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Rapid Communication

Pim kinases are upregulated during Epstein–Barr virus infection and enhance EBNA2 activity

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Abstract

Latent Epstein–Barr virus (EBV) infection is strongly associated with B-cell proliferative diseases such as Burkitt's lymphoma. Here we show that the oncogenic serine/threonine kinases Pim-1 and Pim-2 enhance the activity of the viral transcriptional activator EBNA2. During EBV infection of primary B-lymphocytes, the mRNA expression levels of *pim* genes, especially of *pim*-2, are upregulated and remain elevated in latently infected B-cell lines. Thus, EBV-induced upregulation of Pim kinases and Pim-stimulated EBNA2 transcriptional activity may contribute to the ability of EBV to immortalize B-cells and predispose them to malignant growth.

Keywords: EBV; EBNA2; Pim; transcription

Introduction

Pim proteins constitute a family of small serine/threonine-specific cellular protein kinases which efficiently cooperate with Myc family oncoproteins in murine lymphomagenesis (Allen et al., 1997; van Lohuizen et al., 1989). Similar cooperation is likely to occur also in humans, since expression of *pim*-1 has been observed to be elevated, e.g., in leukemias (Amson et al., 1989). In hematopoietic cells, *pim*-1 or *pim*-2 genes can be upregulated by interleukins and other cytokines (Allen et al., 1997; Lilly et al., 1992; Matikainen et al., 1999), suggesting a role for Pim kinases in cytokine receptor-initiated signaling. Indeed, Pim-1 has been shown to protect cytokine-dependent cells from apoptosis (Aho et al., 2004; Fox et al., 2003; Lilly et al., 1999; Yan et al., 2003) and to regulate activities of several hematopoietic cell transcription factors including c-Myb (Leverson et al., 1998), NFATc1 (Rainio et al., 2002), and STAT5 (Peltola et al., 2004). Pim-1 stimulates c-Myb activity via its interaction with the p100 protein (Leverson et al., 1998), which is also known as the tudor staphylococcal nuclease (Tudor-SN; Callebaut and Mornon, 1997) recently implicated in RNA processing (Caudy et al., 2003), but which was first described as a coactivator for the Epstein–Barr virus (EBV) nuclear antigen EBNA2 (Tong et al., 1995). EBNA2 is able to activate transcription of several viral and cellular target genes through its acidic transactivation domain and is absolutely essential for the EBV-induced immortalization and transformation of primary B-cells (Cohen and Kieff, 1991). Here we demonstrate that infection of primary B-lymphocytes by EBV results in upregulation of *pim* family gene expression, and that the Pim kinases in turn enhance transcriptional activity of EBNA2.

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Results and discussion

Pim-1 and Pim-2 kinases enhance EBNA2-mediated transcriptional activation

To examine the potential effects of Pim kinases on EBNA2 activity, EBV-negative BJAB B-lymphoblasts were transiently transfected with Gal4-luciferase and ß-galactosidase reporter constructs, a plasmid expressing the EBNA2 acidic transactivation domain fused to the Gal4 DNA-binding domain (pGal4-E2), and different amounts of plasmids encoding Pim-1 (pSV-*pim*-1), Pim-2 (pSV-*pim*-2), or an N-terminally truncated, kinase-deficient mutant of Pim-1 (pSV-*NT81*). Two days after transfection, cells were collected and their luciferase and ß-galactosidase activities analyzed. As expected (Cohen and Kieff, 1991), expression of the Gal4-EBNA2 fusion protein alone strongly induced Gal4-dependent luciferase activity (Fig. 1A). Coexpression of either Pim-1 (Fig. 1A) or Pim-2 (Fig. 1B) further enhanced reporter activity up to 10-fold in a dose-dependent fashion, while the kinase-deficient mutant did not have any significant effect (Fig. 1A). To control for specificity, we compared effects of Pim-1 on Gal4-dependent luciferase activities in transfected BJAB cells coexpressing Gal4 or Gal4-fusion proteins containing transactivation domains from EBNA2, VP16, and PEBP2αB1. However, activities of only Gal4-EBNA2 and to a lesser extent Gal4-VP16 were stimulated by Pim-1 (Fig. 1C). Interestingly, the acidic transactivation domain of VP16 can also interact with the p100 coactivator, although less efficiently than that of EBNA2 (Tong et al., 1995), which may explain the weak effects of Pim-1 on Gal4-VP16.

