The M Type K15 Protein of Kaposi’s Sarcoma-Associated Herpesvirus Regulates MicroRNA Expression via Its SH2-Binding Motif To Induce Cell Migration and Invasion

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Kaposi’s sarcoma (KS) associated herpesvirus (KSHV) is the etiological agent of KS. In vivo, KS is a tumor capable of spreading throughout the body, and pulmonary metastasis is observed clinically. In vitro, KSHV induces the invasiveness of endothelial cells. The KSHV open reading frame K15 is a KSHV-specific gene encoding a transmembrane protein. Two highly divergent forms of K15, the predominant (P) and minor (M) forms (K15P and K15M, respectively), have been identified in different KSHV strains. The two K15 alleles resemble the latent membrane protein 2A (LMP2A) gene of Epstein-Barr virus (EBV) in their genomic locations and protein topology. Also, both K15 proteins have motifs similar to those found in the EBV LMP1 protein. K15 therefore appears to be a hybrid of a distant evolutionary relative of EBV LMP1 and LMP2A. Since both LMP1 and LMP2A proteins are capable of inducing cell motility, we sought to determine whether K15 has similar abilities. In this study, we show that K15M is latently expressed in KSHV-positive PEL cells and knockdown of K15M in PEL cells reduces cell motility. K15M localizes to lysosomal membranes and induces cell migration, invasion, and NF-κB (but not AP-1) activity via its conserved SH2-binding motif. K15M also induces the expression of microRNAs miR-21 and miR-31 via this conserved motif, and knocking down both these microRNAs eliminates K15M-induced cell motility. Therefore, K15M may contribute to KSHV-mediated tumor metastasis and angiogenesis via regulation of miR-21 and miR-31, which we show here for the first time to be a specific regulator of cell migration. In light of these findings, the targeting of K15 or the downstream microRNAs regulated by it may represent novel therapies for treatment of KSHV-associated neoplasia.

Cell migration plays an important role in many diverse biological processes ranging from embryogenesis to immune response (44). Aberrant activation of cell migration in neoplastic cells results in tumor metastasis, which is the principal event leading to death in the majority of cancer patients (16). The metastatic phenotype is a highly complex series of steps called the metastatic cascade, including the ability to break through local physical barriers such as basement membrane, migrate from the primary tumor to blood or lymphatic vessels, survive in circulation, invade distant tissues, and establish distant metastatic nodules (16).

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi’s sarcoma (KS), a tumor of lymphatic endothelial lineage (47) and is also linked to the pathogenesis of certain lymphoproliferative disorders such as primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) (1, 3). At the far right-hand end of the KSHV genome, open reading frame (ORF) K15 encodes a putative transmembrane protein in the same genomic location as the Epstein-Barr virus (EBV) latent membrane protein 2A (LMP2A) (10, 13, 19). K15 also resembles LMP2A in its splicing pattern and predicted protein structure (10, 13). In addition, the C terminus of K15 has sequences similar to those found in EBV LMP1, including a putative tumor necrosis factor (TNF) receptor-associated factor (TRAF) binding site (5, 13). K15 therefore appears to be a hybrid of a distant evolutionary relative of both EBV LMP1 and LMP2A.

Two highly divergent forms of K15 have been identified: the predominant (P) and minor (M) forms (K15P and K15M, respectively) (19, 28, 37, 48). These two alleles possess only 33% amino acid identity and yet retain 12 transmembrane spanning domains and a putative cytoplasmic signal transducing carboxyl terminus (C terminus) (10, 48). The C termini of both K15 proteins have potential signaling motifs including Src homology 2 and 3 binding domains (SH2-B and SH3-B, respectively) (10, 48). The K15P protein interacts with cellular proteins, TRAF, and Src kinases and activates AP-1, NF-κB, and the mitogen-activated protein kinase c-Jun-N-terminal kinase 1 (JNK1), and extracellular signal-regulated kinase 1/2 (ERK1/2) (5). This signaling activity of K15P is dependent on phosphorylation of Y381 of the K15P SH2-B motif YEEV (5, 10). A CD8-K15P C-terminal chimeric protein was constitutively tyrosine phosphorylated at Y381 of the YEEV SH2-B
motif. Like EBV LMP2A, this CD8-K15 chimeric protein modulates B-cell receptor signal transduction in B cells (10). This motif was phosphorylated by the tyrosine kinases Src, Lck, Yes, Hck, and Fyn (5). Similarly, K15M also induces the activation of ERK2, JNK1, and NF-κB, as well as the expression of a similar range of cellular invasion and inflammatory genes, including MMP-1 and MMP-3 (48). The activation of most K15M target genes is impaired by mutagenesis of Y490 in its SH2-binding motif Y490EVE, a position equivalent to the Y461EVE SH2-B motif in K15P (48).

Human gammaherpesviruses have been shown to promote cell migration and invasion (7, 22). KSHV promotes invasion of primary human umbilical vein endothelial cells by inducing matrix metalloproteinase 1 (MMP-1), MMP-2, MMP-9, and AP-1 activity (38). The K1 protein of KSHV can induce the expression of angiogenic and invasion factors, including VEGF and MMP-9 (49). EBV infection has also been implicated in enhancing invasion and migration of NPC cells (50). Several EBV proteins such as LMP1, LMP2A, and Zta proteins are capable of regulating cell mobility (9, 35, 43). LMP1 downregulates E-cadherin via activation of DNA methyltransferases, hence inducing cell migration (43). LMP1 also upregulates MMP-1 and MMP-9, whereas LMP2A enhances the expression of c-Jun through the ERK pathway (9, 29, 51). Since the KSHV K15 P and M alleles and protein products share many structural and/or functional characteristics with EBV LMP1 and LMP2A, such as induction of MMP-1 and MMP-3 expression in epithelial cells (48) we postulated that the K15 proteins may therefore also induce cell migration and invasion.

The K15P protein is expressed in PEL cells and in KSHV BAC36-infected epithelial cells (6, 40). However, it is not known whether the K15M protein is expressed in KSHV-latently infected tumor cells. The size of endogenous K15M protein, its exact subcellular localization and its function have also not been determined. In the present study, we generated and used a specific monoclonal antibody against K15M to show its expression in PEL cells. In addition, the K15M protein was found to localize to lysosomal membranes when ectopically expressed and induced cell migration and invasion in vitro. Although K15M did not induce AP1 activity like that of K15P, we show the Y490 residue of the SH2-binding motif of K15M to be critical for induction of cell motility.

Finally, we show that expression of K15M resulted in the deregulation of motility-related microRNAs (miRNAs), which in part explains how K15M induces cell migration and invasion. miRNAs are small RNAs that can moderate cellular gene expression, thereby contributing in the regulation of various crucial biological processes including cancer metastasis. Among cancer-related miRNAs (oncomiRs), miR-21 and miR-22, which are transcribed from the same pri-microRNA cluster, have been reported to affect the expression of stem cell factor receptor c-Kit and, as a consequence, block endothelial cell migration, proliferation, and angiogenesis (36). Enhanced MR-21, which is in the same cluster with miR-22, increases tumor cell proliferation, migration, and invasion in both human hepatocellular cancer cells and normal human hepatocytes (30). EBV can induce the expression of a variety of cellular miRNAs, including miR-21 (32). miR-21 also stimulates invasion, intravasation, and metastasis in colorectal carcinoma (CRC) (2). Similar to miR-21, miR-24 and miR-31 were also upregulated in CRC (41, 45). In the present study, we determined the expression level of the above miRNAs by quantitative reverse transcription-PCR (RT-PCR) in the presence of wild-type or mutant K15M. We show that miR-21 and miR-31 are induced by K15M via its conserved SH2-binding motif and that these two miRNAs also contributed in K15M-induced cell migration. Our data therefore establish K15M as a KSHV-encoded latently expressed protein that has the capacity to modulate host cell signaling pathways, which ultimately results in changes in gene expression patterns to promote cell motility and invasiveness. These K15M functions in turn may contribute to KSHV-induced angiogenesis, KS tumor metastasis, and the extravasation of PEL cells from the circulatory system into body cavities.

**MATERIALS AND METHODS**

**Plasmids.** Plasmids expressing wild-type or mutant K15P proteins have previously been described (40). LMP1 expression vector was kindly provided by Ching-Hwa Tse (29). Full-length KSHV K15M cDNA was cloned by RT-PCR. A 1-μg portion of total RNA from Ficoll-treated unstimulated PEL cell line BC-1 (KSHV inside contains K15M allele) was reverse transcribed by using Superscript II RT (Gibco-BRL, Paisley, United Kingdom) in a total reaction volume of 50 μl as described previously (46). Then, 5 μl of this cDNA was used for PCR with K15M-specific primers: 5′ specific primer 5′-CGGCTTCGACGTTAGTTACAAAGATACC-3′ (the CPO I site is underlined, and the predicted start codon is indicated in boldface) and the 3′ specific primer 5′-CAACGACCTGTCGGAACACAAACATCC-3′ (the CPO I site is underlined). The DNA fragment product was agarose gel purified and then ligated into similarly cut pLenti4-V5 vector (Invitrogen, Groningen, The Netherlands) to give pLenti4-K15M construct, which can express the 8-exon M form protein of K15 in cells. pGEX6p-1-K15M-CT was used to produce recombinant GST-K15M-CT protein (see below) and was constructed as follows. cDNA encoding amino acids 351 to 498 was PCR amplified from the full-length K15M wild type previously cloned (see above) using the 5′ primer 5′-ACTGTTATCTTATCTTACCGGAGA-3′ (the EcoRI site is underlined) and the 3′ primer 5′-CGTGGACTCATTAGTCTGTTGGAACAAACATCC-3′ (the CpoI site is underlined). The PCR product was purified, restriction enzyme digested with EcoRI and SalI, and ligated in-frame into similarly cut pGEX6p-1 vector (Amersham Pharmacia Biotech, Bucks, United Kingdom).

