The replication and transcription activator of murine gammaherpesvirus 68 cooperatively enhances cytokine-activated, STAT3-mediated gene expression

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Gammaherpesviruses (γHV) have a dynamic strategy for lifelong persistence, involving productive infection, latency, and intermittent reactivation. In latency reservoirs, such as B lymphocytes, γHV exist as viral episomes and express few viral genes. Although the ability of γHV to reactivate from latency and re-enter the lytic phase is challenging to investigate and control, it is known that the γHV replication and transcription activator (RTA) can promote lytic reactivation. In this study, we provide first evidence that RTA of murine γHV68 (MHV68) selectively binds and enhances the activity of tyrosine-phosphorylated host STAT3. STAT3 is a transcription factor classically activated by specific tyrosine 705 phosphorylation (pTyr705-STAT3) in response to cytokine stimulation. pTyr705-STAT3 forms a dimer that avidly binds a consensus target site in the promoters of regulated genes, and our results indicate that RTA cooperatively enhances the ability of pTyr705-STAT3 to induce expression of a STAT3-responsive reporter gene. As indicated by cotransfection in latently infected B cells that are stimulated to reactivate MHV68, RTA bound specifically to endogenous pTyr705-STAT3. An in vitro binding assay confirmed that RTA selectively recognizes pTyr705-STAT3 and indicated that the C-terminal transactivation domain of RTA was required for enhancing STAT3-directed gene expression. The cooperation of these transcription factors may influence both viral and host genes. During MHV68 de novo infection, pTyr705-STAT3 promoted the temporal expression of ORF59, a viral replication protein. Our results demonstrate that MHV68 RTA specifically recognizes and recruits activated pTyr705-STAT3 during the lytic phase of infection.

Viruses strategically modulate specific host factors to promote their persistence or their replication. Gammaherpesviruses (γHV), like all herpesviruses, undergo lytic replication, yet establish a lifelong infection predominantly characterized as latency with intermittent reactivation (1–4). Latent γHV infections are associated with cancer, and viral reactivation can cause severe pathologies. Specific γHV-host interactions, such as modulation of immune responses, play a crucial role in viral infection, latency, and virus-induced pathogenesis. Murine gammaherpesvirus 68 (MHV68/MuHV-4) is a natural pathogen of rodents and is closely related to the well-known oncogenic human γHV, Epstein-Barr virus (EBV/HHV-4), and Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8). The critical reservoir of latency for these γHV is most commonly the B cell compartment. We have shown previously that animals with a targeted B cell deletion of signal transducer and activator of transcription-3 (STAT3) have a dramatic reduction in latency establishment and persistence in an animal model of MHV68 infection (5). In addition, in EBV and KSHV cell culture systems, high levels of STAT3 correlate with latent infection rather than effective lytic replication (6–8). These results indicate that cellular STAT3 helps to promote latent viral colonisation of the host. Yet in this study, we find that STAT3 is also influenced by a viral lytic transcription factor.

Reactivation of γHV lytic replication from a latent state can be triggered by expression of the viral protein, replication and transcription activator (RTA) (9–11). The RTA proteins of KSHV and MHV68 share homology, and KSHV RTA can substitute for MHV68 RTA during viral reactivation (12, 13). RTA has been shown to bind specific DNA sequences in regulated genes, and to enhance the activity of other transcription factors (14–18). To probe the potential involvement of STAT3 during γHV lytic infection, we evaluated the ability of MHV68 RTA to influence STAT3. STAT3 is a member of the STAT family of transcription factors that are activated by tyrosine phosphorylation in response to cytokines, growth factors, or oncogenic tyrosine kinases (19–22). Tyrosine phosphorylation promotes STAT dimerization of monomers via their reciprocal phospho-
RTA targets tyrosine-phosphorylated STAT3

tyrosine and Src homology 2 (SH2) domain interactions. The STAT dimers effectively bind a DNA consensus sequence often found in the promoter of regulated genes, designated a γ interferon–activated site (GAS) (23). A classical activator of STAT3 is the interleukin-6 (IL-6) cytokine, originally cloned as a B cell differentiation factor (24–27). Notably, cellular IL-6 production has been reported in response to γHV infection (28–31). In addition, EBV and KSHV encode cytokine homologues (vIL-10 and vIL-6) that activate STAT3, as well as other viral factors that lead to an increase in STAT3 tyrosine phosphorylation (32–43).

