May 2004

Multiple signal transducers and activators of transcription are induced by EBV LMP-1

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Published in *Virology* **323**:1 (May 20, 2004), pp. 141–152; doi:10.1016/j.virol.2004.03.007

Submitted October 28, 2003; revised December 12, 2003; accepted March 4, 2004; published online April 26, 2004.

Multiple signal transducers and activators of transcription are induced by EBV LMP-1

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Abstract

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP-1) is required for EBV immortalization of primary B cells in vitro. Signal transducers and activators of transcription (STATs) play a pivotal role in the initiation and maintenance of certain cancers. STAT proteins, especially STAT-1, -3, and -5, are persistently tyrosine phosphorylated or activated in many cancers. We show here that EBV-infected type III latency cells, in which the EBV oncoprotein, LMP-1 is expressed, express high levels of four STATs (STAT-1, -2, -3, and -5A) and that LMP-1 is responsible for the induction of three (STAT-1, -2, and -3). In addition, the C-terminal activator region 1 (CTAR-1) and CTAR-2 of LMP-1 cooperatively induced the expression of STAT-1. The cooperativity was evident when CTAR-1 and CTAR-2 were present in *cis*, but not in *trans*. Furthermore, NF-κB is an essential factor involved in the induction of STAT-1. Most of the induced STATs were not phosphorylated at the critical tyrosine residue activated by many cytokines. However, the induced STATs, at least STAT-1, were functional because it could be activated by interferon (IFN) and could upregulate an IFN-inducible gene. Finally, expression of STAT-1, but not STAT-2 and -3, is associated with EBV transformation. The association of the expression of STAT-1, -2, -3, and -5A with EBV type III latency and the expression of STAT-1 in the EBV transformation process may be part of the viral programming that regulates viral latency and cellular transformation.

Keywords: LMP-1, STAT-1, Epstein-Barr virus, latency

Introduction

Epstein-Barr virus (EBV) is a human herpesvirus of increasing medical importance. EBV infection is associated with the development of nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL). In addition, EBV infection is an important cause of lymphomas in severely immunocompromised persons, especially patients with AIDS and organ-transplant recipients (Kieff, 1996; Pagano, 1991; Pagano, 1999; Raab-Traub, 1996; Rickinson and Kieff, 1996).

The biologic hallmark of the EBV–lymphocyte interaction is latency. Three types of latency have been described, each having its own distinct pattern of gene expression. Type I latency is exemplified by BL tumors *in vivo* and earlier passages of cultured cell lines derived from BL biopsies. EBNA1 is the major viral protein synthesized in this form of latency. Latent membrane protein 2A (LMP2A) may also be expressed. Type II latency is exemplified by NPC and Hodgkin's disease. EBNA1, LMP-1, LMP2A, and LMP2B proteins are expressed in type II latency. Type III latency is typical of early phases of EBV lymphoproliferative syndromes and is captured in lymphoblastoid cell lines (LCLs). Nine viral proteins are expressed, including all six nuclear proteins (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP) and the three integral membrane proteins (LMP-1, LMP-2A, and LMP-2B) (reviewed in Kieff, 1996 and Rickinson and Kieff, 1996).

EBV immortalizes and transforms B cells from cord and adult blood into LCLs and concomitantly establishes type III latency in vitro. LMP-1 expression is required for the im-

mortalization process (Kaye *et al.*, 1993; Kilger *et al.*, 1998). LMP-1 can induce a variety of cellular genes that enhance cell survival as well as adhesive, invasive, and angiogenic potential (Fries *et al.*, 1996; Henderson *et al.*, 1991; Miller *et al.*, 1995; Murono *et al.*, 2001; Wakasaka and Pagano, 2003; Wakasaka *et al.*, 2002; Wakasaka *et al.*, 2004; Wang *et al.*, 1985; Wang *et al.*, 1990a; Yoshizaki *et al.*, 1998).

LMP-1 is an integral membrane protein with six transmembrane-spanning domains and a C-terminal domain located in the cytoplasm (Kieff, 1996; Liebowitz *et al.*, 1986). LMP-1 acts as a constitutively active receptor-like molecule that does not need a ligand (Gires *et al.*, 1997). The transmembrane domains mediate oligomerization of LMP-1 molecules in the plasma membrane, a prerequisite for LMP-1 function (Floettmann *et al.*, 1996; Gires *et al.*, 1997). Two regions in its C terminus initiate signaling processes, the C-terminal activator region 1 (CTAR-1, amino acids 194–231) and CTAR-2 (amino acids 332–386) (Figure 1; Huen *et al.*, 1995; Mitchell and Sugden, 1995).

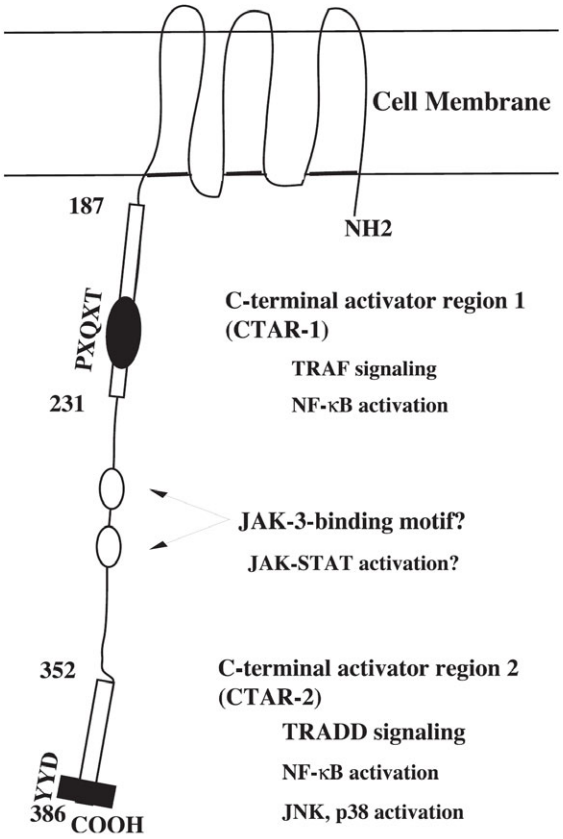

Figure 1. Molecular structure and locations of functional domains in LMP-1. LMP-1 contains a short cytoplasmic amino terminus, a transmembrane hydrophobic domain, and a long cytoplasmic carboxy terminus that contains three major signaling domains. CTAR-1 mediates interaction with the TRAFs, and is the minor NF-κB-activating region. The location of the TRAF-interacting motif, PXQXT, is indicated. CTAR-2 is the major NF-κB-activating region. Also, CTAR-2 can activate JNK and p38 molecules. Two JAK3-binding sites are also indicated; the JAK-STAT pathway might be activated by interaction between JAK3 and LMP-1. The amino acid numbers are shown. The drawing is not on scale.

