

Kaposi's Sarcoma-associated Herpesvirus Open Reading Frame 50 Stimulates the Transcriptional Activity of STAT3*

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Kaposi's sarcoma-associated herpesvirus (KSHV) is an important pathogen in Kaposi's sarcoma and abnormal lymphoproliferation. KSHV open reading frame 50 (ORF50), a homolog of the Epstein-Barr virus immediate-early gene product RTA, activates early and late gene transcription in the KSHV lytic cycle, and its expression is closely correlated with KSHV-related diseases. ORF50 interacts with the cellular proteins CBP and histone deacetylase and represses p53-induced apoptosis through a CBP-related mechanism. We show here that KSHV ORF50 also interacts with STAT3. ORF50 stimulated transcription of STAT-driven reporter genes, and interleukin-6 and v-Src further activated this stimulating effect of ORF50. Physical association of STAT3 and ORF50 required the carboxyl-terminal transactivation domain of ORF50 and multiple regions within STAT3. ORF50 recruited STAT3 to the nucleus and induced the dimerization of STAT3 monomers in the absence of STAT3 phosphorylation. We show here that KSHV ORF50 activates STAT3-mediated transcription through direct interaction without mediating tyrosine phosphorylation.

Kaposi's sarcoma-associated herpesvirus (KSHV),¹ also known as human herpesvirus 8, is an important pathogen in Kaposi's sarcoma and multicentric Castleman's disease (1, 2). In a KSHV infection, the viral DNA is found principally in B lymphocytes, and KSHV is in fact associated with abnormal lymphoproliferation such as primary effusion lymphoma (3). In a Kaposi's sarcoma tumor, KSHV DNA is found in spindle cells, and viral lytic replication is important for Kaposi's sarcoma tumor development (4). KSHV is a member of the γ -herpesvirus family and has homology to the other herpesviruses, herpesvirus saimiri, and Epstein-Barr virus (EBV).

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¹ The abbreviations used are: KSHV, Kaposi's sarcoma-associated herpesvirus; EBV, Epstein-Barr virus; ORF50, open reading frame 50; CBP, cAMP-responsive element-binding protein-binding protein; STAT, signal transducer and activator of transcription; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; IL-6, interleukin-6; CRM1, chromosome region maintenance-1; v, viral.

An important step in the KSHV life is the switch from latency to lytic replication. Upon chemical induction, KSHV produces RNA transcripts from its immediate-early genes. These transcripts encode viral transcriptional activator proteins such as open reading frame 50 (ORF50) and K8, which are necessary to induce lytic replication (5). ORF50 is a homolog of the EBV immediate-early gene product RTA. It has been reported that ORF50 activates the lytic cycle of KSHV and is expressed earlier than K8, a homolog of the EBV ZTA protein, which induces the lytic cycle of EBV (6, 7). ORF50 activates expression of the early and late genes in the KSHV lytic cycle (8). Demethylation of the ORF50 promoter closely correlates with expression of the ORF50 gene and induction of the lytic phase (9). Biopsies from patients with KSHV-related diseases such as Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma show demethylation of the ORF50 promoter, whereas samples from a latently infected KSHV carrier show a methylated ORF50 promoter. These data suggest that the expression of ORF50 is very important for the development of KSHV-associated diseases. ORF50 binds to the cellular proteins CBP and histone deacetylase, and these binding events activate and repress ORF50-activated viral transcription, respectively (10). By sequestering CBP and thus preventing its interaction with the tumor suppressor p53, ORF50 represses the transcriptional activation function of p53 and p53-induced apoptosis (11).

