Molecular Mechanisms of the Effect of *Herpesvirus saimiri* Protein StpC on the Signaling Pathway Leading to NF-κB Activation*

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*Herpesvirus saimiri* (Saimiriine herpesvirus-2) causes lethal T lymphoproliferative diseases in the susceptible species and transforms T lymphocytes to continuous growth in vitro. *H. saimiri*-induced transformation of T cells is becoming an important experimental tool of biomedical research. Two proteins of *H. saimiri* subgroup C, Tip and StpC, are essential for T cell transformation by this virus. It has been shown previously that StpC transforms fibroblasts, activates NF-κB, and binds to tumor necrosis factor (TNF)-receptor-associated factor (TRAF) proteins, but the molecular mechanism of its action remains insufficiently understood. This study further characterized the effect of StpC on NF-κB. First, StpC activates NF-κB via the consensus pathway involving activation of IκB kinase and subsequent phosphorylation and degradation of IκB in both T lymphoid and epithelial cells. Second, triggering of this pathway by StpC in both T lymphoid and epithelial cells is dependent on the presence of functional NF-κB-inducing kinase (NIK). Third, StpC physically interacts with TRAF in epithelial cells, and the effect of StpC on NF-κB activity in these cells requires the presence of functional TRAF. Finally the effect of StpC is completely independent of TNF-α, a well described stimulus of NF-κB activity. Moreover it appears that StpC uncouples stimulation of NF-κB activity from TNF-α stimulation. Overall these results argue that the effect of StpC on NF-κB is similar to the effects of other viral proteins, “usurping” the TRAF/NIK/IκB kinase pathway, and reinforce the notion that the role of StpC in cell transformation by *H. saimiri* may be mediated by signaling that results in NF-κB activation.

*Herpesvirus saimiri* (Saimiriine herpesvirus-2) causes transformation of T lymphocytes, thus inducing lethal T lymphoproliferative diseases in the susceptible species. The strains of group C of this virus are capable of transforming human T cells in vitro (for reviews, see Refs. 1–6). Although this transformation renders T cells capable of proliferating in a T cell antigen receptor/CD3 stimulation-independent manner similar to that

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The present study was focused on the detailed analysis of the effects of StpC on NF-κB activity. