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Virology 476 (2015) 377–385

Contents lists available at ScienceDirect

Virology

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Polyomavirus T antigens activate an antiviral state

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ARTICLE INFO

Article history:
Received 17 October 2014
Returned to author for revisions
18 November 2014
Accepted 19 December 2014
Available online 10 January 2015

Keywords:
Polyomavirus
SV40
BKV
JCV
Large T antigen

ABSTRACT

Ectopic expression of Simian Virus 40 (SV40) large T antigen (LT) in mouse embryonic fibroblasts (MEFs) increased levels of mRNAs encoding interferon stimulated genes (ISGs). The mechanism by which T antigen increases levels of ISGs in MEFs remains unclear. We present evidence that expression of T antigen from SV40, Human Polyomaviruses BK (BKV) or JC (JCV) upregulate production of ISGs in MEFs, and subsequently result in an antiviral state, as determined by inhibition of VSV or EMCV growth. The first 136 amino acids of LT are sufficient for these activities. Furthermore, increased ISG expression and induction of the antiviral state requires STAT1. Finally, the RB binding motif of LT is necessary for activation of STAT1. We conclude that the induction of the STAT1 mediated innate immune response in MEFs is a common feature shared by SV40, BKV and JCV.

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Introduction

Members of Polyomaviridae are circular dsDNA viruses near 5 kb in length, and sometimes establish persistent infections within a permissive host. Some viruses from the family have been linked with diseases in humans. The human polyomaviruses BKV and JCV are reported to be present in up to 80% of adults, and can incite serious disease in immunocompromised patients (Pinto and Dobson, 2014). Specifically, BKV has been implicated as an etiologic protagonist of hemorrhagic cystitis in bone marrow transplant patients (Arthur et al., 1986), and JCV infection has been linked to the incidence of progressive multifocal leukoencephalopathy in immunosuppressed patients (Padgett et al., 1976). While the SV40 has not been clearly linked to human disease, it bears similarity to the human polyomaviruses (BKV and JCV) and is well known for its contribution to the study of tumorigenesis in mammalian models (Black and Rowe, 1964).

SV40, BKV, and JCV share a relatively high degree of genome sequence homology (approximately 70%) and T antigen transcripts are produced from their early regions (Pinto and Dobson, 2014; McNees et al., 2005). The viral early regions express multiple T antigen mRNAs as a result of differential splicing. All three viruses encode a large T antigen (LT) and a small t antigen (sT) (Fig. 1A). Additional T antigen products include: a 17kT antigen (17kT) from SV40, a truncated T antigen (truncT) from BKV, and three T prime (T′) T antigens (T′135, T′136, T′165) from JCV (Trowbridge and Frisque, 1995; Gjoerup and Chang, 2010; Abend et al., 2009). The T antigens are the first proteins expressed from the viral genome, and LT initiates viral DNA replication and transcription. LT is needed to drive the host cell into S phase so as to aid in amplification of the viral genome and facilitate progeny assembly (Pipas, 2009; Damania and Pipas, 2009).

The LT from SV40, BKV, and JCV are known to induce oncogenic transformation via the disruption of cellular tumor suppressor p53 and retinoblastoma protein (pRB) family members: pRB, p107, and p130 (An et al., 2012). LT abrogates the activity of p53 by binding to it through its ATPase domain (Bargonetti et al., 1991) and blocking the p53 DNA binding domain (Bargonetti et al., 1992). LT also binds the pRBs via its LXCXE motif (Dyson et al., 1990, DeCaprio et al., 1988), and releases cellular E2F transcription factors; thus driving the cell into S phase (Sullivan et al., 2001). The LTs from SV40, JCV, and BKV are very similar in domain structure (Fig. 1B).

Previous studies have shown that SV40 LT (Forero et al., 2014; Rathi et al., 2010) or JCV genomic DNA (Verma et al., 2006) alter the expression of interferon stimulated genes (ISGs) in mouse and human cells. Moreover, expression of SV40 LT in primary mouse embryonic fibroblasts (MEFs) results in the upregulation of many ISGs (Rathi et al., 2010). Interestingly, this phenotype is independent of viral infection, and was activated in the absence of interferon (IFN) production (Rathi et al., 2010). During normal viral infection, a virus is recognized by pathogen recognition receptors (PRRs), which bind to the pathogen-associated molecular patterns (PAMPs) specific for the virus. This binding induces a signaling cascade leading to the production of IFN. Secreted IFN will then bind to the IFN receptor expressed on the cell surface,

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http://dx.doi.org/10.1016/j.virol.2014.12.032
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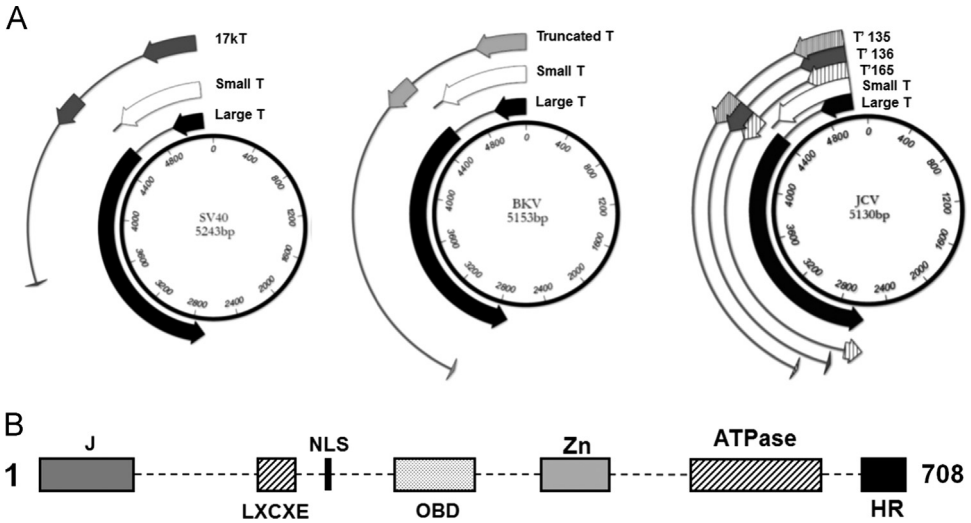

Fig. 1. Polyomavirus early region products. (A) Circular genomes for each virus are labeled with the T antigen products of the early region. Arrows indicate coding sequence, and lines indicate introns. (B) Domain map of SV40 large T antigen. Notable features of large T antigen such as the J domain (J), pRB binding motif (LXCXE), nuclear localization signal (NLS), origin binding domain (OBD), zinc finger (Zn), ATPase domain (ATPase), and host range region (HR) are indicated in the diagram.

and stimulate type I or II IFN-mediated signaling pathways. The STAT transcription factors (Signal Transducers and Activator of Transcription) are phosphorylated and thus activated as a result of IFN pathway stimulation. At this point, STAT molecules dimerize and translocate to the nucleus, where they bind to gene promoters and activate the transcription of ISGs (Platanias, 2005; Kawai and Akira, 2006). Interferon stimulated genes are known to have various roles, but are often associated with antiviral activities (Samuel, 1991; Darnell et al., 1994; Schoggins et al., 2011).

