gene was PCR-amplified from the MHV-68 genome using the primers 5’CCG TTA GCG AAT TCA CTG TT and 3’TCA GAT CCA GTC TGA TGA GG, corresponding to bases 4010 – 4641 of MHV-68 genome, and cloned into the BamHI/EcoRI sites of pGEX-4T1 (Amersham Biosciences), and designated pGEX-M2. Plasmids for expression of proteins in eukaryotic cells: pCMV-FLAG-Vav1 was a kind gift of Dr. Martin Turner, Brabaham Institute, Brabaham, UK (Doody, et al. (48)). M2 gene was PCR-amplified from MHV-68 genome using the primers 5’ACG GTA CCT TCA CTG TTA TTC and 3’TAT CCA ATT CAT GAG ATT CAT GGC, representing coordinates 4017–4623 of MHV-68 genome, and cloned into the EcoRI/KpnI sites of pCMV-Myc (Clontech) MCS in-frame with the Myc tag, and this plasmid was named pCMV-Myc-M2. M2ΔP1 and M2ΔP2 genes were PCR-amplified from pGEB9-M2ΔP1 and pGEB9-M2ΔP2, respectively, using the primers 5’TAC GGA ATT CCA CTT GGC CAT ACC TCA ATT CAT GGC, representing coordinates 4017–4623 of MHV-68 genome, and cloned into XhoI/KpnI sites of pCMV-Myc-M2, pCMV-Myc-M2ΔP1, and pCMV-Myc-M2ΔP2, respectively, into pCMV-HA (Clon-


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...tech) MCS by digestion with the same restriction enzymes used for cloning into pCMV-Myc, and these plasmids were designated pCMV-HA-M2, pCMV-HA-M2ΔP1, and pCMV-HA-M2ΔP2, respectively. vav2 gene was subcloned from pACT2-Vav2 into pCMV-HA and pCMV-Myc MCS, in-frame with the HA and Myc tags, into the EcoRI sites, and these plasmids were designated by pCMV-Vav-HA and pCMV-Myc-Vav2, respectively.

**Retroviral Plasmids**—M2 gene was PCR-amplified from the MHV-68 genomic DNA, M2ΔP1 gene was PCR-amplified from pGBl9-M2ΔP1 and M2ΔP2 gene was PCR-amplified from pGBl9-M2ΔP2, using the primers 5′: AAA TTC AAA ACA ATG GCA TCA ATG CAG AAG CTG ATC TCA GAG GAG GAC CTG GCC CCA ACA CCC CCA CAA GGA AGG AT and 3′: AAA CTC GAG TTA CTC CTC GCC CCA CTA CAC; the respective genes were cloned into the EcoRI/XhoI sites of pMSCV-Zeo MCS (28), these plasmids were designated pMSCV-Zeo-M2, pMSCV-Zeo-M2ΔP1, and pMSCV-Zeo-M2ΔP2.

**Yeasts Two-hybrid System**

All experiments were performed in the yeast reporter strain Y190. The yeast vector pGBT9 is a Gal4 DNA-binding domain (Gal4BD) encoding vector that allows fusion of desired protein to the Gal4BD. The yeast vector pACT2 is a Gal4 activation domain (Gal4AD) encoding vector that allows fusion of desired protein to the Gal4AD. The yeast two-hybrid library screen and protocols were as recommended (Clontech).

**Cell Culture, Transfections, and Cell Lines**

WEHI-231 and WEHI-bclXL (described in Ref. 29) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin/streptomycin, and 50 μΜ β-mercaptoethanol, and were stimulated with 10 μg/ml of monoclonal anti-IgM (clone b.7.6 purchased from Cappel/ICN, Costa Mesa, CA). 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. For each transfection, 8 × 10⁵ 293T cells were seeded in 60-mm plates and incubated at 37 °C, 5% CO₂ for 16 h. Cells were transfected with plasmid DNA using the FuGENE Reagent (Roche Applied Science) according to the manufacturer’s instructions. The WEHI-231-M2, WEHI-231-M2ΔP1, and WEHI-231-M2ΔP2 cell lines were obtained by viral infection with high titer viral supernatants derived from 293T cells co-transfected with pMSCV-Zeo-M2, pMSCV-Zeo-M2ΔP1, or pMSCV-Zeo-M2ΔP2 retroviral vectors (28) and the packaging vector pEQPAM3 (30) selected in zeocin (Invitrogen, UK) for 2 weeks. WEHI-bclXL-M2 cells were obtained by infection with retrovirus encoding for M2, as described above for WEHI-231-M2 cells. For growth curves, cells were seeded in triplicated flasks at about 0.4 × 10⁶ cells/ml and cultured in growth medium in the presence of absence of 10 μg/ml of anti-IgM.

**Western Blot Analysis and Antibodies**

Western blot experiments were prepared by lysing cells with two times packed cell volume of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 5 mM iodoacetamide, 1 mM sodium fluoride, and Complete mini protease inhibitors (Roche Applied Science)) for 15 min on ice. 293T cell lysates (500 μg) were pre-cleared for 1 h with protein G-Sepharose, then incubated with the antibody of interest for 1 h, and then with protein G-Sepharose for 1 h. Beads were washed three times with 1 ml of lysis buffer and resuspended in 50 μl of 2× SDS-PAGE loading buffer. Immunoprecipitates were then Western blotted as before.

**Cell Cycle Analysis**

Cell cycle analyses were performed by propidium iodide staining as described previously (29). Briefly, cells were washed with phosphate-buffered saline and fixed in 90% ethanol, 10% phosphate-buffered saline. Following fixation, cells were washed again and then incubated with 500 μg/ml DNase-free RNase A (Sigma) and 20 μg/ml propidium iodide (Sigma) for 30 min at 37 °C prior to analysis using a FACScan flow cytometer (BD Biosciences).

**GST-PAK Pull-down Assays**

WEHI-231, WEHI-231-M2, WEHI-231-M2ΔP1, or WEHI-231-M2ΔP2 cells (5 × 10⁶) were lysed in 750 μl of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10 mM NaMoO₄, 1 mM NaVO₄, 1 mM NaF, 5 mM iodoacetamide, 1 tablet of Complete mini protease inhibitors (Roche Applied Science), per 10 ml of buffer). Cell lysates were incubated with 30 μl of glutathione-agarose beads and 5 μg of GST-PAK protein, at 4 °C for 1 h with rotation. Pull down assays were analyzed by Western blotting as described previously. The levels of pulled-down Rac were measured by band densitometry using the program, Image J (National Institutes of Health).

**RESULTS**

M2 Interacts with Vav1 and Vav2 Guanine Nucleotide Exchange Factors in the Yeast Two-hybrid Screens—M2 is a unique viral protein with no known mammalian or viral domain homology. To determine its function and cellular targets, we performed a yeast two-hybrid screen using M2 as bait. Full-length M2 protein was fused in-frame with the DNA binding domain of the GAL4 (GAL4-BD) yeast transcription fac-
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### Table ONE
Screening of an MHV-68 latently infected B-cell cDNA library with the latency-associated M2 protein

| B-cell cDNA library | Ga4AD clone | pACT2-unknown cDNA |
|---------------------|-------------|-------------------|
| First screen        | pACT2-unknown cDNA |
|                     | pACT2-Vav1-SH (a.a. 532–845) |
|                     | pACT2-Vav1-SH (a.a. 532–845) |
|                     | pACT2-Vav1-SH (a.a. 532–845) |
| Second screen       | pACT2-unknown cDNA |
|                     | pACT2-Vav1-SH (a.a. 532–845) |

a. a.a., amino acids.

MHV-68 latency-associated M2 protein

MHV-68 latency-associated M2 protein was cloned from a CD4+ T cell cDNA library using a filter lift assay. The cloned cDNA was then sequenced using an ABI Prism sequencer. The resulting sequence was used to search for homologous proteins in the National Library of Medicine using the BLAST algorithm (TABLE ONE). A total of 8.7 x 10^6 transformants was analyzed in two independent rounds of screening. The seven positive clones selected for sequence analysis, four were found to contain the C-terminal region of Vav (amino acids 532–845), encoding for a C-terminal zinc finger (ZF) (32). The other three clones did not match any of the published cDNAs, and their identities remained undefined. The yeast family of guanine nucleotide exchange factors is composed of three highly homologous proteins, namely Vav1, Vav2, and Vav3. Additional interaction experiments using the yeast two-hybrid system showed that M2 is also able to bind to the yeast two-hybrid system showed that M2 is also able to bind to the C-terminal region of Vav (amino acids 536–868) encoding for the SH3-SH2-SH3 domains but not to Vav3.

The PXXP Motifs Located at the C Terminus of the Viral Protein Are Important for M2-Vav Interaction—After confirming interaction of Vav1 and -2 with M2, we examined the amino acid sequence of M2 required for binding to Vav. We observed that M2 protein contains 9 PXXP motifs, where R represents a proline residue and X any amino acid. These loosely defined motifs have been proposed to have a role in signaling molecules through their SH3 domains (32). Given that the C terminus region of Vav proteins contain two SH3 domains, it is possible these moieties are mediating the M2 binding. To determine if these PXXP motifs were important for binding to Vav, we constructed several M2 deletion mutants lacking specific PXXP motifs. These mutants were cloned in-frame with the binding domain of GAL4 and used in a yeast two-hybrid experiment to study for their interactions with Vav1 and Vav2 SH3-SH2-SH3 regions. These results showed that amino acids 160–179 of M2 are essential for Vav1 and Vav2 SH3-SH2-SH3 binding (Fig. 1). This region of M2 contains three PXXP motifs, at amino acid residues 162–165, 165–170, and 174–176. To further investigate the importance of these motifs in Vav binding, we generated two M2 mutants in which the proline residues at positions 167 and 170 (M2ΔP1 mutant) and 165, 167, 170, and 174 (M2ΔP2 mutant) were replaced by alanine residues. As shown in Fig. 1, the M2ΔP1 and M2ΔP2 mutations partially or totally abrogated the binding of M2 to Vav1 and Vav2, respectively. These results demonstrated that the three PXXP motifs located at the C terminus of M2 are essential for binding to the SH3-SH2-SH3 region of both Vav1 and Vav2 proteins.

![Schematic representation of the MHV-68 latency associated M2 protein.](image)

**A)** Schematic representation of the MHV-68 latency associated M2 protein. A, amino acid structure of M2 protein. PXXP motifs, where R represents a proline residue and X any amino acid, are indicated inside gray boxes; B, deletion and point mutation effects on M2-Vav1/Vav2 interaction in the yeast two-hybrid system. Schematic diagram of M2 with the locations of the PXXP motifs denoted by white boxes. Thick lines represent M2 coding region present in each construct, and the dashed lines represent the deleted region. Mutation of proline residues (P) for alanines (A) are indicated. The binding ability of each of the M2 deletion or mutant proteins to Vav1/Vav2 is represented by (+) for strong binding, (+) for weak binding, and (−) for no detectable binding.

**B)** M2 Interacts with Vav1 and Vav2 Proteins in Vivo—We next studied the in vivo interaction between M2 and Vav proteins in mammalian cells by co-immunoprecipitation (Fig. 2, A and B). To this end, 293T cells were transiently transfected with Vav mammalian expression vectors, either alone or in combination with M2, M2ΔP1, or M2ΔP2. 48 h after transfection, cell lysates were immunoprecipitated with either an anti-M2 serum or with the pre-immune serum. The immunoprecipitates were then resolved by SDS-PAGE and immunoblotted with anti-Vav antibodies. As shown in Fig. 2 (A and B), M2 co-immunoprecipitated with both Vav1 and Vav2 proteins in vivo, respectively. These results also showed that the binding of M2ΔP1 and M2ΔP2 to Vav proteins was partially abrogated, consistent with previous results obtained using the yeast two-hybrid system. Nevertheless, the yeast two-hybrid experiments have shown complete abrogation of binding of M2ΔP2 to Vav1 and Vav2 SH3-SH2-SH3 domains. One possible explanation for the co-immunoprecipitation results is that the full-length Vav protein might bind to another domain in M2. Another explanation could be that, although direct interaction between Vav proteins and M2 has been abrogated in the M2ΔP2 mutant, Vav is able to interact with other proteins (e.g. endogenous Vav proteins) that reside in the complex with M2. Accounting for this second hypothesis is the fact that M2 has 9 PXXP motifs that can potentially interact with other signaling proteins.

Because B-lymphocytes constitute the main reservoir of MHV-68 during long term latency, we reasoned that it would be important to study M2 interaction with Vav proteins in B-cells. To this end, we sought to generate WEHI-231 and WEHI-bcl-XL cell lines expressing M2 protein (29). WEHI-231 and WEHI-bcl-XL were infected with...
retroviruses expressing M2 or control empty retroviruses, and selected with Zeocin (Invitrogen) for 2 weeks. As shown in Fig. 3A, both the M2-infected cells, but not the controls, expressed significant amounts of viral M2 protein.