STATs are a family of latent cytoplasmic proteins that are activated by various cytokines and growth factors (12–16). STATs have an amino-terminal protein/protein interaction domain, a DNA interaction domain, an SH2 domain, and a single tyrosine phosphorylation site. Phosphorylation of this tyrosine residue stabilizes the association of two STAT monomers through the interaction between the phosphotyrosine and SH2 domain (12–16). These activated STAT dimers translocate into the nucleus, where they bind to their cognate DNA response elements. In addition to their normal roles in cell signaling, a relationship between the activation of STATs and oncogenesis has been shown (15, 16). A number of oncoproteins can activate specific STATs, and the activated STAT protein participates in oncogenesis by stimulating cell proliferation and preventing apoptosis. Several tumor viruses are known to be associated with STAT activation. STAT3 and STAT5 are constitutively activated in human T cell lymphotropic virus I-transformed T cells (17). The herpesvirus saimiri tyrosine kinase-interacting protein Tip-484 also activates STAT3 through up-regulation of p56^{lck}, a non-receptor tyrosine kinase (18). Constitutively activated STATs are also found in lymphoid and myeloid leukemia cells and in EBV-related lymphoma cell lines (19, 20). Taken together, these observations indicate that certain viruses modify STAT activity (particularly STAT3) to increase the persist-

ence, replication, or oncogenic potential of the viruses. In this report, we show that KSHV ORF50 activates STAT3-mediated transcription without mediating tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid that contains FLAG-tagged STAT3 and the reporter plasmid p3Xly6e-luc were kind gifts from Dr. James E. Darnell. The vector pBabe-v-Src and reporter plasmids ptk-luc and pSIE-luc were gifts from Dr. Richard Jove. ORF50 and the ORF50 deletion mutants were subcloned into pcDNA3 (*EcoRI* and *XhoI* sites) (Invitrogen, Carlsbad, CA) using PCR. Green fluorescent protein (GFP) fusion vectors were constructed using pEGFP-C1 (CLONTECH, Palo Alto, CA). The ORF50 and STAT3 fragments were introduced into pGEX4T-1 (Amersham Biosciences, Inc., Uppsala, Sweden), and the glutathione *S*-transferase (GST) fusion proteins were expressed and purified according to the manufacturer's instructions.

Transient Transfection Assays—Transfection assays were performed in 293T cells using the calcium phosphate method. Transfection assays in NIH3T3 cells were performed using LipofectAMINE Plus reagent (Invitrogen). In all assays, the luciferase activity derived from the reporter plasmids was determined after normalizing to the β -galactosidase activity from a cotransfected pRSV- β gal control plasmid. All experiments were performed in triplicate. Equivalent expression of each plasmid was verified by Western blot assays (data not shown). 293T cells were transfected with 1 μ g of reporter plasmid, either 20 ng (in 293T cells) or 1 μ g (in NIH3T3 cells) of pRSV- β gal control plasmid, and the amounts of the expression plasmids indicated in the figure legends. The total amount of each expression vector was kept constant by adding empty pCMV expression plasmid. BJAB cells were transfected by electroporation as described previously (8).

GST Pull-down Assays—ORF50, STAT3, and their deletion mutants were *in vitro* transcribed and translated using a T7-coupled transcription/translation system (Promega Corp., Madison, WI). The labeled proteins were incubated with 1 μ g of a GST fusion protein in binding buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, and 0.5% Nonidet P-40 supplemented with protease inhibitors). Glutathione beads were then added, and the reaction mixture was incubated at 4 °C overnight. The beads were washed four times with binding buffer; SDS-PAGE sample buffer was added; and the proteins were analyzed by SDS-PAGE and visualized by autoradiography or phosphorimaging (BAS-1500, Fuji Film Co., Tokyo, Japan).

In Vivo Co-immunoprecipitation—293T cells were transfected with 7 μ g of each expression plasmid using the calcium phosphate method. The cells were harvested 48 h after transfection and lysed in the binding buffer (containing 1% Triton X-100) used in the GST pull-down assays. The cell lysates were rotated in the buffer for 1 h at 4 °C before the cell debris was removed by centrifugation. The appropriate lysates were immunoprecipitated with the addition of antibody to GFP, hemagglutinin (HA), or FLAG (Sigma) and protein G resin (Santa Cruz Biotechnology, Santa Cruz, CA). The beads were washed four times, and the proteins were analyzed by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized with ECL reagent (Amersham Biosciences, Inc.) according to the manufacturer's instructions.

Immunofluorescence—GFP/ORF50 (1 μ g) and expression vectors containing FLAG-tagged STAT3 were transfected into 293T cells (1 μ g). Cells were fixed and immunostained 48 h after transfection. FLAG-tagged STAT3 was detected using a rhodamine-conjugated secondary antibody.

