Functional Organization of MIR2, a Novel Viral Regulator of Selective Endocytosis*

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Kaposi's sarcoma-associated herpesvirus encodes two related proteins, MIR1 and MIR2, that lead to reduction of the cell surface levels of major histocompatibility complex class I and other polypeptides involved in immune recognition. MIR1 and MIR2 do not affect the assembly or transport of their target proteins through the secretory pathway; rather, they act to enhance the selective endocytosis of target chains from the cell surface. Sequence inspection reveals that the modulator of immune recognition (MIR) proteins contain an NH$_2$-terminal zinc finger of the plant homeodomain (PHD) subfamily, two transmembrane (TM) domains, and a C-terminal conserved region (CR). Here we examine the transmembrane topology and functional organization of MIR2. Both the PHD domain and the CR are disposed cytosolically and are essential for MIR-mediated endocytosis. MIR proteins form homo-oligomers; this activity is independent of the PHD and CR elements and maps instead to the TM regions. Analysis of chimeras between MIR1 and MIR2 reveals that the TM regions also mediate target selectivity. Mutations that ablate the PHD or CR regions generate dominant negative phenotypes for major histocompatibility complex class I endocytosis. These findings suggest a domain organization for the MIR proteins, with the TM regions involved in target selection and the cytosolic PHD and CR domains involved in the possible recruitment of cellular machinery that directly or indirectly regulates internalization of target molecules.

Herpesviruses are a family of large DNA viruses that are able to induce a persistent (usually lifelong) infection. To facilitate the production of such long term infections, herpesviruses have evolved multiple strategies to evade immune detection. Most commonly, this is achieved by interrupting the synthesis, assembly, or function of major histocompatibility complex class I (MHC-I)$^1$ molecules, key proteins involved in the recognition of infected cells by cytotoxic T lymphocytes (1, 2). Kaposi's sarcoma-associated herpesvirus (also known as human herpesvirus 8) is the etiologic agent of Kaposi's sarcoma and several other AIDS-related proliferative disorders (1, 2). We (3) and others (4–6) have shown that Kaposi's sarcoma-associated herpesvirus possesses two genes, K3 and K5, that encode protein products termed MIR1 and MIR2, respectively (for modulator of immune recognition). MIR1 and MIR2 are homologous proteins that are localized predominantly in the endoplasmic reticulum (ER) and lead to reduction of the levels of MHC-I chains present at the cell surface (3). Despite their predominantly ER localization, MIR1 and MIR2 do not affect the assembly, glycosylation, or transport of MHC-I chains in that organelle. Rather, they act to enhance the endocytosis of MHC-I chains from the cell surface, with the endocytosed chains subsequently targeted to the lysosome for proteolytic destruction (3).

This enhanced endocytosis of MHC-I does not reflect a generalized induction of endocytosis, since many other surface proteins known to undergo internalization, including the transferrin receptor and MHC class II molecules, are unaffected by the MIR proteins (3). However, MHC-I is not the sole target of the MIR protein family. Two other molecules involved in immune recognition can be down-regulated by MIR2 expression: B7.2, a cell surface protein involved in helper T cell activation (co-stimulation), and intracellular adhesion protein-1 (ICAM-1), an adhesion protein important for the formation of the immunological synapse (7, 8). Interestingly, neither of these two proteins is targeted by MIR1, indicating that there has been functional specialization of the MIRs during Kaposi's sarcoma-associated herpesvirus evolution.

Although the overall fate of the target surface immunoregulatory proteins is clear, our understanding of the molecular mechanisms by which MIR1 and MIR2 target these chains and mediate their endocytosis remains fragmentary. Analysis of the primary amino acid sequence of the MIR proteins shows that they contain a zinc finger domain of the plant homeodomain (PHD) subfamily at their amino terminus, along with two transmembrane (TM) domains near the center of the proteins (see Fig. 1A). Distal to TM domain II is a short sequence (termed the conserved region (CR)) that is also highly conserved between MIR1 and MIR2. PHD family zinc fingers, also known as leukemia-associated protein zinc fingers (9), have been implicated in protein-protein interactions (9–11) and are structurally similar to RING fingers (12). Mutations in the zinc finger region abrogate MIR function (8), but beyond this, little is known of structure-function relationships in the protein.