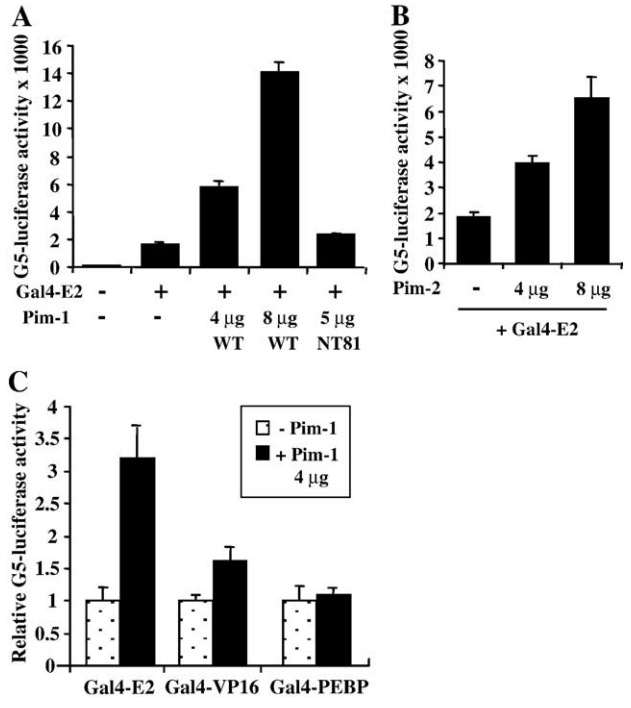

Fig. 1. Both Pim-1 and Pim-2 kinases upregulate activity of the acidic transactivation domain of EBNA2. BJAB cells were transiently transfected with 2 µg of pG5-LUC, 1 µg of pSV-β-GAL, 0.1 µg of pGAL4-E2, -VP16 or -PEBP2αB1, and indicated amounts of wild-type (WT) pSV-*pim*-1, pSV-*pim*-2, or the pSV-*NT81* mutant of Pim-1. Shown are means and standard deviations of either actual luciferase activities normalized against β-galactosidase activities (A and B) or activities relative to those obtained with Gal4-fusion vectors alone (C).

The viral *LMP1* gene encoding a latent membrane protein is one of the physiological target genes of EBNA2 that are turned on during latent EBV infection (Alfieri et al., 1991). To evaluate potential effects of Pim kinases on the activity of full-length EBNA2 and on the expression of LMP1, BJAB cells were cotransfected with a luciferase reporter driven by the LMP1 promoter (pLMP1-LUC) and plasmids expressing Pim-1, Pim-2, or EBNA2 (pSG5-EBNA2). As shown in Fig. 2A, Pim-1 and Pim-2 only slightly affected the basal LMP1 reporter activity. By contrast, EBNA2 upregulated the LMP1 reporter by approximately 10-fold, and the stimulatory effects of EBNA2 were further enhanced by coexpression of either Pim kinase. Western blot analyses confirmed that the exogenous Pim proteins were expressed to a similar extent irrespective of the presence or absence of coexpressed EBNA2 (Fig. 2B). Taken together, our results from both Gal4- and LMP1-dependent luciferase assays suggest that both Pim-1 and Pim-2 potentiate EBNA2-mediated gene expression in a dose- and kinase activity-dependent manner by enhancing the activity of the acidic transactivation domain of EBNA2.

Pim kinases phosphorylate p100, but not EBNA2

Since the kinase activity of Pim-1 was needed for upregulation of EBNA2 activity (Fig. 1A), the ability of Pim-1 to phosphorylate the acidic transactivation domain of EBNA2 was investigated in an in vitro kinase assay. However, Pim-1 was unable to phosphorylate a bacterially expressed GST-fusion protein containing the EBNA2 acidic domain (data not shown). Thus, the phosphorylation-dependent effects of Pim-1 on EBNA2 activity were more likely mediated via other components in the EBNA2-containing protein complex, such as p100, which is a phosphoprotein in vivo (data not shown) and which can be phosphorylated in vitro by Pim-1 (Leverson et al., 1998). To examine whether p100 is a substrate also for Pim-2, we carried out in vitro kinase assays with bacterially produced GST-fusion proteins expressing Pim-1, Pim-2, p100, or the Pim-1-interacting S3 domain of p100 (aa 449–554) identified in a yeast two-hybrid assay (Leverson et al., 1998). When the phosphorylated protein products were analyzed by SDS–PAGE followed by autoradiography, it became evident that both Pim-1 and Pim-2 can phosphorylate p100 or the S3 fragment to a similar extent (Fig. 3B). Both kinases also autophosphorylated themselves but did not target the GST sequences. Silver staining of the gel confirmed that all the GST-fusion proteins were expressed at comparable levels (data not shown).

