The EBV-encoded Latent Membrane Proteins, LMP2A and LMP2B, Limit the Actions of Interferon by Targeting Interferon Receptors for Degradation

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Abstract

Although frequently expressed in EBV-positive malignancies, the role that Latent membrane protein 2A and 2B (LMP2A, LMP2B) play in the oncogenic process remains obscure. Here we demonstrate a novel function for these proteins in epithelial cells, namely, their ability to modulate signalling from type I/II interferon receptors (IFNRs). We show that LMP2A and LMP2B-expressing epithelial cells display decreased responsiveness to interferon (IFN)α and IFNγ, as assessed by STAT1 phosphorylation, ISGF3 and GAF-mediated binding to ISRE and GAS elements and luciferase reporter activation. Transcriptional profiling highlighted the extent of this modulation, with both viral proteins impacting "globally" on interferon-stimulated gene expression. Whilst not affecting the levels of cell surface IFNRs, LMP2A and LMP2B accelerated the turnover of IFNRs through processes requiring endosome acidification. This function may form part of EBVs strategy to limit anti-viral responses and defines a novel function for LMP2A and LMP2B in modulating signalling from receptors that participate in innate immune responses.

INTRODUCTION

Epstein-Barr virus (EBV) is a human gammaherpesvirus which is found as a widespread and largely asymptomatic infection throughout the world. The virus exploits the physiology of normal B cell differentiation to persist within the memory B cell pool of the immunocompetent host as a life-long latent infection. EBV replication occurs in both B cells and in mucosal epithelium lining the nasopharynx. It is the aberrant establishment of latent EBV infection at these sites that results in the development of both lymphoid and epithelial tumours (Young and Rickinson, 2004). Of these malignancies, nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma (EBV-aGC) are numerically of greatest importance in global health terms. Both tumours have a multifactorial aetiology involving virological (EBV), genetic and environmental components (Tao and Chan., 2007; Uozaki and Fukayama., 2008). In NPC and EBV-aGC, viral gene expression restricted to EBNA1, EBER RNAs, the *BamHI*A transcripts and variable expression of the membrane proteins LMP1, LMP2A and LMP2B. This pattern of latent protein expression is distinct from that observed in EBVtransformed lymphoblastoid cell lines (LCL).

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In EBV-infected B cells, LMP2A functions to promote viral latency, providing signals to ensure cell survival in the setting of compromised BCR signalling (Longnecker, 2000; Hammerschmidt, 2007; Anderson and Longnecker, 2008a/b). Although consistently detected in EBV-associated carcinomas, the role that LMP2A or LMP2B play in epithelial cell infection is unclear (Raab-Traub, 2002; Uozaki and Fukayama, 2008). Although LMP2A does not affect the growth or differentiation of normal keratinising epithelium (Longan and Longnecker, 2000), when expressed in immortalised epithelial cell lines, it can enhance cell growth, survival and cellular differentiation through activation of the Ras/ PI3K/Akt, Notch and β-catenin/Wnt signalling pathways (Scholle et al., 2000; Morisson et al., 2003; Ikeda and Longnecker, 2007; Anderson and Longnecker, 2008a/b). Thus, LMP2A appears to have a central function in the natural history of EBV infection whether it is the maintenance of persistent infection in the B lymphocyte pool or the more controversial contribution to epithelial cell transformation. The detailed function of LMP2B remains to be elucidated, although recent findings suggest that it serves to modulate the effects of LMP2A on BCR function, thereby rendering latently infected B lymphocytes susceptible to lytic reactivation (Rechsteiner et al., 2007, 2008; Rovedo and Longnecker, 2007).

To date, much of the work on LMP2A function in epithelial cells has focussed on its role in cell transformation. However, our previous studies have demonstrated an ability of LMP2A to attenuate STAT activity in EBV-infected epithelial cells (Stewart et al., 2004), raising the possibility that LMP2A may function to modulate signalling from IFNRs. This function may form part of EBVs strategy to limit anti-viral responses and defines a novel function for LMP2A in modulating signalling from receptors that participate in innate immune responses.

IFNs function as a first line of defence against viral infection (Katze, 2002), serving to integrate innate and adaptive immune responses and limit the replication and spread of viruses. IFNα and IFNβ (type I IFNs) block viral replication by inhibiting cellular protein synthesis, stimulating the expression of pro-inflammatory cytokines and orchestrating cellular immune responses. Similarly, IFNγ (type II IFN) plays a key role in "fine-tuning" the adaptive immune response. Whereas IFNα and IFNβ share a common receptor, IFNAR, IFN-γ binds to IFNGR. IFNα/β and IFNγ stimulate distinct classes of genes whose expression is regulated by binding of ISGF3 and GAF transcription factor complexes to the ISGF3-responsive IFN-stimulated response element (ISRE) and the IFNγ-activated factor sequence (GAS) respectively, located within the promoter regions of interferon-stimulated genes (ISGs).

The complex nature of the human immune system has driven virus evolution, forcing viruses to develop strategies that override the growth inhibitory effects of IFNs. This is particularly relevant to persistent viruses, which, by definition, establish life-long infections within the infected host. Most, if not all viruses encode proteins which function to target and inactivate the IFN-signalling pathways, albeit temporarily. Certain viruses, such as Myxomavirus, express soluble decoy receptors that sequester IFNs, whereas the vast majority encode proteins, which target and inactivate the JAK/STAT signalling proteins. For example, the Sendai virus C and Hpiv3 V proteins prevent STAT phosphorylation, whereas the Simian virus 5 and Mumps virus V proteins target the STAT1 proteins for ubiquitin-mediated degradation. The V proteins encoded by the Nipah and hendra viruses block nuclear translocation of STAT proteins, thereby blocking the transcription of ISGs (Randall and Goodbourn, 2008). Herpesviruses, which persist long-term within the host, have also developed mechanisms to counteract the effects of interferon. Herpes simplex virus (HSV)-encoded US11 and ICP34.5, like the EBV-encoded EBER RNAs, target and inactivate the double stranded RNA activated Protein kinase R (PKR) (Cassady and Gross, 2002; Nanbo and Takada, 2002). The BZLF1 protein of EBV has previously been reported to down

regulate transcription of the IFNGR during viral entry into lytic cycle, thereby protecting lytically-infected cells from immune surveillance (Morisson et al., 2001). KSHV has developed multiple strategies to counteract the effects of IFNs, expressing membrane proteins (K3, K5) which target cell-surface IFNRs (Li et al., 2007) and viral homologues of the interferon regulatory factors (IRFs), which function to antagonize host IRF function (Offermann, 2007). Here we show that the EBV-encoded latent membrane proteins LMP2A and LMP2B attenuate interferon responses by targeting the IFNAR and IFNGR for degradation. Our findings define a novel role for these proteins in EBV biology that is distinct from those previously described.