To gain a better understanding of which regions of the MIR proteins are important for function, we have examined the transmembrane topology and functional organization of MIR2. These studies show that the transmembrane domains of the protein play a key role in determining the target specificity of the protein. The PHD and CR elements are also critical for
function, and mutations targeted to these regions result in dominant negative inhibitors of MIR2 action. These findings suggest a domain organization for the MIR proteins, with its TM regions involved in target selection and the cytosolic PHD and CR domains involved in the possible recruitment of cellular machinery that could directly or indirectly regulate internalization of target molecules.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—CV-1, Phoenix, and 293T cells were grown in Dulbecco’s modified Eagle’s medium (DME-H21), and BJAB lymphoma cells were grown in RPMI medium 1640. All media were supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin. Transfections of CV-1 and Phoenix cells were performed using Fugene6 (Roche Molecular Biochemicals) according to the manufacturer’s suggested protocol. BJAB cells were transfected by electroporation (250 V, 950 microfarads) of 20 μg of plasmid DNA into 10^7 cells in 0.5 ml of serum-free medium (13). The cells were then transferred to complete RPMI, which was preincubated to 37 °C. The transfection efficiency of BJAB cells was routinely 30–50% under these conditions.

**Antibodies**—For immunofluorescence analysis, mouse anti-FLAG (Sigma) was used at a concentration of 20 μg/ml and rabbit anti-HA (Sigma) was used at a dilution of 1:50. Sheep anti-mouse IgG FITC conjugate and donkey anti-rabbit rhodamine conjugate. The proposed orientation of MIR2 within the ER membrane is shown in G.

**FIG. 1.** Orientation of MIR2 in the ER membrane. A, MIR1 and MIR2 amino acid sequences are aligned, with the PHD zinc finger, transmembrane domains 1 (TM1) and 2 (TM2), and the CR boxed. CV-1 cells were stably transduced with retrovirus vectors expressing FLAG-MIR2-HA (shown in B). The plasma membrane was selectively permeabilized with 5 mg/ml digitonin (C and D), which allows access of the antibody only to the cytosolic compartment, or the cells were completely permeabilized with 0.25% saponin (E and F). All samples were then coincubated with mouse anti-FLAG and rabbit anti-HA, washed, and coincubated with donkey anti-mouse IgG FITC conjugate and donkey anti-rabbit rhodamine conjugate. The proposed orientation of MIR2 within the ER membrane is shown in G.
CONSTRUCTION—All constructs were made by PCR amplification of MIR1, MIR2, or EGF2. Chimeric fusions between EGF2 and MIR2 truncation or MIR1 and MIR2 were made by amplification of overlapping PCR fragments. All expression constructs were introduced into the BamHI and NotI site of pCRII (Invitrogen). The amino FLAG-tagged/unaltered HA-tagged version (see Fig. 2A) of the MIR2 gene was subcloned from pCRII-FLAG-MIR2-HA vector into pBMM-ZIN (kindly provided by G. Nolan, Stanford University). This retroviral vector permits the expression of pCRII-FLAG-MIR2-HA and the neomycin resistance gene from a single bicistronic mRNA.

RESULTS

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B7.2, ICAM-1, and B7.1 (data not shown).

MIR2 truncation or MIR1 and MIR2 were made by amplification of overlapping PCR fragments. All expression constructs were introduced into the BamHI and NotI site of pCRII (Invitrogen). The amino acid sequence of MIR2 was cloned into the pBMM-ZIN vector (kindly provided by G. Nolan, Stanford University). This retroviral vector permits the expression of pCRII-FLAG-MIR2-HA and the neomycin resistance gene from a single bicistronic mRNA.

Retroviral Infection—Upon transfection with the retroviral vectors (pBMN), the Phoenix packaging cell line produces replication-defective viral particles that can be used to stably transduce CV-1 cells. Phoenix cells were transduced, and the virus-containing supernatant was harvested 48 h after the transfection, filtered through a 0.45-μm filter, and diluted with Polybrene (4 μg/ml final dilution). CV-1 cells (in six-well dishes) were infected with mouse anti-FLAG M2, mouse anti-HA, or mouse anti-GFP and then incubated with either mouse anti-FLAG M2 antibody, mouse anti-GFP, or rabbit anti-HA antibody. The antibody-bound proteins were then incubated with protein A/–agarose beads (Santa Cruz Biotechnology) to immunoprecipitate the antibody-bound tagged versions of MIR2. Immunoprecipitated proteins were separated on a 4–20% polyacrylamide gel; transferred to nitrocellulose; and Western blotted with either mouse anti-FLAG M2, mouse anti-HA, or mouse anti-GFP and then incubated with goat-anti-mouse Ig HRP and visualized with Luminol (Santa Cruz Biotechnology).

Flow Cytometry Analysis—Cells were washed in 1% PBS plus BSA and incubated with specific mAbs (1 μg/106 cells) for 60 min at 4°C. Cell surface fluorescence was analyzed with a Becton Dickinson FACScalibur (Becton Dickinson, San Jose, CA), and cell populations were analyzed with CELLQuest (Becton Dickinson).

Dominant Negative Assay—293T cells in six-well dishes were transduced with 2 μg of vector alone or 0.5 μg of pCRII-FLAG-MIR2-EGFP and either 1.75 μg of pCRII.1 vector or pCRII-FLAG-MIR2-ΔPHD. After 36 h, the cells were removed from their dishes by using cell dissociation buffer (Invitrogen) according to the manufacturer’s instructions. The cells were washed with PBS, incubated in mouse anti-FLAG (APC-conjugated; Caltag Laboratories) in PBS plus 1% BSA at a dilution of 1:1500. As shown in Fig. 1B, a form of MIR2 was constructed in which a FLAG epitope tag was placed at the amino terminus of the molecule and an HA tag was placed between the two transmembrane domains. To determine the transmembrane topology of the MIR2 protein, we employed a recently described system involving selective permeabilization-based immunofluorescence (14, 15).

As shown in Fig. 1B, a form of MIR2 was constructed in which a FLAG epitope tag was placed at the amino terminus of the molecule and an HA tag was placed between the two transmembrane domains. This doubly tagged protein is fully functional for B7.2 and ICAM-1 down-regulation (data not shown). CV-1 cells were stably transduced with a retroviral vector encoding this version of MIR2. These cells were fixed and subjected to permeabilization with either (i) digitonin, which selectively permeabilizes the plasma membrane but leaves the ER membrane intact, or (ii) saponin, which under these conditions permeabilizes both the plasma membrane and the intracellular membranes of the cell (16, 17). Following detergent treatment, cells were stained for the FLAG tag (detected by FITC-conjugated secondary antibody) or the HA tag (stained with Texas Red-conjugated secondary antibody). Digitonin permeabilization concentration of 4 μg/ml. For FACS analysis, mouse anti-HLA class I (APC-conjugated or FITC-conjugated; Caltag Laboratories) and mouse anti-B7.2 (phycoerythrin-conjugated; Caltag Laboratories) were used at a dilution of 1:300. Western blotting used mouse anti-FLAG M2 (Sigma) at a dilution of 1:400, mouse anti-GFP (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:300 or rabbit anti-HA (Sigma) at a dilution of 1:256 or mouse anti-GFP. Secondary antibodies for Western blots were HRP-conjugated goat anti-mouse Ig and donkey anti-rabbit (Santa Cruz Biotechnology) that were both used at dilutions of 1:1500.

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allows immunofluorescent tagging of only the FLAG tag (Fig. 1C) and not the HA tag (Fig. 1D), suggesting that the amino terminus of MIR2 is in the cytosol while the intermembranous loop is in the lumen. This is confirmed by permeabilization with saponin (Fig. 1, E and F), which allows immunofluorescent staining of both of the tags. This orientation disposes the zinc finger/PHD domain on the cytoplasmic face of the membrane (Fig. 1G). Since PHD domains are known to mediate protein-protein interactions (9, 10, 16), this would place it in a context where it could be available for interactions with cytosolic constituents. Similar studies with a C-terminal tagged version of MIR2 show that the C terminus is also in the cytoplasm (not shown).

Western blotting of MIR2 in whole cell lysates often shows high molecular weight bands (Fig. 2A), raising the possibility that MIR2 may form homo- or hetero-oligomeric structures. To test if MIR2 can homo-oligomerize, the protein was tagged with either a FLAG epitope or an HA epitope at its amino terminus. The tagged versions of MIR2 were transiently transfected with the indicated constructs from Fig. 3A. After 36 h, the cells were stained with mouse anti-HLA Class I conjugated to APC and mouse anti-B7.2 conjugated to phycoerythrin and analyzed by flow cytometry, gating on those cells that were transfected (either EGFP-positive or FLAG-positive cells), using a Becton Dickinson FACS Calibur.

