Virology 489 (2016) 223–232

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Epstein–Barr virus glycoprotein gM can interact with the cellular protein p32 and knockdown of p32 impairs virus

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ARTICLE INFO

Article history:
Received 28 September 2015
Returned to author for revisions
23 December 2015
Accepted 29 December 2015
Available online 13 January 2016

Keywords:
Epstein–Barr virus
Glycoprotein gN
Glycoprotein gM
p32
Virus egress

ABSTRACT

The Epstein–Barr virus glycoprotein complex gMgN has been implicated in assembly and release of fully enveloped virus, although the precise role that it plays has not been elucidated. We report here that the long predicted cytoplasmic tail of gM is not required for complex formation and that it interacts with the cellular protein p32, which has been reported to be involved in nuclear egress of human cytomegalovirus and herpes simplex virus. Although redistribution of p32 and colocalization with gM was not observed in virus infected cells, knockdown of p32 expression by siRNA or lentivirus-delivered shRNA recapitulated the phenotype of a virus lacking expression of gNgM. A proportion of virus released from cells sedimented with characteristics of virus lacking an intact envelope and there was an increase in virus trapped in nuclear condensed chromatin. The observations suggest the possibility that p32 may also be involved in nuclear egress of Epstein–Barr virus.

Introduction

The envelope proteins of herpesviruses play important roles at several points in infection. They are critical to virus entry and to assembly and egress. They influence virus spread and they can modify the host innate and adaptive immune response. The envelope of the oncogenic human gammaherpesvirus, Epstein–Barr virus (EBV), contains eleven glycoprotein species including three heterodimeric complexes, gHgL, BDLF2/BMRF2 and gMgN. In all cases one member of the complex is dependent on the other for trafficking out of the endoplasmic reticulum (Yaswen et al., 1993; Lake et al., 1998; Gore and Hutt-Fletcher, 2008). Two of the complexes, gHgL and gMgN, are conserved herpesvirus glycoproteins. The EBV gHgL complex, as in all herpesviruses, is required for virus-cell fusion (Hutt-Fletcher, 2007). The roles of the conserved gMgN complexes are more diverse. In many herpesviruses the glycoproteins are classified as non-essential, though in general they have been implicated in some way in assembly and egress and spread and they all bear structural similarities. The gM partners are multispan membrane proteins, whereas the gN homologs are smaller type 1 integral membrane proteins.

Herpes simplex virus gM is important for the efficient incorporation of gHgL into the virion membrane (Ren et al., 2012) and although a gM-null virus has a only small reduction in replication rate (MacLean et al., 1993; Browne et al., 2004), virus which lacks both gM and the membrane associated tegument protein pUL11 is significantly impaired in secondary envelopment (Leege et al., 2009). Deletion of the homologous proteins in the alphaherpesvirus pseudorabies virus results in a similar but even more severe phenotype (Kopp et al., 2004). Defects in virus egress or cell to cell spread are also found in equine herpesvirus 1 (Osterrieder et al., 1996), varicella zoster virus (Yamagishi et al., 2008) and equid herpesvirus 4 (Ziegler et al., 2005) lacking gM, and the gM homologs of Marek's Disease virus (Tischer et al., 2002) and murine gammaherpesvirus 68 (May et al., 2005) are essential for production of virions. Perhaps the most extensively studied gMgN complex, however, is that of the human cytomegalovirus (HCMV), where gN and gM are not only both essential (Hobom et al., 2000; Mach et al., 2005), but are also the most abundant glycoproteins in the virion (Varnum et al., 2004). HCMV gN plays a critical role in secondary envelopment (Mach et al., 2007) and the cytoplasmic

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http://dx.doi.org/10.1016/j.virol.2015.12.019
0042-6822/

tail of gM, which is essential for replication (Krzyzaniak et al., 2007), interacts with the RabII effector protein FIP4 (Krzyzaniak et al., 2009) and is important for transport of the gMgN complex to the assembly compartment.

We previously made a recombinant EBV in which the gN open reading frame was interrupted by a neomycin resistance cassette. The virus was found to be phenotypically null for both gN and gM (Lake and Hutt-Fletcher, 2000). Cells induced to make the recombinant virus released fewer particles than cells making wild type virus and they lysed more rapidly. Some virions accumulated in condensed chromatin in the nucleus, few enveloped particles were seen in the cytoplasm and the majority of virions that were released lacked an intact envelope as judged by isopycnic sedimentation. The few enveloped virions that were harvested were impaired in infectivity at a step following fusion. We had no explanation for the accumulation of virions in chromatin, however, we speculated that, as had been suggested for pseudorabies virus gM (Brack et al., 1999, 2000), where gM serves a redundant function with gE/gI, the role for EBV gM might be in directing capsids to the site of envelopment by recruiting capsid-associating tegument proteins. The cytoplasmic tail of EBV gM, which is predicted to be 79 amino acids long, is highly charged and rich in proline residues. We therefore here first explored whether the 79 amino acids were important to formation of the gNgM complex and second, whether, like the carboxy-terminal sequences of HCMV gM, they mediated interactions with other cell or viral proteins.

Results

The cytoplasmic tail of gM is not required for its association with gN

Glycoprotein M is essential to the processing and transport of the gMgN complex. To determine if complex formation requires the predicted C-terminal cytoplasmic domain of gM, CV-1 cells were infected with vaccinia virus expressing T7 polymerase, transfected with pTM1gN and either pTM1-HA-gM or pTM1-HA-gMΔ79, lacking the 79 amino acids carboxyterminal to the last predicted transmembrane domain of gM, labeled biosynthetically with [^{3}H]leucine, lysed and immunoprecipitated with antibody to HA or to gN. Both antibodies precipitated a complex of gN, which runs as two bands of approximately 15 and 10 kDa, and either full length or truncated gM (Fig. 1).

The cytoplasmic tail of gM interacts with p32

Since the predicted long cytoplasmic domain of gM was not required for formation of the complex with gN we sought to determine whether it interacted with any other virus or cell proteins. The C-terminal 79 amino acids of gM proved to be toxic to yeast and were thus unsuitable for a yeast two-hybrid screen. Instead they were cloned in frame for expression as a fusion protein with GST. GST-gM and GST bound to glutathione-Sepharose were added to lysates of Akata cells and Akata cells that had been induced to make EBV. The cells had been biosynthetically labeled with [^{3}H]leucine and the proteins that precipitated with the beads were analyzed by electrophoresis and autoradiography. A protein with a mobility of approximately 32 kDa was uniquely pulled down with GST-gM from both induced and uninduced Akata cells (Fig. 2). The mobility of this protein was almost exactly the same as the mobility of the GST-gM fusion protein, which would complicate its mass spectroscopic analysis. The same sequences of gM were therefore cloned for expression as a larger fusion protein with MBP. MBP-gM, but not MBP alone, pulled down a protein from EBV-negative Akata cells which had the same mobility as that pulled down by GST-gM (Fig. 3). Since the carboxyterminus of gM includes 9 proline residues and therefore might be considered an intrinsically “sticky” protein we also included as additional control a GST fusion with the amino-terminal 169 residues of the BDLF2 protein, which are also cytoplasmic, and include 13 proline residues. The GST-BDLF2 fusion did not interact with the 32 kDa protein.