The K15M Y490F point mutation created in the pLenti4-K15M was created by using a QuickChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions and the following mutation primer pair: 5′ primer-CATTTGCGCGTACAGATGAACTTC-3′ and 3′ primer-AATTACAAAAATACCG-5′. The CPO I and SalI sites were added to the mutant gene.

**Cell culture, transfection, and reporter assays.** Human embryonic kidney 293 (HEK-293), HEK-293T, NIH 3T3, and HeLa cells were cultured in Dulbecco modified Eagle medium (Gibco-BRL, Paisley, United Kingdom) supplemented with 10% fetal bovine serum or newborn calf serum (for NIH 3T3 cells), 2 mM glutamine, and 50 μg of penicillin and 50 μg of streptomycin/ml. BC-1 and BCBL-1 cells were cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 50 μg of penicillin/ml, and 50 μg of streptomycin/ml in the presence of 5% CO2. HMEC-1 cells were cultured in endothelial cell growth medium MV (catalog no. C-22020; PromoCell, Heidelberg, Germany) supplemented with 1% fetal bovine serum or newborn calf serum. At 48 h posttransfection, cells were harvested and lysed in passive detergent lysis buffer (Promega). The Renilla luciferase was used as the internal control.

**RNA extraction and RT-PCR.** RNA extraction and RT-PCR were performed as previously described (46). The sense primer for specific amplification of K15M

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Generation of recombinant GST-K15M-CT fusion protein. Amino acids 351 to 498 of K15M, which contain the predicted cytoplasmic carboxyl terminus, were expressed as a recombinant glutathione S-transferase (GST) fusion protein from the pGEXP-1-K15M-CT plasmid. A total of 800 ml of mid-log-phase Escherichia coli BL21(DE3)/pLysS expressing the recombinant GST-K15M-CT was harvested, and cell extracts were produced by resuspension of the cells in 20 ml phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonyl fluoride. After centrifugation at 12,000 × g for 30 min to remove debris, 600 μl of 50% glutathione bead slurry was added to 20 ml of supernatant, followed by incubation for 1 h at 4°C, and then the beads were washed twice with PBS. The GST-K15M-CT fusion protein was eluted with 50 mM Tris-HCl, pH 8.0 and used to produce anti-K15M monoclonal antibody. To obtain K15M-CT protein for further studies, the beads were washed in PBS, followed by one wash in PreScission protease (Amersham Pharmacia Biotech, Bucks, United Kingdom) cleavage buffer (50 mM Tris-HCl [pH 7.0], 150 mM EDTA, 1 mM diithiothreitol), and resuspended in 300 μl of cleavage buffer. PreScission protease was added to beads at a final concentration of 80 U/ml, followed by incubation at 4°C for 4 h with rotation. This step cleaved the K15M-CT from the GST bound to beads and released the K15M-CT into the supernatant, which was stored at −80°C for future use. Anti-K15M monoclonal antibody production and purification. To produce a female 8-week-old BALB/c mice were subcutaneously immunized with 50 μg of GST-K15M-CT recombinant protein in RAG deficient (Sigma-Aldrich) at three sites. Every 3 weeks, the animals were boosted with 25 μg of GST-K15M-CT proteins in RAG deficient. Test bleeds were taken and analyzed by Western blotting using the recombinant GST-K15M-CT fusion protein. At 3 days prior to fusion, the chosen mouse was injected intravenously with 5 μg of GST-K15M-CT recombinant protein in saline. The serum of the next day was used to inject nude mice with subcutaneous inoculation of 5 × 10^4 hybridomas. Hybridomas were initially identified by enzyme-linked immunosorbent assay, followed by Western blotting with recombinant K15M-CT protein. Cell lines of interest were cloned by limiting dilution, retested for a positive and specific signal, and then scaled up for affinity purification. Anti-K15M MAbs were purified from culture media supernatant (500 ml) by using an nProtein A-Sepharose 4 FastFlow 0.5 ml column (Amersham Biosciences). Once the supernatant had been applied to the column, it was washed with sodium borate buffer (washing buffer 1 consisted of 3 M NaCl and 50 mM sodium borate [pH 8.9]; washing buffer 2 consisted of 3 M NaCl and 10 mM sodium borate [pH 8.9]) and then eluted with 0.1 M glycine (pH 3). Peak fractions were pooled, concentrated with an Amicon Ultra (Millipore), and then adjusted to 0.2% sodium azide for further manipulation.

The specificity of the anti-K15M MAb was examined by testing the cross-react with K15P protein. K15M MAb did not recognize K15P protein (see supplemental Fig. 1 at http://infobio.ym.edu.tw/downloads/K15M/K15MSuppl.pdf), indicating that it recognizes a specific epitope within the K15M cytoplasmic tail.