We have investigated the role of T antigen in the upregulation of ISGs observed in MEFs. Activity within the J domain and the downstream linker of LT from SV40, BKV, and JCV is sufficient to increase transcription of ISGs. The induction of ISGs by T antigen results in an antiviral state, as seen by the attenuation of virus growth. The upregulation of ISGs and the antiviral state depends on STAT1 transcription factor, with activation of STAT1 dependent upon T antigen activity via the pRB binding motif.

Results

Polyomavirus T antigens upregulate ISGs

To determine if T antigens produced from viral early regions (ER) upregulate ISGs, whole cell extracts and total RNAs were examined from MEF pools stably expressing SV40, BKV, or JCV ERs. Fig. 1A shows the possible transcripts expressed by the ER. Our analysis confirmed previous results that SV40 T antigen expression upregulated STAT1 and ISGs when compared to wild-type MEFs (Rathi et al., 2010; Forero et al., 2014). Additionally, we show by western blot that total STAT1, activated STAT1, and also total STAT2 protein levels are increased in the presence of SV40, BKV, and JCV ERs (Fig. 2A). The ISGs also exhibited elevated transcription levels in the pools expressing any of the viral ER, as detected by reverse transcription PCR (RT-PCR) (Fig. 2B).

The T antigen proteins produced by each genomic early region are shown in Fig. 2C. Although each ER is capable of inducing the transcription of ISGs, Fig. 2C reveals that the different ERs produced various T antigen products, and not all possible products were detected. SV40-ER produced both LT and sT, as shown by western blot using antibodies capable of detecting all known T antigens produced by SV40 (Harlow et al., 1981; Fu et al., 1996; Bollag et al., 2000; Munoz-Marmol et al., 2004). However, we were unable to detect the production of LT from BKV-ER. Instead, BKV-ER preferentially produced either the sT, or truncT antigen based on the apparent size of the detected T antigen (Seneca et al., 2014). Examination of pools expressing JCV-ER showed production of both LT and other alternative splice products which may include sT, or the T's. These results demonstrate that the products from ERs of SV40, JCV, and BKV are capable of activating the cellular interferon response and upregulating production of ISGs. Additionally, the data suggests that ISG upregulation may not be solely dependent on the LT as previously shown (Rathi et al., 2010; Forero et al., 2014).

Small t antigen is not necessary to induce upregulation of ISGs

To evaluate the contribution of sT activity to the upregulation of ISGs in MEFs, we generated MEF pools expressing LT cDNA from SV40, BKV, and JCV, which were unable to produce sT (Fig. 3). Under these conditions, MEFs expressed the corresponding LTs from SV40, BKV, and JCV, as well as other non-sT T antigen products (Fig. 3A). BKV LT cDNA also produced truncated T antigen, and JCV LT cDNA produced at least two T's. Transcription of ISGs was upregulated in the LT cDNA pools relative to wild-type MEFs, and ISG levels were similar to those observed in MEFs expressing the SV40-ER (Fig. 3B). We conclude that the upregulation of ISGs in MEFs by SV40, BKV, and JCV does not depend on the presence of sT.

The J-domain and downstream linker region of large T antigen is sufficient for upregulation of ISGs

To map the activity of LT necessary for ISG upregulation, truncation mutants of the LT were examined. MEF pools expressing truncation mutants of LT, either the first 136 amino acids of SV40 (SV40-N136), or the equivalents in BKV (BKV-N138) or JCV (JCV-N137) were produced. The amino region truncations contain a J domain, LXCXE motif, and nuclear localization signal (Fig. 4A). The expression of the truncation mutant proteins in the MEF pools is shown by western blot (Fig. 4B). The pools expressing the truncation mutants retain the ability to produce sT via alternative splicing; however no sT was detected by the western blot shown in Fig. 4B.

Examination of cell pools expressing the amino terminal region truncations revealed increased levels of interferon response

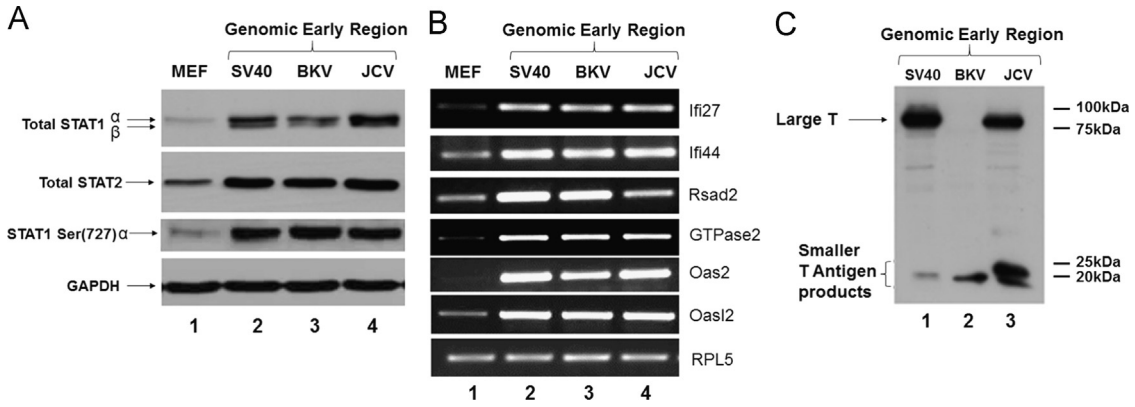

Fig. 2. Polyomavirus early region upregulates ISGs. (A) Western blots were performed with 30 μg of whole cell extract from wild-type MEF and MEF pools expressing the early region of SV40, BKV, and JCV. Total STAT1 and STAT2 and activated STAT1 (Ser 727) levels were detected with the appropriate antibodies. GAPDH served as the loading control (B) The products of RT-PCR for various ISGs using cDNA made from 2 μg of total RNA from wild-type MEFs and the viral early regions expressing MEF pools were resolved on a 1% agarose gel and visualized by ethidium bromide. (C) Steady state protein level of the T antigens produced by the viral early regions was determined by western blot using 30 μg of whole cell extract from MEF expressing the various early regions. The data shown is representative of all pools tested.