We next performed co-immunoprecipitation assays to examine M2-Vav interaction in B-cells. Because binding of M2 to Vav proteins in B-lymphocytes could be dependent on B-cell receptor activation, cells were either unstimulated or stimulated with anti-IgM for 10 min before harvesting. Because the WEHI-231 cells were infected with an M2 containing a Myc tag, the B-cell lysates were immunoprecipitated using an anti-Myc antibody, resolved by SDS-PAGE, and immunoblotted using anti-Vav antibodies. The results showed that M2 interacts with Vav1 in B-lymphocytes (Fig. 3B). However, we failed to show interaction between M2 and Vav1 (data not shown). This might be explained by the fact that Vav2 is the predominant Vav family member in B-lymphocytes and plays a main role in cell proliferation, survival, and development, whereas Vav1 is more important for T-lymphocyte signaling. That M2 would at least preferentially interact with Vav2 in B-lymphocytes is supported by the observation that these cells are long term reservoirs for MHV-68 viruses during latent infection. We further observed that B-cell receptor activation did not have any effect on M2-Vav2 co-immunoprecipitation, indicating that this interaction is not dependent upon B-lymphocyte activation.

M2 Promotes Vav Activation in B Cells—Because M2 interacts with Vav1 and -2 proteins it is therefore possible that M2 can modulate Vav activity. To investigate this possibility, we examined Vav activity using a GST-PAK pull-down assay. Rac is a Vav substrate and can only bind to its own substrate, PAK, when converted by Vav into its active GTP-associated form. Thus, quantification of the amount of Rac pulled down by GST-PAK would allow us to indirectly gauge the activity of Vav. Equivalent amounts of WEHI-231, WEHI-231-M2, WEHI-bcl-XL, and WEHI-bcl-XL-M2 were resolved by SDS-PAGE and analyzed by Western blotting with anti-M2 serum; B, cell lysates from WEHI-231, WEHI-231-M2, WEHI-bcl-XL, and WEHI-bcl-XL-M2 either untreated or treated with anti-IgM were immunoprecipitated using an anti-Myc antibody. The immunoprecipitated Myc-tagged M2 was resolved by SDS-PAGE and Western blotting. The membranes were probed with an anti-Vav2 antibody for Vav2 detection and with the anti-Myc antibody for M2.

Because Vav proteins are the only known guanine nucleotide exchange factors regulated by tyrosine kinases, we next studied if inhibition of tyrosine kinase activity would interfere with the M2-induced up-regulation of Rac activity. To this end, we incubated WEHI-231 and WEHI-231-M2 cells with 0, 100, 200, and 400 μM genistein, a known tyrosine kinase inhibitor, prior to performing the GST-PAK pull-down. The results showed that increasing amounts of genistein caused reduction in the amount of activated Rac in WEHI-231 cells not expressing M2. This induction of Rac activity was not detected in WEHI-231 cells expressing M2ΔP1 or M2ΔP2 mutants, indicating that interaction between M2 and Vav is essential for the observed increase in Rac activity (Fig. 4A).

To further confirm that M2 enhances Vav activity, we prepared cell lysates from WEHI-231 cells transfected with M2 or the control empty expression vector, and probed them with a phospho-Vav (Tyr-174) antibody, which recognizes Vav in its activated form. Because Vav activity is modulated by B-cell receptor-mediated signaling, the WEHI-231
cells were treated with anti-IgM for 0, 4, 8, and 24 h. The results showed that there was an increase in Vav phosphorylation in the B-cells expressing M2 indicating that ectopic expression of M2 enhances the activity of Vav proteins (Fig. 4C). Because Vav activates phosphatidylinositol 3-kinase/Akt activity in B-cells, we also examined the activity of Akt using an active phosphorylation-specific antibody. Consistent with the earlier results, we also detected higher levels of Akt phosphorylation in the WEHI-231 cells expressing M2.