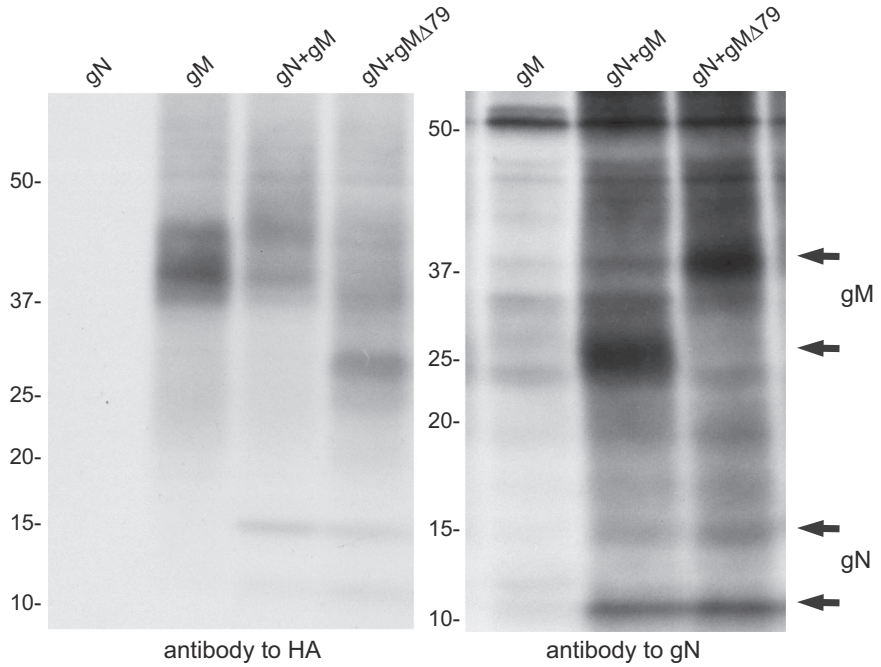


Fig. 1. Interaction of gN and truncated gM. SDS-PAGE and autoradiography of extracts of CV-1 cells infected with vaccinia virus expressing T7 polymerase, transfected with pTM1 plasmids encoding HA-gM, gN, or HA-gMΔ79 as indicated, labeled with [^{3}H]-leucine and immunoprecipitated with antibody to peptides in the ectodomain of gN or with antibodies to HA. Arrows indicated the positions of gM and gN.

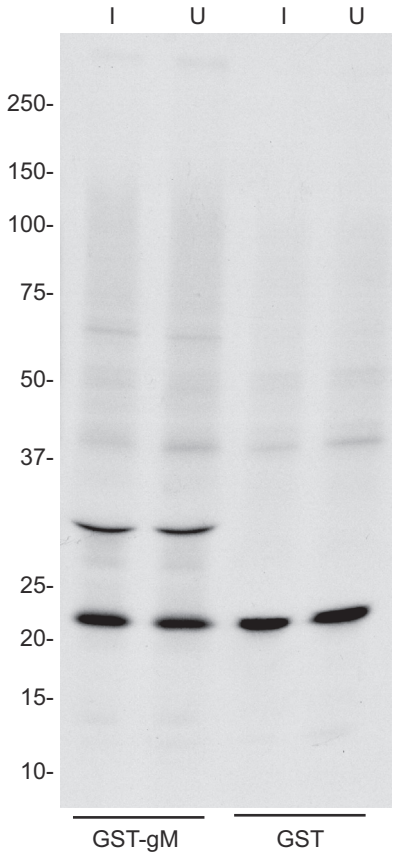


Fig. 2. Proteins pulled down by GST or GST-gM. SDS-PAGE and autoradiography of extracts of induced (I) or uninduced (U) Akata cells labeled with [^{3}H]-leucine and pulled down with GST or GST-gM bound to glutathione-Sepharose.

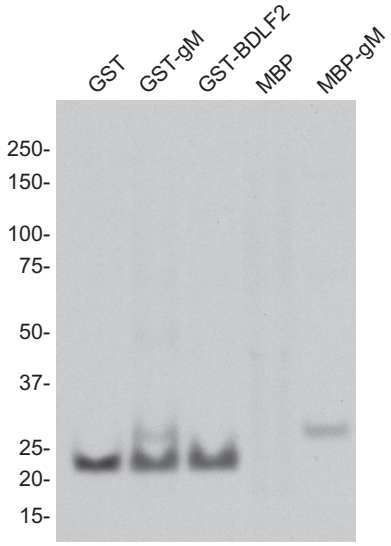
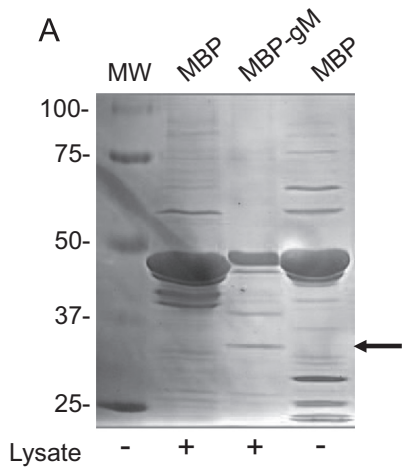


Fig. 3. Proteins pulled down by MBP or MBP-gM. SDS-PAGE and autoradiography of extracts of uninduced Akata cells labeled with [^{3}H]-leucine and pulled down with MBP or MBP-gM bound to amylose resin.

To determine the identity of the 32 kDa protein, unlabeled EBV-negative Akata cell lysates were precipitated with MBP-gM and the proteins were visualized by staining with Coomassie Blue. The region of the gel containing the protein (Fig. 4A) was excised and analyzed by reverse phase HPLC–tandem mass spectrometry. A search of the NCBInr human protein database identified four tryptic peptides that corresponded to the sequence of p32/gC1qR



B

>HUMAN P32 PROTEIN
MLPLLRCVPRVLGSSVAGLRAAAPASPFRQLL
QPAPRLCTRPFGLLSVRAGSERRPGLLRPRGP
CACGCGCGSLHTDGD***K*AFVDFLSDEIKEER**KI
QKHKTLPK***MSGGWELELNGTEAKL***VRKVAGEK
ITVTFNINNSIPPTFDGEEEPSQGQK***VEEQEP***
ELTSTPNFVVEVIKNDDGKKALVLDCHYPEDE
VGQEDEAESDIFSIREVSFQSTGESEWKDTNY
TLNTDSLDWALYDHLMDFLADR***GVDNTFADEL***
VELSTALEHQEYITFLEDLKSFVKSQ

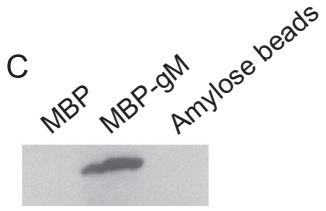


Fig. 4. Interaction of gM with p32. (A) Coomassie-stained SDS-PAGE analysis of proteins pulled down by MBP or MBP-gM from uninduced Akata cell lysates. MW = molecular weight markers. Arrow indicates protein excised for analysis. (B) Tryptic peptides (bold) identified by mass spectroscopy of the excised protein which correspond to the sequence of p32/gC1-q-R. (C) Western blot analysis with antibody to p32 of proteins pulled down from Akata cell lysate by MBP or MBP-gM.

(Fig. 4B), a multifunctional, multicompartmental protein. The identity of the protein was confirmed by Western blotting of protein pulled down from EBV-negative Akata cells by MBP-gM (Fig. 4C). To determine if the interaction could be recapitulated with full-length gM, cells were nucleoporated with vectors pCAGGS-HA-gM and pCAGGS-p32, lysed and either immunoprecipitated with antibody to HA and western blotted antibody to p32, or vice versa. The interaction of gM and p32 could be seen by both protocols (Fig. 5); an unrelated HA-tagged EBV protein, BFLF2, did not interact with p32, serving as an additional control for specificity. Despite the fact that our previous GST-pull downs clearly indicated that endogenous p32 could interact with the gM tail, we were, however, unable to immunoprecipitate detectable levels of endogenous p32 with transfected gM. In addition, there was no discernable colocalization of p32 and gM when the two proteins were co-expressed in nucleoporated cells and the distribution of p32 was unchanged in induced Akata cells making virus (Fig. 6).

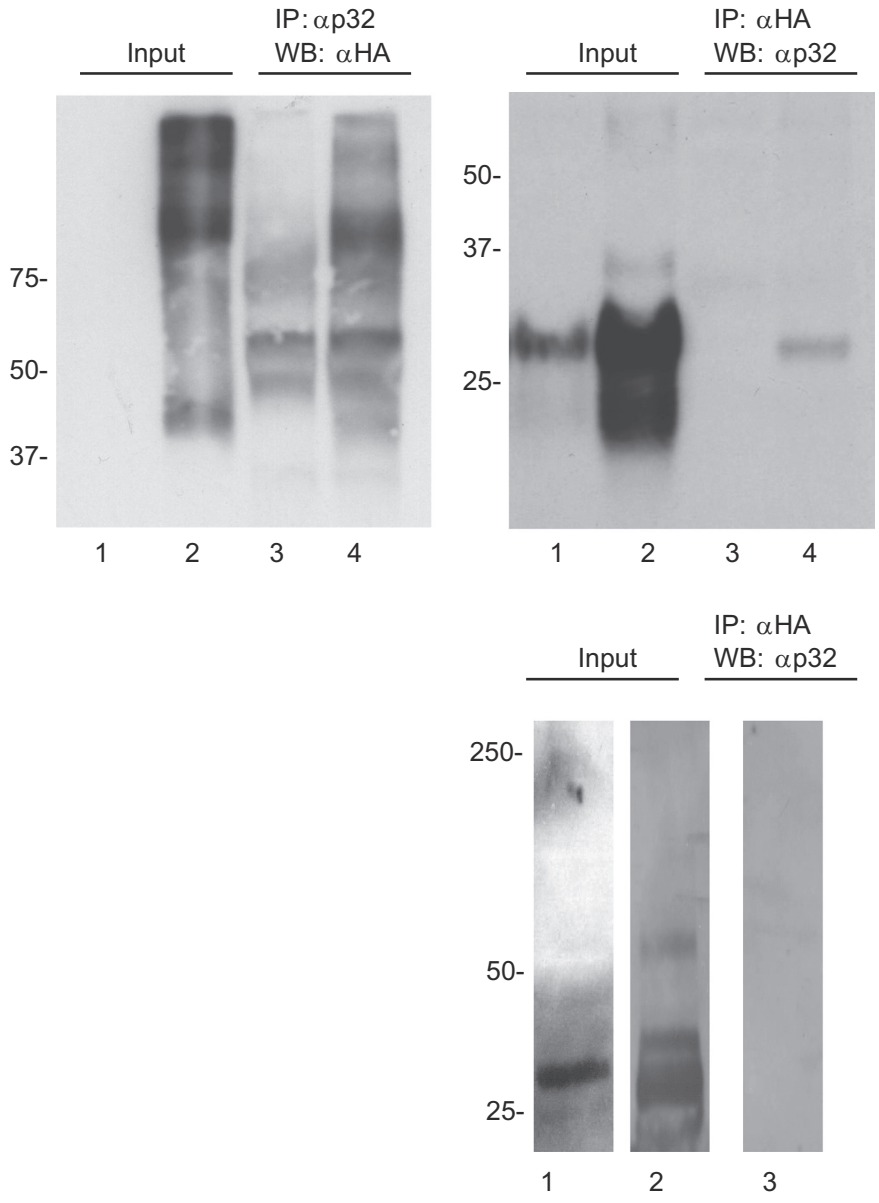


Fig. 5. Interaction of full-length gM with p32. Upper panels: SDS-PAGE and western blot analysis of lysates of AGS epithelial cells nucleoporated with vector pCAGGS (lanes 1 and 3), pCAGGS expressing full length HA-tagged gM and pCAGGS expressing p32 (lanes 2 and 4). Lysates in lanes 3 and 4 were either immunoprecipitated (IP) with antibody to p32 and western blotted with antibody to HA, or immunoprecipitated with antibody to HA and western blotted with antibody to p32 as indicated. Lower panels: SDS-PAGE and western blot analysis of lysates of AGS cells nucleoporated with pCAGGS-HA-BFLF2 and pCAGGS-p32. Lysates in lanes 3 were immunoprecipitated (IP) with anti-HA. Lysates in lanes 2 and 3 were western blotted with antibody to p32; lysates in lane 1 were western blotted with anti-HA.