CTAR-1 is a contributor to the activation of nuclear factor κB (NF-κB) by LMP-1. The PXQXT motif localized within CTAR-1 is involved in the interaction with tumor necrosis factor receptor (TNFR)-associated factors (TRAFs). TRAF-1, -2, -3, and -5 associate with LMP-1 with different affinities and are responsible for NF-κB activation by CTAR-1 (Devergne *et al.*, 1996; Devergne *et al.*, 1998; Miller *et al.*, 1997; Sandberg *et al.*, 1997). CTAR-1 is responsible for induction of epidermal growth factor receptor (EGFR) and TRAF-1 (Devergne *et al.*, 1998; Miller *et al.*, 1997). CTAR-1 is required for the transformation of B cells by EBV, and the PXQXT motif is essential for this process (Izumi *et al.*, 1997; Kaye *et al.*, 1999).

CTAR-2 is also a contributor to the activation of NF-κB by LMP-1. CTAR-2, through its interaction with TNFR-associated death domain protein (TRADD), activates NF-κB (Izumi and Kieff, 1997; Izumi *et al.*, 1999). Also, c-jun N-terminal kinase (JNK) and p38 are activated by CTAR-2 (Eliopoulos and Young, 1998; Eliopoulos *et al.*, 1999; Kieser *et al.*, 1997). The final three amino acids (YYD) play an essential role in the signal transduction pathways of CTAR-2.

Interestingly, consensus janus kinase 3 (JAK3) binding sites between CTAR-1 and CTAR-2 have been identified (Figure 1). However, whether JAK3 can bind to these sites and is responsible for the activation of signal transducer and activator of transcription 1 (STAT-1) or other STATs is controversial (Brennan *et al.*, 2001; Fielding *et al.*, 2001; Gires *et al.*, 1999; Higuchi *et al.*, 2002).

STATs are a family of latent transcription factors that become activated by phosphorylation on a single tyrosine, typically in response to extracellular ligands (Darnell *et al.*, 1994; Stark, 1997). Virtually every cytokine and growth factor can cause STAT phosphorylation through receptor or associated kinases. Once phosphorylated, STATs can form homo- or heterodimers that accumulate in the nucleus, recognize specific DNA sequences, and activate transcription (Darnell *et al.*, 1994; Stark, 1997).

In this report, the relation between EBV and STATs is examined. We show that high levels of expression of STAT-1, -2, -3, and -5A are associated with EBV type III latency in which LMP-1 is expressed. LMP-1 stimulates the expression of STAT-1, -2, and -3, but not STAT-5A. Interestingly, the maximum induction of STAT-1 is a result of a cooperative interaction between the LMP-1 CTAR-1 and CTAR-2 domains. However, after induction by LMP-1, STAT-1 is not activated by phosphorylation. Induction of STATs, especially STAT-1, -3, and -5A, by EBV may be relevant to viral transformation processes as well as the pathogenesis of EBV-associated tumors.

Results

Expression of STAT-1 is correlated with LMP-1 protein in type III latency

We first scanned the expression pattern of STAT-1 in various EBV-infected cell lines with type I or type III latency

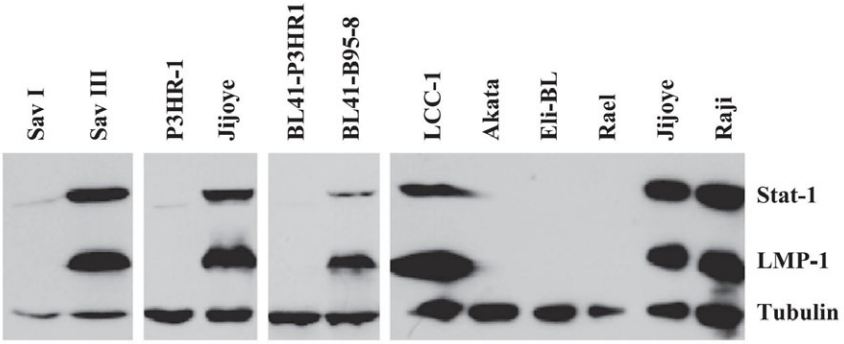

Figure 2. High expression levels of STAT-1 are associated with the expression of LMP-1. Equal amounts of protein lysates from cell lines were electrophoresed in 8% SDS-PAGE and stained with Ponceau S Red after transfer of protein to the membrane. Western blotting with STAT-1, LMP-1, and tubulin antibodies was performed simultaneously. The names of cell lines are as labeled. SAV I and SAV III are genetically identical cell lines derived from the same parental line.

profiles. Sav I and Sav III are sister BL lines each derived from a single parental cell line. The paired lines differ only in their types of latency (Nonkwelo *et al.*, 1996; Zhang and Pagano, 1997). The Jijoye (type III) cell line has all the latency genes in its viral genome, whereas its derivative, the P3HR1 line, lacks the EBNA-2 gene and a portion of EBNA-LP (Adldinger *et al.*, 1985). As a result of the deletion, P3HR1 cells do not express EBNA-2, and consequently because EBNA-2 transactivates the LMP-1 promoter (Abbot *et al.*, 1990; Ghosh and Kieff, 1990; Tsang *et al.*, 1991; Wang *et al.*, 1990b), express a very low level of LMP-1 apparently through induction by IRF-7 (Ning *et al.*, 2003). BL41-P3HR1 and BL41-B95-8 are converted by infection of EBV-negative BL41 cells with either P3HR1 or B95-8 virus, respectively (Calender *et al.*, 1987). In addition to these paired cell lines, other available EBV latently infected cell lines were also examined. As shown in Figure 2, STAT-1 protein was expressed at high levels in type III cell lines with high levels of LMP-1. In type I cells in which LMP-1 is not expressed, STAT-1 was expressed at much lower levels. These data indicate that the expression of STAT-1 correlated with the expression of LMP-1 in type III latency.