RESULTS

KSHV ORF50 Increases the Transcriptional Activity of STAT-regulated Promoters—To assess whether ORF50 modifies the STAT pathway, we tested the effect of ORF50 on the expression of STAT-regulated reporter plasmids. The pSIE-luc reporter plasmid (high affinity mutant of the c-Fos SIS-inducible element, mutant m67) and the p3Xly6e-luc reporter plasmid (containing three copies of STAT-responsive elements (TTCCTGTAA) in the promoter of the murine surface antigen) are generally used as reporter plasmids for determining the activity of STAT family proteins (21–23). The ptk-luc reporter plasmid, which contains the herpes simplex virus thymidine kinase promoter, was used as a negative control. KSHV ORF50 stimulated pSIE-luc expression by 45- and 58-fold in 293T cells that had been transfected with 0.5 and 1 μ g of an ORF50 expression plasmid, respectively. ORF50 also activated the

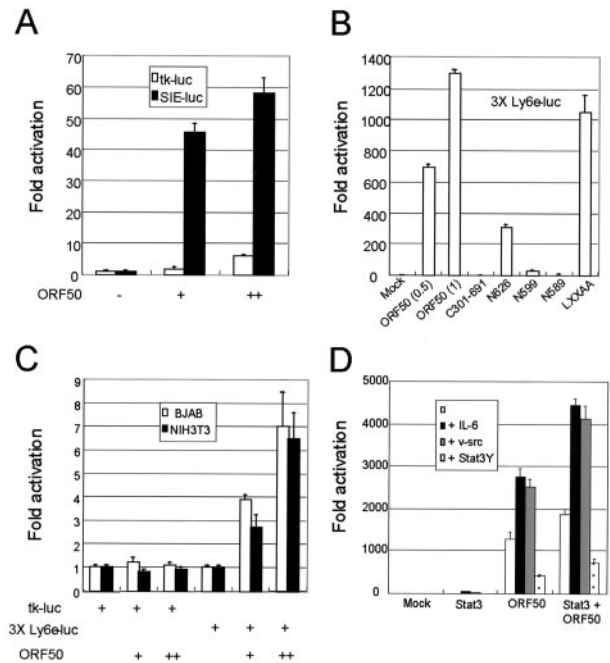


FIG. 1. KSHV ORF50 activates the transcription of STAT-regulated promoters. *A*, 293T cells were transiently cotransfected with a reporter gene construct (ptk-luc or pSIE-luc) and increasing amounts of ORF50-containing expression vectors (pcDNA3/ORF50; 0 (–), 0.5 (+), and 1 (++) μ g). The luciferase activity of the reporter gene in the absence of the other expression vectors was normalized to a value of 1. *B*, the effects of KSHV ORF50 (0.5 and 1 μ g) and its deletion mutants (1 μ g each) on the transcriptional activation function of STAT were determined by measuring the luciferase activity of the p3Xly6e-luc reporter gene. *C*, to confirm the STAT promoter activation function of ORF50, luciferase activity in the presence of ORF50 was also determined in a B cell lymphoma cell line (BJAB) and in NIH3T3 cells. We transfected NIH3T3 cells with 1 μ g of the reporter gene and 0.5 (+) or 1 (++) μ g of ORF50 expression vectors. BJAB cells were cotransfected with 5 μ g of the reporter gene and 5 (+) or 10 (++) μ g of ORF50. *D*, the effects of IL-6 and v-Src on the transactivation of STAT3 by ORF50 were determined in 293T cells using the p3Xly6e-luc reporter. *Mock*, mock transfected.

ptk-luc reporter, but only by ~5-fold (Fig. 1A). We then measured the effect of ORF50 on the p3Xly6e-luc reporter. Cotransfection of p3Xly6e-luc and an ORF50 expression plasmid resulted in a 1300-fold activation of luciferase activity (Fig. 1B).