Reduction of endogenous p32 levels reduced the amount of enveloped virus released

Because we were only able to pull down endogenous p32 with GST-gM we were concerned that the interaction was not biologically relevant. Since deletion of the gN gene and loss of the gMgN complex had previously been shown to impair virus egress, we therefore examined the effect of partially silencing p32 on virus yields. Akata cells were transfected with siRNA to p32 or non-targeting control siRNA, incubated for 96 h and then induced to make virus. Twenty four h later virus was harvested from the supernatant and the relative amounts of virus DNA in the cell and the supernatant were measured by QPCR. Western blotting of cells revealed a reduction in p32 expression by siRNA to a level of approximately one third of the level in cells treated with non-targeting siRNA (Fig. 7) and the viability of cells treated with either siRNA was similar (not shown). In three separate knockdowns the average amount of virus DNA released from cells treated with siRNA was 53 $\pm$ 10% of the amount released from cells treated with non-targeting siRNA. The virus that had been released after deletion of the gN gene was in large part virus that lacked an envelope (Lake and Hutt-Fletcher, 2000). Its ability to bind to CR2-positive cells, with which the major virus attachment protein interacts, was 10% of that of wild type virus and its sedimentation rate increased. The properties of the virus released from cells treated with siRNA to p32 or non-targeting siRNA were therefore compared. Virus harvested from cells treated with siRNA to p32 in two separate knockdowns was reduced in its ability to bind to SVKCR2 cells by 37.6% and 38.9% respectively. This is much less than the impairment of virus lacking the gNgM complex. However, we do not know if we have reduced p32 completely in 66% percent of cells or more than that in a subpopulation of cells, induction of virus replication occurs only in around 50% of cells and the two populations are unlikely to overlap completely. We had previously

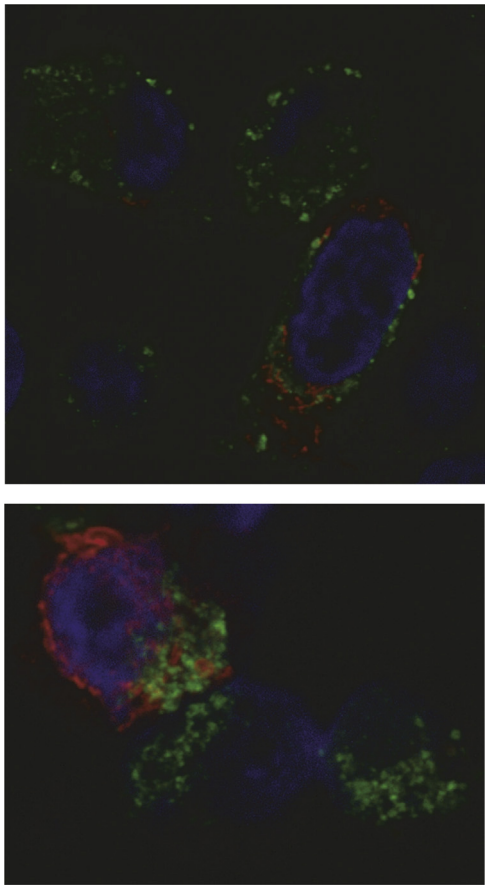


Fig. 6. Confocal images of cellular localization of p32 after nucleoporation or virus induction. Top panel: cells were nucleoporated with pCAGGS-p32, pCAGGS-HA-gM, pCAGGS-gN and stained with DAPI, antibody to p32 (red) and antibody to HA (green). Bottom panel: Akata cells were induced to make virus and cells were stained with DAPI, antibody to gB (red) and antibody to p32 (green).

found that intact virus capable of binding to CR2 sediments predominantly in fractions with a refractive index of 1.36 whereas virus unable to bind, because of a damaged or completely lost envelope, sedimented in fractions with a refractive index greater than 1.38. We now found that the sedimentation rate in a 24–42% Nycodenz gradient of much of the virus released from cells treated with siRNA to p32 also increased relative to virus released from cells treated with non-targeting siRNA (Fig. 7).

In an attempt to establish stable clones of Akata that lacked p32 we transduced cells with a pool of three lentiviruses expressing shRNA targeting p32 or lentiviruses expressing non-targeting shRNA and selected the cells with puromycin. Clones were screened by western blotting for p32 and p32 negative clones were obtained. However, when the clones were expanded to numbers that enabled virus production it was determined that all had regained expression of p32. To complement the studies in which p32 expression was knocked down with siRNA cells were then transduced with the targeting and non-targeting lentiviruses and selected for only 8 days on puromycin, to enrich for transduced cells, before induction of virus. Thirty six hours after induction virus was harvested, cells were analyzed by western blotting to determine levels of expression of p32 and virus was harvested for sedimentation analysis. The expression of p32 in cells transduced with lentivirus targeting p32 was again reduced to approximately one third of that in cells transduced with non-targeting lentivirus and, again, the sedimentation profile of the virus released was consistent with a significant proportion lacking an intact envelope (Fig. 8). We also examined the induced and

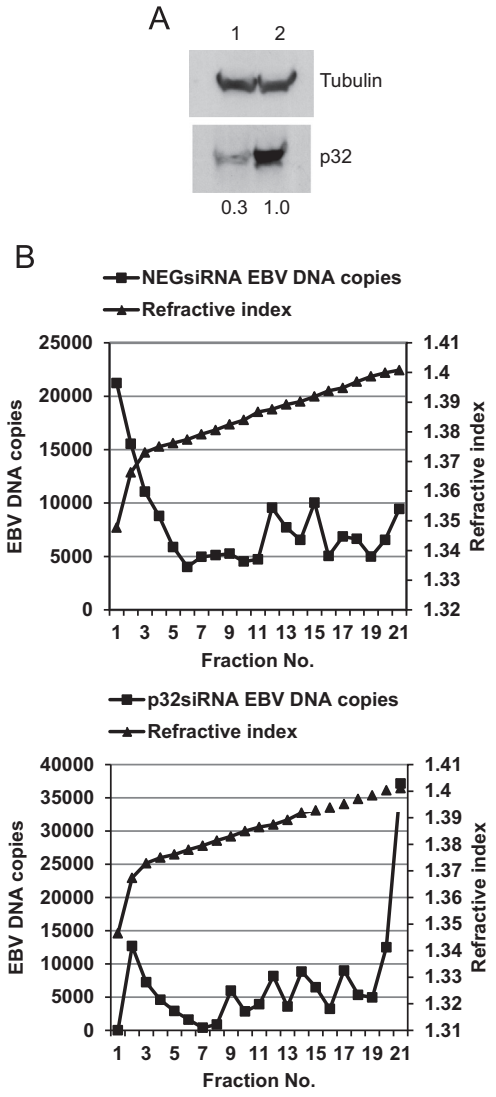


Fig. 7. Sedimentation analysis of virus produced by cells treated with siRNA to p32. A. Western blot of virus-producing Akata cell lysates transfected with siRNA to p32 (lanes 1) or non-targeting siRNA (lanes 2) with antibody to p32 or tubulin as indicated. B. Sedimentation profiles in 24–42% Nycodenz of virus produced from cells transfected with non-targeting siRNA (upper panel) or siRNA targeting p32 (lower panel). Virus DNA in each fraction was measured by QPCR.

transduced cells by electron microscopy (Fig. 9). In only 4 out of 60 virus positive cells transduced with non-targeting lentivirus, EBV could be seen in condensed chromatin whereas nucleocapsids were found in condensed chromatin in 13 out of 60 cells transduced with lentivirus targeting p32.