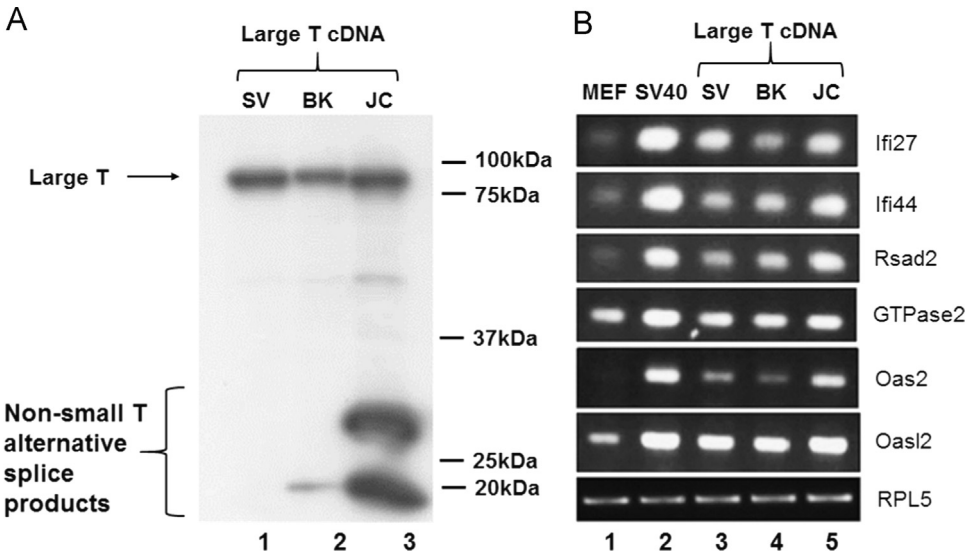

Fig. 3. Large T antigen cDNA upregulates ISGs. (A) Western blots were performed with 30 μg of whole cell extract for expression of the T antigen proteins produced from Large T cDNA MEF pools. T Antigens were detected with appropriate antibodies. (B) The products of RT-PCR for various ISGs using cDNA produced from 2 μg of total RNA from wild-type MEFs, or the Large T cDNA MEF pools are shown. Lane 2 shows ISG transcription levels of the SV40-ER MEF pool for comparison. The data shown is an average of all pools tested.

mediators, STATs (Fig. 4C), and downstream products, ISGs (Fig. 4D), when compared to wild-type MEFs. However, BKV-N138 expressing pools did not upregulate total STAT2 production (Fig. 4C, lane 3). This was in contrast to cells expressing the BKV-ER. The total STAT2 levels were increased in MEF pools expressing SV40-N136 or JCV-N137. We conclude that activity by N136 equivalent truncation mutants is sufficient for the upregulation of the ISGs in MEFs.

A functional retinoblastoma protein binding motif in T antigen is necessary to activate STAT1

To further narrow down the activity of the LT necessary to induce the interferon response leading to the upregulation of ISGs, we examined additional mutants within the full length LT of SV40, and the SV40-N136. We produced two full length SV40 LT expressing MEF clones, and a pool of MEFs expressing SV40-N136 containing a mutated LXCXE motif. Protein expression of the mutant T antigens is shown in Fig. 5B. The new mutant exhibits two point mutations at E107K/E108K (E107/8K). The E107/8K mutant is unable to bind pRB family members (DeCaprio et al., 1988). In all MEFs expressing E107/8K mutants, the steady-state levels of activated STAT1 S727 were not upregulated relative to MEFs expressing normal LT or SV40-N136 (Fig. 5A). The phosphorylation of STAT1 S727 results in translocation of STAT1 to the nucleus and promotion of ISG transcription (Wen et al., 1995; Levy and Darnell, 2002). These results indicate activation of STAT1 S727 in T antigen expressing MEFs is abrogated with a mutated LXCXE motif. This suggests that the LXCXE motif is critical for activation of STAT1, and may be necessary for ISG production.

The carboxy terminal region of large T antigen is defective for the upregulation of ISGs

We next examined carboxy-terminal truncation mutants of the LT from SV40 to determine if additional activities downstream of the LXCXE motif were needed to upregulate the ISGs in MEFs. Two pools of MEFs expressing a truncation mutant of the LT containing amino acids 257–708 (C257, Fig. 5C) were generated. Fig. 5D shows the protein expression of the C257 mutant via western blot. Additional analyses were performed to assess the protein levels

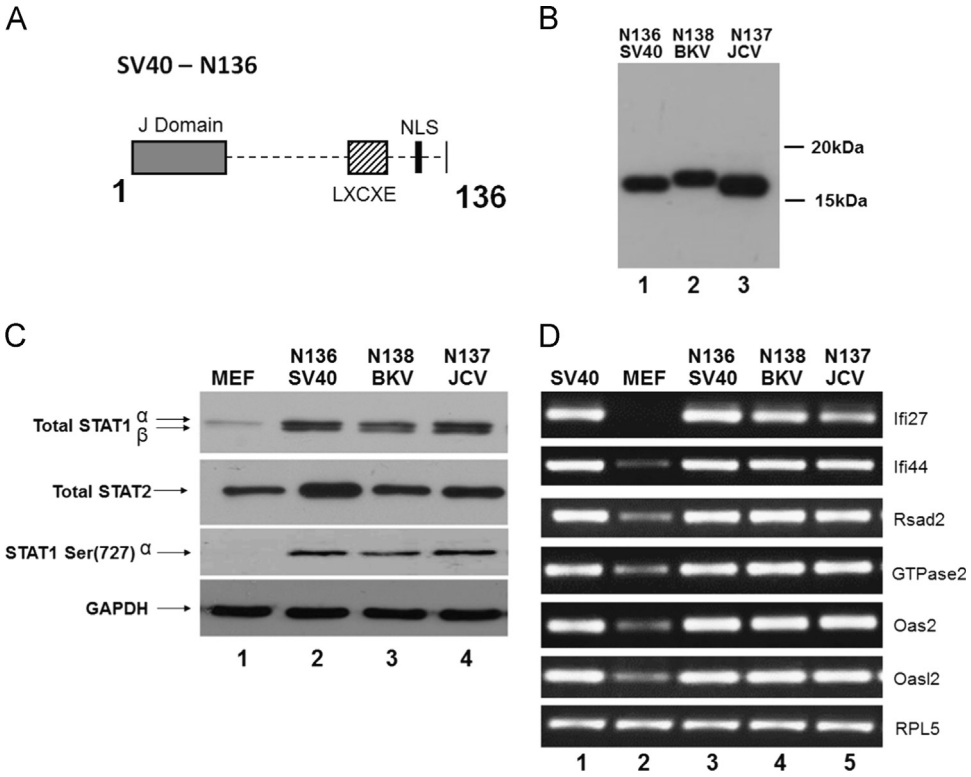

Fig. 4. J-domain and downstream linker region of large T antigen upregulate ISGs. (A) A diagram of the first 136 amino acids of SV40 Large T antigen (N136). The features shown (as indicated in Fig. 1) are also shared with the equivalent amino truncation mutant of BKV (N138) and JCV (N137). (B) Western blot was performed with 30 μg of whole cell extract to show the production of N136 equivalent truncation mutants in MEF pools. (C) Western blot showing the production of STAT1 and STAT2 in whole cell extract from wild-type MEFs (lane 1) or MEF pools expressing N136 equivalents (lanes 2–4). D) RT-PCRs for various ISGs using cDNA made from 2 μg of total RNA from wild-type MEFs (lane 2), or the N136 equivalent mutant MEF pools (lane 3–5). The SV40-ER MEF pool is included for comparison (lane 1). The data shown is an average of all pools tested.