Immunoprecipitation and immunobLOTS. A total of 4 × 10^7 BC-1 and BCBL-1 cells treated with 3 mM sodium butyrate for 48 h were harvested and lysed with 500 μl of lysis buffer A (140 mM NaCl, 50 mM Tris [pH 8.0], 0.5% Triton X-100, 0.2% NP-40, 2 mM diithiothreitol, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride protease inhibitor [Roche]). Lysates were incubated with rotation at 4°C for 2 h and then centrifuged at 13,000 rpm for 5 min to remove debris. Protein extracts were first precleared with Protein G beads (50 ml slurry; Amersham Pharmacia Biotech) for 1 h at 4°C with rotation, followed by the addition of 1 μg of anti-K15M MAb or the isotype control (immunoglobulin G1). Then 30 μl of protein G beads was added for pulldown at 4°C for 16 h. Beads were washed twice in NET buffer (0.25% NP-40, 0.5 mM EDTA, 50 mM Tris-HCl [pH 7.4], 0.15 mM NaCl) and protein denatured by the addition of 40 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. No further boiling step was performed. Proteins were separated by SDS–15% PAGE and Western blotted with the indicated primary antibody.

Immunofluorescence and determining the subcellular localization of K15M. Cells transfected with the indicated expression plasmids were washed three times with ice-cold PBS, fixed with 4% paraformaldehyde–PBS for 20 min at room temperature, and then permeabilized with 0.2% Triton X-100–PBS for 15 min at room temperature. Cells were washed three times with PBS and incubated at 25°C with blocking buffer (PBS with 1% bovine serum albumin). Cells were then washed with PBS and incubated with primary antibody in blocking buffer for 60 to 120 min at 25°C. Cells were washed again with PBS and incubated with the indicated secondary antibody at 25°C, washed as described above, and then mounted on slides using 20 μl of mounting fluid (0.1% p-phenylendiamine, 0.15 M Tris-HCl [pH 8.8], 90% glycerol). Endoplasmic reticulum staining for immuno-fluorescence assay was performed by incubating fixed cells with the anti-calreticulin MAb (catalog no. Ab4; Abcam, Cambridge, MA) for 1 h at 25°C. pDsRed2-Mito (catalog no. 632421; Clontech, Mountain View, CA), pEYFP-Endo (catalog no. 6936-1; Clontech), pEYFP-Golgi (catalog no. 6909-1; Clontech), and pDsRed2-Lysos (kindly provided by Yu-May Lee at the Institute of Biological Chemistry, Academia Sinica, Taiwan) were cotransfected with K15M expression vector to label mitochondria, endosomes, Golgi bodies, and lysosomes, respectively. Images of localized or colocalized proteins were obtained by using confocal laser scanning microscopy (Olympus V1000). Subcellular fractionation was performed as described previously (46).

RNA interference-mediated knockdown of K15M and miRNA expression. To knock down K15M and miRNAs expression, specific small interfering RNA (siRNA) oligonucleotides were electroplated into cells by using a MicroPorator MP-100 (NanoEnTek, Inc., Seoul, South Korea) according to the manufacturer’s instructions. In brief, 10^6 cells were resuspended in 10 μl of R buffer (provided in microporation kit) and mixed with 1 μl of specific siRNA oligonucleotide (50 nm) before microporation. After pulse analysis, the samples were transferred into fresh medium and then further incubated for 48 h before subjecting them to further assays. Anti-K15M siRNA was designed in house and then purchased from Sigma-Proligo (The Woodlands, TX). The sequences used for si-K15M were as follows: sense, 5′-UACGAGUGCUCCGUUUATT-3′; and antisense, 5′-UAAACCGCGAACUUAAGATT-3′. For miRNA knockdown, we used commercial available hairpin inhibitors specific to human miR-21 (catalog no. IH-300492-05-0005; Dharmacon) or human miR-31 (catalog no. IH-300507-06-0005; Dharmacon). Transwell migration assay and Matrigel invasion assay. Cell invasiveness was examined by using a Matrigel basement membrane matrix invasion assay (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer’s instructions. The degree of cell migration was evaluated by using the Costar Transwell polycarbonate permeable supports (Corning, NY). For the NIH 3T3 cells, a total of 10^5 cells resuspended in 100 μl of culture medium containing 10% FBS was applied to the upper chamber of the device, and 600 μl of medium containing 20% FBS was added to the lower chamber. A polycarbonate membrane with a pore size of 8 μm was placed between the two chambers. After 12 h of incubation at 37°C, the membrane was fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 at room temperature for 15 min, and then stained in 0.1% Hoechst 33342 salt solution for 10 min. Migrated cells on the membrane were counted under a microscope. For HMEC-1 cells the procedures for Transwell migration assays were the same as those for the NIH 3T3 cells except that instead of a serum gradient between the upper and lower chambers we used hVEGF added to the lower chamber as a chemoattractant at a final concentration of 10 ng/ml. A total of 10^5 4C1 cells, 5 × 10^5 4C1/ARC cells, and 0.25-μm-pore-size membrane Transwells were used to perform a Transwell migration assay, following a procedure similar to that used for the NIH 3T3 cells.