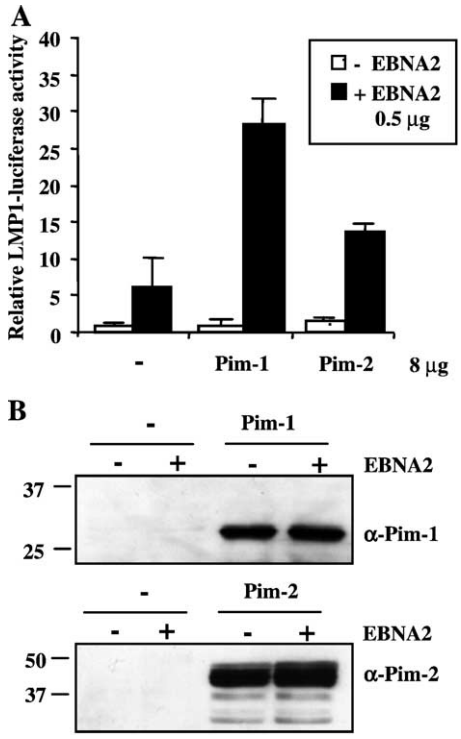

Fig. 2. Pim kinases enhance EBNA2-mediated transactivation of the LMP1 promoter. (A) BJAB cells were transfected with 2 μg of pLMP1-LUC, 1 μg of pSV-β-GAL, and indicated amounts of plasmids encoding Pim-1, Pim-2, and/or full-length EBNA2. Shown are normalized luciferase activities relative to those obtained with LMP1 reporter alone. (B) Pim-1 and Pim-2 protein levels were determined by Western blotting from the same cell lysates.

To investigate the role of p100 in the transactivation assays, we coexpressed FLAG-tagged full-length p100 or two deletion mutants (Fig. 3A) with Gal4-EBNA2 and/or Pim-1 in BJAB cells and measured Gal4-dependent luciferase activities. However, ectopically expressed p100 did not significantly affect Gal4-EBNA2-mediated transactivation or the ability of Pim-1 to enhance that activity (Fig. 3C), suggesting that the levels of endogenous p100 protein were already sufficient in BJAB cells for maximal stimulation under our assay conditions. Furthermore, when we analyzed possible negative interfering effects of two p100 deletion mutants lacking intact Pim-1-interacting domains and one or more of the N-terminal nuclease folds, we noticed that they reduced both the basal and the Pim-stimulated Gal4-EBNA2 activity (Fig. 3C). Western blot analysis confirmed that all the p100 constructs were expressed at comparable levels and that coexpressed Pim-1 or p100 proteins did not significantly affect the expression of each other (data not shown). These results implicate that full-length p100 is needed for the full activity of the EBNA2 acidic domain, as previously demonstrated with an EBNA2 mutant which is unable to interact with p100 and is also transcriptionally inactive (Tong et al., 1995). While it remains to be elucidated whether p100 is essential also for the enhancing effects of Pim-1 and Pim-2, it is possible that by recruiting Pim kinases to the EBNA2 transcriptional complex, p100 may enable them to phosphorylate and regulate some other components of the complex, such as c-Myb which interacts with p100 independently of EBNA2 (Leverson et al., 1998). Via its recently reported DNase and RNase activities (Caudy et al., 2003), p100 might also link Pim kinases to other cellular processes such as chromatin modifications to facilitate EBNA2-mediated transactivation.

EBV infection enhances expression of pim genes

Since our data suggested involvement of Pim kinases in upregulation of EBV-induced gene expression, we wanted to investigate the physiological relevance of our findings by