of STATs and ISG transcription levels (Fig. 5D and E). Both MEF pools expressing C257 were defective for the upregulation of total and activated STAT1 relative to SVT-ER T antigen expression (Fig. 5D, lanes 3 and 4), as well as the ISGs (Fig. 5E). Moreover, total STAT2 levels varied between the two pools examined, i.e., STAT2 was upregulated in the second MEF pool tested not the first. These results indicate that the carboxy-terminal region of LT is defective for the upregulation of ISGs.

Polyomavirus T antigen activates an antiviral state

As the interferon response is upregulated following viral infection, and results in a defensive (antiviral) state by the cell against the pathogen (Samuel, 1991), we hypothesized that the increase in ISGs by T antigen would establish an antiviral state. To test this, we challenged the MEF pools expressing T antigen with vesicular stomatitis virus (VSV), a positive sense RNA virus, and performed plaque assays to quantify viral growth. RNA viruses have been shown to be attenuated by the production of ISGs (e.g. OAS2) (Silverman, 2007; Lin et al., 2009; Fensterl et al., 2012), and thus served as a good candidate for assessment of antiviral state.

VSV was grown on either wild-type MEFs or MEFs expressing the SV40, BKV, or JCV ERs. Supernatants containing viruses were harvested from MEF pools at various time points, and subjected to plaque assay to generate virus growth curves. Fig. 6A shows the average of VSV production by four different lineages of wild-type MEFs over a period of 48 h post-infection. Fig. 6B shows the average production of VSV growth from multiple infections of ER-expressing MEF pools normalized against the wild-type levels. That is, the VSV production from ER expressing MEFs relative to VSV production from wild-type MEFs displayed as a proportion. Infections were performed with at least two different lineages MEFs. These results reveal clearly that VSV growth is inhibited by the presence of T antigen produced from the ERs. ER expressing MEFs show less than ten percent of the VSV production observed in wild-type from 12 h post-infection and later time points. Similar results were obtained from MEFs expressing the N136 equivalents Fig. 6C. Specifically, greater than 90% of VSV production was blocked in SV40-N136 and JCV-N137 from 12 to 48 h post-infection. BKV-N138 reduced VSV growth to similar levels as SV40-N136 and JCV-N137 except at 48 h where viral growth was inhibited 2 fold. Together, these data suggest that the rate of VSV growth is substantially attenuated in the presence of T antigen.

In order to determine if the antiviral state established by T antigen was specific to VSV, we prepared additional virus growth curves with encephalomyocarditis virus (EMCV). Wild type, SV40-ER MEFs, and N136 MEFs, were challenged with EMCV. The cellular supernatant was harvested at various time points, and viral replication was quantified by plaque assay. Fig. 6D shows the production of EMCV growth (pfu/mL) in SV40-ER, SV40-N136, or wild-type MEFs. We observed that EMCV growth was attenuated by at least three to four $\log_{10}$ in the T antigen expressing MEFs compared to wild-type at three hours post-infection. Based on the evidence from both VSV and EMCV viral growth curves, we conclude that expression of T antigen in MEFs activates an antiviral state.

STAT1 transcription factor is necessary to establish the T antigen induced antiviral state

Given that the STAT1 transcription factor is a critical mediator of IFN signaling pathways and for the establishment of the

