BclAF1 restriction factor is neutralized by proteasomal degradation and microRNA repression during human cytomegalovirus infection

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Cell proteins can restrict the replication of viruses. Here, we identify the cellular BclAF1 protein as a human cytomegalovirus restriction factor and describe two independent mechanisms the virus uses to decrease its steady-state levels. Immediately following infection, the viral pp71 and UL35 proteins, which are delivered to cells within virions, direct the proteasomal degradation of BclAF1. Although BclAF1 reaccumulates through the middle stages of infection, it is subsequently down-regulated at late times by miR-UL112-1, a virus-encoded microRNA. In the absence of BclAF1 neutralization, viral gene expression and replication are inhibited. These data identify two temporally and mechanistically distinct functions used by human cytomegalovirus to down-regulate a cellular antiviral protein.

innate immunity | intrinsic immunity | proteasome | UL82 | miRNA

Human cytomegalovirus (HCMV) is a widespread β-herpesvirus that can cause disease in immunologically immature or compromised individuals (1). Its genome contains about 200 protein-coding genes (2), large noncoding RNAs (3), and miRNAs (4–6); and numerous HCMV gene products have been shown to function in immune evasion (7).

Recently, the importance of cell-intrinsic defenses against invading pathogens (8), as opposed to the more global adaptive and the more regional innate immune functions, has become increasingly apparent. Multiple intrinsic defenses have been characterized that protect against viruses, including restrictions imposed by Trim 5 alpha (9), Apobec-3G (10), Tetherin/Bts-2 (11, 12), Daxx (13), PML (14), and Sp100 (15). In each case, a single mechanism has been identified by which the virus counteracts the cellular defense protein, most commonly by viral proteins that induce their proteasomal degradation (16–18). For example, the HCMV pp71 protein degrades Daxx, inactivating a cellular intrinsic immune defense and initiating lytic replication (19).

Here, we identify a previously unknown impediment to HCMV infection instituted by BclAF1 (Bcl-2 associated factor 1; also called Btf for Bcl-2 associated transcription factor), a nuclear protein implicated in apoptosis, transcriptional regulation, RNA processing, and the export of mRNA from the nucleus (20, 21). BclAF1 protein levels drop at the start of infection due to targeted degradation by virion-delivered pp71 and UL35. BclAF1 levels reaccumulate as infection proceeds, but decrease again at late times due to down-regulation by the virus-coded miRNA, miR-UL112-1. Reduced BclAF1 enhances HCMV gene expression, and elevated levels inhibit viral replication. This work identifies BclAF1 as a viral restriction factor that is targeted by two mechanistically independent HCMV functions.

Results

Global Screens Predict BclAF1 Is a Target of an HCMV Protein and miRNA. HCMV pp71 and miR-UL112-1 both modulate viral immediate-early (IE) gene expression. The protein activates IE genes at the onset of the viral replication program (13, 22) and the miRNA attenuates IE expression during the late phase of infection (23, 24). To further define mechanisms through which these factors control HCMV replication, we performed unbiased searches for factors involved in their function.

The first screen was designed to detect cellular proteins associated with pp71. We isolated S-affinity-tagged pp71 from HeLa nuclear extracts using S-protein agarose, and separated the captured proteins by electrophoresis and identified them by mass spectrometry (MS) (Fig. S1). Tagged GFP was analyzed as a control. In two experiments, we identified at least 2 peptides corresponding to 55 different proteins (Table S1). We performed a second screen to detect cellular proteins whose levels decreased in the presence of miR-UL112-1. Human fibroblasts were transduced with a lentivirus encoding the miRNA or a control empty vector, labeled using the SILAC (stable isotope labeling of amino acids in cell culture) procedure and analyzed by MS (Fig. S2). A total of 154 proteins whose levels are decreased in the presence of miR-UL112-1 were identified in each of two experiments (Table S2).

The two screens identified four proteins in common: the transcriptional repressor BclAF1, the RNA helicase DHX9, the phosphatase subunit PP1-B, and the vesicular transport-associated myosin 1 light chain. We focused our efforts on BclAF1, because it is known to suppress the efficiency with which the γ-herpesvirus, KSHV, reactivates from latency and it is targeted by a KSHV-encoded miRNA (25). As both pp71 (13, 26–29) and miR-UL112-1 (23, 24, 30, 31) decrease the steady state levels of their targets, we tested their effects on BclAF1 in fibroblasts infected with a laboratory-adapted strain (AD169) or clinical isolates (TB40/E and FIX) of HCMV.

HCMV Virion Proteins Induce BclAF1 Degradation. pp71 delivered to fibroblasts by the virion directs the degradation of Daxx and the retinoblastoma protein (19). To determine whether BclAF1 suffers the same fate, we used low multiplicity infections, which extend the pre-IE phase of infection but nevertheless deliver virion proteins to most cells in the culture due to the high percentage of noninfectious particles in virus preparations (13). BclAF1, like Daxx, levels dropped rapidly and substantially before detectable IE1 accumulation after infection with each of the HCMV strains tested, then rebounded to a level approximating mock-infected

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cells as infection proceeded (Fig. 1*A*). The drop in BclAF1 levels resulted from proteasomal degradation, because the amount of the protein remained constant in infected cells treated with the proteasome inhibitor lactacystin (Fig. 1*B*). Proteasome inhibition caused a decrease in IE1 protein accumulation, consistent with the ability of BclAF1 (see below) and Daxx (13) to inhibit its expression. Virion constituents, as opposed to de novo synthesized proteins, were responsible for BclAF1 degradation as its levels were decreased in cells infected in the presence of cycloheximide, an inhibitor of translation (Fig. 1*C*).