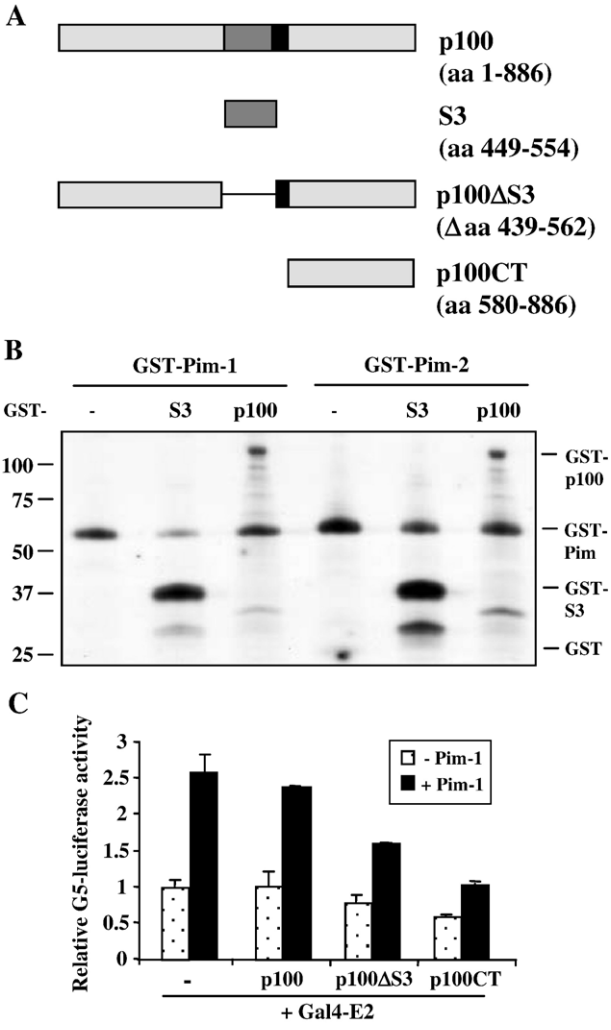

Fig. 3. p100 is phosphorylated by both Pim-1 and Pim-2 kinases in vitro and is required for full EBNA2 activity. (A) Schematic representation of the GST- or FLAG-tagged p100 proteins showing the locations of the Pim-1-interacting domain (S3, dark grey) and the conserved EVES domain (black). (B) GST-fusion proteins expressing Pim-1 or Pim-2 proteins were incubated with GST-S3 or GST-p100 in in vitro kinase assays. The phosphorylation products were analyzed by SDS–PAGE followed by autoradiography. GST alone (−) was used as a negative control. (C) BJAB cells were transfected with 2 μg of pG5-LUC, 1 μg of pSV-β-GAL, 0.1 μg of pGAL4-E2, 3 μg of pSV-*pim*-1, and/or 2.5 μg of FLAG-tagged p100 derivatives. The luciferase activities were measured relative to those induced by Gal4-EBNA2 alone.

analyzing the expression patterns of *pim* genes in EBV-infected cells. We had previously compared the gene expression profile of an EBV-negative Burkitt's lymphoma cell line (BL41) with that of its EBV-infected counterpart (BL41/B95) or an EBV-transformed lymphoblastoid cell line (IB4) (Carter et al., 2002). When data from similar transcriptional profiling experiments were analyzed in further detail, we found also *pim* family genes amongst the EBV-induced genes. In three independent experiments, *pim*-2 mRNA expression was upregulated by 5- to 12-fold in EBV-infected BL41 cells and by 2- to 3-fold in IB4 cells as compared to BL41 cells (data not shown). By contrast, *pim*-1 transcripts were only slightly more abundant in the EBV-positive BL41 cells than in the EBV-negative parental cells and were not significantly elevated in IB4 cells.

To be able to directly evaluate the effects of EBV infection on *pim* gene expression in primary B-cells, purified peripheral blood B-lymphocytes were infected with the EBV B95-8 strain and RNA samples were collected for Northern blot analysis at time points indicated in Fig. 4.