Discussion

Assembly and egress of EBV have not been as extensively studied as have the same processes in several other herpesviruses, largely due to difficulties in studying a virus that is not reproducibly reactivated in a large majority of latently infected cells and produces virus particles asynchronously, at less than robust levels, over a period of several days. Most efforts have focused on understanding capsid assembly (Henson et al., 2009; Wang et al., 2011), which occurs at promyelocytic leukemia nuclear bodies (Wang et al., 2015), and initial nuclear egress. Nuclear egress requires the dimeric complex of BFRF1 and BFLF2 (Lake and Hutt-Fletcher, 2004; Farina et al., 2005; Granato et al., 2008), homologs

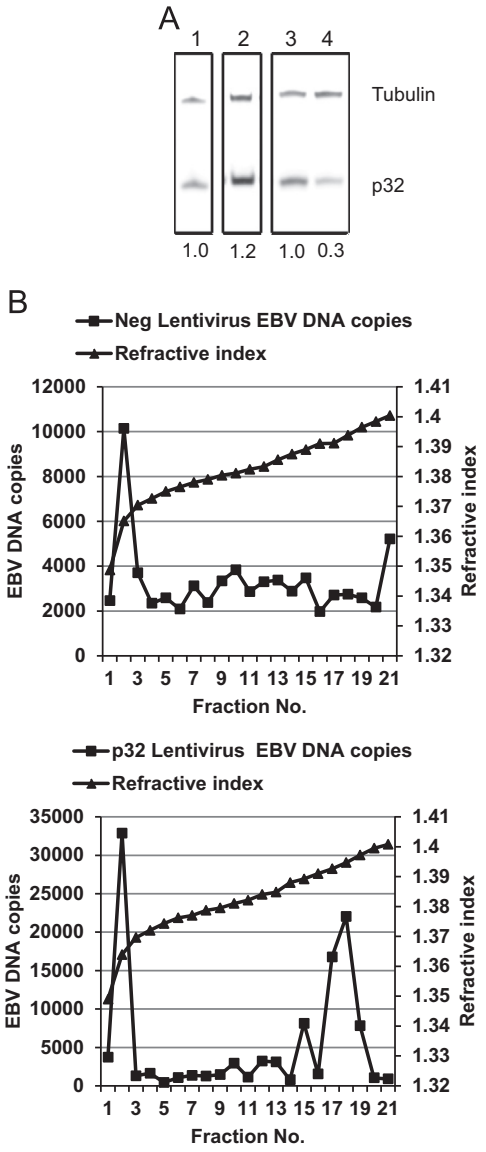


Fig. 8. Sedimentation profile of virus produced by cells transduced with lentivirus expressing shRNA to p32. A. Western blot of uninduced Akata cells (lane 1), induced Akata cells (lane 2), induced Akata cells transduced with non-targeting lentivirus (lane 3) or induced Akata cells transduced with lentivirus targeting p32 (lane 4). Blots were probed with antibodies to tubulin (upper bands) and p32 (lower bands). B. Sedimentation profiles in 24–42% Nycodenz of virus produced from cells transduced with non-targeting lentivirus (upper panel) or lentivirus targeting p32 (lower panel). Virus DNA in each fraction was measured by QPCR.

of the nuclear egress complex proteins of alpha and beta-herpesviruses, which are critical for budding into the perinuclear space, and the viral kinase BGLF4 (Gershburg et al., 2007). Much less is known about movement of EBV into, through and out of the cytoplasm, although the assumption is generally made that it follows an deenvelopment, reenvelopment pathway similar in outline to that of other herpesviruses (Johnson and Baines, 2011). Our previous work with the gN-null recombinant virus had implicated gNgM in the process, possibly at the stage of secondary envelopment (Lake and Hutt-Fletcher, 2000) and we had speculated that the role for EBV gM might be in directing capsids to the site of envelopment by recruiting capsid-associating tegument proteins. Our expectation therefore was that the predicted cytoplasmic tail of gM, which, like that of HCMV (Mach et al., 2005), was not required for formation of the gNgM complex, would interact with one or more of the EBV tegument proteins. It was thus a surprise that the protein we identified as binding to the 79 carboxyterminal residues of gM, was p32.

Human p32 is also known as gC1q-R/p33, a receptor for the complement component C1q, p32/TAP and SF2-associated p32. It is an enigmatic, evolutionarily conserved, widely expressed molecule that has been variously described as a mitochondrial matrix protein (Jiang et al., 1999) and a multi-compartment protein expressed both intracellularly and on the cell surface (Ghebrehiwet et al., 2001). It has been shown to recognize diverse ligands, both microbial, cellular and of course those found in plasma. It has been reported to interact with a confusingly large number of disparate virus proteins, including HIV-1 Tat and Rev, EBV EBNA1, ORF P of herpes simplex virus, rubella capsid protein and core protein V of adenovirus (reviewed in (Ghebrehiwet and Peerschke, 2004)) and has also been described as a general regulator of protein kinase C location and function (Robles-Flores et al., 2002). Of particular interest, it has recently been implicated in nuclear egress of human cytomegalovirus (Marschall et al., 2005; Milbradt et al., 2007) and herpes simplex virus (Wang et al., 2014; Liu et al., 2015). The human cytomegalovirus protein kinase, pUL97, interacts with p32 and accumulates at the nuclear membrane. The model is then that the cell protein acts as an adapter for recruitment of proteins to the nuclear lamin where it interacts with the lamin B receptor. Recruitment of virus and cellular protein kinases leads to the disintegration of nuclear lamina which is required for virus egress.

The phenotype seen here for EBV is at least partially consistent with the observations made for alpha and beta herpesviruses. An EBV protein, gM, which has been implicated in egress, can interact with p32. Unlike the observations made with herpes simplex virus infected cells (Wang et al., 2014; Liu et al., 2015), however, we were unable to detect any change in the punctate location of endogenous p32 in cells that had been induced to make virus and saw no colocalization of p32 and gM when the gNgM complex was expressed together with exogenous p32. This raised concern as to the biologic significance of the finding. In support of its relevance, the effects of reducing expression levels of p32 did, nevertheless, recapitulate findings made with the genotypically gN-null virus that we had previously made. This virus, phenotypically null for the entire gNgM complex, produced extracellular particles which sedimented in isopycnic gradients at rates characteristic of those either lacking an envelope or being incompletely enveloped. The same was true of virus particles made in cells partially knocked down for p32 expression by either siRNA or lentivirus expressed shRNA. We cannot know in how many cells the levels of p32 are reduced, which confounds interpretation of the electron microscopy done. However, it would appear that the phenotype of the gN-null virus, which in part remained associated with condensed chromatin, was also partially reproduced by p32 knockdown.