To determine the domains of ORF50 necessary for STAT activation, we tested the transcriptional activation function of ORF50 deletion mutants whose functions have been described (10). The C301–691 mutant, which has the carboxyl-terminal half of ORF50, is unable to bind CBP and form an ORF50 homodimer (8, 10). However, the C301–691 mutant lost its ability to stimulate the STAT-regulated promoters in 293T cells (Fig. 1B). The N626 mutant (containing amino acids 1–626 of ORF50), which binds to CBP and can form a homodimer, was still able to stimulate the above STAT-regulated reporter plasmids in 293T cells, but to a lower degree (300-fold). The N599 and N589 mutants were unable to stimulate the p3Xly6e-luc reporter in 293T cells. The LXXAA mutant, which has mutations in the CBP-binding motif, is unable to bind CBP and is also unable to repress p53-induced transcription and apoptosis (10, 11). However, this mutant retained its ability to activate p3Xly6e-luc in 293T cells. We can assume that the CBP-binding domain (LXXLL motif) in the carboxyl terminus is not involved in the activation of the STAT-related promoters. From these results, we conclude that (i) the carboxyl-terminal transcriptional activation domain is necessary for the activation of STATs and (ii) the amino-terminal domain

necessary for ORF50 homodimerization and CBP binding is also important for this stimulating function.

To test whether ORF50 can activate STATs in other cell lines, we measured the activation of ptk-luc and p3XLY6e-luc in mouse fibroblast NIH3T3 cells and B cell lymphoma BJAB cells. In the presence of the ORF50 gene, the transcriptional activity of p3XLY6e-luc increased in a dose-dependent manner, whereas the activity of ptk-luc did not change (Fig. 1C). The STAT-regulated promoters used in our experiments are known to be responsive to almost all STATs, but we narrowed our scope of research to STAT3 because it is the STAT protein most closely related to proliferation and apoptosis. When we either treated cells with interleukin 6 (IL-6) or transfected them with v-Src expression plasmids (two well characterized activators of the STAT3 pathway), ORF50 activation of p3XLY6e-luc expression was increased by 2.2- and 2-fold, respectively (Fig. 1D). As a control, cells were either treated with IL-6 or transfected with v-Src expression vectors in the absence of STAT3 and ORF50. p3XLY6e-luc reporter gene expression was not much increased by IL-6 or v-Src. When cells transfected with STAT3 expression vectors were either treated with IL-6 or co-transfected with v-Src expression vectors, expression of the p3XLY6e-luc reporter was increased by 19- and 11-fold, respectively. The stimulating activity of ORF50 was reduced to one-third when cells were cotransfected with STAT3Y (Fig. 1D), a dominant-negative mutant form of STAT3 that cannot be phosphorylated at tyrosine 705 and that cannot be homodimerized upon stimulation (22). Cotransfection of STAT3 and ORF50 stimulated the luciferase activity by 1.5–1.8-fold compared with ORF50 alone. The stimulating activity was also reduced to one-third with STAT3Y. In contrast to IL-6, interferon- γ , which activates the STAT1-related pathway, did not affect the stimulating activity of ORF50 (data not shown). These data suggest a relationship between ORF50 and STAT3 function, although we cannot exclude the possibility that ORF50 modifies the STAT-regulated reporters through interaction with other STAT family pathways.

ORF50 and STAT3 Associate *in Vivo*, and This Interaction Induces Dimerization and Nuclear Localization of STAT3—We next sought to determine whether ORF50 modifies the transcriptional activity of STAT3 by stimulating phosphorylation of STAT3. A FLAG-tagged STAT3 expression vector and either an ORF50 or v-Src expression vector were cotransfected into 293T cells. Whole cell extracts of the transfected cells were precipitated with anti-FLAG antibody, and the precipitates were analyzed by Western blotting with antibody to the phosphotyrosine residue within STAT3. v-Src did stimulate the tyrosine phosphorylation of STAT3, whereas ORF50 did not (Fig. 2A). When STAT3 is tyrosine-phosphorylated and dimerizes, it acts as a transcriptional activator; thus, it is possible that ORF50 associates directly with STAT3 to modify its transcriptional activity. To test this hypothesis, we cotransfected 293T cells with a FLAG-tagged STAT3 expression vector and a vector that expresses either GFP alone or GFP/ORF50. Whole cell extracts were then precipitated with either anti-FLAG or anti-GFP antibody and analyzed by Western blotting (Fig. 2B). Comparable amounts of STAT3 were precipitated with anti-FLAG antibody from both types of cell extracts. When the membrane that had been probed with anti-FLAG antibody was stripped and re-probed with anti-GFP antibody, GFP/ORF50 was detected, whereas the GFP protein was not detected in the precipitates. Thus, we conclude that ORF50 binds to STAT3 *in vivo*.