miRNA expression. The expression plasmid for human hsa-mir-31 miRNA, as well as its control empty vector pmiR-GFP-Zeo (catalog no. MIFCZ312PA-1 and MIFCZ3000PA-1, respectively), was purchased from System Biosciences (Mountain View, CA). The expression of mature human miRNAs was determined by a stem-loop real-time PCR system (8) with the indicated primer pairs (see supplemental Table 1 at http://infobio.ym.edu.tw/downloads/K15M/K15MSuppl.pdf). The PCR reverse primer for miR-21 was GTCGAGGTCC CGAGGT. miRNA expression data were normalized to that of U6 snRNA, which was amplified with the specific forward primer 5′-CTCGCCCTCGCC GAC-3′ and the reverse primer 5′-AACGGTCTCAGATTGGC-3′.

RESULTS

K15M is latently expressed in vivo. To test our investigation into the function of K15M compared to K15P, we performed amino acid sequence and domain organization comparisons of both K15 alleles (Fig. 1A). Both P and M forms of the K15 protein possess similar motifs, including SH3-binding (SH3-B), SH2-B, and HAX1-binding motifs (40). A mitochondrial targeting sequence (MTS) could only be found in the

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C-terminal part of K15P protein (40), indicating K15M and K15P may locate in different subcellular compartments. To obtain a better idea about similarities and differences between the P and M forms of the K15 protein, bioinformatics tools were applied to analyze secondary structure of both proteins. The SH3-B motifs of both K15 alleles were located in a loop area and both SH2-B YEEL motifs in an α-helical region (Fig. 1B).

The first biochemical analysis of K15M we performed in the present study was to determine whether expression was latent or lytic in KSHV-infected cells. BC-1 PEL cells were treated with the lytic inducing agent sodium butyrate, and cells were harvested at the indicated time intervals for RT-PCR analysis of endogenous K15M mRNA. In the absence of sodium butyrate treatment, K15M mRNA was detected at low levels (Fig. 1C) and upon addition of sodium butyrate we observed an increased expression of K15M mRNA over 24 h (Fig. 1C). The KSHV lytic gene vGPCR was analyzed as a control for lytic cycle induction (Fig. 1C). We next examined the size of endogenous K15M protein by using our anti-K15M specific MAb (see Materials and Methods) (Fig. 1D). The predicted size for full-length (498 amino acids) form of K15M protein is 55 kDa with a theoretical isoelectric point (pI) of 8.45. Previously, expression of K15M in HeLa cells resulted in a protein product of 40 kDa (48). In the BC-1 PEL cell line we observed a specific 45-kDa K15M protein band that was not detected by the isotype antibody control (Fig. 1D, upper panel). Western blotting for actin in input lysate was shown as an internal control (lower panel, Fig. 1D).

K15M is partially localized to lysosomal membranes. Protein function is intimately linked to its subcellular localization.
FIG. 2. Cellular localization of K15M. (A) Immunofluorescence staining for K15 proteins. HeLa and NIH 3T3 cell were grown on coverslips and transfected with expression vectors encoding the full-length M or P form of K15. At 48 h posttransfection cells were fixed, and K15 proteins
and vice versa. We therefore determined the location of K15M protein within the cell. The eukaryotic expression vectors pLenti4-v5 containing cDNA encoding the full-length M or P form of K15 were used to transiently transfet NIH 3T3 cells and HeLa cells (Fig. 2A). After transfection, expression of the K15M and K15P proteins was examined via immunofluorescence assay using the K15M- and K15P-specific MAb we developed (40). Cells transfected with vector only (negative control) gave only background staining with these MAb. Cells transfected with K15P produced a staining pattern that localized within the cytoplasm and around the nuclear membrane (Fig. 2A). This pattern is similar to the mitochondrial localization pattern previously observed for K15P (40). For K15M transfected cells the distribution was different, with the localization being predominantly cytoplasmic with a granular and/or vesicle-like staining pattern (Fig. 2A). Cellular fractionation experiments confirmed that the 45-kDa form of K15M was located in the heavy organellar fraction (Fig. 2B). Succinate dehydrogenase and lysosome-associated protein 1 were used as markers for mitochondria and lysosomes, respectively (Fig. 2B).

The initial immunofluorescence staining and biochemical fractionation indicated a possible subcellular organelle localization of K15M. We therefore used various organelle-specific staining markers in further colocalization experiments (Fig. 2C to F). Figure 2C and Fig. 2E to G show that the staining patterns between K15M and several organelles (including mitochondria, Golgi bodies, endoplasmic reticulum, and endosomes) did not colocalize. However, the colocalization of K15M with the lysosome marker indicated clear colocalization to this organelle (Fig. 2D; also supplemental Fig. 2A at http://infobio.ym.edu.tw/downloads/K15M/K15MSuppl.pdf).