RESULTS

LMP2A and LMP2B inhibit both basal and IFN-stimulated transcription

In agreement with our previous observations (Stewart et al., 2004), EMSA confirmed reduced basal STAT activity in EBV-infected cells (Figure 1A). That this repression was relieved in cells infected with a rEBV deleted for the LMP2A gene (LMP2A-rEBV) suggested that LMP2A might function to negatively regulate the JAK/STAT pathway in EBV infected epithelial cells. To confirm that this effect was directly attributable to LMP2A, Ad/AH and CNE2 cells stably expressing LMP2A or LMP2B were generated to examine the impact of LMP2A/2B on STAT activity in isolation from the whole virus (Allen et al., 2005).

As shown in Figure 1B, a significant reduction in basal nuclear STAT (GAF) activity was observed in Ad/AH and CNE2 cells expressing LMP2A and LMP2B, confirming that this effect was attributable to expression of these proteins. That the non-signalling LMP2B isoform was also capable of modulating STAT activity indicated the cytosolic amino-terminus of LMP2A is not required for this effect.

Given that IFNs activate the JAK/STAT pathway as part of a host cell response to viral infection, we sought to examine the effects of LMP2A and LMP2B on IFN signalling in more detail. Nuclear extracts were prepared from IFNα or IFNγ stimulated control and LMP2A or LMP2B-expressing cells and EMSAs performed using ISRE or GAS probes respectively. As shown in Figures 1C and 1D, EMSA revealed that both LMP2A and LMP2B suppressed basal and IFN stimulated ISRE and GAS activity in both cell lines. In agreement with our previous observations, reduced nuclear ISRE and GAS binding activity was also observed in EBV infected CNE2 cells (Figures 1E and 1F).

To confirm that the decreased nuclear ISGF3/GAF binding observed by EMSA translated into attenuated IFN-mediated transcription, luciferase reporter assays were employed to assay ISGF3 and GAF activity. Representative reporter assays, Figure 2A, show that LMP2A and LMP2B significantly reduced the ability of IFNα and IFNγ to stimulate both ISRE and GAS luciferase reporter activity in LMP2A and LMP2B-expressing CNE2 cells. Representative reporter assays, Figure 2B, also show similar reductions in ISRE and GAS luciferase reporter activity in EBV infected CNE2 cells.

LMP2A and LMP2B attenuate JAK/STAT1 phosphorylation

Given the ability of LMP2A and LMP2B to reduce the DNA binding and transcriptional activity of ISGF3 and GAF complexes, we sought to investigate the integrity of the JAK/STAT pathway in LMP2A and LMP2B-expressing cells. Immunoblotting was performed on lysates prepared from IFNα and IFNγ-stimulated cells using an antiserum specific for phosphorylated STAT1 (p-STAT1[701]). As shown in Figure 3A and 3B, in LMP2A and LMP2B-expressing cells, IFNα and IFNγ respectively failed to induce STAT1 phosphorylation to the same extent as it did in control cells. Densitometric scanning

revealed a 50% and 90% reduction in p-STAT1 30 minutes post IFNα and IFNγ stimulation respectively in LMP2A and LMP2B-expressing cells. Similar reductions in JAK and TYK2 phosphorylation were also observed in response to IFNα or IFNγ stimulation (Figure S1A). Although the levels of total STAT1 protein were similar between cell lines, the levels of JAK1, 2 and particularly, TYK2 were lower in LMP2A and LMP2B-expressing cells (Figure S1B).

LMP2A and LMP2B exert global effects on IFN-induced transcription

The ability of LMP2A and LMP2B to attenuate IFN signalling in Ad/AH and CNE2 epithelial cells prompted us to examine their impact on IFNα and IFNγ-stimulated gene transcription. Gene transcription profiling was performed on IFNα or IFNγ stimulated CNE2 cells using unstimulated counterparts as a reference. This analysis allowed us to determine whether the effects of LMP2A and LMP2B were 'global' or whether they target specific subsets of ISGs. The complete gene list obtained after microarray profiling is presented in Tables ST1 and ST2.

When control cells were stimulated with IFNα for 6 hours, 167 genes were significantly induced (Figure 4A). Of these 167 genes, only 99 genes were increased in LMP2A-expressing cells, one gene was significantly decreased, and expression of the remaining 67 genes unchanged relative to un-stimulated cells. A similar trend was observed in LMP2B-expressing cells where only 113 out of the 167 genes were shown to be increased 6 hours after IFNα stimulation. The activity of the remaining 54 genes was unchanged compared to the unstimulated LMP2B-expressing cells. This analysis indicated that LMP2A/2B attenuated the induction of a significant proportion of IFNα stimulated genes.

Similar effects were observed when LMP2A or LMP2B-expressing cells were stimulated with IFNγ. When control cells were stimulated with IFNγ for 6 hours, the expression of 432 genes was significantly increased (Figure 4B). Of these 432 genes, only 290 genes were increased in LMP2A-expressing cells, with the remaining 142 genes remaining unchanged. A similar trend was observed in LMP2B-expressing cells where only 272 out of the 432 IFN-stimulated genes were increased after 6-hour stimulation with IFN-γ. The activity of the remaining 160 genes was unchanged when compared to the unstimulated LMP2B-expressing cells. This further indicated that LMP2A and LMP2B were capable of attenuating the induction of IFNγ induced genes. Examination of the lists of the top 50 differentially-regulated genes (Figure S2 and S3) revealed that LMP2A and LMP2B repressed the induction of many IFNα and IFNγ stimulated targets whose expression was left unchanged or were unresponsive to IFNα or IFNγ stimulation.

Plotting the distribution of fold changes of IFN-stimulated genes clearly highlighted the ability of LMP2A and LMP2B to attenuate the induction of global IFN-stimulated target genes. As shown in Figure 4A, IFNα stimulation of control cells resulted in a rightward shift in the distribution of fold changes. Here, the greater the fold change induction of ISGs, post IFN stimulation, the more significant the rightward shift. Comparing the distribution of fold changes in IFNα stimulated LMP2A or LMP2B-expressing cells, to that observed in IFNα stimulated control cells, demonstrated retardation in the rightward shift. A similar trend was witnessed with IFNγ stimulation (Figure 4B). This further highlighted the ability of LMP2A and, to a lesser degree LMP2B, to attenuate global ISG activity in response to IFNα and IFNγ stimulation.