To determine whether MHV68 RTA influences the activity of STAT3, we tested the effect of its expression on STAT3-directed gene induction. Our results indicate that RTA enhances STAT3 activity, in the context of STAT3 tyrosine phosphorylation. The mechanism of enhancement appears to stem from the ability of MHV68 RTA to specifically bind the tyrosine-phosphorylated conformation of the STAT3 dimer. Together with our previous finding that STAT3 is required for the establishment of MHV68 latency and persistence in an animal model, these results indicate that the virus also uses STAT3 during the lytic phase of infection. RTA may re-direct STAT3 to influence genes that promote viral replication, either at early times of reactivation or infection. Knowledge of this RTA-STAT3 interface may provide a new target of intervention.

Results

Synergistic induction of gene expression by STAT3 and MHV68 RTA

The intimate relationship of gammaherpesviruses with the infected host can be different during establishment of a latent infection and during productive infection. We recently reported that the STAT3 cellular transcription factor is essential for establishment of viral latency by MHV68 in the natural host (5). To determine whether a viral factor critical for acute replication can influence STAT3 function, we examined the effect of the essential viral lytic protein transactivator RTA on gene expression regulated by STAT3 in Hep3B cells. These cells respond to IL-6, a classical cytokine that activates STAT3 tyrosine phosphorylation. In addition, MHV68 replicates in liver, and infection of mice significantly alters liver cell gene expression (44–46). We evaluated gene expression regulated by STAT3 in the presence or absence of IL-6 and RTA.

A STAT3-responsive promoter sequence from the α2-macroglobulin gene was used to generate a responsive luciferase reporter gene (25). Expression of this reporter in Hep3B cells is induced ~10-fold with expression of STAT3 and stimulation with IL-6 (Fig. 1A). To determine the influence of MHV68 RTA on STAT3, the ORF50 gene that encodes RTA was coexpressed with STAT3 and the STAT3-responsive reporter. RTA enhanced the ability of STAT3 to induce gene expression in a dose-responsive and synergistic manner, and the enhancement was dependent on stimulation of cells with IL-6. STAT3-specific tyrosine phosphorylation is required for the effect because a STAT3 with tyrosine 705 to phenylalanine mutation does not cooperate with MHV68 RTA (Fig. 1B). The RTA encoded by the human γHV KSHV/HHV-8 was also found to enhance STAT3-dependent gene expression in response to IL-6 (Fig. 1C) (40). IL-6 is a classical activator of STAT3, but cooperation with RTA is not limited to IL-6. RTA can enhance STAT3-mediated gene expression in response to STAT3 tyrosine phosphorylation by interferon-α (supplemental Fig. 1S). These results indicate that viral RTA promotes transcriptional activation by tyrosine-phosphorylated STAT3.

Endogenous STAT3 and MHV68 RTA are binding partners

To investigate the molecular mechanism by which RTA augments the ability of tyrosine-phosphorylated STAT3 to induce gene expression, we tested the ability of RTA to bind endogenous STAT3. The murine B cell line HE-RIT harbors the latent MHV68 genome and contains a tetracycline-inducible (Tet-On) RTA gene tagged with the FLAG epitope (47). FLAG-RTA expression is driven by the Tet derivative doxycycline (DOX) in these B cells (Fig. 2A). RTA expression is required for reactivation of latent virus, and viral DNA replication in these cells is shown following stimulation to reactivate with DOX and TPA (Fig. 2B). The HE-RIT cells are also responsive to IL-6 as measured by STAT3 tyrosine phosphorylation (Fig. 2C).