**M2 Inhibits Apoptosis/Cell Cycle Arrest of Immature WEHI-231 B-lymphocytes, Induced by B-cell Receptor Activation**—We have previously demonstrated that Vav proteins play an important role in mediating the B-cell receptor-induced signaling cascade, which is essential for B-cell proliferation and survival (39, 41). To further delineate the molecular mechanisms of M2 we investigated the modulation of cell cycle arrest and apoptosis in the murine immature WEHI-231 B-cells expressing M2. To study the effects of M2 on B-cell receptor-induced cell cycle arrest independently of apoptosis, a WEHI-231 cell line overexpressing bcl-XL (WEHI-bcl-XL), which arrests at G0/G1 following B-cell receptor activation but does not undergo apoptosis was also used (29). WEHI-231, WEHI-231-M2, WEHI-bcl-XL, and WEHI-bcl-XL-M2 cells were cultured with a monoclonal antibody directed against anti-IgM and collected at 0, 6, 12, 24, 48, and 72 h after treatment. The cell cycle distribution profiles of propidium iodide-stained cells were measured by flow cytometry. The results showed that M2 expression inhibits G0/G1 cell cycle arrest and apoptosis induced by B-cell receptor-stimulation (Fig. 5A). It was evident that the number of apoptotic WEHI-231 cells at 24 and 48 h after anti-IgM treatment was approximately twice that of the M2-expressing WEHI-231 cells. The fraction of WEHI-231-M2 and WEHI-bcl-XL-M2 cells going through the S and G2/M phase at 24 and 48 h after anti-IgM treatment was also approximately three and two times higher when compared with WEHI-231 and WEHI-bcl-XL cells, respectively.

To confirm the cell cycle results, we performed an analysis of cell number of the four WEHI-231 cell lines in the presence or absence of anti-IgM (Fig. 5B). The result showed that anti-IgM treatment caused a decrease in cell number of the WEHI-231 over the time course of 72 h. The anti-IgM treated WEHI-231 expressing M2 continued to increase in number, albeit at a slower rate compared with the untreated cells. In the presence of anti-IgM, the number of WEHI-bcl-XL cells remained constant for 48 h, whereas the WEHI-bcl-XL cells expressing M2 continued to proliferate throughout the time course. The number of WEHI-bcl-XL cells increased marginally at 72 h, and this probably reflected the fact that the anti-IgM became inactive over time. These results confirmed the earlier cell cycle analysis results as well as the pro-survival and pro-proliferative effects of M2.

To establish the molecular mechanisms whereby M2 controls cell proliferation and survival, we investigated the expression of proteins important in B-cell proliferation and survival in WEHI-231 and WEHI-bcl-XL with or without M2 following anti-IgM treatment (Fig. 6). Due to high levels of apoptosis in the WEHI-231 and WEHI-231-M2 cells after anti-IgM treatment, their protein expression was only followed up to 24 h. Consistent with previous results (29), we observed that anti-IgM treatment up-regulated p130 and the CKI p27kip1 and down-regulated cyclin D2 expression in both WEHI-231 and WEHI-bcl-XL cells. Upon anti-IgM stimulation, down-regulation of cyclin D2 expression was detectable as early as 6 h in both WEHI-231 and WEHI-231-M2 cells. However, the level of cyclin D2 continued to decline after 6 h of anti-IgM treatment in WEHI-231 cells, whereas further cyclin D2 down-regulation was not observed in the WEHI-231 cells expressing M2.

The use of an antibody specific for the CDK4/6 phosphorylation site of pRB, showed a down-regulation of pRB-directed CDK4/6 activity 24 h after anti-IgM in WEHI-231 but not in the WEHI-231-M2 cells. In WEHI-231 cells, the pocket protein p130 and the CKI p27kip1 were both induced at 24 h after anti-IgM, concomitant with cycle arrest. In contrast, no induction of p130 and p27kip1 were detected in the WEHI-231 cells expressing M2. The results also showed that there was no appreciable difference in the expression of pRB and p107, cyclin A and -E, and CDK2, -4, and -6 between WEHI-231 and WEHI-231-M2 cells.

In WEHI-bcl-XL cells, the expression of cyclin D2 was down-regulated at 6 h after anti-IgM treatment, reaching a nadir at 24 h, before recovering at 48 and 72 h. In comparison, the level of cyclin D2 remained constant throughout the time course in the WEHI-bcl-XL expressing M2. Similarly, induction of p27kip1 and p130 expression was detected at 24 h in WEHI-bcl-XL but not in WEHI-bcl-XL-M2 cells. In WEHI-bcl-XL, the initial down-regulation of cyclin D2 expression correlated with a decline of CDK4/6-dependent pRB phosphorylation, but no recovery of CDK4/6 kinase activity was detected at 48 and 72 h. This is probably due to the accumulation of the CKI p27kip1 at 48 and 72 h after anti-IgM in these cells. In the WEHI-bcl-XL cells expressing M2, the CDK4/6 kinase activity declined after anti-IgM treatment, but remained at a higher level compared with WEHI-bcl-XL cells. It was notable that the expression of cyclin A was also down-regulated at 48 and 72 h following anti-IgM treatment in the WEHI-bcl-XL but not in the WEHI-bcl-XL-M2 cells. This down-regulation of cyclin A expression is likely to be the consequence of the down-regulation of cyclin D2 and induction of p130 and p27kip1 expressions. The expression levels and patterns of other cell cycle regulators, including p107, cyclin E,
CDK2, -4, -6, and E2F1 were comparable between the two lines, WEHI-bcl-XL and WEHI-bcl-XL-M2.