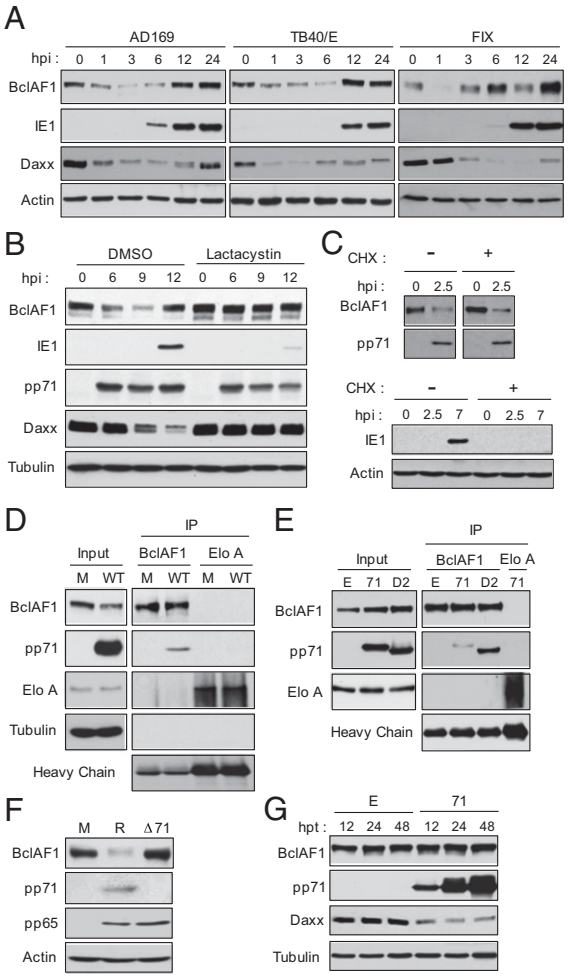

Fig. 1. BclAF1 is degraded during the pre-IE phase of HCMV infection in a pp71- and proteasome-dependent manner. (*A*) Fibroblasts were infected with the AD169, TB40/E, or FIX strain of HCMV (0.1 pfu per cell), and proteins were analyzed after various time intervals (hpi) by Western blot with the indicated antibodies. Actin serves as a loading control. (*B*) Fibroblasts were infected with AD169 (0.5 pfu per cell), treated with solvent, DMSO, or the proteasome inhibitor Lactacystin at the time of infection and proteins were analyzed by Western blot. (*C*) Fibroblasts were either treated (+) or not (-) with cycloheximide (CHX) beginning 3 h before infection with AD169 (0.1 pfu per cell), and proteins were analyzed at the indicated times by Western blot. (*D*) Fibroblasts were mock infected (M) or infected (WT) with AD169 (3 pfu per cell) and at 1 hpi analyzed by Western blot with the indicated antibodies (input) or subjected to immunoprecipitation (IP) with a BclAF1- or Elongin A (Elo A)-specific antibody before Western blot analysis. Tubulin and the antibody heavy chain serve as loading controls. (*E*) Fibroblasts were transduced with recombinant adenoviruses (rAD) expressing the indicated protein (E, empty; 71, wild-type pp71; D2, Did2-3 mutant pp71), and proteins were analyzed 24 h later by Western blot. (*F*) Fibroblasts either mock infected (M) or infected with the pp71-null (Δ71) or revertant (R) virus, and proteins were analyzed by Western blot 6 h later with the indicated antibodies. pp65 serves as a control for viral entry. (*G*) Fibroblasts were transduced with the indicated rADs, and proteins were analyzed by Western blot.

These results mimic the pp71-mediated disappearance and subsequent reappearance of Daxx and the retinoblastoma protein after HCMV infection (13, 32). To validate the interaction of ectopically expressed pp71 with BclAF1 that was detected by MS, we reversed the immunoaffinity purification and monitored the association in HCMV-infected fibroblasts (Fig. 1*D*) and in cells transduced with a recombinant adenovirus that expresses pp71 (Fig. 1*E*). The viral protein interacted with BclAF1 both in the context of infection and in transduced cells, independently of other viral factors. A pp71 mutant unable to associate with (33) or degrade (13) Daxx coprecipitated with BclAF1 more efficiently than the wild-type protein (Fig. 1*E*), showing that pp71 interacts with the two cell proteins through different sequence elements. BclAF1 failed to coprecipitate with Elongin A, a negative control. We next asked whether pp71 was both necessary and sufficient for BclAF1 degradation, as is the case for Daxx (13). A pp71-null virus (34) did not alter the levels of BclAF1, whereas a revertant virus expressing pp71 induced its degradation (Fig. 1*F*), showing that pp71 is required. However, pp71 expressed from a recombinant adenovirus was unable to induce BclAF1 degradation, although it was still able to degrade Daxx (Fig. 1*G*).