Fig. 3. Regions of MIR2 required for function. A, schematic depiction of MIR2 deletion mutants. These constructs were fused to EGFP (or tagged with FLAG in the case of MIR2ΔTMΔC) and ligated into the pCR3.1 eukaryotic expression vector for the studies of B–G. BJAB cells were transiently transfected with the indicated constructs from Fig. 3A. After 36 h, the cells were stained with mouse anti-HLA Class I conjugated to APC and mouse anti-B7.2 conjugated to phycoerythrin and analyzed by flow cytometry, gating on those cells that were transfected (either EGFP-positive or FLAG-positive cells), using a Becton Dickinson FACS Calibur.

2 L. Coscoy, unpublished data.

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The Zinc Finger and Downstream CR: Cytosolic Domains Required for MIR2 Function—To map regions of MIR2 that are important for its function, we generated a series of MIR2 truncations and examined their ability to down-regulate surface MHC-I and B7.2 (Fig. 3A). As outlined in the legend to Fig. 3, most of the truncations were expressed as MIR2-GFP fusion proteins. (Wild-type MIR2 is fully functional when fused to GFP in this fashion (Fig. 3C).) Following transfection, the FACS analysis was gated on transfected (e.g. GFP-positive) cells, whose surface MHC-I and B7.2 levels were then exam-
ined. All truncations were stably expressed as judged by immunofluorescence analysis and flow cytometry (not shown). As expected from previous results (8), a deletion of the amino terminus to the first transmembrane region (thereby removing the PHD zinc finger) abrogates the ability of MIR2 to down-regulate either MHC class I or B7.2 from the cell surface (Fig. 3D). However, the PHD zinc finger by itself is not sufficient to induce the removal of target molecules from the cell surface (Fig. 3E). Consistent with this, removal of the entire C-terminal cytosolic tail also destroys function, indicating that even a PHD zinc finger tethered to the membrane does not suffice to up-regulate endocytosis (Fig. 3F). This is despite the fact that it is stably expressed and accumulates in the ER as efficiently as WT MIR2 (not shown). However, the addition of a small portion of the downstream cytosolic tail encompassing the CR sequences restores full function for B7.2 down-regulation and partial function for MHC-I down-regulation (Fig. 3G). Thus, each cytosolic domain contains conserved sequences that are necessary, but not sufficient, for MIR function.

**The Transmembrane Regions of MIR2 Are Involved in Target Selection**—To map regions of MIR responsible for determining the selection of its targets, we took advantage of the fact that MIR2 can down-regulate B7.2 and ICAM-1 as well as MHC-I, whereas MIR1 can only down-regulate MHC-I chains (7, 8). To determine which regions of MIR2 account for its expanded target range, we constructed chimeras between these two highly related viral proteins (Fig. 4A) and analyzed the ability of the resulting chimeras to down-regulate surface MHC-I, B7.2, and ICAM-1 following transient transfection of BJAB cells (Fig. 4B). (Unlike in Fig. 3, the FACS analysis of Fig. 4B does not gate on the transfected cells; it displays the entire BJAB population, about 30–50% of which are transfected). A chimeric molecule that replaces the amino terminus of MIR2 with that of MIR1 does not affect the down-regulation of B7.2 (Fig. 4B, construct I). Similarly, replacement of both the N- and C-terminal cytosolic domains of MIR2 with their cognate regions of MIR1 does not affect the target selectivity of the protein; like WT MIR2, this chimera also down-regulates both MHC-I and B7.2 (Fig. 4B, construct III). Thus, this selectivity must reside in the TM domains and intermembranous loop of MIR2. As expected, the chimera encoded by construct II, which bears the TM and intermembranous regions of MIR1, has the target range of MIR1. Finally, replacement of the intermembranous loop of MIR2 with that of MIR1 has no impact on MIR2 function (data not shown), suggesting that it is the TM domains of MIR2 that account for its target selectivity. In all cases, the abilities of the chimeras to down-regulate B7.2 and ICAM-1 were inseparable (data not shown), so only the B7.2 data are depicted here.