To validate findings from the microarray profiling of IFN-stimulated cells a number of IFNα and IFNγ target genes were selected and their expression analysed by Q-PCR and western blotting. Representative analyses showed that the induction of a number of key IFNα and IFNγ targets, including STAT1, IRF9, 2-5 OAS, MX2, GBP1 and IRF1 were all

reduced at the mRNA level in LMP2A and LMP2B-expressing cells in response to IFNα or IFNγ stimulation (Figure 4C). Analysis of IFN-stimulated EBV-infected CNE2 cells revealed a similar reduction in selected ISG expression (Figure 4C). At the protein level, expression of IRF1, IRF7 and IRF9 were all significantly reduced in LMP2A and LMP2B-expressing cells both basally, and in response to IFNα or IFNγ stimulation (Figure S4A-C).

LMP2A and LMP2B attenuate IFN signalling by enhancing IFN receptor turnover

The ability of LMP2A and LMP2B to attenuate IFN-mediated transcription indicates disruption of the IFN signalling pathway, yet the mechanism involved in this modulation remained unclear. The reduced levels of JAK1 and TYK2 in LMP2A and LMP2B-expressing epithelial cells raised the possibility that LMP2A and LMP2B may attenuate IFN signalling by influencing the stability of IFNRs (Ragimbeau et al., 2003). Immunoblotting of total cell lysates revealed a reduction in IFNAR1 and IFNGR1 levels in LMP2A and LMP2B-expressing cells compared to control cells (Figure 5A). To analyse this in more detail, immunofluorescence staining and confocal imaging was performed on cells cultured in situ. As shown in Figure 5B, a significant reduction in the pools of intracellular IFNAR1 and IFNGR1 were observed in LMP2A and LMP2B-expressing cells compared to control cells (Figure 5B), with a degree of overlap observed between LMP2A or LMP2B and the IFNRs in vesicular compartments (Figure 5B - right panel). Despite the apparent reduction in intracellular pools of IFNAR1 and IFNGR1 in LMP2A and LMP2B-expressing cells, flow cytometric analysis demonstrated similar levels of expression on the surface of both control and LMP2A or LMP2B-expressing cells (Figure 5C). These findings suggested that LMP2A and LMP2B might influence the turnover and/or stability of intracellular pools of IFNAR and IFNGR rather than affecting cell surface stability or export to the plasma membrane. To investigate whether LMP2A and LMP2B accelerated the transit of IFNRs from the plasma membrane into lysosomes for subsequent degradation, the rate of IFNGR internalisation was examined *in situ*. As shown in Figure 6A, incubation of cells with the anti-IFNGR1 antiserum at 4°C decorated the surface of control, LMP2A and LMP2B-expressing cells equally (left panels). After 2 hours incubation at 37°C, the majority of cell surface IFNGR1 had been internalised into large intracellular vesicles in control cells, whereas a weaker, more diffuse pattern of staining was observed in LMP2A and LMP2B-expressing cells (right panels).

The half-life of the IFNAR and IFNGR were then analysed using a cycloheximide (CHX)-based chase assay (Ragimbeau et al., 2003). Total cellular lysates were prepared from CNE2 control and LMP2A or LMP2B-expressing cells treated for various time points (0 - 6 hours) with Cycloheximide (CHX). Immunoblotting of these lysates with antisera to IFNAR1 or IFNGR1 revealed that the stability of IFNRs was dramatically reduced in cells expressing LMP2A and, to a lesser extent LMP2B (Figure 6B). Compared to IFNAR1, IFNGR1 was much less stable as indicated by a more rapid reduction in protein levels over the 6-hour time course. Quantitation of the fluorescence intensities of IFNGR1 and IFNAR1 protein, normalising against β-actin, revealed that the half-life of the IFNGR1 was approximately 4 hours (3 hours 46 minutes) in control cells (Figure 6C). Although the basal levels of IFNGR1 were lower in cells expressing LMP2A or LMP2B, the half-life of IFNGR1 was significantly reduced, being of the order of one and a half hours (Figure 6C). A similar trend is observed with IFNAR1. In control cells, the half-life of the IFNAR1 was approximately 3 hours (2 hours 59 minutes), yet this was significantly reduced in LMP2A-expressing cells. Surprisingly, although the basal levels of IFNAR1 in LMP2B-expressing cells was much lower than that observed in control cells, its half-life more closely resembled that in control cells (Figure 6C).

To investigate the possibility that LMP2A or LMP2B-mediated turnover of IFNRs required endosomal targeting, the half-life of IFNGR1 was assayed under conditions in which

endosome and lysosome function were compromised. In these experiments, cells were treated with CHX for 2 hours in combination with chloroquine and ammonium chloride (NH$_4$Cl), compounds that inhibit endosomal and lysosomal acidification (Adachi et al., 2007). Treatment of cells with chloroquine had a profound effect on IFNGR1 stability in all cell lines (Figure 6D). Compared to CHX treatment alone, chloroquine treatment significantly blocked degradation of the IFNGR1 in LMP2A and LMP2B-expressing cells, whereas treatment with the lysosomal inhibitor, NH$_4$Cl, had little or no effect. Similar effects to chloroquine were also observed with the broad-spectrum protease inhibitor, MG132, which also increased stability of the IFNGR1 in all cell lines. Densitometric scanning of these blots confirmed the ability of chloroquine and MG132 to block the turnover and degradation of IFNGR1 in LMP2A/2B (Figure 6E) where the expression levels returned to those observed in the control cells.

DISCUSSION

In this report we demonstrate a novel function for LMP2A distinct from those previously reported, namely, an ability to attenuate signalling from type I and type II interferon receptors. We also show that this function is not unique to LMP2A, as the so-called "signalling-defective" LMP2B protein is also capable of attenuating IFN signalling. The latter observation is intriguing given that the only known function of LMP2B is to antagonise LMP2A function in B cells (Rovedo and Longnecker, 2007). The ability of these viral proteins to modulate IFN signalling is another example of a viral strategy for circumventing the innate immune response, an effect that is generally exploited in persistent virus infections but which can, in the context of oncogenic viruses such as EBV, contribute to tumour development. Thus, while the ability to inhibit IFN signalling may form part of the virus's strategy to persist naturally in latently infected B lymphocytes, the inadvertent expression of these proteins in NPC, EBV-positive gastric carcinoma and Hodgkin's lymphoma tumour cells may contribute to oncogenic transformation by stimulating cell growth and limiting cellular responses to virus-infected cells.