The potential protein interaction between RTA and STAT3 was measured by their coimmunoprecipitation from lysates of the HE-RIT cells (Fig. 2D). Cells were either left untreated or treated with DOX to induce expression of FLAG-RTA, and IL-6 was added to activate endogenous STAT3 by tyrosine phosphorylation. Immunoprecipitation of FLAG-RTA demonstrated the presence of the tyrosine-phosphorylated form of endogenous STAT3 in the immunocomplexes (Fig. 2D, lane 2). The interaction of viral RTA with cellular STAT3 was also demonstrated in transient transfections with tagged proteins (data not shown).

Because both RTA and STAT3 function in the nucleus as transcription factors, we evaluated a potential influence of coexpression on their nuclear accumulation (supplemental Fig. 2S) (9, 48). A previous study indicated KSHV RTA promoted nuclear accumulation of STAT3 (40). Fluorescence microscopy of STAT3-GFP or FLAG-RTA did not detect a change in nuclear localization with expression of the partner protein, although following IL-6 stimulation, both STAT3 and RTA accumulate in the nucleus.

Preferential binding of MHV68 RTA with tyrosine-phosphorylated recombinant STAT3

The coimmunoprecipitation of RTA with STAT3 from mammalian cell lysates indicates a complex exists of RTA bound to the tyrosine-phosphorylated form of STAT3 (Fig. 2). We next used a more stringent assay that reconstitutes complex formation in vitro to evaluate the contribution of STAT3 tyrosine phosphorylation. An in vitro binding assay was performed with recombinant STAT3 protein linked to maltose-binding protein (MBP) to enable protein isolation by binding to an amylose resin. MBP-STAT3 was expressed as unphosphorylated protein in the BL21 bacterial strain or as tyrosine-phosphorylated protein in the TKB-1 strain that express the Elk tyrosine kinase. Western blot with antibody to STAT3 phosphorytrosine 705 indicated specific phosphorylation of STAT3 in TKB-1 cells (supplemental Fig. 3SA). Unphosphorylated STAT3 (U-STAT3) and
tyrosine-phosphorylated STAT3 (pTyr705-STAT3) have been shown by crystallography to have a similar monomer structure; however, tyrosine-phosphorylated STAT3 forms a parallel homo-dimer that effectively binds DNA target sites (49, 50).

To evaluate the specificity of the RTA-STAT3 interaction, recombinant proteins MBP and MBP-U-STAT3 were expressed in BL21 cells, and MBP, MBP-pTyr705-STAT3, or MBP-STAT3 containing a tyrosine 705 site-directed mutation...
RTA targets tyrosine-phosphorylated STAT3

Figure 2. Endogenous STAT3 and MHV68 RTA are binding partners. A, Western blot of FLAG-RTA protein expression induced with doxycycline (DOX) in murine MHV68 HE-RIT B cells. B, MHV68 viral replication during reactivation in HE-RIT cells measured by quantitative PCR. The -fold increase in viral ORF46 gene normalized to cellular GAPDH gene. Representative experiment is shown; error bars indicate ± S.E. C, Western blot of tyrosine705-phosphorylated STAT3 (pTyr-STAT3), serine727-phosphorylated STAT3 (pSer-STAT3) and total STAT3 (STAT3) in response to IL-6 in HE-RIT cells. D, communoprecipitation of RTA with endogenous STAT3. HE-RIT cells were treated with DOX or IL-6 or both DOX and IL-6, as indicated. FLAG-RTA was collected on anti-FLAG magnetic beads and bound endogenous STAT3 was detected by Western blotting. Western blots shown for bound pTyr-STAT3, STAT3, FLAG-RTA, and β-actin.

Figure 3. Preferential binding of MHV68 RTA to tyrosine-phosphorylated STAT3 is seen in vitro. A, recombinant MBP and unphosphorylated STAT3 (MBP-U-STAT3) were prepared from BL21 control bacteria. Elk tyrosine kinase expressing bacteria (TKB-1), were used to prepare MBP, MBP-STAT3 Y705F mutant (MBP-Y705F-STAT3), and tyrosine-phosphorylated STAT3 (MBP-pTyR-STAT3). Recombinant proteins were bound to amylose beads and incubated with FLAG-RTA from HE-RIT cell lysates. Western blot analysis detected bound RTA, pTyr STAT3, and total STAT3. B, linear diagram of STAT3 protein with noted functional domains and deletions used in binding assay. MBP-pTyR-STAT3 and N-terminal deletion proteins were bound to amylose and incubated with FLAG-RTA expressed in HEK-293T. Bound RTA was detected by Western blotting (top panel). Input pTyr-STAT3 and total STAT3 are shown.