Thus, pp71 is necessary but not sufficient to degrade BclAF1. pp71 interacts with at least three other virion proteins: UL32, UL35, and UL94 (35, 36). Like pp71, UL35 enhances IE gene expression (35), so we explored a potential role for UL35 in BclAF1 degradation. Antibody to BclAF1 coprecipitated both viral proteins after transfection of cells with expression vectors (Fig. 2*A Left*); and a greater reduction in BclAF1 levels was evident in cells expressing UL35 or UL35 and pp71 together compared with cells with pp71 alone (Fig. 2*A Right*). We compared endogenous BclAF1 levels in cells transfected with vectors expressing viral proteins to an empty vector control (Fig. 2*B*), and densitometric analysis revealed that cells expressing only pp71 contained statistically indistinguishable levels of BclAF1 compared with control cells, cells expressing UL35 had significantly reduced levels of the protein, and cells receiving both viral proteins exhibited the greatest reduction (Fig. 2*C*). To confirm the requirement for UL35, we infected fibroblasts with a UL35-deficient virus (37), and found that the mutant reduced BclAF1 levels to a lesser extent than its wild-type parent (Fig. 2*D*). We infected cells at two multiplicities with the UL35-mutant virus to ensure efficient delivery of pp71 and, even when excess pp71 was delivered, BclAF1 was reduced to a lesser extent than seen for the wild-type virus (Fig. 2*D*). In total, these experiments indicate that pp71 and UL35 delivered by infecting virions function together to bind and induce the proteasomal degradation of BclAF1.

HCMV miR-UL112-1 Reduces BclAF1 Levels. In addition to the role of virion proteins in BclAF1 degradation discovered in the proteomic screen, the SILAC screen predicted a second mode of BclAF1 modulation via miR-UL112-1, and the BclAF1 mRNA 3′ UTR contains a potential miR-UL112-1 binding site (Fig. 3*A*). To test whether miR-UL112-1 can reduce BclAF1 levels, we initially generated fibroblasts over expressing the miRNA from a lentivirus vector. BclAF1 protein levels were substantially reduced in miR-UL112-1–expressing cells, but not in cells receiving a control lentivirus (Fig. 3*B*). To further test for an effect of miR-UL112-1, we cloned the wild-type BclAF1 3′UTR or a derivative of the UTR containing a mutation within the miR-UL112-1 seed sequence, downstream of a luciferase reporter gene (Fig. 3*C*). The miRNA significantly inhibited the wild-type but not the mutant 3′ UTR, demonstrating that the 3′UTR of BclAF1 is a direct target of miR-UL112-1.

Although the pp71/UL35-mediated degradation of BclAF1 occurs at pre-IE stages, potential effects of miR-UL112-1 on the levels of this protein should not be evident until much later after

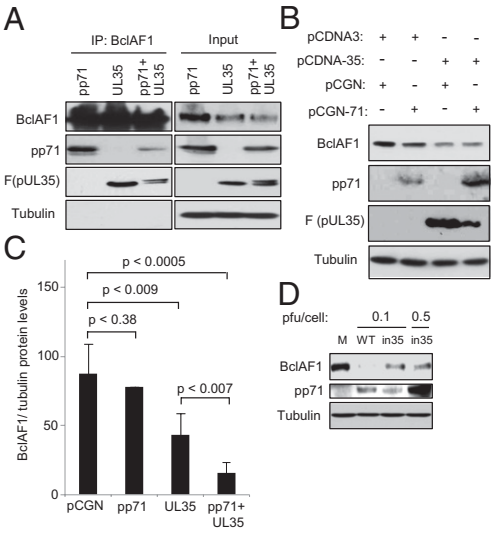

Fig. 2. pp71 and UL35 cooperate to induce the degradation of BclAF1. (*A*) 293 cells were transfected with expression plasmids and proteins were analyzed 48 h later by Western blot either directly (input) or after immunoprecipitation (IP) with BclAF1-specific antibody. (*B*) 293 cells were transfected with expression plasmids and proteins were analyzed 48 h later by Western blot. pCDNA3 and pCGN are empty vectors. (*C*) Protein bands from three independent experiments (as in *B*) were quantified with ImageQuant software. The level of BclAF1 relative to tubulin for each transfection condition is displayed with average deviation and Student's *t* test *P* value. (*D*) Human fibroblasts were mock infected (M) or infected with wild-type AD169 (WT) or a UL35 transposon insertion mutant (in35). Proteins were analyzed at 3 hpi by Western blot.