The original assumption that the EBV gNgM complex was involved in secondary envelopment rather than nuclear egress was based on visualization of unenveloped capsids in the cytoplasm of cells making the gN-null virus. In view of the current findings and those made with alpha and betaherpesviruses, however, it seems possible that the primary defect may actually have been in nuclear egress and that the cytoplasmic particles represented some kind of nuclear breakdown, even though none was obvious (Lake and Hutt-Fletcher, 2000). At this point it is premature to speculate as to whether p32 is part of an EBV nuclear egress complex, but the function conservation throughout the herpesvirus subfamilies of other virus proteins involved in egress, such as the homologs of BFRF1, BFLF2 and BGLF4, suggests that the possibility should be explored further.

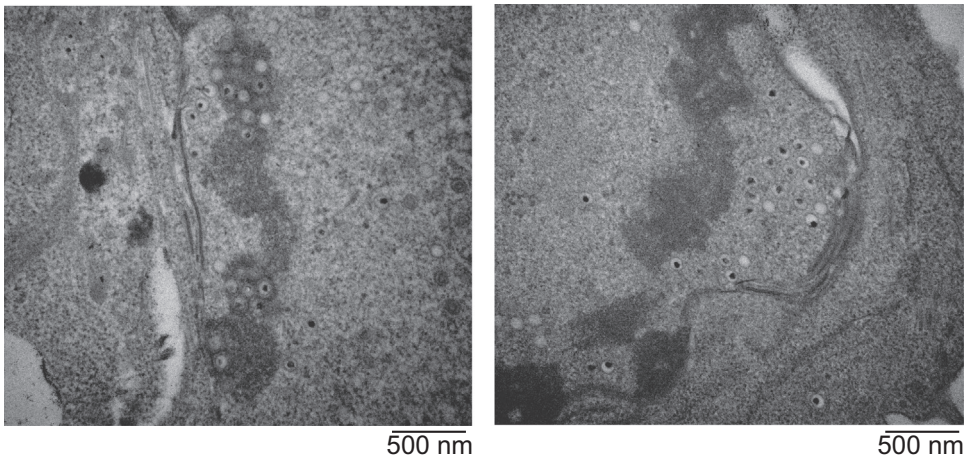


Fig. 9. Electron micrographs of induced Akata cells which had been transduced with lentivirus targeting p32 (left panel) or non-targeting lentivirus (right panel). Cells were harvested after being induced for 24 h.

Materials and methods

Cells, virus and antibodies

Akata B cells, Burkitt lymphoma-derived cells that carry and can be induced to make EBV (Takada, 1984), and EBV negative-Akata B cells were grown in RPMI 1640 (Sigma). CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium, AGS gastric carcinoma cells (American Type Culture Collection) were grown in F-12 Nutrient Mixture (Gibco) and SVKCR2 cells, an SV40-transformed keratinocyte cell line engineered to express CR2 (Li et al., 1992) were grown in Joklik's modified Eagle's medium supplemented with 10-ng/ml cholera toxin (Sigma) and 10% calf serum (Hyclone). All other media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Akata cells at a concentration of 10^6/ml were induced to make virus by incubation with 100 μg/ml goat anti-human globulin (MP Biomedics). Vaccinia virus expressing T7 polymerase was made as described (Lake et al., 1998). GIPZ lentiviruses expressing shRNA targeting p32 (V2LHS_112642, V3LHS_379794 and V3LHS_379796) and non-targeting lentiviruses were obtained from ThermoFisher and used to transduce Akata cells according to the manufacturer's instructions. Antibodies used were monoclonal antibodies 72A1 to gp350 (Hoffman et al., 1980), CL55 to gB, sc23885 to p32 (Santa Cruz Biotechnology), 16B.12 to HA (Covance) and to tubulin alpha Ab-2 (DMIA) (NeoMarkers) and rabbit polyclonal antibodies sc48795 to p32 (Santa Cruz Biotechmology), HA.11 to HA (Covance) and to peptides corresponding to residues 44–69 of gN (Lake and Hutt-Fletcher, 2004). Secondary antibodies included goat anti-mouse IgG1 conjugated to AlexaFluor 488, goat anti-IgG2a conjugated to AlexaFluor 594, goat anti-mouse IgG conjugated to AlexaFluor 488 and goat anti-rabbot conjugated to AlexaFluor 594 (Invitrogen). All non-commercial antibodies were purified by affinity chromatography on Protein A-Sepharose 4B (Sigma).

Expression plasmids

The predicted cytoplasmic tail of gM, residues 327–405, was cloned for expression in bacteria as a fusion protein with glutathione S-transferase (GST-gM) and as a fusion protein with maltose-binding protein (MBP-gM) using vectors pGEX-5X-3 (GE Healthcare) and pMAL-c4 (New England Biolabs) respectively. To make pGEX-gM, sequences were amplified from Akata virus DNA with forward primer 5′-CATTAGGATCCTCAAAA GCATGCGCCAG-3′ which included a BamHI site and reverse primer 5′-CAATACTC GAGTTAGGGGAAGATCTCCTCC-3′, which included an XhoI site and inserted into pGEX-5X-3 cut with BamHI and XhoI. To make pMAL-c4-gM sequences were amplified with forward primer 5′-CATTAGGATCCAAAAGCATGCGCCA-3′ which included a BamHI site and reverse primer 5′-TGAGAAGCTTTTAGGGGAAGATCTCCTC-3′ which included a HindIII site and inserted into pMAL-c4x vector cut with BamHI and HindIII. The 169 residue cytoplasmic tail of BDLF2 was also cloned for expression as a fusion protein with glutathione S-transferase (GST-BDLF2) as described (Gore and Hutt-Fletcher, 2008). For expression in mammalian cells, genes were either cloned into the pTM1 vector under control of the T7 promoter (Moss et al., 1990), or cloned into the pCAGGS/MCS vector (a gift of Martin Muggeridge, LSUHSC) under control of the β-actin promoter in cooperation with the HCMV-IE enhancer (Niwa et al., 1991). Plasmids pTM1 gM and pTM1 gN were previously described (Lake et al., 1998). To make pTM1-HA-gM, gM sequences were amplified from pTM1-gM with forward primer 5′-GGCCTCAGCATATGAAGTCCTCCAA-3′ containing an NdeI site and reverse primer 5′-GGCGGATCCTTAGGGGAAGACT-3′ which contained a BamH1 site. The amplified fragment was cloned into pGADT7 cut with NdeI and BamHI to make pGADT7-HA-gM. An NcoI/BamHI fragment containing HA-gM was then cloned back into pTM1. To make pCAGGS-HA-gM an NcoI/XhoI fragment from pTM1-HA-gM, in which the NcoI site had been blunted, was cloned into pCAGGS cut with SmaI and XhoI. To make pTM1-HAgMΔ79gM sequences were amplified from pTM1-HAgM with forward primer 5′-GGCCTCAGCATATGAAGTCCTCCAA-3′ and reverse primer 5′-GATCTCGAGTTAGAAAATCCGGCATATCC-3′. The amplified fragment was cut with NdeI and XhoI and cloned into pTM1-HAgM cut with the same enzymes. To make pCAGGS-p32, cDNA was made from RNA extracted from Akata cells and amplified with forward primer 5'-CAGTGAATTCGCCACCATGCTGC-CTCTGCTGCGC-3' which included an EcoRI site and reverse primer, 5′-TTACAGATCTCTACTGGCTCTTGACAAAACTCTTG-3′ which included a BglII site and was inserted into pCAGGS which had been cut with EcoRI and BglII. Plasmid pCAGGS-HA-BFLF2 was recloned from the previously described pTM1-HA-BFLF2 (Lake and Hutt-Fletcher, 2004). All PCR amplifications were done with Pfx (Invitrogen) and constructs checked by sequencing.