Here we show that LMP2A and LMP2B attenuate IFN responses by targeting both the IFNRs. This strategy is more powerful than targeting individual signalling components such as the JAKs or STATs as exploited by viruses such as HSV, HCMV and HCV, as it produces a global blockade of IFN signalling. Immunoblotting, with "activation-state" specific antiserum confirmed that IFN-α/β and IFN-γ's ability to stimulate JAK and STAT phosphorylation and the formation of nuclear ISGF3 and GAF transcription factor complexes was severely impaired in LMP2A and LMP2B-expressing cells. This resulted in a robust attenuation of both IFN-α/β and IFN-γ-mediated transcription and a global repression in the induction of ISGs. The magnitude of this effect was clearly evident when transcriptional profiling was performed on IFN-β and IFN-γ stimulated cells. Here, both qualitative and quantitative reductions in ISG expression were observed and further characterisation of selected ISGs with established anti-viral activities were weakly induced in LMP2A and LMP2B-expressing cells as well as EBV infected CNE2 cells. The observation that EBV infected CNE2 cells exhibit attenuated ISG activity (Figure 4C), when compared to control cells, indicates that the low level LMP2A expression observed in these virally infected cells (Stewart et al., 2004) is likely to be sufficient to attenuate ISG activity.

Further investigations revealed that both LMP2A and LMP2B target IFNAR1 and IFNGR1 for degradation. Although it is presently unknown whether this involves direct physical interaction of LMP2A or LMP2B with IFN receptors, it appeared to relate to the ability of these viral proteins to enhance the rate of IFN receptor degradation. Although LMP2A or LMP2B did not influence the levels of cell-surface IFNAR1 and IFNGR1, immunofluorescence staining and immunoblotting revealed that the intracellular pools of

IFNAR1 and IFNGR1 were greatly reduced in LMP2A and LMP2B-expressing cells. The partial co-localization of LMP2A and LMP2B with IFNRs in late endosomes/lysosomes suggests that these viral proteins may modulate the fate of internalised IFNRs, forcing them into a degradative arm of the endocytic pathway. This effect is reminiscent of the ability of the lytic cycle K3 and K5 proteins of KHSV to down-regulate the IFNGR which is achieved via the E3 ubiquitin ligase activity of these viral proteins (Rezaee et al., 2006; Li et al., 2007).

A role for endosome-mediated degradation in IFNR turnover was confirmed as studies revealed decreased half-lives of both IFNAR1 and IFNGR1 in LMP2A and LMP2B-expressing cells. Key roles for IFNR ubiquitination and endosomal targeting were established as treatment with MG132, a proteasomal inhibitor, and chloroquine, an endosome acidification inhibitor, completely blocked LMP2A and LMP2B-mediated degradation of the IFNGR. Taken together these data suggest that LMP2A and LMP2B influence receptor stability by inducing receptor ubiquitination and the trafficking from endosomes to lysosomes. Although the mechanism by which LMP2A and LMP2B influence the endosome network is unclear, it may be linked to their association with lipid rafts (Dykstra et al., 2001; Ikeda and Longnecker, 2007), cholesterol rich microdomains that act as signalling platforms for many growth factor receptors (Ikeda and Longnecker, 2007). As LMP2A and, presumably LMP2B, signal in a ligand-independent manner (Longnecker, 2000), they may induce the turnover and degradation of receptors from these structures by enhancing early endosome formation. Such a scenario has been reported for the herpes virus saimiri (HVS) Tip protein, which induces the turnover of the T-cell receptor (TCR) and CD4 on T lymphocytes by promoting their endocytosis from lipid rafts (Cho et al.,2006). Like the TCR and CD4, the IFNRs, particularly IFNGR1, are recruited to lipid rafts upon activation from where they are endocytosed (Claudinon et al., 2007). Recently, a ligand-independent mechanism of IFNAR internalisation and degradation has been reported (Liu et al., 2008). Although phosphorylation and ubiquitination are required, it requires the activity of an as yet unidentified kinase distinct from TYK2. As LMP2A and LMP2B accelerate the turnover of IFNRs in the absence of IFN stimulation, they may stimulate the activity of this kinase to promote phosphorylation and ubiquitin-mediated lysosomal degradation.

An alternative possibility is that they modulate endosome function directly. Both proteins are highly hydrophobic transmembrane proteins that self-oligomerise and localise to endosomal compartments (Dawson et al., 2001). Such a scenario is not without precedent, as the HPV-encoded E5 proteins have also been reported to modulate endosome function by associating with the vacuolar H^+-ATPase (Conrad et al., 1993).

The fact that LMP2A and LMP2B are expressed in a significant proportion of NPC tumours, suggests that, apart from their role in the maintenance of EBV persistence in healthy seropositive individuals, these proteins contribute to the continued growth of NPC tumour cells. A previous study has identified defects in antigen processing machinery and down-regulation of HLA Class I antigen expression in EBV-positive NPC tumours (Ogino et al., 2007) suggesting that during tumour progression NPC cells acquire mechanisms to escape immune recognition. Although it is presently unclear as to whether these two phenomena are linked, the ability of LMP2A and LMP2B to attenuate IFNα and IFNγ signalling, as demonstrated in this study, may constitute an additional immune escape mechanism by which EBV protects malignant tumour cells from immune recognition and destruction by the hosts' immune system.

In conclusion, our data demonstrate a novel function for LMP2A and LMP2B in evading the IFN response thereby contributing to the persistence of EBV in healthy individuals and to the pathogenesis of virus-associated malignancies. A deeper understanding of the precise

mechanisms responsible for this effect could lead to innovative approaches to treating EBV-positive tumours and to eradicating persistent virus infection.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture

CNE2 and Ad/AH cells were cultured in RPMI 1640 supplemented with 5% FCS and antibiotics (Sigma-Aldrich). Cell stably expressing LMP2A, LMP2B or a neomycin resistance cassette were generated as previously described (Allen et al., 2005).

Antibodies and Reagents

Antibodies to the phosphorylated forms of STAT1, JAK1, JAK2, TYK2, or IRF1 and IRF7 were purchased from Cell Signalling Technology. Antibodies to STAT1, IRF9, IFNAR1, IFNGR1 and β-actin were purchased from Santa Cruz Biotechnology. LMP2 staining was performed as previously described using human serum (Ba) displaying reactivity to LMP2A/2B (Allen et al., 2005). AlexaFluor-conjugated anti-mouse, anti-Rabbit or anti-Human immunoglobulins and IRDye700-conjugated Goat anti-mouse or anti-rabbit immunoglobulins were purchased from Invitrogen. HRP-conjugated anti-mouse or anti-rabbit immunoglobulins were purchased from DAKO (Denmark).

IFNα and IFNγ (Peprotec, UK) were diluted to a final concentration of 100ng/ml. Cycloheximide, NH_4Cl, Chloroquine (Sigma Aldrich, UK) and MG132 (Calbiochem, UK) were used at final concentrations of 100μM, 50nM, 50μM respectively.