LMP-1 stimulates the expression of STAT-1 protein

Because EBNA-2 is the primary inducer of LMP-1 mRNA (Abbot *et al.*, 1990; Ghosh and Kieff, 1990; Tsang *et al.*, 1991 and Wang *et al.*, 1990b), and because of the consistent association between STAT-1 and LMP-1 expression (Figure 2), it is possible that either EBNA-2 and/or LMP-1 are responsible for the induction of STAT-1. Both EBV-negative DG75 and EBV-positive Akata cells were used to determine which viral gene could directly induce the expression of STAT-1. LMP-1 or EBNA-2 and a CD4-expression plasmid were transfected into cells, and the levels of STAT-1 were determined by Western blotting after selection of the transfected cells by the use of CD-4 antibody-conjugated magnetic beads (see Materials and methods). As shown in Figure 3, LMP-1 expression causes a marked increase in STAT-1 protein levels in both DG75 and Akata cells; however, EBNA-2 seems to have no effect on the induction of STAT-1 in DG75 cells. Therefore, LMP-1 is probably responsible for the induction of STAT-1 in type III latency cells. If EBNA2 is involved in the induction of STATs, it is likely to do so indirectly via induction of LMP-1 in EBV-infected cells.

LMP-1 induces the expression of STAT-1, STAT-2, and STAT-3

Whether LMP-1 increases STAT-1 at the RNA level was examined by RNase Protection Assays (RPA) with specific probes. The probe set is capable of detecting RNA of all STATs (see "Materials and methods" for details). Pairs of the genetically identical Sav I and Sav III cell lines, as well as P3HR1 cells and its parental line, Jijoye, were used for the experiments. As shown in Figure 4, STAT-1 RNA levels were higher in Sav III and Jijoye lines (lanes 2 and 7). Therefore,

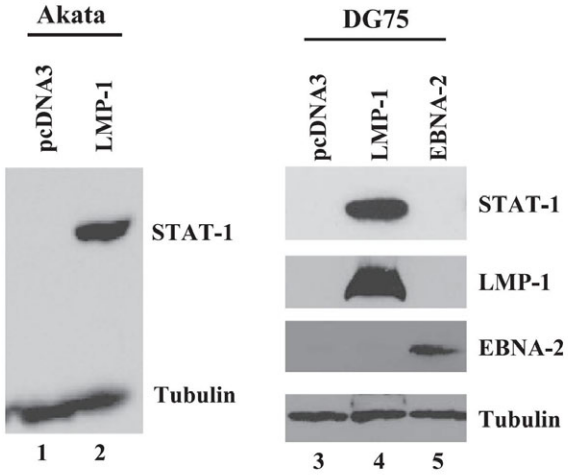

Figure 3. LMP-1 induces the expression of STAT-1 protein. Lysates from cells transfected with pcDNA3 (lanes 1 and 3) or LMP-1 expression plasmid (lanes 2 and 4), or EBNA2 expression plasmid (lane 5) were used. Western blots with STAT-1, LMP-1, EBNA-2, and tubulin antibodies were performed. Lanes 1 and 2, Akata cells were used for transfection; lanes 3–5, DG75 cells used. The identity of proteins is as shown.

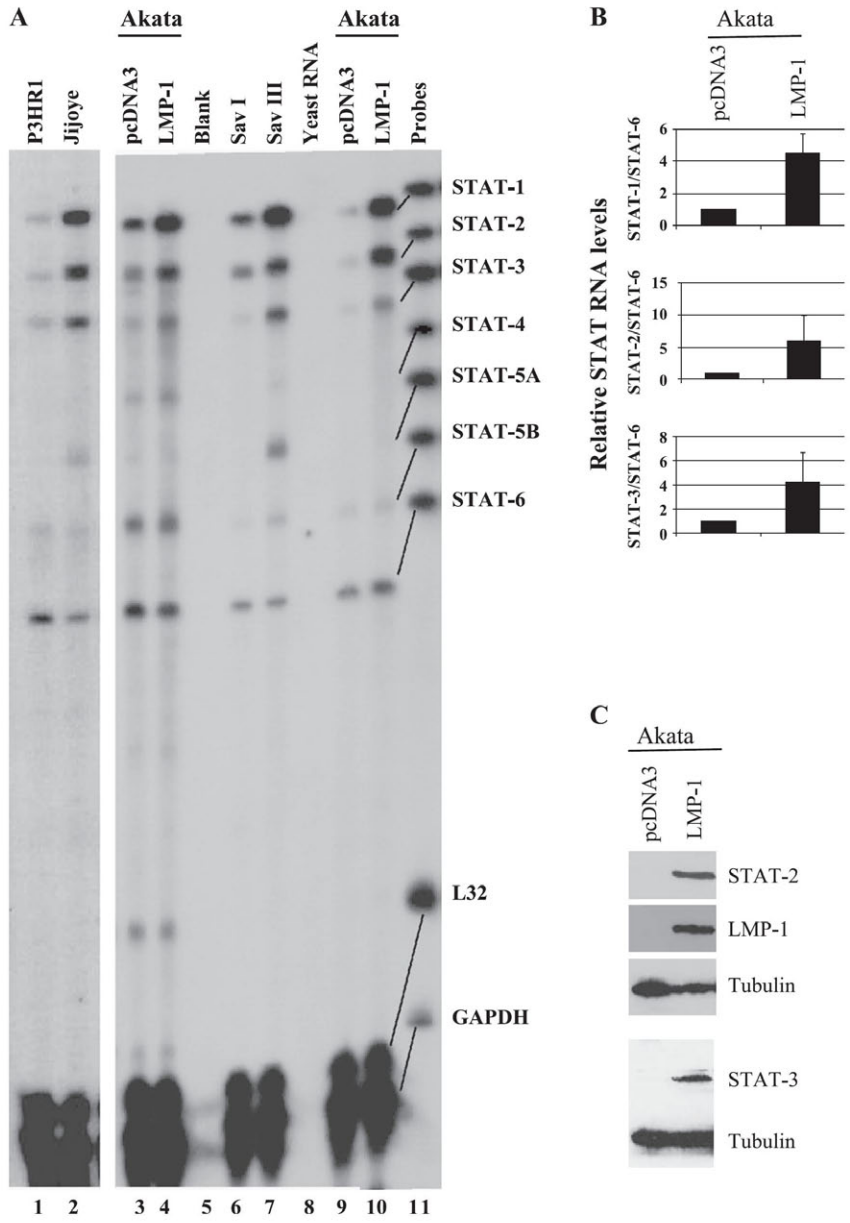

Figure 4. LMP-1 induces the expression of endogenous STAT-1, -2, and -3. (A) LMP-1 induces the expression of endogenous STAT-1, -2, and -3 RNA. Human STAT RPA probes were labeled with α-^{32}P-UTP and used for RPA. Lane 11, undigested STATs probes; lanes 1, 2, 6, and 7, RNAs from P3HR1, Jijoye, Sav I and Sav III cells, respectively; lane 8, yeast tRNA; lane 5, blank. Lanes 3, 4, 9, and 10, RNAs from transfected and concentrated Akata cells; lanes 3 and 9, pcDNA3; lanes 4 and 10, LMP-1 expression plasmid. The identity of the STATs is as indicated. (B) The relative levels of STAT RNAs induced by LMP-1. The autoradiography was examined by the Gene Genius Bioimaging System, and the intensity of bands was recorded and analyzed. The results from three independent experiments (two of which are shown in lanes 3, 4, 9, and 10 in panel A) were used for calculations of the induction. STAT-6 was used as an internal control. The relative levels of STAT-1, -2, -3, and standard deviations are shown. (C) LMP-1 induces the expression of endogenous STAT-2 and -3 proteins. Cell lysates from transfected and concentrated Akata cells were used for Western blot analysis. The identity of proteins is as shown.