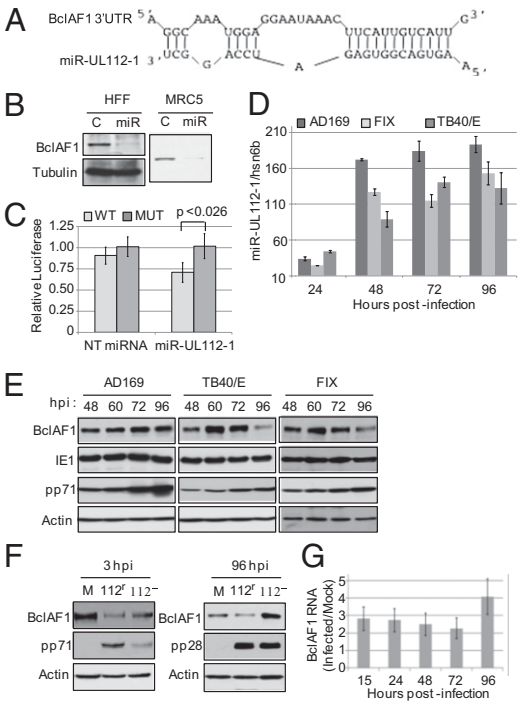

Fig. 3. miR-UL112-1 expression reduces the levels of BclAF1. (*A*) Sequence complementarity between miR-UL112-1 and the 3′ UTR of the BclAF1 message as determined by TargetScan analysis. (*B*) Primary fibroblasts (HFF) or MRC5 cells were transduced with either a control (C) lentivirus or one expressing miR-UL112-1 (miR), and proteins were analyzed by Western blot. Tubulin serves as a loading control. (*C*) HeLa cells were transfected with luciferase reporters containing either a wild-type BclAF1 3′ UTR or a derivative in which the putative miR-UL112-1 seed sequence was mutated, as well as either a control RNA (NT) or a miR-UL112-1 precursor RNA for 48 h. Relative luciferase activities (firefly/renilla) are presented with SDs and Student's *t* test *P* value. (*D*) Fibroblasts were infected with the AD169, TB40/E, or FIX strain of HCMV (3.0 pfu per cell), and miR-UL112-1 levels were determined after various time intervals by qRT-PCR and normalized to the steady state levels of a cellular small nucleolar RNA, hsn6B. (*E*) Cells were infected as in *D* and proteins were analyzed by Western blot. Actin serves as a loading control. (*F*) Fibroblasts were mock infected (M) or infected (3 pfu per cell) with BFX*sub*112-1^{-} (112^{-}) or its revertant BFX*sub*112-1^{r} (112r), and proteins were analyzed by Western blot after 3 or 96 h. (*G*) Fibroblasts were infected with the FIX strain (3 pfu per cell), and BclAF1 RNA was quantified by qRT-PCR at the indicated times, normalized to GAPDH, and displayed relative to mock-infected cells with the SEMs.

infection, as the miRNA accumulates to substantial levels beginning at 48 h postinfection (hpi) (24) (Fig. 3*D*). miRNAs curtail de novo protein expression but do not affect protein stability, and thus a detectable drop in the steady-state level of a targeted protein can be delayed from the time when the miRNA first accumulates. Therefore, we monitored BclAF1 levels during the late phase of infection (Fig. 3*E*). BclAF1 levels decreased at 96 hpi with the TB40/E and FIX clinical strains of HCMV. Surprisingly, however, this late drop was not evident upon infection with the AD169 laboratory strain, even though the sequence of miR-UL112-1 is identical in the three strains and its expression level was similar (Fig. 3*D*). A clinical HCMV isolate deleted for miR-UL112-1 (24) failed to down-regulate BclAF1 levels late but not at pre-IE times after infection (Fig. 3*F*), when BclAF1 loss is mediated by pp71 and UL35. A revertant, in which the miRNA mutation was repaired, down-regulated BclAF1 normally. Finally, BclAF1 mRNA levels are not decreased during infection (Fig. 3*G*), consistent with the view that miRUL112-1 inhibits the translation of BclAF1 mRNA.

BclAF1 Restricts HCMV IE Gene Expression and Spread. We next assayed the consequences of BclAF1 function on the viral replication cycle in cells engineered to contain reduced or elevated levels of the protein. We tested three BclAF1-specific siRNAs, and observed decreased BclAF1 and increased IE1 protein levels following HCMV infection in each case (Fig. 4*A*). Reduced BclAF1 did not affect the levels of pp71 protein, which was delivered to cells in virions. We then infected cells expressing an shRNA directed against BclAF1, and observed increased IE1 expression at each time tested after HCMV infection (Fig. 4*B*). Although knockdown of BclAF1 partially restored IE1 expression upon infection with a pp71-null virus (Fig. 4*C*), it failed to rescue the growth defect (i.e., the production of progeny) for either the pp71-null or UL35-mutant virus (Fig. 4*D*). Presumably, additional substrates of these proteins contribute to the mutant phenotypes. We further probed the restrictive role of BclAF1 in HCMV infection by generating cells that expressed a BclAF1 transcript with a 3′UTR lacking miR-UL112-1 binding sites (Fig. 4*E Left*). Expression of BclAF1, which was resistant to miR-UL112-1 repression, prevented HCMV spread without noticeably altering cellular viability (Fig. 4*E Right*). We conclude that BclAF1 represses viral IE gene expression and as such is a physiologically relevant substrate for neutralization by pp71 and UL35.

Discussion

Here, we identify BclAF1 as an HCMV restriction factor targeted by two mechanistically and temporally distinct viral countermeasures (Fig. S3). The steady-state levels of BclAF1 are reduced at the start of HCMV infection through proteosome-dependent degradation by virion-delivered pp71 and UL35 (Figs. 1 and 2). pp71 is well known to induce the proteasomal degradation of proteins to which it binds through a mechanism that apparently does not require ubiquitination (38). The UL35