Immunofluorescence Staining and Immunoblotting

Cells grown insitu on microscope slides (Hendley-Essex) were fixed, permeabilised and stained as previously described (Allen et al., 2005). For examination of IFNAR1 and IFNGR1 half-life, immunoblotting was performed using IRD-700 or 800-labelled anti-mouse or anti-rabbit antibodies and membranes scanned using the Li-COR Odyssey infrared imaging system. Fluorescence intensity was quantified and normalised to intensities of β-actin using the associated Odyssey software. All immunoblots were repeated on three independent occasions.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as recommended by the manufacturer's instructions (LI COR™ Biosciences, UK). 5μg of nuclear lysates (prepared using the NE-PER nuclear extraction reagents (Pierce Biotechnology, UK)) were mixed with 50 nM of 5′-IRDye-700-labeled oligonucleotides in LI COR™ IRDye EMSA reagents for 30 min at room temperature. The 5′-3′ sequences for the EMSA probes are as follows:

ISRE (5′-GATCAGGAAATAGAAACTG-3′)

GAS (5′-CATGTTATGCATATTCTTGTAAGTGCATG-3′)

Generic STAT (5′-GACATTTCCCGTAAATCAT-3′)

Reaction mixtures were resolved on 6% acrylamide-TBE gels and scanned using the LI COR™ Odyssey infrared imaging system. Fluorescence intensity was quantitated using the associated Odyssey software. EMSAs were repeated on at least three independent occasions.

ISRE and GAS luciferase Reporter Assays

ISRE and GAS reporter assays were performed using the dual luciferase reporter assay (Promega). The reporter plasmids pTAL-ISRE-luc (BD Biosciences) and GRR-5 (Beadling

et al., 1996) or the control plasmids, pTAL-luc or pBL-luc were cotransfected with the *Renilla* plasmid to control for transfection efficiency. All dual luciferase assays represent an average of three independent experiments. Data is presented as the mean ± SD.

Flow cytometry

Flow cytometry was performed as described previously (Dawson et al., 2000). Briefly, single-cell suspensions were resuspended in 50μl of diluted primary antibody (IFNAR1 and IFNGR1 or isotype control) and incubated on ice for 60 minutes. Labelled receptors were visualised using AlexaFluor488-conjugated anti-mouse or anti-rabbit immunoglobulin (Invitrogen, UK). Immunofluoresence staining of cell surface IFNR expression was analysed by flow cytometry using a Coulter EPICS XL FACSCAN. Data was processed using the WinMDI software package to produce the relevant histograms.

Microarray analysis

CNE2 cells were serum-starved for 16 hours and either left un-stimulated, or treated with IFNα or IFNγ for 6 hours. RNA from a series of biological triplicates was pooled and 10μg of each was processed, labelled according to the Affymetrix protocol (Affymetrix, 2004) and hybridised to Affymetrix human genome U133 plus 2 arrays. The scanned images of microarray chips were analysed using the GCOS (GeneChip Operating Software) from Affymetrix, Inc. (Santa Clara, California, USA) with the default settings except that the target signal was set to 100. The gene expression profile of IFNα or IFNγ stimulated cells was compared with that of unstimulated cells at the 6 hour time point. Significantly differentially expressed probe sets were identified using GCOS pairwise comparison algorithm. No fold change cut off criterion was applied. The distribution of the data was analysed using the density function in R (http://www.r-project.org). Gene expression heat maps were generated using dChip (http://www.dchip.org) with the default settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

REFERENCES

Adachi K, Ichinose T, Takizawa N, Watanabe K, Kitazato K, Kobayashi N. Inhibition of betanodavirus infection by inhibitors of endosomal acidification. Arch Virol. 2007; 152:2217–2224. [PubMed: 17891330]

Allen MD, Young LS, Dawson CW. The Epstein-Barr virus-encoded LMP2A and LMP2B proteins promote epithelial cell spreading and motility. J Virol. 2005; 79:1789–1802. [PubMed: 15650203]

Anderson LJ, Longnecker R. An auto-regulatory loop for EBV LMP2A involves activation of Notch. Virology. 2008a; 371:257–266. [PubMed: 17980397]

Anderson LJ, Longnecker R. Epstein Barr virus latent membrane protein 2A exploits Notch1 to alter B cell identity in vivo. Blood. 2008b

Beadling C, Ng J, Babbage JW, Cantrell DA. Interleukin-2 activation of STAT5 requires the convergent action of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. EMBO J. 1996; 15:1902–13. [PubMed: 8617237]

Cassady KA, Gross M. The herpes simplex virus type 1 U(S)11 protein interacts with protein kinase R in infected cells and requires a 30-amino-acid sequence adjacent to a kinase substrate domain. J Virol. 2002; 76:2029–2035. [PubMed: 11836380]

Cho NH, Kingston D, Chang H, Kwon EK, Kim JM, Lee JH, Chu H, Choi MS, Kim IS, Jung JU. Association of herpesvirus saimiri tip with lipid raft is essential for downregulation of T-cell receptor and CD4 coreceptor. J Virol. 2006; 80:108–118. [PubMed: 16352535]

Claudinon J, Monier MN, Lamaze C. Interfering with interferon receptor sorting and trafficking: impact on signalling. Biochimie. 2007; 89:735–743. [PubMed: 17493737]

Conrad M, Bubb VJ, Schlegel R. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. J Virol. 1993; 67:6170–6178. [PubMed: 7690419]

Dawson CW, Eliopoulos AG, Blake SM, Barker R, Young LS. Identification of functional differences between prototype Epstein-Barr virus-encoded LMP1 and a nasopharyngeal carcinoma-derived LMP1 in human epithelial cells. Virology. 2000; 272:204–217. [PubMed: 10873763]

Dawson CW, George JH, Blake SM, Longnecker R, Young LS. The Epstein-Barr virus encoded latent membrane protein 2A augments signaling from latent membrane protein 1. Virology. 2001; 289:192–207. [PubMed: 11689042]

Dykstra ML, Longnecker R, Pierce SK. Epstein-Barr virus coopts lipid rafts to block the signalling and antigen transport functions of the BCR. Immunity. 2001; 14:57–67. [PubMed: 11163230]

Ikeda M, Longnecker R. Cholesterol is critical for Epstein-Barr virus latent membrane protein 2A trafficking and protein stability. Virology. 2007; 360:461–468. [PubMed: 17150237]

Katze MG. Interferon, PKR, virology, and genomics: what is past and what is next in the new millennium? J Interferon Cytokine Res. 2002; 22:283–286. [PubMed: 12034034]

Li Q, Means R, Lang S, Jung JU. Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. J Virol. 2007; 81:2117–2127. [PubMed: 17166914]

Liu J, Plotnikov A, Banerjee A, Suresh Kumar KG, Ragimbeau J, Marijanovic Z, Baker DP, Pellegrini S, Fuchs SY. Ligand-independent pathway that controls stability of interferon alpha receptor. Biochem Biophys Res Commun. 2008; 367:388–93. [PubMed: 18166147]