To study the potential mechanism employed by M2 to inhibit apoptosis, we also examined the expression patterns of the Bcl-2 family of apoptosis regulators. We observed that anti-IgM treatment caused the down-regulation of the anti-apoptotic Bcl-2 protein Bcl-XL in WEHI-231 cells, thereby confirming previous results. We also found that the expression levels of the BH-3 only protein Bim was lower in B-cells ectopically expressing M2 than controls, suggesting that increases in the ratio of Bcl-XL to Bim caused by M2 render cells less susceptible to anti-IgM-induced apoptosis.

**DISCUSSION**

The MHV-68 viral protein, M2, is expressed during the establishment and long term maintenance of viral latency, indicating that it plays a vital role in viral persistence (26, 33). Infection experiments using an M2-deficient MHV-68 virus in mouse models have shown that M2 is essential for the establishment and maintenance of latent infection in vivo (34). The initial establishment of MHV-68 latency involves a transient expansion of germinal center GC B-cells in the spleen, whereas the long term latency is maintained primarily in memory B-cells as well as germinal center B-cells (27). It is believed that like other γ-herpesvi-
interactions between M2 and Vav proteins were also confirmed present at the C-terminal region of both Vav proteins (32, 36, 37). These grated into “P motifs, which are known to bind to SH3 domains proximally with cellular tyrosine kinases. The fact that M2 has nine viral protein enhances Vav activity through bringing Vav into close proximity. M2 expression leads to increased activity of Vav substrate, Rac, in WEHI-231 B-cells. This increase in Rac activity is not observed in cells expressing M2 mutants, M2P1 or M2ΔP2 that are compromised in Vav-binding, indicating that the M2-Vav interaction is essential for the induction of Vav activity. This result was further confirmed using a phospho-specific antibody, which specifically recognizes the activated form of Vav proteins. Studies of Vav1- and -2-deficient B-cells showed that phosphatidylinositol 3-kinase/Akt signaling pathway is defective (39), indicating that Vav proteins integrate B-cell receptor signals with the phosphatidylinositol 3-kinase/Akt signaling pathway. Consistent with this, we also detected a comparatively higher basal Akt activity (as revealed by the phospho-Akt antibody) in M2-expressing B-cells. The observation that the increase in Vav/Rac1 activity by M2 can be blocked by the addition of the tyrosine kinase inhibitor, genistein, suggests that tyrosine kinase activity is involved. Because M2 has no obvious tyrosine kinase catalytic domain, it is most likely that this viral protein enhances Vav activity through bringing Vav into close proximity with cellular tyrosine kinases. The fact that M2 has nine PXXP motifs, which are known to have a main role in recruiting proteins in signaling cascades through its interaction with SH3 domains (32), further supports this hypothesis.