K15M expression induces cellular migration and invasion. In light of the similarities in biochemical, structural, and genomic location between the K15 and EBV LMP2A genes and proteins (5, 10, 37), we hypothesized that these membrane proteins of gammaherpesviruses may share the same biological functions, i.e., are all able to induce cell migration and invasion (35). To test this hypothesis, NIH 3T3 cells transfected with K15M expression vector or vector-only control were subjected to the Transwell cell migration assay. As shown in Fig. 3A, significantly more K15M-transfected NIH 3T3 cells migrated through the Transwell membrane than those transfected with vector only control (Fig. 3A), demonstrating that K15M expression enhances cell migration.

We also tested the extracellular matrix invasion ability of K15M-expressing cells. NIH 3T3 cells transfected with K15M, EBV LMP1, or pLenti4-v5 vector were subjected to the Matrigel cell invasion assay. As shown in Fig. 3B, K15M- and LMP1-transfected cells invaded into the other side of membrane at similar efficiencies (Fig. 3B), indicating that K15M protein can also induce the invasiveness of cells.

Since exogenous expressed K15M protein in NIH 3T3 cells can enhance cell motility, we next examined whether endogenous K15M protein also contributes to cell migration. K15M-positive BC-1 PEL cells were transfected with siRNA against K15M to knock down endogenous K15M expression. At 24 h after transfection, cells were harvested for Transwell migration assay (Fig. 3C). Knocking down K15M expression led to the reduction of cell motility (Fig. 3C). Real-time PCR was performed to validate the knock-down effect (Fig. 3D). K15M was also overexpressed in BC-1 cells as a positive control (Fig. 3C and D).

The SH2-binding motif is required for K15M-induced cell migration. K15M has been shown to transduce signals and induce inflammation-related genes via its SH2-B Y490EEL motif (48). Similarly to K15P, K15M induces the activation of ERK2 kinase, JNK1 kinase, and the NF-κB transcription factor in human HEK-293T cells via this conserved motif (5, 48; also supplemental Fig. 3 at http://infobio.ym.edu.tw/downloads/K15M/K15MSuppl.pdf). The P form of K15 has been reported to activate AP-1 transcription factor in HEK-293T cells (5). We therefore analyzed K15M for activation AP-1 by cotransfecting 293T cells with the corresponding K15 expression vectors and a luciferase reporter plasmid containing three AP-1-responsive elements. K15P and EBV LMP1 proteins were included as positive controls. K15M was not able to activate the AP-1 pathway (Fig. 4A). Mutation at the Y481 residue reduced the AP-1 activation ability of K15P (Fig. 4A), a finding consistent with a previous report (5). Western blots were performed for the indicated K15 forms and mutants to control for protein expression levels (Fig. 4B).

We further examined whether the SH2-B motif of K15M was involved in its proinvasive function. As depicted in Fig. 4C, K15M induced cell migration but the K15M Y490F mutant was significantly impaired in its ability to induce cell migration (Fig. 4C). Invasiveness induced by K15M was also examined by a Matrigel invasion assay (Fig. 4D). Similar results were observed for the Y490F mutant, which had impaired invasive ability compared to wild-type K15M.

K15M regulates a specific subset of miRNAs involved in cell motility and invasiveness. It is thought that the K15P allele regulates signal transduction pathways (such as AP-1 and NF-κB) that ultimately converge on the activation of proproliferative and transforming transcriptional profiles (5, 6). We therefore wanted to examine the downstream targets of K15M to determine a possible mechanism for the K15M-induced motility and invasiveness. Recent studies have shown that the miRNA members of cellular transcriptome can regulate cell migration and/or invasion, and therefore deregulation of
miRNA expression may contribute to angiogenesis and cancer metastasis (26, 27, 42). We analyzed a subset of miRNAs in order to determine those that might be regulated by K15M. Cancer-related miRNAs (oncomiRs) were selected based on published studies: miR-221 and miR-222, which are transcribed from the same pri-microRNA cluster, have been reported to affect the stem cell factor receptor c-Kit expression and, as a consequence, block endothelial cell migration, proliferation, and angiogenesis in vitro (36). miR-222/221 also regulate endothelial nitric oxide synthase protein levels (27). Enhanced miR-21, which is in the same cluster with miR-22, increases tumor cell proliferation, migration, and invasion in both human hepatocellular cancer cells and normal human hepatocytes (30). The infection with EBV induces the expression of a variety of cellular miRNAs such as miR-21 (32). miR-21 also stimulates invasion and intravasation, and metast-
tasis in CRC (2). Similar to miR-21, miR-24 and miR-31 were also upregulated in CRC (41, 45). miR-16, which is in the same pri-miRNA cluster with miR-15, has been implicated in anti-angiogenesis by targeting Bcl-2 to induce apoptosis (11). Using quantitative PCR, we examined the above set of miRNAs in K15-M-transfected HEK-293T cells. K15M upregulated miR-21 and miR-31, and its SH2-B motif was essential for this effect (Fig. 5A and C). In contrast, miR-16, miR-22, miR-24, and the miR-221/222 cluster were not affected (Fig. 5A to C).