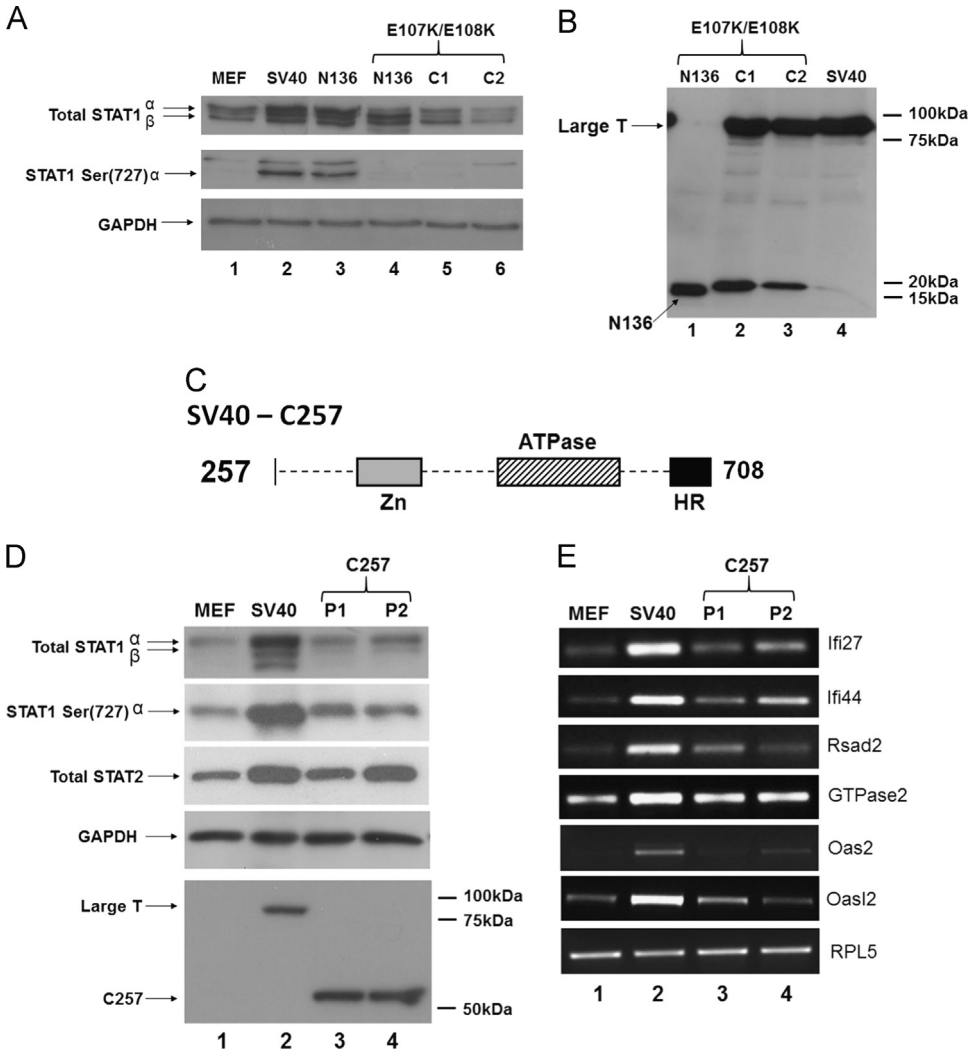

Fig. 5. T antigen mutants C257 and E107/E108K are defective for the upregulation of ISGs. (A) Western blot of total and activated STAT1 levels. Wild-type MEF (lane 1), MEFs expressing SV40-ER (lane 2), and MEFs expressing N136 (lane 3) are included for comparison to E107/8K mutant STAT1 production/ activation in lanes 4–6. Lane 4 is a MEF pool expressing N136 E107/8K, and lanes 5–6 are full length SV40 T antigen E107/8K mutant expressing clones (C1 and C2). (B) Steady state levels of E107/8K mutant T antigens (lanes 1–3) and an SV40-ER expressing MEF pool T antigen were determined by western blot. (C) Diagram of the SV40-C257 mutant and its features are shown. (D) Western blot showing the production of STAT1 and STAT2 in whole cell extract from wild-type MEFs (lane 1) or two MEF pools—P1 or P2–expressing C257 lanes 3–4). SV40-ER expressing MEFs are included for comparison (lane 2). E) RT-PCRs for various ISGs using cDNA made from 2 μg of total RNA from wild-type MEFs (lane 1), or MEF pools expressing C257 (lane 3–4).

antiviral state (Horvath and Darnell, 1996), we hypothesized that T antigen may be inducing ISG production via STAT1 signaling. To determine if the upregulation of ISGs by T antigen requires STAT1, pools of STAT1(−/−) MEFs expressing T antigen were examined. Fig. 7A and B shows the production of various T antigens in STAT1 (−/−) MEFs expressing either SV40-ER or the N136 equivalents. As described earlier, whole cell extract and total RNA from these pools were evaluated. Results from both western blot and RT-PCR showed that none of the T antigen expressing pools examined (SV40-ER or N136 equivalents) exhibited increased levels of ISGs (Fig. 7C and D).

Next, we sought to determine if STAT1 was required by T antigen to induce the antiviral state. We quantified VSV production from STAT1(−/−) MEFs by plaque assay. We did not obtain a robust difference between the level of VSV produced from STAT1(−/−) MEFs and SV40-ER expressing STAT1(−/−) MEFs (Fig. 7E). Furthermore, Fig. 7E reveals that the SV40-ER STAT1(−/−) MEFs produced nearly the same amount of VSV(pfu/mL) as wild-type MEFs from 12–48 h (Fig. 6A). Together, our data suggests that STAT1 transcription factor is necessary for the upregulation of ISGs by T antigen, and that ISG production is necessary to induce the antiviral state.

Discussion

Expression of the ERs from SV40, BKV, and JCV in MEFs results in the production of various T antigen proteins via alternative splicing of the early pre-mRNA transcript. In our system, MEF pools expressing the ER of SV40 or JCV produced LT, were able to upregulate ISGs, and generate an antiviral state as assessed by VSV and EMCV infection. Interestingly, we could only detect what appears to be truncated T antigen or sT, but not LT protein from the MEFs expressing BKV-ER. We have shown that this was not a failure of the assay to detect LT from BKV-ER MEFs because we can detect large amounts of BKV LT in the BKV LT cDNA expressing MEFs (Fig. 3A). We conclude that alternative splicing favors the production of alternative mRNAs when the entire early region is expressed. The results suggest that T antigen's ability to activate the antiviral state, at least with BKV, must be limited to activity found within its small or truncated T antigens.

Because the sT and truncated T antigens share characteristics (e.g. J-Domain and Rb binding motif) with the N136 equivalent mutants described above, we hypothesized that N136, N137, and N138 would be able to induce the antiviral state as seen in MEF