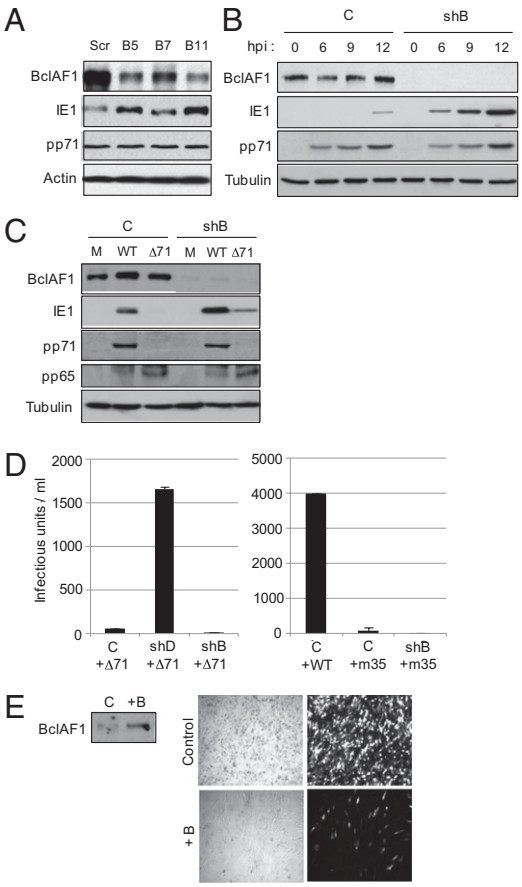

Fig. 4. BclAF1 restricts HCMV IE gene expression and replication. (*A*) Fibroblasts were transfected with the indicated BclAF1-specific (B5, B7, or B11) or nonspecific (scr) siRNAs, and infected with the AD169 strain (0.1 pfu per cell) 24 h later. At 7 hpi, proteins were analyzed by Western blot. (*B*) Control (C) or BclAF1-knockdown (shBclAF1) fibroblasts were infected with AD169 (0.1 pfu per cell), and proteins were analyzed at the indicated times after infection by Western blot. (*C*) Control or shBclAF1 fibroblasts were mock infected (M) or infected with wild-type (WT) or pp71-null (Δ71) AD169 (0.05 pfu per cell), and proteins were analyzed by Western blot 24 h later. (*D*) Fibroblasts, C, or derivatives expressing shRNAs targeted to Daxx (shD) or BclAf1 (shB) were infected (0.1 pfu per cell) with AD169 WT, Δ71 or in35, and the virus yield was quantified 8 d later on TERT-HF cells. Average pfu/mL released after 8 d of infection are displayed with SE. (*E*) MRC5 cells were transduced with a control lentivirus, C, or one expressing BclAF1 (+B), and analyzed by Western blot with the indicated antibodies (*Left*). Cells were infected with FIX*wt*-GFP, and confocal and epifluorescence images of representative cultures were captured at 9 d postinfection (*Right*).

protein has not been previously implicated in protein degradation, although it recently was reported to interact with members of a ubiquitin ligase complex (39). After the IE phase, BclAF1 levels reaccumulate, but subsequently decline again late after infection due to translational repression by miR-UL112-1 (Fig. 3). BclAF1 was identified in screens (Figs. S1 and S2) for pp71-interacting proteins and miR-UL112-1–regulated genes. Both screens, although not exhaustive, provide a wealth of targets (Tables S1 and S2) that may provide further clues to the roles pp71 and miR-UL112-1. Several known substrates of these viral factors (Rb and Daxx for pp71; MICB for miR-UL112-1) were not identified, indicating that the screens are not comprehensive. Furthermore, the levels of pp71 and miR-UL112-1 in the cells used for screening do not necessarily mimic the changing levels observed during viral infection. Thus, the predicted targets of pp71 and miR-UL112-1 require further validation in HCMV-infected cells, as provided here for BclAF1.

At the start of infection, BclAF1 prevents the accumulation of the IE1 protein, and as such is a component of the intrinsic cellular defense against HCMV (13) along with the PML-NB resident proteins PML, Sp100, and Daxx (40). Our data provide a clear rationale for the degradation of BclAF1 by virion proteins: to promote IE gene expression and initiate lytic replication. It is unclear whether the reaccumulation of BclAF1 during the middle stages of HCMV infection indicates that it plays some positive role at this time or simply mimics other known pp71 substrates such as the retinoblastoma protein and Daxx (13, 32) that, for unknown reasons, also reaccumulate as infection proceeds. Additionally, because it regulates the reactivation of latent KSHV infections (25), it will be interesting to determine whether BclAF1, and perhaps newly expressed virion proteins, also regulate reactivation from HCMV latency (41).

A physiologic rationale for the reduction of BclAF1 levels late after infection is more enigmatic. Whereas intrinsic immune proteins suppress IE gene expression even at late times (42), miR-UL112-1 itself inhibits IE gene expression (24), so we consider it unlikely that negative effects of BclAF1 on IE gene expression are relevant at late times. Perhaps other processes that BclAF1 has been implicated in, including apoptosis regulation, mRNA metabolism, and nuclear lamina biology (21), are more relevant during the late phase of infection. Interestingly, the previously known targets of miR-UL112-1, HCMV IE1 and the stress-induced natural killer (NK) cell receptor ligand MICB (23, 24, 30), suggest this miRNA has a role in immune evasion. Down-regulation of IE1 may inhibit presentation through MHC molecules, as has been demonstrated for T antigen, a target of the miRNA encoded by SV40 (43). Furthermore, reduction of MICB by miR-UL112-1 prevents NK cell-mediated immune detection of HCMV-infected cells (31). As miRNAs are known to target multiple genes affecting individual biological processes (44), it follows that BclAF1 may play an as yet unidentified role in facilitating immune clearance of infected cells, thus explaining why its down-regulation by miR-UL112-1 during the late phase could promote HCMV infection.