Longan L, Longnecker R. Epstein-Barr virus latent membrane protein 2A has no growth-altering effects when expressed in differentiating epithelia. J Gen Virol. 2000; 81:2245–2252. [PubMed: 10950982]

Longnecker R. Epstein-Barr virus latency: LMP2, a regulator or means for Epstein-Barr virus persistence? Adv Cancer Res. 2000; 79:175–200. [PubMed: 10818681]

Mancao C, Hammerschmidt W. Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. Blood. 2007; 110:3715–3721. [PubMed: 17682125]

Morrison JA, Klingelhutz AJ, Raab-Traub N. Epstein-Barr virus latent membrane protein 2A activates β-catenin signalling in epithelial cells. J Virol. 2003; 77:12276–12284. [PubMed: 14581564]

Morrison TE, Mauser A, Wong A, Ting JP, Kenney SC. Inhibition of IFN-gamma signalling by an Epstein-Barr virus immediate-early protein. Immunity. 2001; 15:787–799. [PubMed: 11728340]

Nanbo A, Takada K. The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis. Rev Med Virol. 2002; 12:321–326. [PubMed: 12211044]

Offermann MK. Kaposi sarcoma herpesvirus-encoded interferon regulator factors. Curr Top Microbiol Immunol. 2007; 312:185–209. [PubMed: 17089798]

Ogino T, Moriai S, Ishida Y, Ishii H, Katayama A, Miyokawa N, Harabuchi Y, Ferrone S. Association of immunoescape mechanisms with Epstein-Barr virus infection in nasopharyngeal carcinoma. Int J Cancer. 2007; 120:2401–2410. [PubMed: 17315195]

Raab-Traub N. Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol. 2002; 12:431–441. [PubMed: 12450729]

Ragimbeau J, Dondi E, Alcover A, Eid P, Uze G, Pellegrini S. The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. Embo J. 2003; 22:537–547. [PubMed: 12554654]

Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol. 2008; 89:1–47. [PubMed: 18089727]

Rechsteiner MP, Berger C, Weber M, Sigrist JA, Nadal D, Bernasconi M. Silencing of latent membrane protein 2B reduces susceptibility to activation of lytic Epstein-Barr virus in Burkitt's lymphoma Akata cells. J Gen Virol. 2007; 88:1454–1459. [PubMed: 17412973]

Rechsteiner MP, Berger C, Zauner L, Sigrist JA, Weber M, Longnecker R, Bernasconi M, Nadal D. Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection. J Virol. 2008; 82:1739–1747. [PubMed: 18057232]

Rezaee SA, Cunningham C, Davison AJ, Blackbourn DJ. Kaposi's sarcoma-associated herpesvirus immune modulation: an overview. J Gen Virol. 2006; 87:1781–1804. [PubMed: 16760382]

Rovedo M, Longnecker R. Epstein-barr virus latent membrane protein 2B (LMP2B) modulates LMP2A activity. J Virol. 2007; 81:84–94. [PubMed: 17035319]

Scholle F, Bendt KM, Raab-Traub N. Epstein-Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J Virol. 2000; 74:10681–10689. [PubMed: 11044112]

Stewart S, Dawson CW, Takada K, Curnow J, Moody CA, Sixbey JW, Young LS. Epstein-Barr virus-encoded LMP2A regulates viral and cellular gene expression by modulation of the NF-kappaB transcription factor pathway. Proc Natl Acad Sci U S A. 2004; 101:15730–15735. [PubMed: 15498875]

Tao Q, Chan AT. Nasopharyngeal carcinoma: molecular pathogenesis and therapeutic developments. Expert Rev Mol Med. 2007; 9:1–24. [PubMed: 17477889]

Uozaki H, Fukayama M. Epstein-Barr Virus and Gastric Carcinoma - Viral Carcinogenesis through Epigenetic Mechanisms. Int J Clin Exp Pathol. 2008; 1:198–216. [PubMed: 18784828]

Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer. 2004; 4:757–768. [PubMed: 15510157]
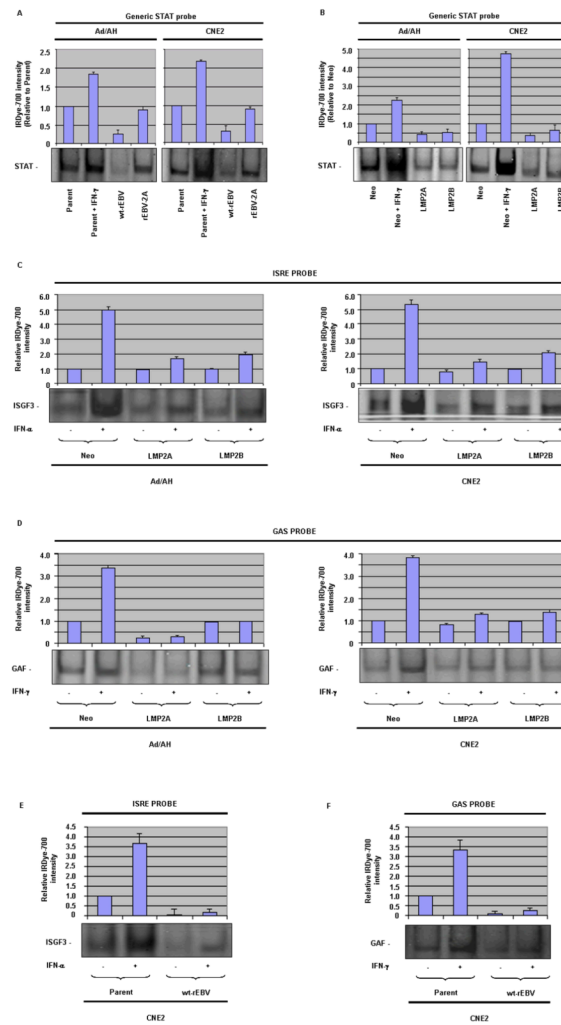
Figure 1. LMP2A and LMP2B attenuate both basal and IFN-stimulated ISRE and GAS activity
(A) EMSA demonstrating reduced nuclear binding of GAF complexes in nuclear extracts from Ad/AH and CNE2 cells infected with wild-type rEBV compared to cells infected with a rEBV deleted for LMP2A (LMP2A-rEBV).
(B) EMSA demonstrating reduced nuclear binding of generic STAT complexes in nuclear extracts from Ad/AH and CNE2 cells stably expressing LMP2A or LMP2B.
(C) and (D) EMSA demonstrating reduced nuclear binding of ISGF3 and GAF complexes in nuclear extracts from Ad/AH and CNE2 cells stably expressing LMP2A or LMP2B, both basally, and in response to IFNα or IFNγ stimulation.
(E) and (F) EMSA demonstrating reduced nuclear binding of ISGF3 and GAF complexes in nuclear extracts from CNE2 cells stably infected with wt-rEBV both basally, and in response to IFNα or IFNγ stimulation.
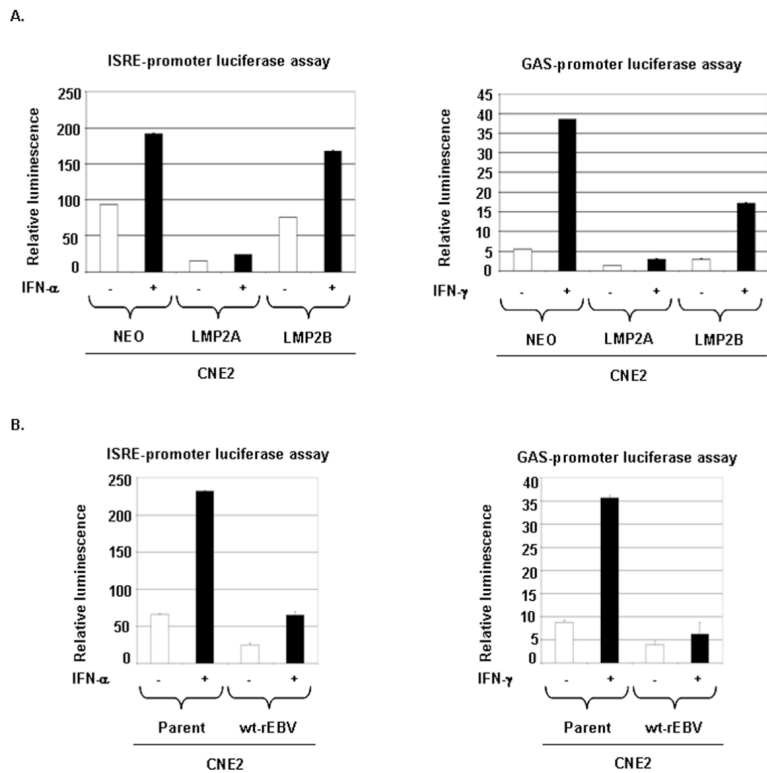
A.