Expression of proteins in mammalian cells

The pCAGGS vectors were nucleoporated into cells (see below). For expression in from pTMI vectors cells were infected with vaccinia virus and transfected with plasmid using Lipofectamine (Gibco) as previously described (Lake et al., 1998).

Expression of fusion proteins in bacteria

NEB5-α cells (New England Biolabs) carrying pGEX-gM or pGEX-5X-3 were induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside when the OD_{600} of the culture reached ~0.5 and were grown for 3 h more at 37 °C before harvesting by centrifugation. The bacteria were resuspended in phosphate buffered saline (PBS) containing 1% Triton X-100 (Sigma) and Complete Mini EDTA-free protease inhibitors (Roche). After sonication cellular debris was removed by centrifugation and GST and GST-gM were purified from the lysates on glutathione-Sepharose (Amersham) following the manufacturer's instructions. Arctic Express RIL cells (Stratagene) carrying pMAL-c4-gM or pMAL-c4 were induced with 0.15 mM isopropyl-β-D-thiogalactopyranoside when the OD_{600} of the culture reached ~0.5 and were grown for 24 h more at 13 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris–HCl [pH 7.4], 200 mM NaCl 1 mM EDTA) containing Complete Mini EDTA-free protease inhibitors. Lysozyme was added (1 mg/ml), the lysates were incubated at room temperature for 15 min, sonicated, centrifuged to remove cell debris and incubated with amylose resin (New England Biolabs) to purify MBP or MBP-gM according to the manufacturer's protocol.

Radiolabeling and precipitation

Two million Akata cells were induced to make EBV by induction with 100 μg goat $(Fab')_2$ anti-human immunoglobulin G (MP Biomedicals). Four h later cells were suspended in leucine-free medium (Gibco) for 2 h and then labeled for 24 h with 200 μg [^{3}H] leucine (100–150 Ci/mmol; Perkin Elmer). Two million EBV-negative Akata cells were suspended in leucine-free medium for 2 h and labeled for 24 h with 200 μg [^{3}H]leucine. Labeled cells were solubilized in radioimmunoprecipitation buffer, (RIPA; 50 mM Tris–HCl [pH 7.2], 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml). Lysates were incubated overnight at 4 °C with ~100 μg GST or GST-gM bound to glutathione-Sepharose, or ~100 μg MBP or MBP-gM bound to amylose resin. Beads were washed 10 times with RIPA and boiled for 3 min in sample buffer containing β-mercaptoethanol. Dissociated proteins were analyzed by SDS-polyacrylamide electrophoresis in either 12% polyacryamide cross-linked with bis-acrylamide, followed by fluorography or in 9–18% gradient gels.

Mass spectrometry

For identification of proteins interacting with MBP-gM, Akata cells (1.5×10^7) were lysed in RIPA. Lysates were incubated either MBP or MBP-gM bound to amylose beads, beads were washed 10 times with RIPA and boiled for 3 min. Dissociated proteins were separated by electrophoresis in a 12.5% Criterion Tris–HCl polyacrylamide gel (BioRad). The gel was stained with coomassie blue and slices of gel containing the protein of interest were cut out and stored in 0.1% formic acid until analyzed by mass spectrometry. Gel slices were reduced, alkylated and digested with trypsin. The peptide extracts were analyzed in triplicate on a LTQ FT mass spectrometer (Therno Fisher Scientific) coupled with a 2D NanoLC (Eksigent Technologies, California, USA). In each run, the sample was injected with 1D pump to a trap-column (100 μm ID fused silica, packed in-house with 3 cm of 100 Å, 5 μ, Magic C18 particles, Michrom Bioresources), and washed with 0.1% formic acid for 15 min at 0.5 μl/min. Before elution of the peptides the trap-column was connected to an analytical column (75 μm ID fused silica, packed in-house with 12 cm of 100 Å, 5 μ, Magic C18 particles, Michrom Bioresources) that was mounted on the electrospray stage of a LTQ FT mass spectrometer (ThermoFinnigan). The mobile phase A and B in the 2D pump were 0.1% formic acid, and 0.1% formic acid in acetonitrile. The gradient profile was as follows: 0–5 min, 5–15% B; 5–60 min, 15-40% B; 60–70 min, 40% B; 70–75 min, 40–75% B; 75–83 min 75% B. At the end the column was washed with 90% B. The flow rate was maintained at 300 nl/min and using an electrospray voltage of 1.9 kV with the ion transfer temperature set to 250 °C. The mass spectrometer was controlled by the Xcalibur software to perform continuously mass scan analysis on the FT in the range of 400–1900 m/z at 50,000 resolution, followed by MSMS scans on the ion trap of the six most intense ions, with a dynamic exclusion of two repeat scans of the same ion, 60 s repeat duration and 90 s exclusion duration. Normalized collision energy for MS/MS was set to 35%. Isolation with was 2. For data analysis all MSMS scans were searched using Proteome Discoverer (version 1.4,ThermoFisher Scientific) running Sequest as the search algorithm. Database search was conducted against a human protein database derived from the NIBInr repository (as on 08 15, 2015), using trypsin cleavage specificity and a maximum of 2 missed cleavages. The following variable modifications were selected: pyroglutamination from Q (N-terminal), oxidation of M and deamidation of N, Q; carboxymethylation of C was selected as a fixed modification, a maximum of 3 modifications/peptide were allowed. Estimation of false positive rate (FDR) was conducted by searching all spectra against a decoy database consisting of the inverted sequences of all proteins in the original (direct) database. A minimum Xcorr score 0f 1.8, 2.5 and 3.8 for ions with 1, 2, and 3 charges, respectively, and a peptide score larger than 0.1 was required for tandem MS selection. For protein identification, a FDR $\leq$ 0.1 was defined and a minimum of two unique peptides per protein was required. Amino acid sequence assignments of all peptides of interest were subsequently inspected manually.