For successful colonization of the host, γ-herpesviruses have to amplify their viral genomes in latently infected host B lymphocytes, while at the same time evade the immune surveillance of the host. The proliferation and survival of the latently infected lymphocytes requires the expression of latent viral antigens. We and others have previously shown that Vav protein is a component of the B-cell receptor-mediated signaling cascade, which is important for proliferation, survival, and development of B-lymphocytes. Our present data show that ectopic expression of M2 in WEHI-231 cells can overcome the B-cell receptor-mediated cell cycle arrest and apoptosis, suggesting that the latent protein M2 can promote B-cell proliferation and survival through the up-regulation of Vav activity. Similar strategies have been employed by other γ-herpesviruses to manipulate B-cell signaling events to induce cell activation, proliferation, survival, and differentiation. For example, the LMP-2A protein of EBV imitates a constitutively activated B-cell receptor (7, 8), whereas another oncogenic EBV protein LMP-1 mimics a deregulated CD40 receptor, which is a co-receptor of the B-cell receptor (5, 6, 40). Although EBV manipulates the B-cell-signaling events at receptor levels via LMP-1 and LMP-2A, MHV-68 influences the signaling molecule downstream of the B-cell receptor via M2, which functions as an activator of Vav. Our previous data shows that Vav has an important role in setting the threshold for antigen receptor-mediated activation of B lymphocytes, which is likely due to its regulation of receptor clustering (41). M2 expression is likely to modulate this threshold to promote cell proliferation, survival, and development through enhancing Vav activity. Consistent with this idea is the observation that the expression levels of the pro-apoptotic Bcl-2 family protein Bim and the cell cycle inhibitors p27Kip1 and p130 are lower in the B-cells expressing M2 compared with their non-M2-expressing counterparts (42). Moreover, cyclin D2 is also expressed at higher and more sustainable levels in WEHI-231 cells expressing M2 following anti-IgM treatment (42). The type mice. Moreover, Vav1 and -2 null B-cells also fail to proliferate in response to B-cell receptor stimulation (38).

Because B-lymphocytes are the main reservoir for MHV-68 during its latent infection, we investigated the functional significance of M2-Vav interaction using the murine B-cell line WEHI-231, a commonly used cell model for studying B-cell receptor-mediated signal transduction, cell cycle arrest, and apoptosis (29). Using WEHI-231 cells, we found that M2 expression augments Vav activity. GST-PAK pull-down experiments showed that expression of M2 protein leads to increased activity of Vav substrate, Rac, in WEHI-231 B-cells. This increase in Rac activity is not observed in cells expressing M2 mutants, M2P1 or M2ΔP2 that are compromised in Vav-binding, indicating that the M2-Vav interaction is essential for the induction of Vav activity. This result was further confirmed using a phospho-specific antibody, which specifically recognizes the activated form of Vav proteins. Studies of Vav1- and -2-deficient B-cells showed that phosphatidylinositol 3-kinase/Akt signaling pathway is defective (39), indicating that Vav proteins integrate B-cell receptor signals with the phosphatidylinositol 3-kinase/Akt signaling pathway. Consistent with this, we also detected a comparatively higher basal Akt activity (as revealed by the phospho-Akt antibody) in M2-expressing B-cells. The observation that the increase in Vav/Rac1 activity by M2 can be blocked by the addition of the tyrosine kinase inhibitor, genistein, suggests that tyrosine kinase activity is involved. Because M2 has no obvious tyrosine kinase catalytic domain, it is most likely that this viral protein enhances Vav activity through bringing Vav into close proximity with cellular tyrosine kinases. The fact that M2 has nine PXXP motifs, which are known to have a main role in recruiting proteins in signaling cascades through its interaction with SH3 domains (32), further supports this hypothesis.

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induction of cyclin D2 expression by M2 could have a similar functional significance as the expression of the cyclin D-related v-cyclin by HHV-8 during latent infection (12, 13). We have shown previously that cyclin D2 is critical for B-cell receptor-mediated proliferation using B-cells derived from cyclin D2-knock-out mice (43, 44). Similarly, the molecular basis for the proliferative defect observed in varL−/− B-cells is their failure to express cyclin D2 (41). These results taken together with our current observations suggest that M2 targets cell cycle regulators, such as cyclin D2 through augmenting Vav activity to promote B-cell proliferation during latent infection. Further supporting this idea, Vav proteins have also been shown recently to have a role in promoting B-cell survival (45). However, constitutively activated B lymphocytes, which are constantly proliferating and have an extended-life span, are predisposed to acquiring mutations in proto-oncogenes or tumor suppressor genes that could result in the development of lymphoproliferative diseases, including lymphoma and leukemia. Indeed, latent EBV infection has been linked with a number of human malignancies, including Burkitt lymphoma, Hodgkin disease, and lymphoproliferative disease (46), whereas 10% of the MHV-68 latently infected mice develop lymphoproliferative disease identified as lymphomas of B-cell origin (47).

In summary, this study has identified the Vav proteins as the cellular targets of the MHV-68 γ-herpesvirus latency-associated M2 protein. Our data also show that binding of M2 to Vav proteins promotes Vav activity as well as B-cell activation, proliferation, and survival. This functional interaction between M2 and Vav could have a key role in the establishment and maintenance of viral genomes during MHV-68 γ-herpesvirus latent infection.

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