ISRE-promoter luciferase assay

GAS-promoter luciferase assay

B.

ISRE-promoter luciferase assay

GAS-promoter luciferase assay

Figure 2. LMP2A and LMP2B attenuated both basal and IFN-stimulated ISRE and GAS luciferase reporter activity

Luciferase reporter assays confirmed that the reduced ISGF3 and GAF activity translates into reduced transcription from generic ISRE and GAS containing promoters; both basally, and in response to IFNα or IFNγ stimulation. Data presented are the mean (±SEM) from five experiments. (A) CNE2 control, LMP2A and LMP2B cells. (B) CNE2 parental and EBV infected cells.
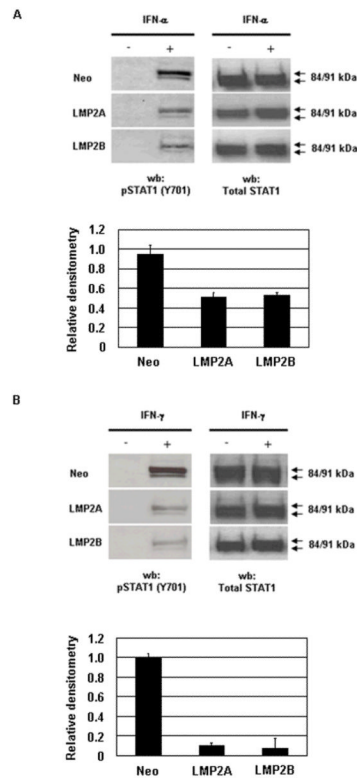
Figure 3. LMP2A and LMP2B impair IFN-mediated phosphorylation of STAT1
(A) and (B). Representative western blotting analysis of phosphorylated STAT1
(STAT1^{Y701}) in CNE2 control, LMP2A and LMP2B-expressing cells stimulated with either
IFNα (upper panel) or IFNγ (lower panel). Reprobing of the blots with antiserum to STAT1
confirmed equal protein loading in all cases. Densitometry was performed on the western
blots in Figure 2A and 2B as shown by the bar graphs. Error bars represent standard
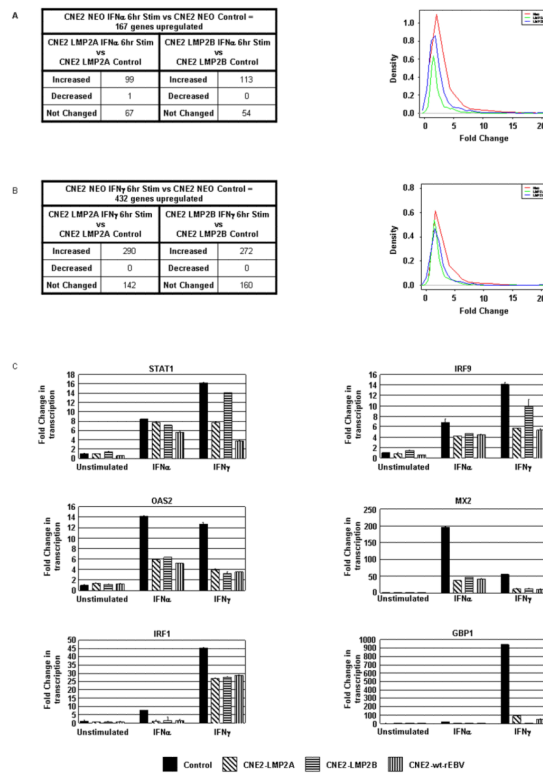deviations taken from three independent readings.

Figure 4. Gene expression profiling confirming global attenuation of interferon stimulated genes (ISGs) by LMP2A and LMP2B

(A) and (B). Analysis of microarray data from CNE2 control, LMP2A and LMP2B-expressing cells 6 hours after stimulation with (A) IFNα or (B) IFNγ. Tables were compiled to analyse ISGs that were significantly differentially increased, decreased or whose expression were unchanged in response to IFNα or IFNγ. The distributions of fold change increase in ISG expression was plotted on a distribution graph using 'R' (www.r-project.org). Data are shown for 6-hour time points following IFNα or IFNγ treatment. CNE2 control (Red line), CNE2 LMP2A (Green line), and CNE2 LMP2B (Blue line).