(MBP-Y705F-STAT3) were expressed in TKB-1 cells. The recombinant proteins were bound to amylose resin and incubated with DOX-induced FLAG-RTA from HE-RIT cell lysates during reactivation (Fig. 3A). RTA showed significant binding to MBP-pTyr705-STAT3 (Fig. 3A, lane 5), and no detectable binding to MBP or MBP-U-STAT3. The low level of binding to MBP-Y705F-STAT3 may be because of residual Elk phosphorylation of WT STAT3 in lysates that dimerizes with MBP-Y705F-STAT3. To ensure that other viral proteins in HE-RIT cells are not needed for the RTA-STAT3 complex formation, FLAG-RTA was expressed in transfected human 293T cells and used in the in vitro binding assays. FLAG-RTA from 293T cells also showed selective binding to the tyrosine-phosphorylated form of STAT3 (supplemental Fig. 3B).

We tested N-terminal deletion mutants of STAT3 to identify the domains of STAT3 that are required for binding to RTA (Fig. 3B). The proteins expressed by the STAT3 deletions are shown relative to the structural elements of STAT3 revealed by crystallography (49). All of the STAT3 deletion proteins were tyrosine phosphorylated when expressed in TKB-1 cells. RTA bound tyrosine-phosphorylated full-length STAT3 (1–770 a.a.), STAT3 lacking the N-terminal domain (127–770 a.a.), and STAT3 lacking both the N-terminal and the coiled coil domains (320–770 a.a.). RTA did not bind to the STAT3 protein lacking the N-terminal, coiled coil, and DNA-binding domains, although this fragment is tyrosine phosphorylated (495–770 a.a.) as shown by Western blotting. The data suggest that STAT3 tyrosine 705 phosphorylation alone is not sufficient for RTA stable interaction, but it requires the conformation of a dimer with the DNA-binding domain of STAT3.

The transactivation domain of MHV68 RTA is required for synergistic induction of a STAT3-responsive gene

RTA functions as a transcriptional activator of a subset of viral genes during productive infection. Homology studies of
γHV RTAs and deletion analyses indicate RTA has a DNA-binding domain at its N terminus and a transactivation domain at the C terminus (Fig. 4A) (9, 51–53). To determine whether the transactivation domain of RTA is needed for its stimulatory effect on STAT3-induced gene expression, we evaluated the action of RTA C-terminal deletions. Two C-terminal deletions, Rd1 and Rd2, have been described previously (53), and we tested their ability to synergize with tyrosine-phosphorylated STAT3 in a reporter assay. We also tested the effect of a fragment of RTA corresponding to the transactivation domain (TAD) that was deleted in Rd2. All the RTA proteins were expressed in the assay (supplemental Fig. 4S), but enhancement of STAT3 induction of the reporter gene was only evident with wild-type (WT) RTA containing the transactivation domain, not with Rd1, Rd2, or the TAD alone (Fig. 4B).

An in vitro binding assay was performed with recombinant STAT3 and the RTA mutants to determine whether binding correlated with transcriptional enhancement. The MBP-STAT3 recombinant protein was expressed as unphosphorylated protein (U-STAT3) or tyrosine-phosphorylated protein (pTyr705-STAT3), bound to amylose resin, and incubated with mammalian cell lysates expressing WT, Rd1, Rd2, or TAD RTA proteins (Fig. 4C). WT RTA bound preferentially to pTyr705-STAT3 as shown in Fig. 3. The Rd1 and Rd2 proteins demonstrated significant binding to STAT3. Rd1 protein showed some preference for pTyr705-STAT3, but Rd2 showed binding to both U-STAT3 and pTyr705-STAT3. The TAD fragment did not bind to STAT3. The results indicate WT RTA displays a selective binding to pTyr705-STAT3, whereas deletion mutants lose selectivity. Binding of the RTA deletions to STAT3 is not sufficient for enhancement of STAT3 gene induction, and the transcriptional activation domain of RTA appears necessary to synergize with STAT3 transactivation.