STAT-1 RNA expression is also associated with LMP-1 expression, in agreement with the data in Figure 2. Surprisingly, in addition to STAT-1, expression levels of STAT-2, -3, and -5A RNAs were also higher in type III latency cells (lanes 1, 2, 6, and 7). STAT-1, -2, -3, and -5A RNAs were also expressed at higher levels in CBC/B95-8 (an LCL, type III latency, with high level expression of LMP-1) and lower in Eli-BL (type I latency, no LMP-1 expression) (data not shown). Thus, the expression of STAT-2, -3, and -5A RNAs is also associated with type III latency.

Whether LMP-1 increases STAT RNAs was examined by transient transfection of LMP-1 and a CD4-expression plasmid into Akata cells and selecting transfected cells as before. As shown in Figure 4, LMP-1 expression produces increased levels of STAT-1, -2, and -3 RNAs; duplicate results are shown in lanes 3 and 4, and lanes 9 and 10. However, LMP-1

did not increase the expression of STAT-5A RNA (lanes 3, 4, 9, and 10). The relative STAT RNA levels are shown in Figure 4B. In addition, we tested if LMP-1 could induce the expression of STAT-2 and -3 proteins. In Akata cells, both STAT-2 and -3 are induced by the expression of LMP-1 as shown in Figure 4C. In DG75 cells, STAT-2 protein was also efficiently induced; however, the induction of STAT-3 protein was not very obvious (data not shown). The data together indicate that LMP-1 increases the expression of STAT-1, -2, and -3, but not STAT-5A.

CTAR-1 and CTAR-2 cooperatively induce the expression of STAT-1

Next, the LMP-1 domain requirement for the induction of STAT-1 was examined in Akata cells by the use of several LMP-1 mutants. Akata cells were used for these experiments because STAT-1 is highly inducible in this cell line (Figure 3 and Figure 4). We tested the role of CTARs in the induction of STAT-1 in the context of the whole LMP-1 molecule. LMP-PQAA has mutations in CTAR1 PXQXT motif that change the proline and glutamine into alanines. PQAA mutation in intact LMP-1 will knock out the function of the TRAF-interaction domain (Devergne *et al.*, 1998; Miller *et al.*, 1998; Sandberg *et al.*, 1997). LMP-IID has mutations in the CTAR-2 YYD motif that change the two tyrosines into isoleucines. The tyrosines (Y) in the last three amino acids of LMP-1 (YYD) have been shown to play an important role in the signaling pathway of CTAR2; mutations of the tyrosine amino acids abolish TRADD binding and the activation of NF-κB and AP-1 by the CTAR-2 region (Floettmann and Rowe, 1997; Izumi *et al.*, 1999; Kieser *et al.*, 1997). LMP-DM has mutations in both CTARs (Figure 5A). As shown in Figure 5B, LMP-PQAA or LMP-IID induces very marginal levels of STAT-1. When both CTARs were mutated in LMP-DM, STAT-1 induction is completely abolished. Because either CTAR alone only marginally induces STAT-1, these data suggest that CTAR1 and CTAR2 cooperatively induce the expression of STAT-1.

Whether the full induction of STAT-1 can be complemented in *trans* was examined by co-transfection of individually mutated CTAR-1 and CTAR-2 mutants. As shown in Figure 5B, co-transfection of LMP-1 plasmids containing individually mutated CTAR-1 (LMP-PQAA) and CTAR-2 (LMP-IID) barely induces STAT-1 and certainly not at a level comparable to that induced by LMP-1wt. These data suggest that CTAR1 and CTAR-2 cooperatively induce STAT-1 only in *cis* configuration, but not in *trans*.

NF-κB is essential for the induction of STAT-1 by LMP-1

Next, the roles of intracellular molecules involved in the induction were examined. Both CTARs can activate NF-κB as shown in Figure 6B. In addition, several other molecules have been shown to be activated by LMP-1. As shown in Figure 6A, LMP-1 alone induced high levels of STAT-1 in either Akata or DG75 cells. However, in the presence of superrepressor IκB

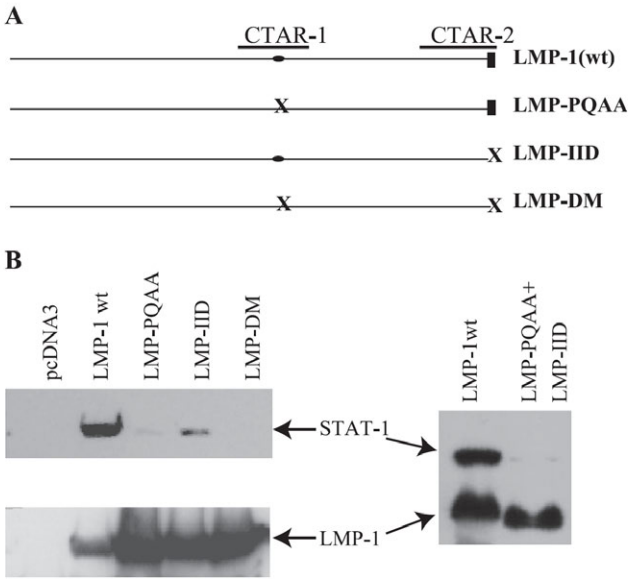

Figure 5. Cooperativity between both CTARs is required for efficient induction of STAT-1. (A) Schematic diagram of LMP-1 and its mutants. Solid ovals, CTAR1 PXQXT motif; solid bars, CTAR-2 YYD motif. X denotes the destruction of the motifs. LMP-PQAA has mutations in the conserved PXQXT motif that change the proline and glutamine into alanines. LMP-IID has mutations in the CTAR-2 YYD motif that change the two tyrosines into isoleucines. LMP-DM has mutations in both CTAR-1 and CTAR-2. (B) Akata cells were transfected with pcDNA-3 vector or the various LMP-1 plasmids shown in A. The cell lysates were used for Western blot analysis. The identity of the proteins is indicated.