(C) Q-PCR validation confirmed reduced expression of selected ISGs (OAS2, IRF9, MX2, STAT1, GBP1 and IRF1) in LMP2A/2B expressing cells and EBV infected cells. Q-PCR data were normalised against GAPDH.
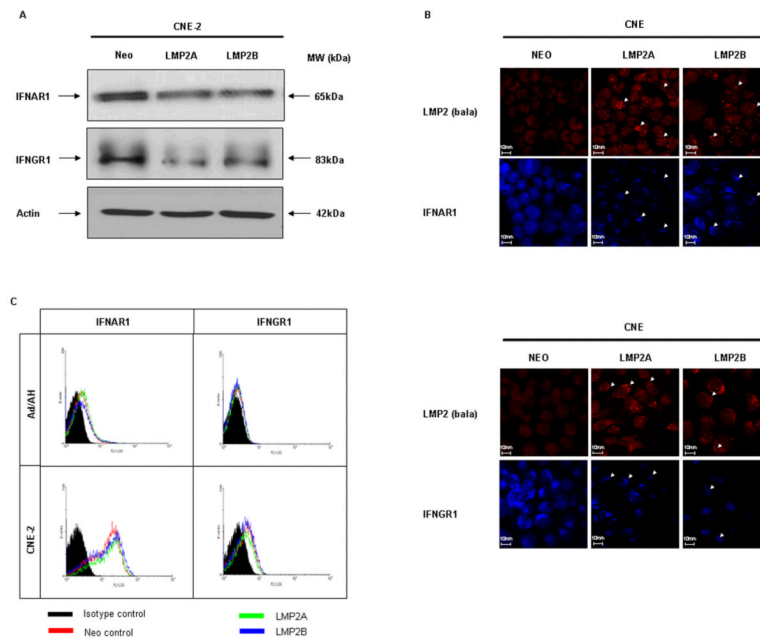
Figure 5. LMP2A and LMP2B target IFNAR and IFNGR in epithelial cells
(A) Representative immunoblots demonstrating reduced levels of IFNAR1 (upper panel) and IFNGR1 (lower panel) in CNE2 control, LMP2A or LMP2B-expressing cells. Reprobing of the blots with a mAb to β-actin confirmed equal protein loading.
(B) Dual immunofluorescence staining and confocal imaging analysis shows decreased expression of IFNAR1 (upper panel) and IFNGR1 (lower panel) in CNE2 cells expressing LMP2A or LMP2B. Bar = 10μm.
(C) A representative flow-cytometric analysis of Ad/AH and CNE2 cells for IFNAR1 and IFNGR1 demonstrating similar levels of cell surface IFNAR1 and IFNGR1 between and control and LMP2A or LMP2B-expressiing cells. IFNAR1 and IFNGR1 expression is compared against the isotype control (solid black peak). Control (Red), LMP2A (Green) and LMP2B (Blue).
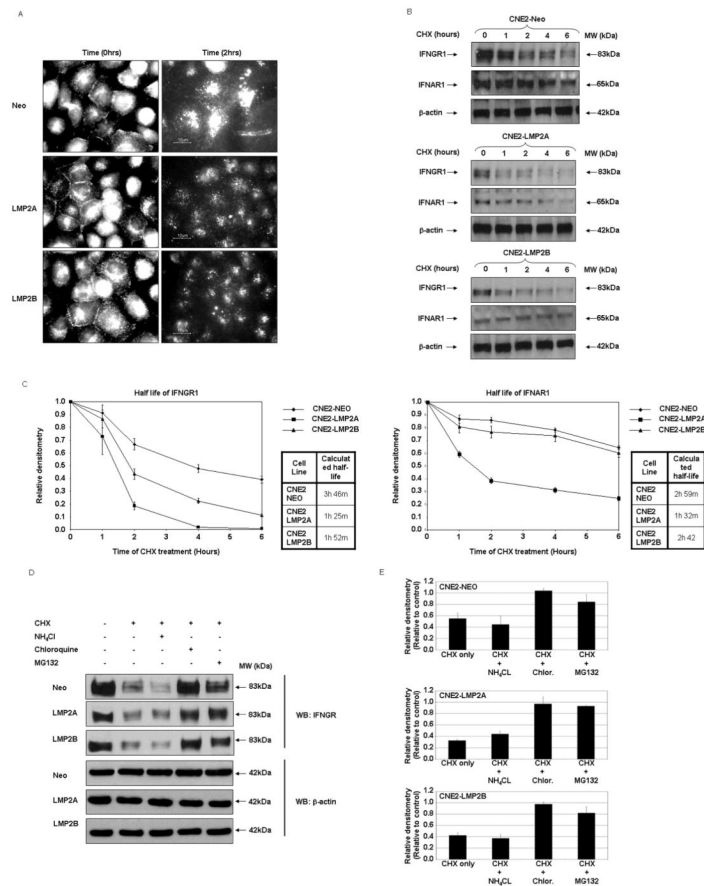
Figure 6. LMP2A and LMP2B increase the turnover and degradation of IFNAR1 and IFNGR1

(A) The extent of IFNGR internalisation was monitored by following the fate of FITC-labelled IFNGR over time at 37°C. Cells were incubated at 4°C to label cell-surface IFNGR1 (Left panels) and the fate of cell surface IFNGR1 examined 2 hours after incubation at 37°C (Right panels). Bar = 10μm.

(B) LMP2A and LMP2B accelerate the turnover of IFNAR1 and IFNGR1. Control, LMP2A and LMP2B-expressing cells were treated with 20μg/ml Cycloheximide (CHX) at 37°C for various time points (0 - 12 hours). Cells were harvested at the indicated time points and total cell lysates immunoblotted with antisera specific for IFNAR1 or IFNGR1 followed by detection with IRDye-700-labelled goat anti-mouse/rabbit immunoglobulin. Immunoblots were analysed on the Odyssey infrared imaging system. To confirm equal protein loading immunoblots were also probed with a mAb to β-actin.

(C) LMP2A and LMP2B reduce the half-life of IFNAR1 and IFNGR1. The immunoblots shown in (B) were subjected to quantitation and graphs drawn to illustrate the half-lives of IFNAR1 and IFNGR1. In two independent experiments IFNGR1 and IFNAR1 were found to have half-lives of 3 h 46 min and 2 h 59 min in control cells, whereas in the presence of LMP2A and LMP2B this was reduced to 1 h 25 min and 1 h 52 min or 1 h 32 min and 2 h 42 min respectively.

(D) LMP2A and LMP2B accelerate endocytic targeting of IFNGR to the lysosomes. Control, LMP2A and LMP2B-expressing cells were treated for 2 hours with 20μg/ml Cycloheximide (CHX) in the presence or absence of Chloroquine, NH$_4$Cl, and MG132. Total cell lysates were subjected to immunoblotting with an antiserum specific for IFNGR1 followed by detection with a HRP-conjugated goat anti-rabbit immunoglobulin. Equal protein loading was confirmed using an antibody to β-actin.

(E) Densitometry performed on western blots in Figure 5D. Error bars represent standard deviations taken from three independent readings.