IL-6 augments MHV68 viral gene expression via the STAT3 pathway

To investigate potential biological consequences of RTA-enhanced STAT3 activity on acute viral infection, we examined the effect of IL-6 on viral gene expression. Hep3B cells respond to IL-6 stimulation with activation of STAT3, and a single-step growth curve showed that Hep3B cells are permissive for MHV68 lytic replication (Fig. 5A). Gammaherpesvirus gene expression follows a programmed and orderly progression: immediate early genes (IE), followed by early genes (E), and finally viral DNA replication and viral late gene expression (L). MHV68 infection expressed LANA (IE), ORF59 (E), and ORF75C (L) during the course of Hep3B lytic infection (Fig. 5B).

The influence of IL-6 on viral protein expression was evaluated by stimulating cells with IL-6 at 10 h after MHV68 infec-
tion and measuring viral protein levels (Fig. 5C). Because tyrosine phosphorylation of STAT3 is known to be transient, a 2-h stimulation with IL-6 was used to detect the activated tyrosine phosphorylation of STAT3. Cells were serum-starved prior to infection to decrease potential tyrosine phosphorylation of STAT3 by serum growth factors. At 12 h post infection, in the absence of IL-6, protein expression of the early viral gene, ORF59, was just detectable. However, the addition of IL-6 led to a substantial increase in ORF59 expression. The expression of another early gene, ORF57, was not affected by addition of IL-6 at this time point.

The advanced kinetics of viral ORF59 expression in response to IL-6 stimulation could be because of the activity of various signaling molecules downstream of IL-6, including STAT3. To investigate the potential role of STAT3, we examined ORF59 protein expression following MHV68 infection of immortalized mouse embryonic fibroblasts expressing STAT3 (stat31626), or deleted for the STAT3 gene (stat3−/−) for 12 or 24 h (Fig. 5D) (5, 54). ORF59 levels were reduced at both times post infection in cells lacking STAT3. Expression of a viral late gene, ORF75C, was not altered by the lack of STAT3, but anti-serum to MHV68 viral proteins indicated that expression of other viral proteins was slightly reduced. Although IL-6 did not have a significant effect on viral replication during reactivation from the latent HE-RIT cell line (supplemental Fig. 5S), cytokine activation of STAT3 in vivo may have an impact during the host response to infection. The results in tissue culture indicate that IL-6 via the STAT3 pathway can augment MHV68 viral protein expression during productive infection.

Discussion

Gammaherpesvirus infections progress to lifelong persistence, primarily residing in the B lymphocyte reservoir. Because lytic and latent phases of infection are distinct, it is expected that the interface between virus and host will differ between these phases. Previously we determined that the cellular STAT3 transcription factor is critical for establishment of MHV68 latency in an animal host (5). In the current study, we find that the same host factor, STAT3, is positively influenced by the MHV68 RTA protein, known to be critical for lytic reactivation. It appears that STAT3 is involved in multiple aspects of the viral life cycle, intriguingly as a host factor likely hijacked during both latent and reactivation phases of infection.

The ability of MHV68 RTA to enhance specific gene expression induced by activated STAT3 was examined with a STAT3-responsive reporter gene. Both MHV68 RTA and KSHV RTA