We next determined the subcellular localization of these two proteins in our system. Because STAT3 exists as a latently cytoplasmic protein and ORF50 is known to be located in the

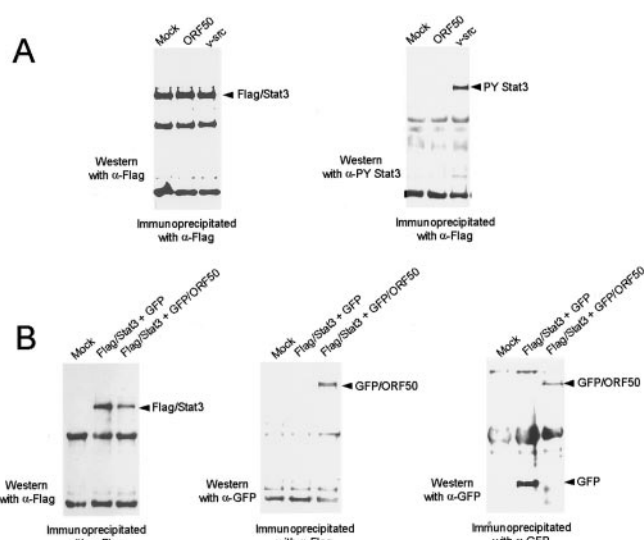


FIG. 2. ORF50 and STAT3 associate *in vivo*. A, ORF50 does not stimulate the phosphorylation of STAT3. Extracts from cells transfected with ORF50 or v-Src were precipitated with anti-FLAG antibody (α -Flag) and Western-blotted with anti-FLAG antibody. The blots were stripped and re-probed with an antibody that recognizes the phosphotyrosine residue in STAT3 (α -PY Stat3). B, 293T cells were transfected with the FLAG-tagged STAT3 expression vector and either a blank vector (GFP) or an expression vector carrying GFP/ORF50. Proteins in the cell lysates were precipitated with either anti-FLAG or anti-GFP (α -GFP) antibody, subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and visualized with either anti-FLAG or anti-GFP antibody. Mock, mock transfected.

nucleus (10), we used confocal microscopy to determine the localization of STAT3 in the absence or presence of ORF50 in cotransfected 293T cells similar to those used for Fig. 2B. GFP was dispersed in both the cytoplasm and nucleus in the transfected 293T cells (Fig. 3A). STAT3 was dispersed mostly in the cytoplasm, in accordance with previous observations (13–16). When these two images were superimposed, STAT3 and GFP did not show any relationship with respect to their location (*i.e.* they did not show any yellow color, which is indicative of co-localization in our system). GFP/ORF50 was located in the nucleus; and, interestingly, most of the STAT3 protein was also located in the nucleus in the presence of GFP/ORF50 even without IL-6 or cotransfection of v-Src expression vectors. To investigate further the co-localization of STAT3 and ORF50, we fractionated extracts from the transfected cells into cytoplasmic and nuclear fractions, and each fraction was analyzed by Western blotting (Fig. 3B). In the absence of GFP/ORF50, STAT3 was localized chiefly in the cytoplasmic fraction. In contrast, nearly 50% of the cellular STAT3 protein was localized in the nucleus in the presence of GFP/ORF50. The amount of nuclear STAT3 in the presence of ORF50 was comparable to that observed in cells transfected with v-Src. These results suggest that ORF50 recruits STAT3 to the nucleus in the absence of any other signal, and this observation is consistent with the fact that ORF50 stimulates STAT-regulated promoters without any other stimulator of the STAT pathway.