Whatever the mechanism by which BclAF1 restricts viral replication at late times, it is unlikely to be a significant impediment to in vitro replication because the late drop in BclAF1 levels is not evident in fibroblasts infected with the AD169 fibroblast-adapted strain, and because the miR-UL112-1–null virus displays no defect in fibroblasts (24). BclAF1 reduction at later times by clinical HCMV isolates may be more important for replication in cell types other than fibroblasts or for in vivo infections. Perhaps miR-UL112-1 serves primarily to restrict BclAF1 expression during latency, when pp71 is not expressed. Reduced BclAF1 activity could be needed to allow transient expression of numerous viral genes during the establishment of latency (45–47) or the small subset of viral genes that continue to be expressed following the establishment phase. Alternatively, its reduction could help to prevent apoptosis (21) during latency.

It is presently unclear why BclAF1 is down-regulated late after infection by the FIX and TB40/E clinical strains but not with the laboratory-adapted AD169 strain. Each of these viruses expresses sequence-identical versions of miR-UL112-1 to similar levels. Because it is not uncommon for multiple miRNAs to target the same message, perhaps additional miRNAs expressed by clinical strain viruses but not AD169 cooperate with miR-UL112-1 to alter BclAF1 accumulation. Alternatively, other factors expressed from the UL/b′ region of the genome that is present in clinical strains but absent in AD169 may contribute to BclAF1 down-regulation at late times.

Finally, the neutralization of BclAF1 by two independent mechanisms during HCMV infection is an example of an intrinsic immune defense protein being targeted by more than one mecha-

nism during a viral infection. Although individual viruses are known to encode independent factors that inactivate different intrinsic or innate immune pathways at unique steps, BclAF1 appears to be a restriction factor targeted by more than one viral mechanism. This likely attests to the importance of BclAF1 down-regulation for efficient HCMV replication and spread. Whether this dual targeting represents a simple redundancy of mechanisms that by themselves are not entirely effective, or if it indicates that independent requirements for BclAF1 neutralization exist at distinct times during the viral life cycle remains to be determined.

Experimental Procedures

Cells and Viruses. Human foreskin fibroblasts (HFFs), lung fibroblasts (MRC5), 293 cells, and HeLa cells were cultured in medium supplemented with 10% (vol/vol) FBS. Three phenotypically wild-type HCMV strains were used: BAD*wt* (48), TB40/E-mCherry (49), and BFX*wt*-GFP (24). The pp71-null (BAD*sub*UL82), UL35-null (BAD*in*UL35), miR-UL112-1–null (BFX*sub*112-1^{-}), and revertant viruses (24, 34, 37), as well as plasmids expressing pp71 (13) and UL35 (36), have been described.

pp71 Interactome Screen. HeLa cells (1.5×10^7) transiently expressing either S-tagged pp71 (50) or S-tagged GFP were washed in PBS and fixed with 1% paraformaldehyde (51) for 20 min at 37 °C. Glycine was then added to a final concentration of 125 mM and incubated at room temperature for 5 min. Nuclear extracts were prepared (52) in buffer with 1 mM sodium vanadate. Proteins captured with an S-tag resin (Novagen) were incubated at 95 °C for 20 min in loading buffer before separation on 4–20% SDS-containing polyacrylamide gels and visualization with Bio-Safe Coomassie Blue (Bio-Rad). Bands unique to pp71-containing extracts were excised, washed three times with water, incubated in 100% acetonitrile for 45 min, reduced with 50 mM DTT at 56 °C for 45 min, and then alkylated with 55 mM iodoacetamide for 1 h at room temperature. The material was dried, rehydrated in a 12.5 ng/μL modified sequencing grade trypsin (Promega), and incubated in an ice bath for 40–45 min. Excess trypsin was removed and replaced with 40–50 μL of 50 mM ammonium bicarbonate/10% (vol/vol) acetonitrile (pH 8.0), and the mixture was incubated overnight at 37 °C. Peptides were extracted twice with 25 μL of 50% (vol/vol) acetonitrile/5% (vol/vol) formic acid and dried. Digests were resuspended in 20 μL of Buffer A (5% (vol/vol) acetonitrile/0.1% formic acid/0.005% heptafluorobutyric acid) and 3–6 μL were loaded onto a 12-cm × 0.075-mm fused silica capillary column packed with 5-μm-diameter C-18 beads (Nest Group) using a N2 pressure vessel at 1,100 psi. Peptides were eluted over 55 min by applying a 0–80% linear gradient of Buffer B (95% (vol/vol) acetonitrile/0.1% formic acid/0.005% HFBA) at a flow rate of 150 μL/min with a precolumn flow splitter resulting in a final flow rate of ∼200 nL/min directly into the source. In some cases, the gradient was extended to 150 min. A LTQ Linear Ion Trap (ThermoFinnigan) was run in an automated collection mode with an instrument method composed of a single segment and five data-dependent scan events with a full MS scan followed by four tandem MS/MS scans of the highest intensity ions. Normalized collision energy was set at 35, and activation Q was 0.250 with minimum full scan signal intensity at 1×10^5 with no minimum MS2 intensity specified. Dynamic exclusion was turned on using a 3-min repeat count of 2 with the mass width set at 1.0 *m/z*. Sequence analysis was performed with TurboSEQUEST (ThermoFinnigan) or MASCOT (Matrix Sciences).