**Truncated Forms of MIR2 Lacking the PHD or CR Regions Can Act as Dominant Negatives**—The above experiments establish that there are several domains of MIR2 that are required for the MIR2-mediated endocytosis of molecules from the cell surface; some of these affect target selectivity, whereas others affect functions common to all targets. In other multifunctional proteins, deletion of a single effector domain often generates dominant negative mutants. Such mutants can have many bases: formation of nonfunctional hetero-oligomers, competition for binding to an essential partner, etc. Accordingly, we sought to establish whether deletion of one domain of MIR2 might engender a dominant negative mutant. For this, we initially selected the MIR2 ΔPHD mutant (see Fig. 3A), which lacks the NH2-terminal cytosolic domain (including the conserved PHD domain). Cells were cotransfected with a fixed quantity of WT MIR2 and either the empty vector or a vector expressing the ΔPHD mutant. As expected, wild-type MIR2 expression reduces the surface level of MHC class I when cotransfected with vector alone (Fig. 5A). When MIR2 was cotransfected with the MIR2 ΔPHD mutant, the surface level of MHC class I was restored to a level comparable with the vector-transfected controls (Fig. 5B), indicating that MIR2 ΔPHD acts as a dominant negative mutant. As a specificity control, expression of MIR2 ΔPHD did not restore surface levels of MHC-I that had been reduced by cotransfection with a vector expressing the herpes simplex ICP47 protein (Fig. 5C). ICP47 acts by inhibiting the TAP transporter, preventing the loading of assembling MHC-I chains with antigenic peptides and thereby blocking egress of MHC-I chains from the ER (17, 18). These results indicate that the negative effects of the ΔPHD mutant are selective for MHC-I down-regulation mediated by MIR.

Similar findings were made with mutant MIR2ΔC1, which lacks the C-terminal cytosolic region including the CR element (see Fig. 3A). As shown in Fig. 6A, WT MIR2 cotransfected with an empty expression vector produced the expected reduction in cell surface MHC-I chains. However, this reduction could be
FIG. 5. An NH$_2$-terminal truncated form of MIR2 can act as a dominant negative to block MIR2 down-regulation of HLA class I from the cell surface. 293T cells were transfected with either pCR3.1, pBKM-MIR2-GFP and pCR3.1 (A) or pBKM-MIR2-EGFP and pCR3.1 MIR2 ΔPHD (B). After 36 h, cells were stained with mouse anti-HLA class I conjugated to APC and analyzed by flow cytometry using a Becton Dickinson FACSCalibur. GFP-positive cells were gated, and the level of HLA class I on the cell surface was measured. C, MIR2ΔPHD does not affect MHC-I down-regulation by HSV ICP47.

FIG. 6. A C-terminal truncation of MIR2 has a dominant negative phenotype. 293T cells were transfected with pCR3.1 (four times), pCR3.1-MIR2 (FLAG-tagged) (one time) (A) or pCR3.1-MIR2 (FLAG-tagged) (one time) and pCR3.1 MIR2 ΔC1 (four times) (B). After 36 h, cells were stained with mouse anti-HLA class I conjugated to APC and analyzed by flow cytometry using a Becton Dickinson FACSCalibur. FLAG-positive cells were gated, and the levels of HLA class I on the cell surface were measured.

abrogated by coexpression of the MIR2ΔC1 mutant (Fig. 6B). Thus, deletion of either the N- or C-terminal cytosolic tail of MIR2 generates mutants that can block the ability of the WT protein to regulate the selective endocytosis of its MHC-I protein target.

**DISCUSSION**

These data establish that MIR2, a novel viral mediator of immune evasion, is an oligomeric, type III membrane protein with two transmembrane domains flanked by cytosolic regions at the N and C termini. Each cytosolic tail contains conserved sequences; the NH$_2$-terminal region harbors a conserved zinc finger of the PHD subfamily, whereas the C-terminal tail contains a shorter CR of unknown function. Analysis of MIR1/MIR2 chimeras indicates that the transmembrane regions are the major determinants of target selectivity. We do not know whether one or both TM regions are required for this activity, nor do we yet understand the biochemical basis for target selection. The simplest model would be that the TM regions of MIR2 interact, directly or indirectly, with the TM domains of its target(s). Consistent with this idea, we and others (6) have recently found that MIR2 can down-regulate human but not mouse MHC-I chains, and chimeras between human and mouse MHC-I implicate the TM domain of the target in conferment of MIR2 regulation (19). However, more indirect models are also possible.

This raises an interesting conundrum, since MIRs localize principally in the ER (3, 5, 20) but their targets are endocytosed from the plasma membrane (3). It is possible that the interaction between effector and target takes place in the ER, leading to “marking” of the target chains for later endocytosis. However, in an earlier study (3), we showed that MHC-I chains made in the absence of MIR proteins could be down-regulated following subsequent introduction of MIR expression vectors. If so, this would make “ER marking” an unlikely model. We think it more likely that a small fraction of MIR chains escape the ER and travel to the plasma membrane, where they can carry out their function. However, since flow cytometric and surface labeling experiments$^3$ have been unable to reproducibly observe such chains above the background level, we estimate that putative surface MIR proteins would have to represent $<$5% of the total intracellular pool.