(sr-IκB), the expression of STAT-1 was completely abolished. Surprisingly, dominant-negative mutants for TRAFs (TRAF-1, -2, -3, and -5 DNs) were not able to block the induction of STAT-1 efficiently even though the level of LMP-1 expressed was similar. In addition, AP-1DN and JAK3DN were not able to block the induction (data not shown).

Because NF-κB is essential for the induction of STAT-1, and both CTARs are capable of activating NF-κB, we asked whether NF-κB activation might be contributing to the cooperative induction of STAT-1 by CTAR-1 and -2. As shown in Figure 6B, the two CTAR mutations could activate NF-κB individually as predicted, and the combination of the two was able to activate NF-κB to a similar level as wild-type LMP-1. These data are in contrast to the requirement for the *cis* configuration of the CTARs for the induction of STAT-1 and suggest that other factors in addition to NF-κB are also involved.

Phosphorylation of Tyrosine 701 in STAT-1 is not detected in EBV latency

STAT-1, -3, and -5 are often activated in human cancers. Phosphorylation at the critical Tyr-701 residue of STAT-1 is a crucial event for its function although the role of activated STAT-1 in oncogenesis is not clear yet. We tested the activation status of STAT-1 by the use of phospho-specific antibod-

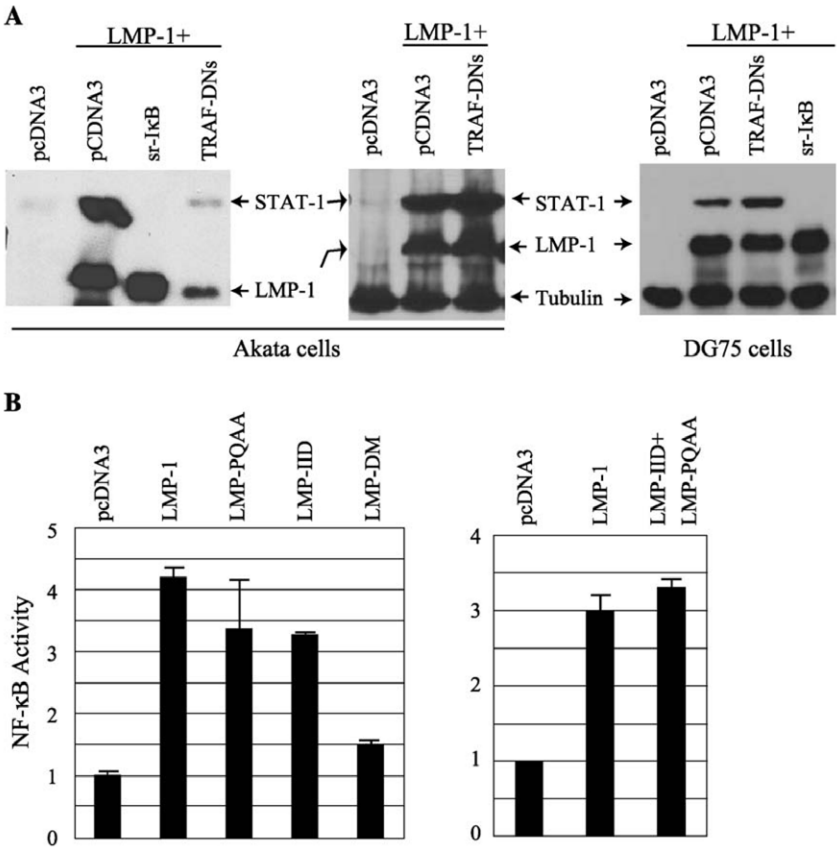

Figure 6. NF-κB is required for the induction of STAT-1. (A) NF-κB is required for the induction of STAT-1. Akata or DG75 cells were transfected with pcDNA-3 vector or LMP-1, or LMP-1 plus sr-IκB, or LMP-1 plus a combination of dominant-negative mutants of TRAF-1, -2, -3, and -5 (TRAF DNs). The cell lysates were used for Western blot analysis. The identity of proteins is as shown. (B) LMP-1 activates NF-κB activity in Akata cells. Akata cells were transfected with NF-κB-reporter construct along with pcDNA-3 or various LMP-1 expression plasmids. Luciferase activities were normalized by β-galactosidase activity. The reporter activity is expressed relative to vector control. Standard deviations are shown.

ies. As shown in Figure 7A, STAT-1 is not phosphorylated, or very marginally activated, at the critical Tyr-701 residue in all the EBV-positive cell lines tested including Akata, Jijoye, and Sav III (lanes 1 and 3, and data not shown). However, STAT-1 in these cells is capable of being phosphorylated in response to IFN-α, suggesting that the STAT-1 is functional (lanes 2 and 4). We also tested the phosphorylation status of serine 727 (Ser-727). Interestingly, the STAT-1 Ser-727 is apparently constitutively phosphorylated (lanes 5–8). Because Ser-727 is also phosphorylated in type I latency cells (data not shown), the results suggest that the phosphorylation of Ser-727 is independent of LMP-1. In addition, the subcellular localization of STAT-1 was examined by immunostaining in Jijoye cells, in which STAT-1 is highly expressed (Figure 2). Without IFN treatment, STAT-1 predominantly localized in the cytoplasm; however, STAT-1 was predominantly localized to the nucleus upon IFN treatment as predicted (data not shown). It is noteworthy that we did not observe any phosphorylation and nuclear translocation of STAT-1 in LMP-1-transfected human B cells (data not shown).

In addition to phosphorylation of the tyrosine residue, IFN can induce its target genes. As shown in Figure 7B, interferon-stimulated gene 15 (ISG-15) could be induced in all four cell lines tested. All these results suggest that STAT-1 in type III latency cells is not activated, or very marginally activated, at the critical tyrosine residue (Tyr-701). However, the induced STAT-1 is functional and capable of responding to IFN.

We also determined the activation status of STAT-3 and -5, both of which are associated with human cancers. Phospho-STAT-3-specific or phospho-STAT-5-specific antibodies were used to determine the activation status of STAT-3 and -5. Neither STAT was phosphorylated, or phosphorylated at very low levels in type III latency cells. However, STAT-3 and -5 could be activated by IL-6 (for STAT-3) or IL-2 (for STAT-5) (data not shown). These data on the activation status of STAT-3 and -5 are in agreement with a recent report (Higuchi *et al.*, 2002).