We next determined the effect of ORF50 on the dimerization of STAT3. ORF50 stimulated the transcriptional activity of STAT3 without affecting the phosphorylation of STAT3. However, STAT3 must be dimerized to bind to its specific DNA elements and thus to be an active transcriptional regulator. Furthermore, in most cases, STAT3 translocates into the nucleus after it forms a dimer. We cotransfected 293T cells with vectors expressing HA- and FLAG-tagged STAT3 and expression vectors for either ORF50 or the N589 mutant, which lost the activity to activate STAT-regulated promoters. The

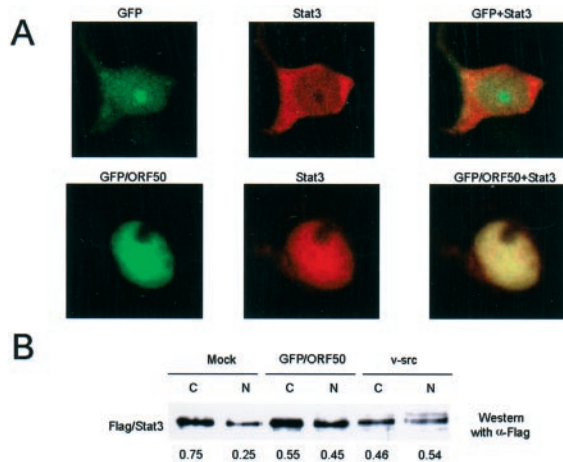


FIG. 3. ORF50 co-localizes with STAT3 and induces nuclear re-localization of STAT3. *A*, GFP/ORF50 (1 μ g) and the FLAG-tagged STAT3 expression vector (1 μ g) were transfected into 293T cells. Cells were fixed and immunostained 48 h after transfection. FLAG-tagged STAT3 was detected using a rhodamine-conjugated secondary antibody against anti-FLAG monoclonal antibody. *B*, cytoplasmic and nuclear extracts from 293T cells transfected with the FLAG-tagged STAT3 expression vector and either GFP/ORF50 or a v-Src expression vector were analyzed by Western blotting. *Mock*, mock transfected; α -Flag, anti-FLAG antibody.

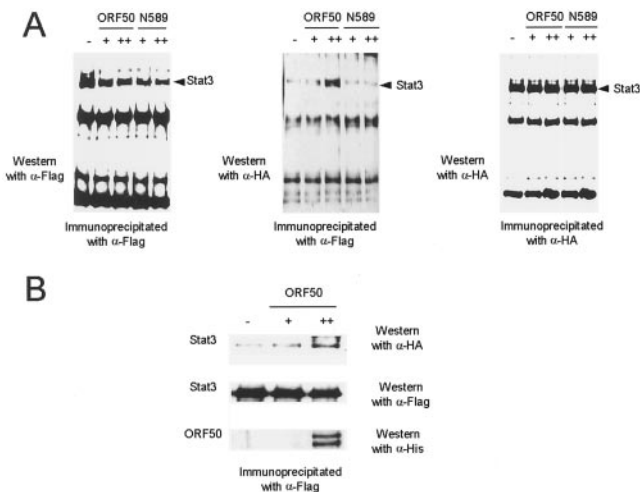


FIG. 4. ORF50 induces dimerization of STAT3. *A*, the dimerization of STAT3 was induced in the presence of ORF50 *in vivo*. Cell extracts from 293T cells transfected with FLAG-tagged STAT3, HA-tagged STAT3, and either ORF50 or N589 expression vectors were immunoprecipitated with anti-FLAG (α -Flag) or anti-HA (α -HA) antibody. These precipitates were analyzed by Western blotting. *B*, ORF50 induces dimerization of STAT3 *in vitro*. Each of the extracts transfected with FLAG- and HA-tagged STAT3 expression vectors was added to GST pull-down buffer in the presence of His-tagged ORF50 (purified from insect cells infected with ORF50 recombinant baculoviruses). These reaction mixtures were precipitated with anti-FLAG antibody and analyzed by Western blotting. α -His, anti-His antibody.