Western blotting

Western blotting of electrophoresed proteins was done as previously described (Molesworth et al., 2000). Membranes were reacted with appropriate concentrations of primary and horseradish peroxidase-conjugated secondary antibodies (Amersham), were washed several times and were developed with ECL plus western blotting detection system (GE Healthcare) according to the manufacturer's instructions.

Virus binding

Virus concentrations were adjusted to contain equal amounts of encapsidated EBV DNA after analysis by Quantitative Real Time-PCR (QPCR). Virus binding was measured by incubating virus with 10^6 SVKCR2 cells for 2 h at 4 °C, after which cells were washed 3 times with PBS and scraped off into ice cold versene (Invitrogen). The cells were pelleted by centrifugation and DNA was isolated with QIAamp DNA Blood Mini Kits (QIAGEN Sciences) for determination of the amount of virus DNA bound by QPCR.

Quantitative Real Time-PCR (QPCR)

Genomic DNA from cells was isolated with QIAamp DNA Blood Mini Kit (QIAGEN Sciences). For isolation of DNA from supernatants (passed through 0.8 μm filter), these were first digested with DNase I (2 U/ 20 μl of supernatant) for 1 h at 37 °C followed by heat inactivation of the enzyme (65 °C for 15 min) after addition of stop solution. Supernatants were diluted (1:10) with water containing proteinase K (1 mg/ml), incubated at 56 °C for 2 h and boiled for 2 min to inactivate the enzyme. The solution was further

diluted 10 times and used for QPCR done as previously described (Turk et al., 2006). Briefly A 76-bp region of the EBV EBNA1 gene in the single copy BamHI K fragment was amplified with primers 5′-GGATGCGATTAAGGACCTTGTT-3′ and 5′-CGTCAAAGCTGCACACAGTCA-3′, base coordinates 109677 and 109753, respectively (NCBI GenBank accession number VO1555) At the same time a 101-bp DNA sequence of the human C-reactive protein (CRP) gene was amplified with primers 5'-CTTGACCAGCCTCTCTCATGC-3′ and 5′-TGCAGTCT TAGACCCCACCC-3′, base coordinates 132705 and 132605, respectively (accession no. AL445528). Amplifications were done with the TaqMan Fluorogenic System (PE Applied Biosystems). The EBNA1 probe (5′-CAAAGCCCGCTCCTACCTGCAATATCA-3′, base coordinate 109703) was labeled with 6-carboxyfluorescein and the CPR probe (5′-TTTGGCCAGACAGGTAAGGGCCACC-3′, base coordinate 132682) was labeled with VIC (PE Applied Biosystems). Reactions were performed in a 50 μl volume with TaqMan Universal Master Mix (PE Applied Biosystems), 300 nM primers, 200 nmol/liter probe, and 400 or 200 ng of DNA. Amplification consisted of 2 min at 50 °C, 10 min at 95 °C, and 50 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample was run in duplicate, together with multiple template-negative controls. Serial dilutions of IB4 DNA, a Burkitt's lymphoma cell line containing five copies of EBV per cell, served as the standard. The EBV copy number per sample was normalized to the amount of CRP DNA representing the actual amount of amplifiable cellular DNA in each sample.

Nucleofection

AGS cells were nucleoporated with pCAGGS, pCAGGS-HA-gM or pCAGGS-p32 proteins following the manufacturer's protocol (Amaxa). Briefly, 8 μg of DNA was nucleoporated per 5×10^6 cells using Kit V and program T-20. Nucleofected cells were incubated at 37 °C in growth media for 48 h before further analysis. For knock down of RNA, 2 μM siRNA (Dharmacon) to p32 (L-011225-01) or a non-targeting control (D-001810-10) were nucleoporated into 10^6 EBV positive Akata cells using Kit V and program G-16. Cells were incubated at 37 °C for 96 h before further analysis or processing.

Immunoprecipitation

For immunoprecipitation, AGS cells were washed with PBS, collected in versene (Invitrogen), washed with PBS again and then lysed in RIPA. Lysates (500 μg at 1 μg/μl concentration) were precleared with either protein A- (Sigma) or protein G-agarose (Pierce) beads for 30 min at 4 °C and then appropriate antibodies (5-8 μg) were added for immunoprecipitation at 4 °C for ~2 h. Lysates were reincubated for 1 h at 4 °C after addition of protein A- or protein G-agarose beads. Pelleted beads were washed twice with RIPA and 3 times with PBS. Immunoprecipitated proteins were dissociated by boiling for 3 min in sample buffer containing β-mercaptoethanol (samples were instead held at 56 °C for 30 min when full length gM was used) and analyzed by SDS-polyacrylamide electrophoresis in 12% polyacryamide cross-linked with bis-acrylamide.

Sedimentation analysis of virus

Virus was harvested from the supernatant of Akata cells that had been nucleoporated with siRNA incubated for 96 h and induced for 24 h or transduced with lentivirus, selected on puromycin for 8 days, induced for 36 h. Five hundred μl of the virus were loaded onto a 24 to 42% continuous gradient of Nycodenz (Sigma) in 1 mM potassium phosphate containing 0.01% bacitracin made, as previously described (Lake and Hutt-Fletcher, 2000), by layering 1 ml of 42, 40, 38, 36, 34, 32, 30, 28, 26 and 24% Nycodenz in a centrifuge tube and allowing the steps to diffuse overnight at 4 °C. The gradient was centrifuged in a Beckman SW41 Ti rotor at 70,000 × *g* for 2 h at 4 °C and 500 μl fractions were harvested from the top. The refractive index of each fraction was measured and the amount of virus in each was determined by QPCR.

Electron microscopy

Ten million cells were pelleted at 350 × *g* and washed with cacodylate buffer (01.5 M sodium cacodylate, 2 mM calcium chloride) at 4 °C. Cells were fixed overnight at 4 °C in cacodylate buffer containing 4% glutaraldehyde, washed 3 times in buffer alone, transferred to a microcentrifuge tube, pelleted at 250 × *g* and resuspended in in 50 μl of cacodylate buffer containing 10% gelatin prewarmed to 37 °C. Cells were pelleted at 250 *g* for 2 min and put on ice. Each gelatin plug was cut into 1mm cubes which were rinsed, postfixed for 1 h with osmium tetroxide, rinsed, dehydrated and embedded in Araldite for sectioning.

Acknowledgments

This research was supported by Public Health Service grant AI061017 from the National Institute of Allergy and Infectious Diseases.

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