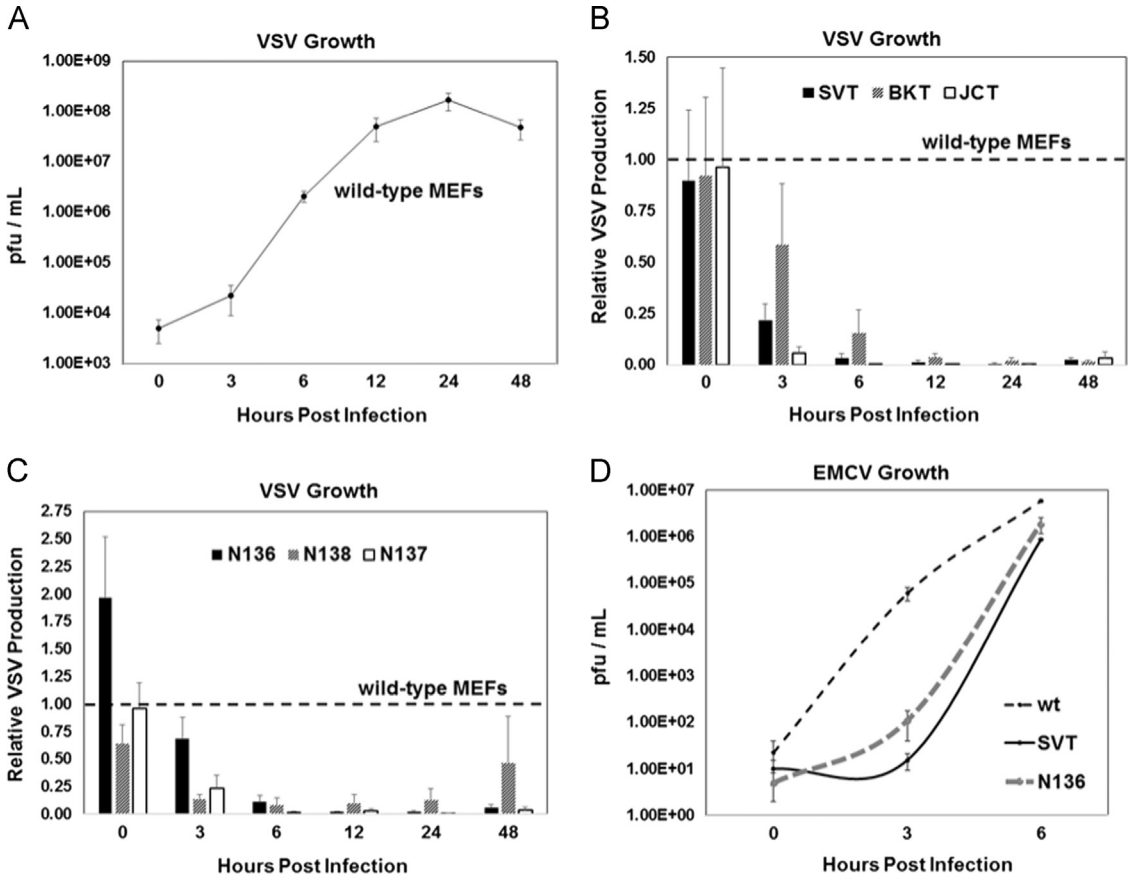

Fig. 6. T antigen inhibits growth of VSV and ECMV. (A) The titer of VSV (pfu/mL) on wild-type MEFs over 48 h. Samples were collected at various time points and the virus titer was determined by plaque assay on BHK21 cells. (B) VSV growth on MEFs expressing the ER of SV40, BKV, or JCV. The *y*-axis values shown are a proportion of VSV produced from ER expressing MEFs to VSV produced from wild-type MEFs at the corresponding time, which are shown in panel A. The dashed line indicates VSV growth on wild-type MEFs. (C) VSV growth on MEFs expressing N136 equivalents (N136, N138, or N137). Again, the dashed line indicates of VSV growth on wild-type MEFs. (D) Titer of EMCV produced (pfu/mL) from SV40-ER expressing MEFs (SVT), and N136 expressing MEFs (N136). ECMV titer in wild-type MEFs is represented as the dotted line (wt). In all panels (A–D), error bars represent standard error of the mean.

pools expressing the early region. Further analysis showed the antiviral state was clearly established in the MEF pools expressing any of the N136 equivalents. We also examined the role of sT in ISG induction. Expression of constructs unable to produce sT still retained the ability to upregulate ISGs. However, we cannot rule out the possibility that sT from SV40, BKV, or JCV expressed independently can activate ISGs.

The activity of T antigen necessary to activate the ISGs requires an intact LXCXE (pRB binding) motif. Mutation of the LXCXE motif, either in full length LT or the N136 truncation, resulted in a failure to upregulate the activation state of STAT1 S727. Therefore, it is possible that the binding of pRB family members is required to induce the upregulation of ISGs. However, it is also possible that the LXCXE motif is capable of binding other partners besides pRB family members, and those are necessary for STAT1 activation. Indeed, previous studies have shown that the LXCXE motif is capable of binding insulin receptor substrate 1 (IRS1) which resulted in the activation of protein kinase B (AKT) (Yu and Alwine, 2008). We examined the effect of AKT activation on ISG upregulation, but concluded that AKT activation by T antigen is not related to the induction of ISGs (data not shown). In an earlier study we reported that the association of SV40 LT with p53 is necessary for ISG induction (Rathi et al., 2010). This study utilized MEF clones expressing N136 rather than early passage cell pools used in the current study. The N136 clones underwent crisis prior to their establishment as stable cell lines. We hypothesize that mutations arising during clonal selection explain the difference in results.

The evidence presented in this study shows that the upregulation of ISGs by T antigen is dependent upon the presence of STAT1, and that LT failed to induce the upregualtion of ISGs as well as an antiviral state in STAT1(−/−) MEFs. This indicates that STAT1 is critical. However, the mechanism by which T antigen manages to induce the activation of STAT1 via the LXCXE motif remains unresolved. Alternatively, the level of LT expression in SVT-ER STAT1(−/−) MEFs is lower relative to normal SVT-ER MEFs (Fig. 7B), and that this may explain the lack of ISG induction in the SVT-ER STAT1(−/−) MEFs. However, we find this highly unlikely because we have observed full ISG induction with very low levels of T antigen. These cases arise because some MEF pools express low T antigen levels or some T antigen mutants lower the steady state levels of T antigen.

We have previously shown that LT also induces ISGs and an antiviral state in human fibroblasts (Forero et al., 2014); however, the mechanism appears to differ from what occurs in MEFs. In human cells ISG induction is mediated by the DNA damage response and the production of secreted interferon signal (Forero et al., 2014). In contrast, in MEFs ISGs are induced irrespective of interferon production (Rathi et al., 2010), and therefore it seems unlikely that the antiviral state in these cells would be established through a similar mechanism. Further more sensitive assays may be necessary to confirm that MEFs upregulate ISGs via an interferon independent mechanism, but in either case our investigation extends upon the previously known phenomena that expression of the LT from SV40 in mouse or human fibroblasts can activate ISGs (Rathi et al., 2010; Forero et al., 2014).