Expression of STAT-1 is associated with EBV transformation

STATs are involved in the pathogenesis of human cancers. STATs, especially STAT-1, -3, and -5, are persistently tyrosine phosphorylated or activated and play a pivotal role in initiation and maintenance of the phenotypes of some cancers

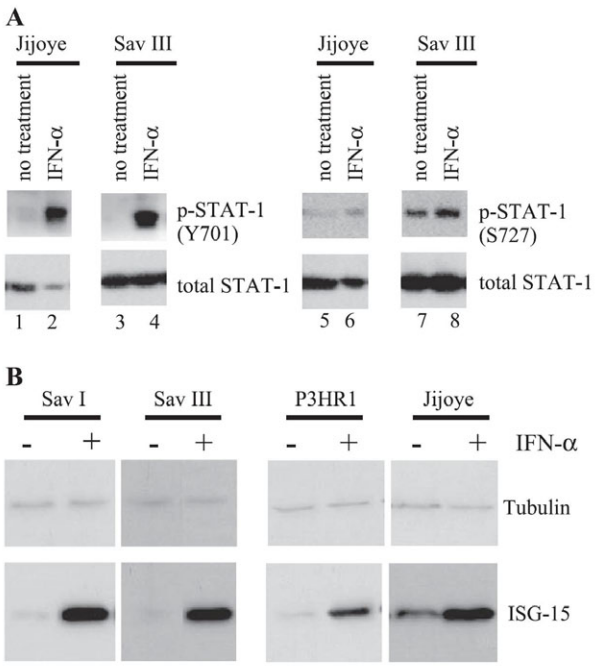

Figure 7. Activation status of STAT-1 in EBV-infected cells. (A) Phosphorylation status of STAT-1 in type III latent cells. Western blot with phospho-specific STAT-1 antibodies was first performed. The membranes were then stripped and antibody against intact STAT-1 was used to determine the total STAT-1 expression. Lanes 2, 5, 6, and 8 are lysates of cells that had been treated with IFN-α for 30 min. Lanes 1, 3, 5, and 7 are untreated lysates. Cell lines are labeled at the top. (B) ISG-15 is inducible in latent cells. Lysates from cells treated with IFN-α (12 h) were used. Western blots with ISG-15 and tubulin antibodies were performed. The identity of proteins is as shown.

(for a review, see Bowman *et al.*, 2000; Bromberg and Darnell, 2000).

EBV can immortalize and transform primary B cells into continually growing lymphoblastoid cell lines (LCLs). We examined if STATs are associated with this immortalization/transformation process. The primary B cells were isolated from fresh blood by CD-19-conjugated magnetic beads (see "Materials and methods" for details). Primary B cells from two individuals were compared with four newly transformed LCLs. As shown in Figure 8, the expression of STAT-1 is associated with the EBV immortalization/transformation process. Interestingly, expression of STAT-2, -3, and -5A is apparently not associated with this process (Figure 8A). Although we have shown clearly that LMP-1 induces STAT-2 and -3, CD-19-positive primary B cells are heterogeneous and therefore differ from the clonal type I latency and EBV-negative Burkitt's lymphoma cell lines. In addition, it has been documented that at least one LMP-1-induced gene, *Bcl*-2, is not associated with EBV transformation (Henderson *et al.*, 1991; Martin *et al.*, 1993), and we confirmed this observation (Figure 8A). Thus, STAT-1 is the only one identified that is associated with EBV transformation.

Next, we examined if the phosphorylation of STATs is associated with EBV transformation processes. Because of the availability of phospho-STAT antibodies, the phosphorylation status of STAT-1, -3, and -5 were examined by Western blot

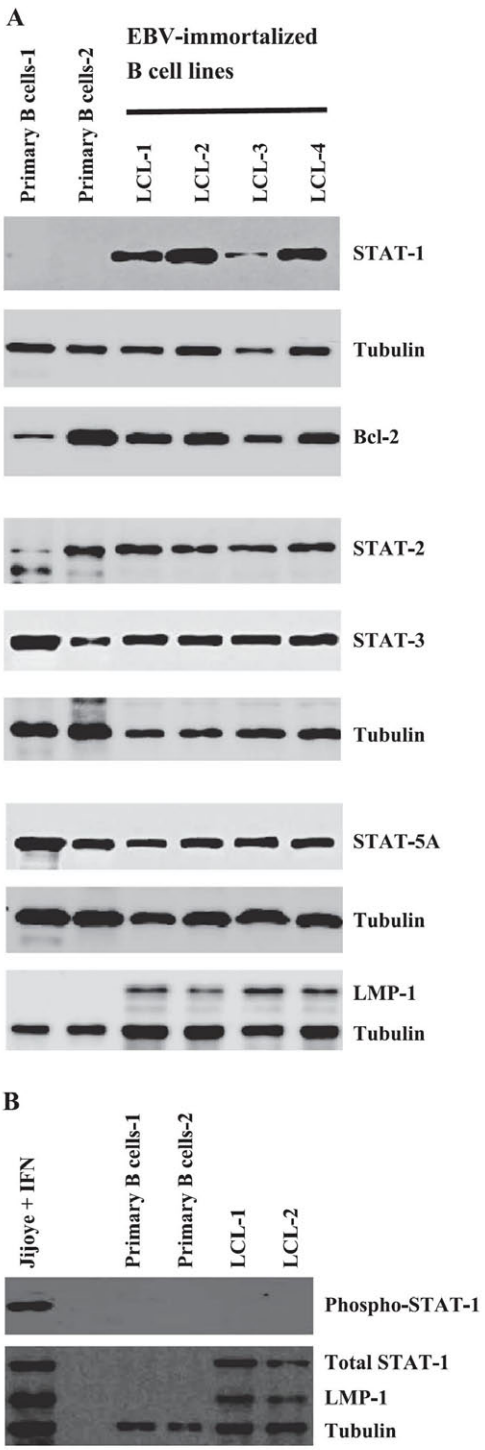

Figure 8. Expression of STAT-1 is associated with EBV transformation processes. (A) STAT-1 expression is induced during the process of immortalization of B lymphocytes by EBV. Primary B cells were isolated from fresh blood. Equal amounts of protein lysates from primary B cells and four newly transformed LCLs were electrophoresed, and Western blotting with various antibodies was performed. The identity of proteins is indicated. (B) STAT-1 is not phosphorylated at a critical tyrosine residue during EBV transformation. Western blot with phospho-specific (Tyr-701) STAT-1 antibodies was first performed. The membranes were then stripped, and antibodies against total STAT-1, LMP-1, and tubulin were used to determine expression of these proteins. The positive control is Jijoye cells treated with IFN-α for 30 min. The identity of proteins is as shown.

analysis. As shown in Figure 8B, STAT-1 was not phosphorylated at the critical Tyr-701 residue. STAT-3 and -5 were not activated either (data not shown), in agreement with the recent report (Higuchi *et al.*, 2002).