miR-UL112–Regulated Protein Screen. HFFs were transduced with an empty lentiviral construct expressing dsRed2 (pFURW-CMV-U6) or the same construct expressing miR-UL112-1. Red cells were isolated by fluorescence-activated cell sorting at 48 h posttransduction and grown in either "light" medium or "heavy" medium containing L-lysine-$^{13}C_6$,$^{15}N_2$-HCl (Lys-8) and L-Arginine-$^{13}C_6$,$^{15}N_2$-HCl (Arg-10) for five cell doublings. Equal amounts of protein from light and heavy lysates were mixed and separated by electrophoresis in a 4–20% SDS-containing polyacrylamide gradient gel. The lanes of the gel were cut into 1-cm bands, digested with trypsin, and subjected to quantitative MS analysis on an LTQ-Orbitrap (53). Arg-10– and Lys-8–labeled peptides were quantified using area under extracted ion chromatograms (XICs). XICs were found and paired using PVIEW (http://compbio.cs.princeton.edu/pview) (54). The ratio of the areas under the paired XICs was reported as the ratio between heavy and light peptides. MS/MS spectra were searched using IPI version 3.62 human protein sequence database, and database searches used a ±20 ppm precursor mass window. Spectra were assigned an amino acid sequence using a 5% false discovery rate, computed using a concatenated and reverse decoy database in which lysine and arginine were swapped to remove precursor mass correlations. For proteins quantified by multiple peptides, the median ratio of all peptides was assigned to the protein to eliminate outlier ratios. Protein ratios were normalized using all detected XIC pairs so the median of their logarithm was zero, correcting for unequal loading of light and heavy sample.

Knockdown of BclAF1. The following siRNAs (Dharmacon) were used: siBclAF1-5 (sense sequence 5′-GGAAAGAGAGAAGAATTTdTdT-3′); siBclAF1-7 (5′-CCTTATGGGTACAGAGGAAdTdT-3′); siBclAF1-11 (5′-GAACATAGTACTCGGCAAAdTdT-3′). HFFs were transfected with siRNA (1.25 μg per 1.2×10^6 cells) using the Basic Nucleofector Kit (Amaxa Biosystems). After 24 h, cells received medium containing 0.1% FBS; cells were infected 24 h after transfection. TERT-immortalized HFFs constitutively expressing a short hairpin RNA directed at BclAF1 were created by transduction with a retrovirus encoding the shBclAF1-5 sequence (13).

Assay of miR Function. miR-UL112-1 premiR precursor (5′-AAGUGACGGUGAGAUCCAGGCU-3′) with a 5′ C6-NH2 modification (Applied Biosystems) was cotransfected with reporter plasmids using FuGENE 6. Lysates were prepared 48 h later and assayed using the Dual-Glo Luciferase Assay System (Promega). Reporter plasmids (pmirGLO, Promega) with a Firefly expression cassette containing a wild-type BclAF1 3′ UTR or a mutant unable to bind miR-UL112-1 were generated using the primers: wt-forward, 5′-tcgataggatccaaggcaaatggaggaataaacttcattgtcattggt-3′; wt-reverse, 5′-ctagaccaatgacaatgaagtttattcctccatttgccttggatcctac-3′; mut-forward, 5′-tcgataggatccaaggcaaatggaggaataaacaagtaacagtaaggt-3′; mut-reverse, 5′-ctagaccttactgttacttgtttattcctccatttgccttggatcctac-3′, where the underlined nucleotides correspond to substituted sequence.

Analysis of Protein and RNA Levels. Equal amounts of protein were analyzed by Western blot (13, 55) using the following antibodies: BclAF1 (A300-608A-1, Bethyl); Daxx (M-112, Santa Cruz; D7810, Sigma); hemagglutinin (Ha.11, Covance); FLAG-M2 (F1804, Sigma); tubulin (DM1A, Sigma); Actin (ab8226-100, Abcam); Elongin A (H-300, Santa Cruz); UL44 (CA006, Virusys); pp65 (10-C50K, Fitzgerald); and pp71, pp28, and IE1 (13, 55). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Chemicon.

Total RNA isolated with TriReagent (Sigma) was processed using the TaqMan reverse transcription kit (Applied Biosystems). BclAF-1 (forward: 5′-CGCGTCGAAGGTAGCTCTAT-3′; reverse: 5′-TTGGAGCGACCCATTTCTTTT-3′). RNA was normalized to GAPDH RNA, and miRNA quantification was performed as described (24).