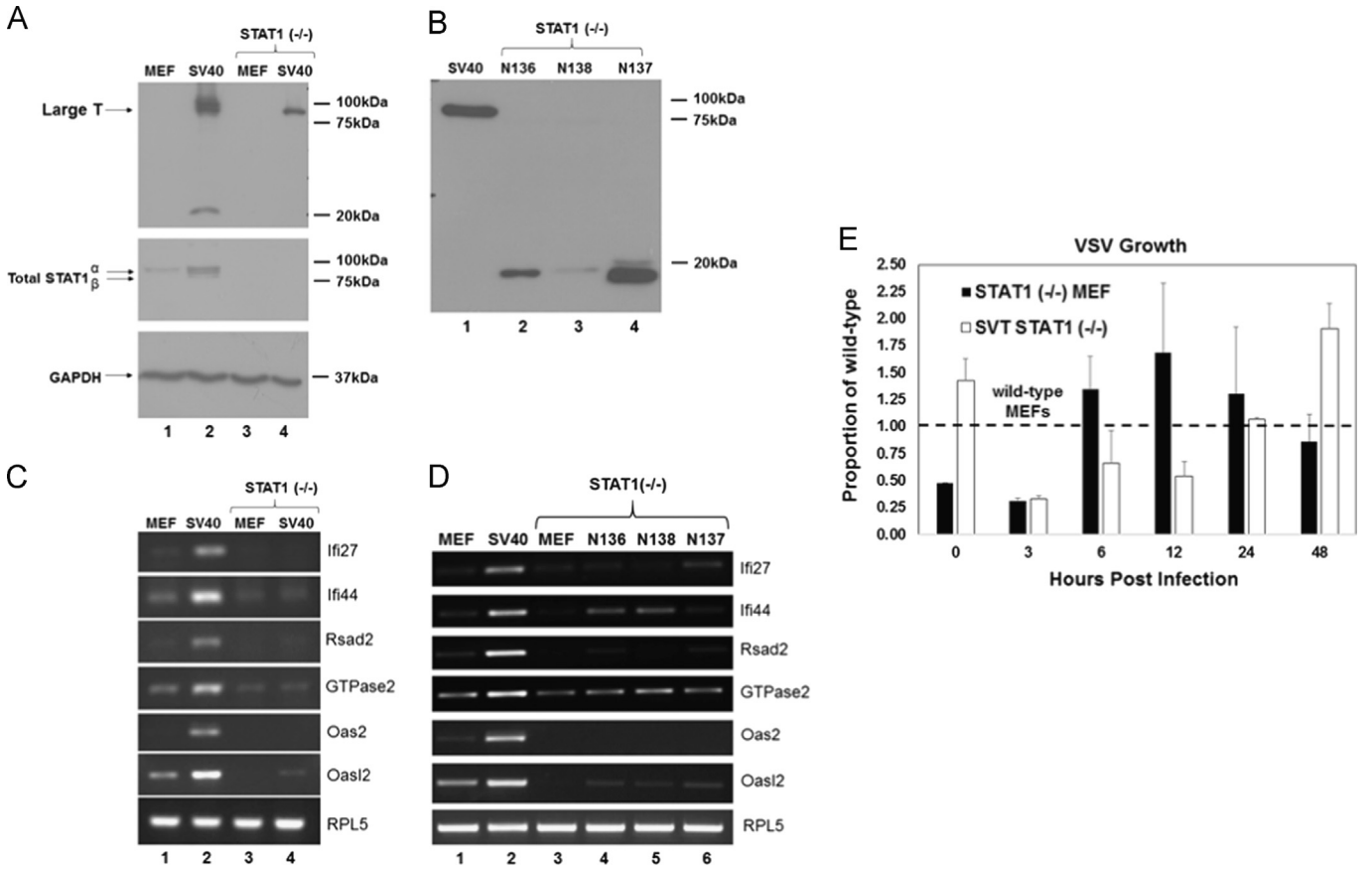

Fig. 7. STAT1 is necessary for induction of ISGs and generation of the antiviral state. (A) Western blots of T antigen production from wild-type MEFs and SV40-ER expressing MEF pools in both normal MEF and in the STAT1(−/−) MEF backgrounds. (B) Western blot of N136 equivalent mutants expressed in STAT1(−/−) MEFs. (C and D) RT-PCR analysis of various ISGs using cDNA made from 2 μg of total RNA from wild-type MEFs and MEF pools expressing T antigen in both the normal and STAT1(−/−) backgrounds. Lane 2 of both C and D are SV40-ER expressing MEFs and serve as a positive control for ISG upregualtion. (E) VSV growth on STAT1(−/−) MEFs. The *y*-axis values shown are a proportion of VSV produced from STAT1 (−/−) MEFs or STAT1 (−/−) MEFs expressing SVT-ER to VSV produced from wild-type MEFs at the corresponding time, which are shown in panel of Fig. 6A. The dashed line indicates VSV growth on wild-type MEFs. In E, error bars represent standard error of the mean. The data shown is representative of all pools tested.

In this study we have described that the stable expression of different polyomavirus T antigens in MEFs triggers the upregulation of ISGs and subsequently establishes an antiviral state. This is the first report, to our knowledge, that describes these phenotypes for BKV and JCV T antigens in mouse cells. Our results suggest that T antigen activity via the LXCXE motif leads to the activation of STAT1, and may be critical to the increase of ISG transcription. Furthermore, our data indicate that STAT1 and ISGs play a crucial role in mediating the antiviral response by T antigen.

Materials and methods

Plasmids and constructs

Cloning of pBABEpuro-SV40-ER, BKV-ER, and JCV-ER involved the conversion of pBABEpuro into a Gateway® destination vector using the Gateway® Vector Conversion System (Invitrogen, catalog #11838-029) by ligating the reading frame cassette C.1 into the SnaBI site. attB-PCR products of the early regions of SV40, BKV and JCV were generated according to the "attB Adapter PCR" procedure in the Gateway® Technology with Clonase II kit (Invitrogen, catalog #12535-029) using template DNA pSVB3 (Peden et al., 1980), pBKV (JF894228) and pJCmad1 (gift from Keith Peden, NC_001699) for SV40, BKV and JCV, respectively. Template-specific primers were designed to contain a Kozak consensus sequence (5′-ACC-3′) immediately upstream of the ATG start site. attB-PCR products were cloned into pDONR221 using the BP recombination reaction and the resulting plasmid was recombined by an LR reaction with pBABEpuroDEST to create the early region expression clone. All procedures were followed as recommended by the manufacturer.

Cloning of pLenti6.3 (Invitrogen #K5315-20) constructs was performed in accordance with the manufacturer's instructions. An additional Kozak sequence (ACC) was introduced directly upstream of the start codon to enhance translation of the construct. N136 equivalent and C257 mutants were previously described (Robles et al., 2013) Additional full length SV40 LT E107K/E108K mutants were described (Rathi et al., 2010). The full length LT cDNA constructs were prepared by amplification via RT-PCR. Restriction sites EcoRI and NotI were introduced with primers during amplification. Following restriction digestion and gel purification the DNA fragments were ligated into the pEF1/V5-His plasmid (Invitrogen #V920-20) at the corresponding sites. Constructs were verified by sequencing.

Isolation of primary mouse embryonic fibroblasts, conditions of cell culture, and establishment of pools

Mouse embryonic fibroblasts (MEFs) and STAT1(−/−) MEFs were harvested from 13.5 day old FVB embryos using the method previously described (Markovics et al., 2005). MEFs were grown in DMEM (Corning #10-013-CV) supplemented with 10% fetal bovine serum (HYCLONE#: SH30070.03) and 1% penicillin/streptomycin (Invitrogen #15140163). MEFs were grown at 37 °C with 5% CO_2.

Plasmids encoding different T antigen mutants were transfected with lipofectamine 2000 reagent (Invitrogen 11668-027) into the producer cell line, 293FT (Life technologies #R700-07) for production of lentivirus in accordance with manufacturer's instructions. Pools of MEF cells expressing different constructs were then generated via lentiviral transduction (Invitrogen ViraPower #K4975-00) with 12 μg/ml polybrene reagent (Sigma #H9268). Lentivirus transduction conveyed the pL6.3 lenti/V5 TOPO plasmid (Invitrogen #K5315-20) expressing a genomic version of different mutant large T antigens (SV40-N136, BKVT-N138, JCVT-N137, SV40 C257-708, or N136 E107/E108K). Following lentiviral infection of MEFs, cells were grown to confluency and selected for 7–10 days in 2 μg/mL blasticidin (Life Technologies #R210-01). Surviving colonies were pooled and passaged until approximately passage four, except for the MEF pool expressing N136 E107/E108K. They could not be passaged beyond selection, as those cells did not transform/immortalize with expression of the mutated N136 construct. Instead they were collected 2 days post-confluency, passage zero. All other selected MEF pools were collected for further analysis at passage $\geq$ four.