The above data obtained for miR-31 represent the first report of its kind to show the ability of this specific miRNA to induce cell motility. We therefore further validated this result by transfecting NIH 3T3 cells with an miR-31-expressing plasmid and subjecting transfectants to Transwell migration assays. miR-31 overexpression significantly increased cell migration in NIH 3T3 cells (Fig. 5D). Next, we knocked down the expression of endogenous miR-31 and miR-21 in HMEC-1 cells in the presence of K15M protein expression and again performed Transwell migration assays (Fig. 5E). The results indicated that K15M-induced cell migration was disrupted if miR21 or miR31 expression was blocked (Fig. 5E). Expression level of each miRNA was determined by real-time PCR to validate the knockdown efficiency (see supplemental Fig. 4 at http://infobio.ym.edu.tw/downloads/K15M/K15MSuppl.pdf).

FIG. 4. K15M induces cell migration and invasion via Y490 residue signaling. (A) HEK-293T cells were transiently cotransfected with AP-1 promoter luciferase reporter plasmid and the indicated K15 or LMP1 expression constructs. Relative fold activation compared to that of vector-transfected cells is shown. The columns indicate the averages of three independent experiments done in duplicate; bars indicate the standard deviations. (B) Similar expression of wild-type and mutant proteins in transfected cells. (C and D) The SH2-B Y490EEL motif of K15M is critical in the induction of cell motility. NIH 3T3 cells transfected with K15M Y490F mutant or wild-type K15M were subjected to Transwell migration assay (C) or Matrigel invasion assay (D) at 24 h posttransfection. Pictures were taken at 8 h after the migrated or invasive cells were counted. The data are means ± the standard deviation (n = 3).

**DISCUSSION**

Cell motility and invasiveness play a central role in tumor progression and metastasis. Many oncogenic viruses have evolved mechanisms to facilitate such processes (23, 29, 35). KSHV has been shown to promote invasion of human primary endothelial cells in vitro (38). However, no specific KSHV gene product has been linked to this viral induced invasive ability of infected cells. The K1 protein of KSHV has been shown to induce the expression of invasion factors such as MMP-9 (49). However, these effects on MMP expression have not yet been examined in functional assays. Here we show for the first time that the K15M protein is latently expressed in the cytoplasm and associated with lysosomal organelles in KSHV-infected cells and is capable of inducing cell migration and invasion via its SH2-binding motif. One possible mechanism through which this is achieved is via signal transduction events ultimately affecting transcription of proinvasive and motility-related miRNAs. These data illustrate an important role for
K15M in KSHV-induced cell invasion and therefore the progression of KSHV-related malignancies.

By use of the specific anti-K15M MAb we have demonstrated that K15M is a novel KSHV latent protein that is inducible by sodium butyrate treatment (Fig. 1). Other KSHV proteins that are latently expressed in KSHV-related tumor cells include the latent nuclear antigen (LANA-1), viral cyclin, viral FLIP, and kaposin (encoded by ORF K-12) (14). LANA-2/vIRF-3, an interferon-regulatory protein homologue encoded by ORF K10.5 (39), and vIL6, the viral-interleukin 6 homologue encoded by ORF K2 (31), are expressed in latently infected PEL and MCD cells but not in KS spindle cells (31, 39). We have previously shown that K15P is latently expressed in PEL and MCD cells and is also inducible by lytic stimulations such as tetradesoxycyclin phorbol acetate treatment (40). Thus, both K15 alleles are expressed at the latent stage of KSHV infection, similar to the expression pattern of EBV LMP1 and LMP2A proteins (13).

The link between the lysosomal localization of K15M to its invasion function is intriguing. Emerging evidence suggests that lysosomal alterations are common in cancer cells. Tumor invasion, angiogenesis, and metastasis are associated with altered lysosomal trafficking and increased expression of lysosomal proteases such as cathepsins (17, 25). Cathepsins and other lysosomal hydrolases participate in cancer progression after their release from lysosomes into the extracellular space (17, 25). Lysosomal-membrane permeabilization has been shown to be regulated by lysosome-associated proteins such as heat shock protein 70 (33). Whether K15M also regulates the permeability of lysosomal membrane and thus affects subsequent release of cathepsins remains an interesting question to elucidate. Targeting these pathways may therefore constitute a novel approach to treat KSHV-related cancers (25).