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Supporting Information

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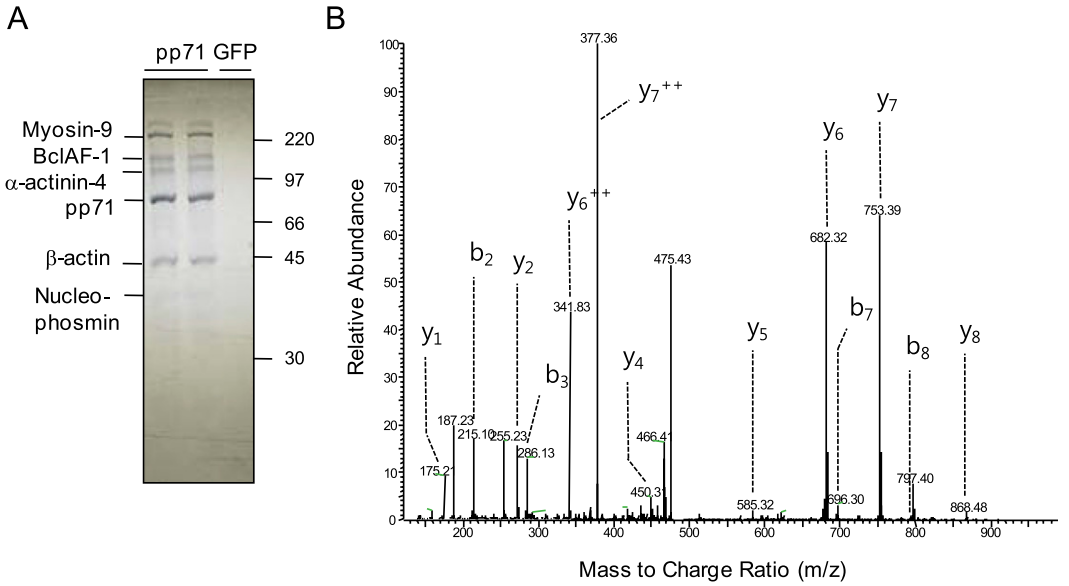

Fig. S1. Cellular proteins that coprecipitate with pp71. (*A*) pp71 and associated proteins purified from nuclear extracts of transiently transfected HeLa cells by S-tag affinity chromatography were separated by electrophoresis, and visualized by Coommassie staining. S-tagged GFP served as a control. The predominant proteins corresponding to each band are indicated as well as the band corresponding to BclAF1. (*B*) Excised bands from *A* were identified by LTQ-tandem mass spectrometry. Shown here is the MS/MS spectrum of the 484.62 *m/z* tryptic peptide K.TIAPQNAPR.D of BclAF1. The peptide score was 39 with an expect score of 0.019. (See related data in Table S1.)

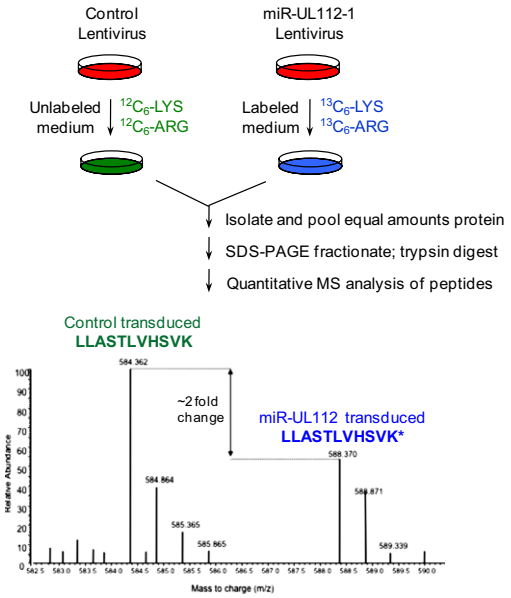

Fig. S2. BclAF1 expression is reduced in cells that express miRUL112-1. Schematic diagram of the stable isotope labeling of amino acids in cell culture (SILAC) protocol that allows for quantitative discrimination of protein levels between two different samples, with a representative MS/MS spectrum of one of the eleven BclAF1 peptides detected at reduced levels in miR-UL112-1–expressing cells. (See related data in Table S2.)

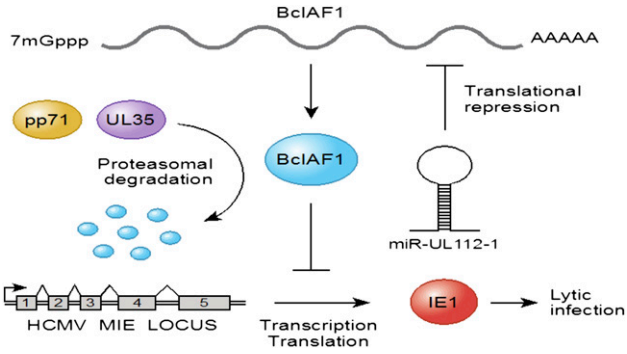

Fig. S3. Model for BclAF1 function and inhibition during HCMV infection. BclAF1 restricts HCMV infection by preventing the accumulation of the HCMV IE1 protein that is essential for efficient viral replication. It is likely that the expression of other IE proteins (e.g., the essential IE2) that share coding exons from the HCMV major immediate early (MIE) locus with IE1 is also inhibited by BclAF1. Furthermore, BclAF1 may have additional and independent effects on HCMV replication. The negative effects of BclAF1 are neutralized first by proteasomal degradation mediated by tegument-delivered pp71 and UL35, and subsequently by miR-UL112-1 through translational inhibition of the BclAF1 mRNA.

Table S1. Candidate proteins comprising the pp71 interactome

Table S1

Proteins affinity purified from HeLa cells with ectopically expressed pp71 were identified by MS. Protein scores were derived from the MASCOT search and represent the sum of the relevant peptide confidence scores. The number of unique peptides identified for each protein is indicated. Only proteins with two or more hits are included. Symbols are from the HUGO gene nomenclature committee.

Table S2. Candidate proteins modulated by miR-UL112-1

Table S2

Proteins whose levels were reduced in the presence or absence of miR-UL112-1 were identified by SILAC labeling and MS/MS with two independent analyses. The log-base-2 score of the heavy/light (H/L) ratio is shown. Proteins are ranked in order of decreasing effect. Symbols are from the HUGO gene nomenclature committee.