Discussion

EBV has the capability of deregulating B-cell growth through activation of endogenous programs of cellular gene expression. We show that high levels of STAT-1, -2, -3, and -5A are associated with EBV type III latency cells. In addition, LMP-1, a key type III latency gene, could induce the expression of STAT-1, -2 and -3; however, it could not induce the expression of STAT-5A RNA (Figure 4).

Some genes can be induced by both LMP-1 CTARs, especially those that are regulated primarily by NF-κB (Mehl *et al.*, 2001; Miller *et al.*, 1997; Takeshita *et al.*, 1999). In this report, we have shown a cooperative induction of STAT-1 by the two CTARs in the *cis* configuration (Figure 5B). The requirement for efficient induction of STAT-1 by both CTARs in *cis* (Figure 5) is interesting and suggests that LMP-1 *in vivo* might assume a functional conformation maintained by both CTARs. The cooperativity is apparently not due to the activation of NF-κB because activation of NF-κB by the CTARs was not cooperative in the same cells (Figure 6B). However, NF-κB is an essential factor for the induction of STAT-1 (Figure 6). It is obvious that another factor(s) resulting from the cooperation of the two CTARs is also involved in the induction of STAT-1. It is somewhat surprising that the combination of TRAF DNs including TRAF-1,-2, -3, and -5 could not efficiently block the induction of STAT-1 (Figure 6A). Because CTAR-1 is apparently involved in the activation (Figure 5B), the results also suggest that another TRAF member(s), or signaling pathway(s) derived from the TRAF binding domain might be responsible for partial induction of STAT-1.

LMP-1 has been reported to activate STAT-1 protein by phosphorylation via JAK-3 based mainly on studies carried out in a fibroblast cell line (Gires *et al.*, 1999). However, we examined the STAT-1 and LMP-1 in native environments in B cells and found that STAT-1 was not phosphorylated in any of the type III cell lines in which both LMP-1 and STAT-1 are highly expressed. Also, we did not observe any phosphorylation and nuclear translocation of STAT-1 by transfecting LMP-1 into human B cells (data not shown). The difference might be due to the cell lines used for the experiments. Activation of JAK-3 by LMP-1 might be due to the particular cell line used (Higuchi *et al.*, 2002).

Although STATs are mostly in their latent form in EBV latency, these STATs may also have functional roles. A well-studied example is the ability of latent STAT-1 to regulate osteoblast differentiation by attenuating Runx2, an essential transcriptional factor in this differentiation process, in the cytoplasm (Kim *et al.*, 2003). Another example is low-molecular weight protein 2 (LMP-2) that is involved in MHC class 1 processing. Latent nonphosphorylated STAT-1 is able to move into the nucleus, bind to DNA, and is responsible for the constitutive expression of LMP-2 (Chatterjee-Kishore *et al.*, 2000). Also, LMP-1 has been reported to activate a STAT-responsive element (Fielding *et al.*, 2001; Richardson *et al.*, 2003); the induction of STAT-1, -2, and -3 could possibly explain that observation (Figure 4).

In addition, high expression levels of STATs might be related to the regulation of the EBV *Bam*HI Q latency promoter (Qp) which is used for the transcription of EBNA-1 in type I latency. Activation of the JAK-STAT pathway has been reported to activate EBV Qp (Chen *et al.*, 1999; Chen *et al.*, 2003). Because Qp is completely inactive in type III latency, and STAT-1, -2, -3, and -5A are highly expressed in type III latency, it is unlikely that any of these STATs function to activate the promoter. Also LMP-1, which is expressed in type III latency, represses the activity of Qp reporter constructs as well as endogenous Qp activity (Zhang and Pagano, 2000; Zhang and Pagano, 2001; Zhang *et al.*, 2001). Therefore, STAT-1, -2, -3, and -5A are likely to be repressors of Qp in type III latency. In addition, these STATs are not activated in type I latency in which Qp is active (data not shown). These observations led us to test the role of STATs in the regulation of Qp. However, STAT-1, -2, and -3, at least in their nonphosphorylated state, neither activate nor significantly repress Qp in Akata cells (data not shown). It is possible that the activation of Qp by STATs might only be observable in NPC cells (Chen *et al.*, 2003). Thus, our data and published reports suggest that the induced but still latent STATs might be mediators that regulate both cellular and EBV genes to the benefit of viral latency. However, the specific targets of these latent STATs are currently unknown.

One of the chief functions of activated STATs is their involvement in the pathogenesis of human cancers. In normal cells and in animals, ligand-dependent activation of the STATs is a transient process, lasting for several minutes to several hours. In contrast, in many cancerous cell lines and tumors, where growth-factor dysregulation is frequent, STAT proteins, especially STAT-1, -3, and -5, are persistently tyrosine-phosphorylated or activated. These activated STAT proteins play a pivotal role in initiation and maintenance of the phenotypes of some cancers (for a review, see Bowman *et al.*, 2000; Bromberg and Darnell, 2000). We have shown in this report that expression of STAT-1 is associated with the EBV-transformation process; however, STAT-2, -3, and -5A are not apparently associated with immortalization of lymphocytes into cell lines (Figure 8). It is also reported that Bcl-2, another LMP-1-inducible gene, is not associated with the EBV transformation (Henderson *et al.*, 1991; Martin *et al.*, 1993). Thus, it is apparent that not all LMP-1-inducible genes are associated with EBV transformation. We suggest that induction of STATs, especially STAT-1, by LMP-1 may be a part of the EBV programming that regulates viral latency and leads to cellular transformation. The overexpression of STAT-1 in EBV transformation may provide a unique scenario that differs from

other cancers in which the tyrosine-phosphorylated STATs are major factors in oncogenesis. The function of STAT-1 in EBV transformation is currently under investigation. Also interesting is that type III latency cells express high levels of two interferon regulatory factors (IRF-2 and IRF-7) (Zhang and Pagano, 1997; Zhang and Pagano, 1999). It is well known that IRFs are involved in the regulation of IFN responsiveness (Nguyen *et al.*, 1997; Pitha *et al.*, 1998; Taniguchi *et al.*, 2001). Thus, it is likely that EBV regulates its latency state by the use of two families of proteins involved in the IFN signaling pathway.