Kaposi’s sarcoma is a highly disseminated and angiogenic tumor of proliferative lymphatic endothelial cells (21, 24). Analyzing the KSHV clonality in different stages of KS have shown that KS begins as a polyclonal disease with a subsequent evolution to a mono/oligoclonal process involving infected spindle cells (18). This model therefore suggests the spreading of a few malignant spindle cells in patient bodies during tumor...
progression. Moreover, distal metastasis, such as pulmonary KS, can be observed in AIDS-KS patients and cause diffuse lung disease (20). Tumor cells of KSHV-associated B-cell neoplasias (PEL and MCD) are also able to migrate into body cavities (3). The ability of KSHV to induce cell migration and invasion is therefore important in disease progression. Furthermore, since in KS lesions and KSHV-induced B-cell neoplasias the majority of tumor cells are latently infected by KSHV (15), the latent proteins should, at least in part, contribute to the invasiveness of KSHV-infected cells. We have shown that K15M is a KSHV latent protein capable of inducing cell invasion. Whether other latent viral proteins also contribute in cell motility and how these genes achieve such effects are exciting questions and represent an important field of further study in KSHV biology.

In addition to contributing on KS metastasis and the extravasation of PEL cells into body cavities, the cell migratory and invasive phenotype induced by K15M may also contribute in angiogenesis and lymphangiogenesis, which in turn may promote tumor progression and metastasis. Both angiogenesis and lymphangiogenesis are the development of new blood/lymph vessels from preexisting vasculature and therefore may be an essential event for tumors to grow beyond an otherwise limited size (≤2 mm³) (12). Angiogenesis and lymphangiogenesis also contribute in metastasis since metastatic tumor cells need to migrate into surrounding blood or lymph vasculature to achieve systemic circulation. Studies on KSHV- and/or K15M-induced endothelial cell migration and invasion may therefore improve our understanding of these two crucial biological events, benefitting the development of new therapeutic approaches to treat metastatic diseases (34).

Using real-time PCR we found K15M upregulated certain oncomiRs. Specifically, K15M upregulates miR-21 and miR-31, but not miR-16, miR-22, miR-24, or the miR-221/222 cluster (Fig. 5). The SH2-B motif of K15-M, and by inference NF-κB activity, was essential for regulating these oncomiRs (Fig. 5A to C). miR-21 has been linked to tumor migration, invasion, and angiogenesis by targeting tumor suppressors PTEN and Pdcd4 (2, 30). Both miR-31 and miR-21 have also been shown to be upregulated in CRC (P = 0.0006 and P = 0.0001, respectively) (4, 41). Furthermore, miR-21 stimulates invasion and intravasation, and metastasis in CRC (2) is associated with lymph node positivity (P = 0.025) and the development of distant metastases (P = 0.009) in CRC patients (41). In addition, the expression level of miR-31 is also higher in stage IV CRC patients than in stage II ones (P = 0.0028) (4), suggesting this miRNA contributes to both CRC tumorigenesis and progression. The upregulation of miR-31 by K15M indicates that miR-31 may directly promote tumor cell migration and invasion.

K15P and K15M proteins share many structural and functional characteristics, including the conservation of SH2- and SH3-binding motifs (40) (Fig. 1A); similar transmembrane structure topology (Fig. 1B); latent expression pattern in PEL cells (Fig. 1C and D); the ability to induce activation of the ERK2, JNK1, and NF-κB canonical pathways (48; also supplemental Fig. 3 at http://infobio.ym.edu.tw/downloads/K15M/K15MSupp.pdf); and, the induction of a similar range of cellular inflammatory genes (48). However, they are distinct from each other in several ways. K15P (but not K15M) localizes to mitochondria with a perinuclear staining pattern (40) (Fig. 2). K15M, on the other hand, localizes to lysosomal membranes (Fig. 2). The different subcellular localizations of these two proteins correlates well with sequence analysis, where there is an MTS on K15P but not on K15M (Fig. 1A). Moreover, only K15P activates AP-1 transcription, and yet both K15 alleles are able to activate NF-κB (Fig. 4A; also supplemental Fig. 3 at http://infobio.ym.edu.tw/downloads/K15M/K15MSupp.pdf). Interestingly, all of the subcellular lysosomal marker colocalized to K15M, but only ~50% of the K15M signal colocalized to the lysosomes (Fig. 2; also supplemental Fig. 2 at http://infobio.ym.edu.tw/downloads/K15M/K15MSupp.pdf), indicating that a proportion of the K15M may be localized to as-yet-undetermined subcellular structures or organelle that may represent additional novel functions.

In summary, we have demonstrated K15M to be a new latent expressed KSHV protein located on lysosomal membrane. K15M activates the NF-κB, but not the AP-1, pathway in vivo. In addition, we have assigned biological function to K15M and found its SH2-B motif to be critical for induction of cellular motility and invasiveness via deregulation of the cellular transcriptome and specifically miR-21 and miR-31 regulation. K15M may therefore contribute in KSHV-mediated tumor cell migration, metastasis, lymphangiogenesis, and angiogenesis.

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