Figure 5. IL-6 augments MHV68 lytic gene expression via the STAT3 pathway. A, MHV68 de novo viral lytic replication in Hep3B cells measured by plaque assay. Data are from one representative experiment with error bars ± S.E. B, protein profile of viral gene expression and STAT3 measured by Western blotting with specific antibodies to viral LANA, ORF59, ORF75C, host STAT3, and GAPDH during single step growth curve. C, diagram of viral infection and IL-6 addition. Hep3B cells were serum-starved and infected with MHV68-H2BYFP at m.o.i. 10. Infected cells were treated with IL-6 at 10 h post infection and harvested after 2 h of IL-6 treatment. Western blotting for viral proteins and STAT3 as indicated. D, murine embryonic fibroblasts corresponding to stat31626 and stat3−/− were serum-starved and infected with MHV68-H2BYFP at m.o.i. 3 without IL-6 addition and harvested after 12 or 24 h of infection. Levels of viral proteins and host STAT3 and β-actin were detected by Western blotting with specific antibodies.
were found to augment STAT3 transcriptional activity, but only with STAT3 tyrosine phosphorylation. To decipher the means by which RTA augments STAT3, we tested the potential interaction of the two proteins. We found that RTA specifically coimmunoprecipitated with endogenous pTyr705-STAT3 in reactivated latently infected B cells. In addition, an in vitro protein-binding assay was developed with recombinant U-STAT3 or pTyr705-STAT3 to evaluate binding of RTA. The results showed a clear preference of RTA binding to pTyr705-STAT3 rather than U-STAT3. This new finding provides insight into the functional nature of the RTA-STAT3 complex, as its role may depend on the microenvironment of the infected cell. The specific lymphatic milieu in which latently infected B cells reside is influenced by local immune and inflammatory cells that produce a variety of STAT3 activating cytokines and growth factors including IL-6, IL-10, IL-21, IFN, and EGF. The RTA-STAT3 complex may regulate viral targeted genes, and/or influence host transcription to enhance viral reactivation from latency in the host.

The MHV68 genome is largely colinear with KSHV, and infection of mice recapitulates aspects of human gammaherpesvirus infection and disease (1). A previous study with KSHV RTA demonstrated stimulation of a STAT-responsive reporter gene in 293T cells; however cotransfection with STAT3 had only a limited increase in reporter expression (40). The study did detect KSHV RTA binding to STAT3, but the in vitro binding assay was performed only with U-STAT3. To interpret their findings, a proposal was put forth that RTA binding promoted STAT3 dimerization and nuclear accumulation. Our results are clearly distinct. We find that RTA has a specific preference for binding pTyr705-STAT3, and analyses of the cellular localization of RTA and STAT3 by microscopic imaging indicate that RTA does not change the nuclear accumulation of STAT3. STAT3 nuclear import is independent of tyrosine phosphorylation, although pTyr705-STAT3 has been shown to accumulate in the nucleus coincident with DNA binding (55–57). Because RTA is a nuclear protein, RTA may associate with pTyr705-STAT3 in the nucleus. RTA is also known to interact with other important cellular transcription factors and transcription cofactors such as CREB-binding protein (CBP) (58), histone deacetylase (59), Oct-1 (60), RBP-Jk (61), and IRF4 (62), and is proposed to modulate both viral and host gene expression (63).

Here, we show that the preference of RTA for transcriptionally active pTyr705-STAT3 reflects a remarkable selectivity of RTA for its host targets.

Gammaherpesvirus persistence in the host is associated with low levels of cellular lytic reactivation. The cellular microenvironment plays a critical role in enforcing latency or driving reactivation (1, 64–67). We have shown previously that STAT3 is required for establishment of MHV68 latency in vivo (5), and other studies with KSHV and EBV showed an inverse relationship between viral reactivation and STAT3 protein levels (6, 8, 68). These findings may seem incongruous with our results on RTA-STAT3 interaction; however, once the balance is tipped from latency to reactivation, RTA may use STAT3 to advance its goal. Perhaps, host STAT3 is involved in multiple aspects of viral life cycle regulation, the selective decision for latency or lytic replication is based on the viral interaction partner.