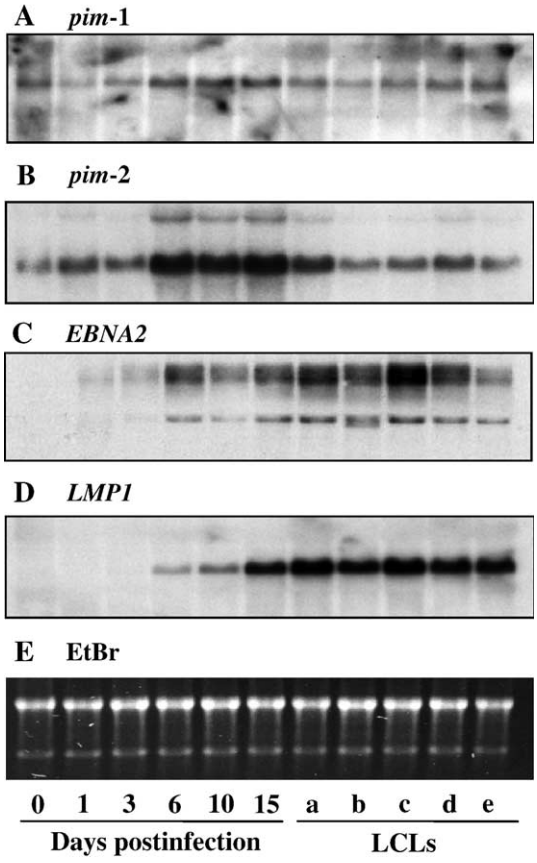

Fig. 4. Levels of *pim*-1 and *pim*-2 mRNAs are upregulated in EBV-infected primary B-cells and LCLs. CD19-positive B-cells were infected with EBV and cell samples were collected at indicated days pre (0)- or postinfection. In addition, five different LCLs were grown for at least 6 weeks after EBV infection. Total cellular RNA samples were isolated and analyzed by Northern blotting using *pim*-1 (A), *pim*-2 (B), *EBNA2* (C), and *LMP1* (D) cDNAs as probes. Equal loading was confirmed by ethidium bromide (EtBr) staining (E).

Again, *pim*-2 mRNA was more significantly induced than *pim*-1 mRNA, following an intriguing kinetics. Its expression was slightly elevated already within the first day, together with *EBNA2* mRNA. However, after 6 days when the infected cells had already begun to proliferate and express also *LMP1* mRNA, levels of *pim*-2 mRNA were strongly upregulated and remained high at least until the day 15 postinfection. We also measured mRNA expression from five EBV-immortalized LCLs, which had been cultured for at least 6 weeks after the initial infection. While these cells still strongly expressed both *EBNA2* and *LMP1*, the levels of *pim* mRNAs had in most cases returned back to those observed during the first days postinfection. Taken together, these data indicate that EBV infection substantially, albeit transiently, upregulates expression of *pim* genes, especially of *pim*-2. Thus, Pim kinases may play an important role during EBV-induced cellular events.

Interestingly, ectopic expression of *LMP1* alone in BL41 cells increases *pim*-2 mRNA levels (data not shown), suggesting that LMP1 might be responsible for EBV-induced upregulation of *pim* expression, possibly via its ability to activate NF-κB (Cahir-McFarland et al., 1999) or support STAT-dependent cytokine signaling (Mosialos, 2001). The transient nature of *pim* gene upregulation may in turn be due to negative feedback via other EBV gene products or via cellular STAT inhibitors such as SOCS proteins whose activities are upregulated by Pim kinases (Chen et al., 2002; Peltola et al., 2004). While further studies are required to fully characterize the transcriptional regulation of *pim* genes in EBV-infected B-cells, their sequential and ultimately coordinated regulation by EBNA2 and LMP1 may be similar to that described for *CD23* (Alfieri et al., 1991) and are likely to be more critical for the establishment than maintenance of the EBV latency.

Pim kinases may promote EBV-induced immortalization and tumorigenesis

Since Pim kinases can support survival of cytokine-dependent hematopoietic cells, the transient upregulation of *pim* genes during early stages of EBV infection may be equally important in protecting infected cells from apoptosis at this critical phase preceding immortalization. Since LMP1-promoted expression of antiapoptotic proteins is essential for the survival of LCLs (Cahir-McFarland et al., 1999), it is possible that Pim kinases are part of a positive autoregulatory loop that ensures adequate expression of LMP1, and subsequently protection from apoptosis in newly infected cells. Thus, inhibition of Pim kinase expression or activity might affect EBV-induced immortalization of B-cells or the survival of LCLs.

Type III EBV latency with EBNA2 and LMP1 expression is observed not only in LCLs, in vitro, but also in EBV-associated lymphoproliferative diseases (LPD), which can further transition into Burkitt's lymphoma (Young et al., 1989) with deregulated c-*myc* oncogene expression. Inter-

estingly, micro-array data at the Genomics Institute of the Novartis Research Foundation Web site (http://expression.gnf.org) indicates that also *pim*-2 expression is upregulated in most BL cell lines. Since both *pim*-1 and *pim*-2 genes strongly cooperate with c-*myc* in development of murine pre-B-cell leukemias (Allen et al., 1997; van Lohuizen et al., 1989), the results presented here suggest that *pim* and *myc* genes may similarly collaborate in EBV-promoted human malignancies.