Our analysis of deletion mutants and chimeras supports the notion that the MIR2 protein is composed of multiple domains, with the TM regions determining target selection and the cytosolic regions mediating other function(s) that are common to the endocytosis of all target proteins. Our experiments do not suffice to define the nature of these cytosolic function(s) but indicate that, whatever they may be, both cytosolic domains are required for their action. Deletion of cytosolic regions not only inactivates MIR2 function but, consistent with the aforementioned domain organization, creates dominant negative mutants. Since only the TM and intermembranous regions are required for oligomer formation, such phenotypes might result from the creation of mixed, nonfunctional oligomers with WT MIR2. Alternatively (or additionally), the mutants might act by titrating away a putative essential cellular cofactor required for either interaction with the substrate or downstream interactions of a putative MIR-substrate complex with the endocytic machinery. We are currently screening for host factors that

$^3$ L. Coscoy and D. Ganem, unpublished observations.
might interact with these regions, using a variety of genetic and biochemical screens. Given the remarkable selectivity of MIR-regulated endocytosis, delineation of the pathways that are engaged by MIR proteins can be expected to further inform our understanding of the cell biology of regulated endocytosis.

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REFERENCES
1. Whitby, D., and Boshoff, C. (1998) Curr. Opin. Oncol. 10, 405–412
2. Ganem, D. (1997) Cell 91, 157–160
3. Coscoy, L., and Ganem, D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8651–8656
4. Haque, M., Ueda, K., Nakano, K., Hirata, Y., Parravicini, C., Corbellino, M., and Yamanishi, K. (2001) J. Gen. Virol. 82, 1175–1180
5. Ishido, S., Wang, C., Lee, B. S., Cohen, G. B., and Jung, J. U. (2000) J. Virol. 74, 5300–5309
6. Stevenson, P. G., Efstathiou, S., Doherty, P. C., and Lehner, P. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8455–8460
7. Coscoy, L., and Ganem, D. (2001) J. Clin. Invest. 107, 1599–1606
8. Ishido, S., Choi, J. K., Lee, B. S., Wang, C., DeMaria, M., Johnson, R. P., Cohen, G. B., and Jung, J. U. (2000) Immunity 13, 365–374
9. Linder, B., Newman, R., Jones, L. K., Debernardi, S., Young, B. D., Freemont, P., Verrijzer, C. P., and Saha, V. (2000) J. Mol. Biol. 299, 369–378
10. Yokochi, G. S., and Ayer, D. E. (2001) Mol. Cell. Biol. 21, 4110–4118
11. Fasová, J., Martinez-Yamout, M., Dyson, H. J., and Wright, P. E. (2000) J. Mol. Biol. 304, 723–729
12. Capilli, A. D., Schultz, D. C., Rauscher, F. I., and Borden, K. L. (2001) EMBO J. 20, 165–177
13. Lagunoff, M., Lukac, D. M., and Ganem, D. (2001) J. Virol. 75, 5891–5898
14. Mazaitis, H., Korza, G., Hand, A. R., Gerard, C., and Ozols, J. (1999) J. Biol. Chem. 274, 14122–14129
15. Lin, S., Cheng, D., Liu, M. S., Chen, J., and Chang, T. Y. (1999) J. Biol. Chem. 274, 23276–23285
16. Schultz, D. C., Friedman, J. R., and Rauscher, F. J., III (2001) Genes Dev. 15, 428–443
17. Hill, A., Jugovic, P., York, J., Russ, G., Bennink, J., Yewdell, J., Ploegh, H., and Johnson, D. (1995) Nature 375, 411–415
18. Fruh, K., Ahn, K., Djballah, H., Sempe, P., van Endert, P. M., Tampe, R., Peterson, P. A., and Yang, Y. (1995) Nature 375, 415–418
19. Coscoy, L., Sanchez, D., and Ganem, D. (2001) J. Cell Biology 155, 1265–1274
20. Haque, M., Chen, J., Ueda, K., Mori, Y., Nakano, K., Hirata, Y., Kanamori, S., Uchiyama, Y., Inagi, R., Okune, T., and Yamanishi, K. (2000) J. Virol. 74, 2867–2875