Understanding the molecular interface of viral host factors is key to designing targeted therapeutic interventions for both lytic reactivation as well as long-term persistence. RTA specifically recognizes the pTyr705-STAT3 parallel dimer formed via phosphotyrosine and SH2 domain reciprocal interactions. This is the classical activated form of STAT3 that recognizes and directly binds a consensus DNA target site first described as the GAS (23). The RTA specificity presumably has a structural basis, and to understand the interaction we evaluated the binding of mutant proteins. In vitro binding of RTA to N-terminal deletion fragments of STAT3 showed the requirement of STAT3 phosphotyrosine 705 and SH2 domains, as well as the DNA-binding domain. The crystal structure of the STAT3 dimer was solved bound to DNA and does not indicate that the monomers interact via the DNA-binding domains (49). However, because our studies were not performed in the presence of STAT3 target DNA, pTyr705-STAT3 monomer interactions in the absence of DNA may involve the DNA-binding domain. We also evaluated the regions of RTA required for STAT3 functional cooperation and physical association. We found that the C-terminal TAD of RTA was needed to enhance STAT3-directed gene expression. The TAD of RTA has been shown to bind to several transcriptional regulators such as p300 and CBP (14, 58, 59), and so it is likely that RTA enhances STAT3 activity by recruitment of transcription factors. The RTA TAD domain alone cannot bind STAT3, and does not enhance STAT3 activity. However, it is needed for RTA to differentiate between U-STAT3 and pTyr705-STAT3, because deletion of the RTA TAD produces a protein that indiscriminately binds STAT3. The crystal structure of RTA has not been solved, but KSHV-RTA has been shown to be a tetramer in solution (69). The stoichiometry of RTA in complex with pTyr705-STAT3 dimers remains to be determined.

IL-6 is a classical activator of STAT3 and is known as an acute-phase response cytokine common to inflammation during infection and injury, and is known to promote the differentiation of B cells (70). For these reasons we evaluated the effect of IL-6 on MHV68 protein expression early during de novo infection. In the presence of IL-6 there was a clear increase in expression of one of the early viral proteins, ORF59, the viral DNA polymerase processivity factor. This effect of IL-6 appears to be a function of STAT3 because fibroblasts without STAT3 (stat3–/–) do not respond to IL-6 with increased ORF59.

Our data demonstrate the ability of a viral lytic switch protein, MHV68 RTA, to specifically recognize activated pTyr705-STAT3 dimers, and to increase gene expression directed from a STAT3-responsive promoter. The complexity of STAT3 function in the γHV life cycle indicates that it has multiple roles in viral replication, persistence, and potentially cellular transformation. The IL-6/STAT3 pathway has been associated with inflammation and the development of cancer, and more pertinently STAT3 activity has been linked to EBV- and KSHV-related cancers (71–73). The interface between the virus and STAT3 during different phases of the infection may vary, dependent on the action of expressed viral regulatory factors, and the phosphorylation state of STAT3 responsive to the micron-
RTA targets tyrosine-phosphorylated STAT3

vironment of the infected cell in vivo. Future studies are needed to determine the impact of RTA and IL-6/STAT3 signaling on viral and host gene expression during reactivation from latency in an animal model.

Experimental procedures

Cell culture

HEK-293T, Hep3B, and NIH3T12 cells were obtained from ATCC, and immortalized stat3<sup>+/+/</sup> and stat3<sup>−/−</sup> MEF cells were gifts from Dr. Valeria Poli (University of Turin) (54, 74). HE-RIT cells are MHV68 latently infected murine A20 B cells inducible for RTA (47). Cells were maintained as described (47, 54, 57). 20 ng/ml of human IL-6 (Cell Signaling Technology, Danvers, MA) was used to stimulate human Hep3B cells. 25 ng/ml of murine IL-6 (PeproTech, Rocky Hill, NJ) with 25 ng/ml of murine IL-6 receptor (IL-6R) (R&D Systems, Minneapolis, MN) was used to stimulate murine HE-RIT cells; IL-6 and IL-6R were combined prior to cell treatment.