amounts of FLAG- and HA-tagged STAT3 in the extracts from the transfected cells were shown to be similar (Fig. 4A, left and right panels). However, in the presence of ORF50, the amount of HA-tagged STAT3, which was associated with FLAG-tagged STAT3, was increased in a dose-dependent manner. N589 had no effect on the dimerization of STAT3 (Fig. 4A, middle panel). To investigate further the effect of ORF50 on STAT3 dimerization, we conducted similar experiments *in vitro*. Extracts from cells transfected with either HA- or FLAG-tagged STAT3 were added to a purified preparation of ORF50 protein and immunoprecipitated with anti-FLAG antibody. ORF50 simulated the association of the STAT3 proteins (Fig. 4B). When the anti-

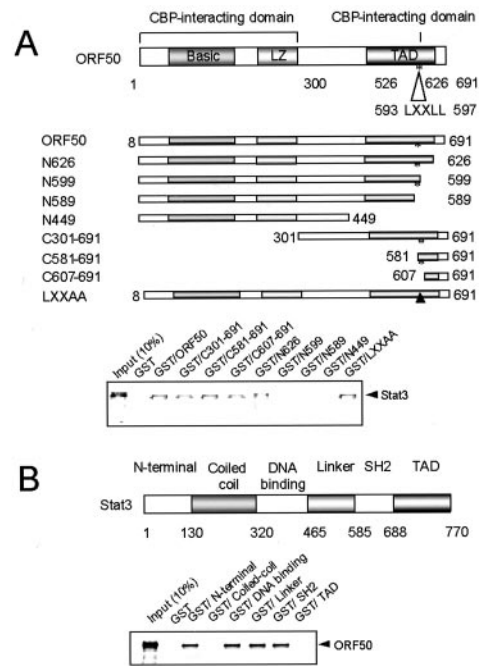


FIG. 5. *In vitro* interaction of ORF50 with STAT3. *A*, the domains within ORF50 and the ORF50 deletion mutants are shown. ORF50 contains the basic domain, the leucine zipper motif (LZ), and the transcriptional activation domain (TAD). The LXXLL motif, which interacts with CBP, is located from amino acids 593 to 597 of ORF50. The GST-fused ORF50 fragments were purified and used in GST pull-down assays performed with glutathione-Sepharose beads. STAT3 was translated *in vitro* and labeled with [³⁵S]methionine. *B*, the domains of STAT3 are indicated. STAT3 contains the DNA-binding domain, the SH2 domain, and the carboxyl-terminal transcriptional activation domain. The GST-fused STAT3 fragments and *in vitro* translated ORF50 were used in GST pull-down assays.

FLAG precipitates were analyzed by Western blotting, we found that the bound fraction of HA-tagged STAT3 was increased, and ORF50 was shown to associate with these STAT3 dimers using anti-His antibody.

Association of ORF50 and STAT3 *In Vitro* and Interaction Domains within STAT3 and ORF50—To detect ORF50 and STAT3 interaction *in vitro* and to decipher the domains within ORF50 and STAT3 that participate in this interaction, we performed GST pull-down assays using *in vitro* translated STAT3 and GST/ORF50 fusion proteins (made with various ORF50 deletion mutants) (Fig. 5A). STAT3 bound to wild-type ORF50 and the ORF50 mutants containing the carboxyl-terminal transcriptional activation domain (C301–691, C581–691, and C607–691). N626, the carboxyl terminus (amino acids 627–691)-truncated mutant, could interact with STAT3. STAT3, however, did not bind to the amino terminus-containing ORF50 mutants (N599, N589, and N449). N599 and N589 also showed a reduction in the ORF50 transcriptional activation function (Fig. 1B). Taken together, these results show that the STAT3-binding domain within ORF50 is in the carboxyl-terminal transcriptional activation domain of ORF50 (amino acids 607–626). We also performed GST pull-down assays using *in vitro* translated ORF50 and GST-fused STAT3 functional domain fragments (Fig. 5B). ORF50 bound to multiple regions within STAT3, including the amino-terminal domain, the DNA-binding domain, the linker domain, and the SH2 fragments, whereas it did not bind to the coiled-coil and transcriptional activation domain fragments.

DISCUSSION

We have shown here that KSHV ORF50 affects the transcriptional activation of STAT3 through direct interaction.

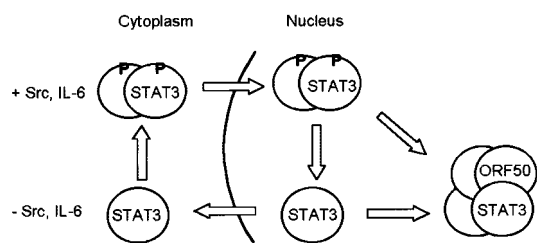


FIG. 6. Model for the stimulation of STAT3-induced transcription by KSHV ORF50.

With respect to viral physiology, this observation is not surprising because many viral proteins such as EBV, human T cell lymphotropic virus I, and herpesvirus saimiri modulate STAT-related activity to enhance viral replication. The ability of these viruses to stimulate STATs is closely related to viral oncogenic potential (17–20). In this report, we focused on the ability of ORF50 to induce STAT3 activity, but it is possible that ORF50 also associates with other STAT family proteins and can affect their function.

ORF50 interacts with multiple regions within STAT3 (Fig. 5, A and B). The amino-terminal domain in STAT3 is a protein/protein interaction domain, and this domain interacts with other dimeric forms of STAT and CBP (12–16). The STAT3 DNA-binding domain interacts with a cellular transcriptional factor (c-Jun) to augment the transcriptional activity of STAT (24, 25). The SH2 domain interacts with a phosphotyrosine residue of another STAT monomer to form the activated dimeric form of STAT proteins (12–16). It is possible that ORF50 modulates these diverse protein/protein interactions, and an example of this is shown herein. The crystal structure of the dimeric STAT1 proteins (coiled-coil, DNA-binding, linker, and SH2 domains) has been published (26). According to the data, the coiled-coil domains project outward from the C-shaped core (DNA-binding, linker, and SH2 domains) around DNA, and the three domains in the core region are in close proximity to each other. This core region is also located in proximity to the other core region in the dimeric structure. So, it seems to be quite logical that ORF50 interacts only with the DNA-binding, linker, and SH2 domains, but not with the coiled-coil domain. It is possible that ORF50 could stabilize the dimeric form of STAT protein through the interaction between the core region of STAT and ORF50. ORF50 induced STAT3 dimerization *in vivo* and *in vitro* without tyrosine phosphorylation of STAT3. This ORF50-induced dimerization of STAT3 seems to be related to the STAT3-stimulating activity of ORF50 because the carboxyl terminus-deleted mutant N589, which completely lost its stimulating activity, also lost its ability to induce STAT3 dimerization. Fig. 6 shows the possible mechanism of ORF50-induced STAT3 activation. A STAT3 monomer is activated with v-Src or IL-6 to form a dimer, translocates into the nucleus, and interacts with the specific DNA elements (12–16). ORF50 recruited STAT3 to the nucleus and induced the dimerization of STAT3 without any additional signal. When cells do encounter other signals such as v-Src and IL-6, a large proportion of STAT3 molecules are located in the nucleus, and this may increase the probability that ORF50 will meet a STAT3 protein. Another possible mechanism of ORF50-induced translocation of STAT3 is that ORF50 could inhibit the interaction of CRM1 and STAT3 because ORF50 and CRM1 both interact with STAT3 at its DNA-binding domain. CRM1 is involved in the nuclear

export of STAT proteins and serves to inactivate the STAT pathway (27). It is possible that ORF50 could inhibit the STAT3/CRM1 interaction and induce the retention of STAT3 in the nucleus.

KSHV ORF50 is a viral transcriptional factor that activates transcription of many viral genes to induce the lytic phase of KSHV. ORF50 modifies the activity of cellular transcription factors such as p53 through association with CBP (10, 11). In addition, the expression of ORF50 is closely associated with the development of KSHV-related diseases (9). We have shown here that ORF50 also has a role in the modulation of cell growth. KSHV vIL-6 has been shown to have transforming activity through activation of the STAT pathway and to be mostly expressed in the lytic phase (28–32). We showed here that STAT3 stimulation of ORF50 cooperates with v-Src and human IL-6. Therefore, it is possible that ORF50 and vIL-6 cooperate to stimulate the STAT pathway.

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