In summary, our results expand the role of LMP-1 as a pleiotropic molecule in effecting deregulation of cellular genes. LMP-1 is now presented as a stimulator of STAT-1, -2, and -3 in EBV-infected cells, and NF-κB is an essential factor for this induction. In addition, the expression of STAT-1 is associated with immortalization and transformation of human lymphocytes and lymphoblastoid cell lines.

Materials and methods

Cells, plasmids, and antibodies

DG75 is an EBV-negative Burkitt's lymphoma (BL) cell line (Ben-Bassat *et al.*, 1977). Akata, Eli-BL, Rael, Sav I, Sav III, P3HR-1, Jijoye, and Raji are all EBV-positive BL lines (Adldinger *et al.*, 1985; Calender *et al.*, 1987; Klein *et al.*, 1972; Ragona *et al.*, 1980; Rooney *et al.*, 1986; Takada, 1984). LCC-1 (gift of Dr. Richard Longnecker) and CBC/B95-8 are EBV-transformed lymphoblastoid cell lines (LCL) (Pagano *et al.*, 1992). BL41-P3HR1 and BL41-B95-8 are cell lines converted by infection of EBV-negative BL41 BL cells with the two different EBV strains (Calender *et al.*, 1987). Four newly transformed LCLs (LCL-1, -2, -3, and -4) are gifts from Dr. Kenneth Izumi at the University of Texas at San Antonio. All cells were maintained in RPMI-1640 plus 10% FBS. The fresh blood was purchased from local Red Cross station. The CD19-positive primary B cells were isolated from fresh PBMC by the use of CD-19 antibody conjugated to magnetic beads according to the manufacturer's recommendation (Dynal, Inc.). The method was used successfully, and the cells isolated were infectable by EBV (Sinclair *et al.*, 1994; Sinclair *et al.*, 1995).

pcDNA/CD4, pcLMP1, the mutant LMP-1 plasmids (LMP-PQAA, LMP-IID, and LMP-DM), EBNA-2 expression plasmid, and NF-κB reporter constructs were all described before (Sung *et al.*, 1991; Zhang and Pagano, 1997; Zhang and Pagano, 2000; Zhang and Pagano, 2001; Zhang *et al.*, 2001). STAT-1 and STAT-3 expression plasmids were gifts from Dr. James Darnell and Dr. Rolf de Groot, respectively. STAT-2 cDNA was from Dr. James Darnell, and the expression plasmid was constructed in pcDNA-3 vector at *Kpn*I and *Xba*I sites. Dominant-negative mutants (DN) for TRAFs (TRAF-1DN, TRAF-2DN, TRAF-3DN, and TRAF-5DN), AP-1DN, and janus kinase 3 (JAK3) DN were described previously (Zhang *et al.*, 2001). The pQ-luc reporter construct has been described (Davenport and Pagano, 1999).

LMP-1 monoclonal antibody (CS1-4) and EBNA2 specific antibody (PE2) were purchased from Dako. STAT-1 (sc-417, sc-591), STAT-2 (sc-1668), STAT-3 (sc-482), and STAT-5A (sc-1081) antibodies were purchased from Santa Cruz. Phospho-STAT-3-specific (Tyr-705; #9131) and phospho-STAT-5-specific (#9351) antibodies were from Cell Signaling Technology. Phospho-Ser-727 STAT-1 antibody (#06-802) and Phospho-Tyr-701 STAT-1 antibody (06-657) were from Upstate Biotechnology. Tubulin antibody was from Sigma. ISG-15 antibody was the gift of Dr. Ernest Borden. Interferon α (IFN-α) was from Hoffmann La Roche.

Western blot analysis with enhanced chemiluminescence (ECL)

Separation of proteins on SDS-PAGE followed standard methods. After the proteins were transferred to a nitrocellulose or Immobilon membrane, the membrane was blocked with 5% non-fat dry milk in TBST (50 mM Tris pH 7.5, 200 mM NaCl, 0.05% Tween-20) at room temperature for 10 min. It was then washed briefly with water and incubated with a primary antibody in 5% milk in TBST for 1–2 h at room temperature, or overnight at 4 °C. After washing with TBST for 10 min three times, the membrane was incubated with the secondary antibody at room temperature for 1 h. It was then washed three times with TBST as before, treated with ECL (Amersham) or SuperSignal (Pierce) detection reagents, and exposed to Kodak XAR-5 film.

Transient transfection, chloramphenicol acetyltransferase (CAT) assays, and isolation of transfected cells

10^7 cells in 0.5 ml medium were used for transfection with the use of a BioRad Gene Pulser (320 Volts and 925 μF). Two days after transfection, cells were collected for reporter assay or for isolation of transfected cells. The luciferase and β-galactosidase assays were essentially the same as described (Zhang *et al.*, 2001).

For isolation of transfected cells, enrichment for CD-4-positive cells was performed with the use of anti-CD-4-antibody conjugated to magnetic beads according to the manufacturer's recommendation (Dynal) as described before (Zhang and Pagano, 1999; Zhang and Pagano, 2000; Zhang and Pagano, 2001; Zhang *et al.*, 2001). The isolated cells were used for the extraction of total RNA with the use of RNase Total RNA Isolation Kit (Qiagen) or for Western blot analysis.

RNA extraction and RNase Protection Assays (RPA)

RPA was performed with total RNA with the use of the RNase Protection Kit II (Ambion, Inc.). The hybridization temperature was 42 °C. The human STAT probe set was purchased from Pharmingen (hSTAT Multi-Probe Template Set,

cat# 558834). The probes were generated with the use of T7 RNA polymerase. RPA autoradiography was analyzed with the Syngene Gene Genius Bioimaging System.

Acknowledgements

We are grateful to Dr. Leslie Huye for a careful reading of the manuscript and helpful suggestions. We thank Dr. Charles Wood for use of laboratory equipment, Dr. James Darnell Jr. for STAT-1 expression plasmids and STAT-2 cDNA, Dr. Rolf P. de Groot for STAT-3 expression plasmid, Dr. Ernest Borden for ISG15 antibody, Dr. Albert Baldwin for the NF-κB reporter construct, Dr. Richard Longnecker for the LCC-1 cell line, Dr. Kenneth Izumi for newly transformed B cell lines, Dr. Jenny Ting for the CD-4 expression plasmid, and Dr. Evan Burkala for the use of the Gene Genius Bioimaging System. The work was supported in part by grants from the National Institute of Allergy and Infectious Diseases (AI 42372-01) and from the National Cancer Institute (CA 19014) to J.S.P., and grants from the Tobacco Settlement Biomedical Research Enhancement Fund, the Layman Foundation, and the Nebraska Center for Virology funded by NIH (RR15635) from the COBRE Program of the National Center for Research Resources to L.Z.

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