Virus and plasmids

MHV68-H2BYFP was a gift from Dr. Samuel Speck (Emory University) (75). pFLAG-murine-RTA (pFLAG-M-RTA) was a gift from Dr. Pinghui Feng (University of Southern California) (76), and pV5-KSHV-RTA was a gift from Dr. David M. Lukac (Rutgers University). pCMV-STAT3 was described (57). Deletion mutants were generated by PCR cloning using pFLAG-M-RTA as a template with the primer sets listed in supplemental Table 1S. STAT3 was cloned into the pT7TEV-HMBP1-maltose-binding protein (MBP) vector, a gift from Dr. Miguel Garcia-Diaz (Stony Brook University) (77), by PCR cloning with primer sets listed in supplemental Table 1S. The STAT3 Y705F mutation was generated by site-directed mutagenesis using primers listed. Recombinant STAT3 expression was induced following isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) addition. MBP and fusion proteins were specifically bound to amylose matrix (New England BioLabs Inc., Ipswich, MA). The STAT3-specific luciferase reporter was engineered based on the bona fide STAT3 GAS target site of the α2-macroglobulin gene promoter (-229 to -215) (25). Three GAS sites (TTACGGGAA) with the flanking sequence (TGGCTC) were cloned into the Hsp70-luciferase reporter, a gift of Dr. David Lukac (78), to generate p3XGAS-Hsp70-Luc reporter. All cloning was confirmed by sequencing.

Transfection and luciferase reporter assays

Hep3B cells were transfected using TransIT-LT1 (Mirus, Madison, WI). Empty plasmid vectors were used to equilibrate DNA input. Cells were placed in serum-free DMEM overnight prior to treatment with 20 ng/ml of human IL-6. Luciferase activity was measured with a Lumat model LB luminometer (Promega, Madison, WI; Roche) and normalized to protein content (DC protein assay, Bio-Rad). Luciferase activity is described relative to vector controls without IL-6 treatment. Values are means of three to six independent experiments.

MHV68 reactivation assay

Viral genome replication was measured in HE-RIT cells during virus reactivation with 10 μg/ml of doxycycline (DOX) and 20 ng/ml TPA. The viral genome copy number was measured by quantitative PCR using the primers listed in supplemental Table 2S. Quantitative PCR analysis was performed using PerfeCTa SYBR Green FastMix ROX (Quanta Bioscience, Beverly, MA) using Applied Biosystems QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific). The -fold increase in viral ORF50 or ORF46 genes as specified was normalized to the cellular GAPDH gene.

Immunassays

For immunoprecipitation assays HE-RIT B lymphocytes (47) were resuspended in growth media with 10 μg/ml of DOX for 12 h and media were changed to RPMI with 0.5% FBS and 10 μg/ml DOX for an additional 12 h. 25 ng/ml murine IL-6/IL-6R mixture was added 15 mins prior to lysis in 20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM PMSF, 1× protease inhibitors (P8340, Sigma-Aldrich), and 1× PhosSTOP (Roche). Lysates were centrifuged at 12,000 g for 15 min at 4 °C. Protein lysate (~2 μg/assay) was reacted with anti-FLAG magnetic beads (Sigma-Aldrich) and incubated overnight, and immunocomplexes were collected according to manufacturer’s recommendation (EMD Millipore, LSKMAG S08). Antibodies to FLAG, STAT3, β-actin, β-tubulin, phosphotyrosine STAT3 (Cell Signaling Technology), phosphoserine STAT3 (Cell Signaling Technology), or ORF59 and ORF75C (Gallus Immunotech) were used in Western blots. ORF73 antibodies were kindly provided by Dr. Scott Tibbetts (University of Florida), and ORF57 antibodies were kindly provided by Dr. Paul Ling (Baylor College of Medicine).

In vitro protein-binding assays

Recombinant MBP-tagged unphosphorylated or tyrosine-phosphorylated STAT3 proteins were generated in BL21-DE (MBP-U-STAT3) or TKB-1 (MBP-pTyr-STAT3 and MBP-Y705F-STAT3) (Agilent Technologies, Santa Clara, CA) and induced with 0.2 mM IPTG at 25 °C. Recombinant MBP-STAT3 was bound to amylose beads in lysis buffer containing 20 mM HEPES, pH 8.0, 200 mM KCl, 1% Triton X-100, 5% glycerol, 1 mM PMSF, and bacterial protease inhibitors. Approximately 40 μg of recombinant STAT3 was incubated with 30 μl of amylose beads for 1 h with rotational mixing at 4 °C. The MBP-STAT3 bound to amylose was washed in the lysis buffer described for immunoprecipitation. Recombinant protein on beads was incubated with FLAG-RTA from cell lysates of HE-RIT cells stimulated for reactivation.

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