Materials and methods

Plasmids

The plasmids pSV-*pim*-1 and pSV-*pimNT81* (Aho et al., 2004), pGal4-EBNA2 and pSG5-EBNA2 (Cohen and Kieff, 1991), pGEX-2T-*pim*-1 (Rainio et al., 2002), pGEX-2T-*pim*-2 (Yan et al., 2003), pGEX-3X-S3 (Leverson et al., 1998), and the LMP1 promoter-driven luciferase reporter (Han et al., 2002) have been described previously. pSV-*pim*-2 was constructed by PCR from the murine *pim*-2 cDNA kindly provided by A. Berns, (Netherlands Cancer Institute, Amsterdam). pGal4-PEBP2αB1 was kindly provided by Y. Ito (University of Kyoto, Japan). pGEX-3X-p100 expressing full-length p100 was subcloned from pET-p100 (Leverson et al., 1998) and pcDNA3 (Invitrogen)—derived constructs expressing full-length and truncated p100-FLAG fusion proteins from pSG5-p100-FLAG (Tong et al., 1995). The Gal4-luciferase (G5-LUC) and the SV40-driven β-galactosidase (SV-ß-GAL) reporters were from Promega and pGal4-VP16 from Clontech.

Cell culture and reporter assays

The EBV-negative BJAB cells were grown in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Gibco) and antibiotics. These cells were transfected by electroporation using reporter and effector plasmids as indicated in figure legends. Total amounts of transfected DNA samples were balanced by adding corresponding empty vectors. Two days after transfection, cells were collected and their luciferase and ß-galactosidase activities analyzed as previously described (Rainio et al., 2002). The transfection efficiencies were normalized against β-galactosidase activities. Shown in figures are means and standard deviations of representative experiments with duplicate or triplicate samples.

Western blotting

100 μg aliquots of protein from luciferase assay samples were resolved on SDS–PAGE, transferred onto PVDF membranes (Millipore), and blocked overnight in PBS containing 0.1% Tween-20 and 5% milk. Proteins of interest were detected with anti-Pim-1 or anti-Pim-2 antibodies (kindly provided by M. Lilly, Loma Linda University, CA), followed by HRP-linked secondary anti-mouse antibodies and ECL+Plus reagents (Amersham).

Phosphorylation assays

GST-fusion proteins expressing Pim-1, Pim-2, p100, or the S3 fragment of p100 (aa 449–554) were grown in bacteria, purified by glutathione sepharose beads (Amersham Biosciences), and eluted from the beads by 30 mM glutathione (Sigma). 2.7 μg aliquots of GST-Pim-1 or -Pim-2 proteins were mixed with 8 μg of GST, GST-S3, or GST-p100 proteins in in vitro kinase reactions as previously described (Rainio et al., 2002) and analyzed by SDS–PAGE followed by autoradiography. The amounts of proteins loaded were visualized by silver staining.

B-cell purification and viral infections

Peripheral blood B-cells were prepared from buffy coats of healthy donors obtained from the Finnish Red Cross Blood Transfusion Service. Lymphocytes were first purified using Ficoll-Paque Plus (Amersham Biosciences), after which the CD19-positive B-cells were selected with CD19 magnetic beads (Miltenyi Biotech) according to manufacturer's instructions. The cells were suspended in Yssel's medium (Irvine Scientific) supplemented with 1% human AB serum (the Finnish Red Cross Blood Transfusion Service) and incubated overnight prior to EBV infection. The B95-8 strain of EBV was obtained from 400× concentrated growth medium of the B95-8 marmoset cell line. For infection, B-cells were pelleted and incubated with the concentrated virus on ice for 1 h with occasional mixing and then resuspended at 2×10^6 cells/ml in serum-supplemented Yssel's medium. Samples for mRNA analysis were collected from EBV-infected B-cells on indicated time points and fresh Yssel's medium was added when cells started to grow and divide.

Northern blot analyses

Total cellular RNA was isolated and analyzed as previously described (Matikainen et al., 1999) by Northern blot hybridization with the *pim*-1, *pim*-2, *EBNA2*, and *LMP1* probes. The *pim*-2 probe was cloned from IL-15-treated NK-92 cells by RT-PCR using oligonucleotides CCCCA-GAGTGGATCCCTCGACACCAGT and AATGTCCATG-GATCCCTGTGA-CATGGC. Ethidium bromide (EtBr) staining of ribosomal RNA bands was used to confirm equal RNA loading.

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