MEF pools expressing viral ER were generated by a similar transduction, using retroviral vectors. The ER were cloned into the pBABE puro plasmid (kindly provided by Ole Gjoerup), utilizing a murine maloney leukemia retrovirus promoter, and transfected into phoenix eco-cells (ATCC #CRL-3214). Retrovirus made from producer cell lines were used to infect MEFs. Subsequent selection was carried out in a similar fashion using 2 μg/mL puromycin for 7–10 days. Surviving clones were pooled and passaged to approximately passage four, and cellular pellets were collected.

MEFs expressing Large T antigen cDNAs were generated via transfection with pEF1/V5-HisA plasmids (Invitrogen) expressing the cDNA of the various LTs. Plasmids were introduced via FUgene 6 (Promega #E2691) transfection in accordance with manufacturer's instructions, and MEF pools were obtained after selection with 200 μg/mL Neomycin,G418, (Cellgro #: 30-234-CR) for 7–10 days. MEF cell lines expressing full length LT E107/108K mutation were previously described (Rathi et al., 2010), and expressed the mutated LT via pRSVneo plasmid (Srinivasan et al., 1997). The clones were isolated, grown, collected, and samples of whole cell extract were prepared as described below.

Virus growth and plaque assays

Wild-type MEFs or MEF pools stably expressing T antigen were plated in 6 cm dishes and infected with VSV the following day at a multiplicity of infection (MOI) of 0.1. At the time of infection, cells were counted to establish the correct MOI. Infection lasted for 1 h in a 37 °C, 5% CO2 incubator, rocking the plates every 15 min. Afterward, the virus was removed and 2.5 mL of growth medium was supplied. Plates were collected at different time points and frozen at −80 °C. Plates were thawed, and the supernatant was collected by centrifugation at 3000 rpm for 15 min. Infections for each condition were performed across at least two different MEF lineages.

Infections with EMCV were performed with wild type MEFs or MEF pools expressing T antigen. The cells were plated and allowed to grow overnight. The following day cells were counted, seeded in plates (~2e6 cells/plate) and infected with EMCV at a MOI of 0.005. Infection lasted for one hour at 37 °C in a 5% CO2 incubator, rocking the plates every 15 min. Afterward, the viruses were removed; plates were washed three times in 1X PBS, and 2.5 mL of growth media was supplied. Plates were collected at 0, 3, and 6 h post-infection, and frozen at −80 °C. Plates were freeze/thawed 2 times, and viral supernatant was collected via centrifugation at 3000 rpm for 15 min.

Infectious virus production was quantified by plaque assay. Supernatant produced from cells infected with VSV was serially diluted in DMEM and used as inoculum for infection of BKH21 cells (ATCC: #CCL-10). BHK21 cells were infected for 1 h in a 37 °C, 5% CO_2 incubator, rocking every 15 min. Media containing virus was removed and cells were overlaid with 1.5% agarose (LONZA #50100) with 2X MEM (LifeTechonologies #61100) and 10% FBS. Twenty-four to thirty-six hours post-infection, viral plaques were visualized with the addition of 1% neutral red solution (Sigma). The same procedure was used to quantify EMCV production with two exceptions: VERO cells (ATCC # CCL-81) were used for viral infection, and plates were stained at 36–48 h post-infection to visualize plaques.

Immunoblot analysis

Western blots were conducted as previously described (Markovics et al., 2005) with the following antibodies: Total STAT1 (Cell Signal #9172), Phospho-STAT1 Ser727 (Cell Signal #9177), and Total STAT2 (Cell Signal #4597) and were prepared in accordance with the manufacturer's instructions. SV40 T Antigen mouse monoclonal antibodies have been described previously: PAb416, PAb419 (Harlow et al., 1981), and PAb901 (Fu et al., 1996). Additional mouse monoclonal antibodies against JCV T antigens (PAb962 and AB2003) were previously described (Bollag et al., 2000; Munoz-Marmol et al., 2004). GAPDH mouse monoclonal was used as the loading control (US Biologicals #G8140-11). Peroxidase-conjugated goat anti-mouse (A2554), and goat anti-rabbit (A0545) from Sigma-Aldrich were used as secondary antibodies. The Luminata Western HRP substrate (Millipore #WBLUF0100) was used in accordance with manufacturer's instructions. A mixture of monoclonal antibodies 416/419/962/2003 (1:1000/1:500/1:1000/1:1000) were used for the detection of T antigen signal in Figs. 2C, 3A, 4B, and 7B. Additionally, SV40-C257 detection used monoclonal Ab901 (1:500) in Fig. 5D, and in Fig. 5B a mixture of 416/419 (1:500/1:250) was used.

Whole cell extract, RNA extraction, and reverse transcription PCR

MEF cells were grown for two days post-confluency, trypsinized, and pelleted by centrifugation. Cell pellets were washed in 1X PBS and frozen at −80 °C. Total RNA was extracted with the Qiagen RNeasy kit (#74104) in accordance with manufacturer's instructions. Samples were quantified using a NanoDrop2000. Proteins extracts from whole cell extract were prepared by lysis in [HEPES pH 7.9 50 mM, KCl 0.4 M, EDTA 0.5 mM, NP40 0.1%, Glycerol 10%], and lysates were centrifuged for 15 min at 4 °C to remove insoluble material. Quantification of extracts was performed by Bradford protein assay (BioRad #500-0006) in accordance with manufacturer's instructions on a BioRad iMArk microplate reader.

Reverse transcription PCR for the production of cDNA from total RNA (2 μg) was reverse-transcribed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen #18080-051) in accordance with manufacturer's instructions. PCR analysis with gene specific primers (Rathi et al., 2010) was performed using equal amounts of cDNA and GOTaq polymerase (Promega #M712). PCR conditions were described previously (Rathi et al., 2010). Rpl5 served as a loading control. The reactions were resolved through 1% agarose gels and visualized with ethidium bromide (Sigma-Aldrich #E1510).

Acknowledgments

This work was supported by the National Institutes of Health Grant 1R21-AI109339 to J.M.P. We thank Saumen Sarkar, Adriana

Forero, Maria-Teresa Sáenz Robles, and Paul Cantalupo for helpful discussions. We also thank Jennifer L. Koffman for technical assistance.

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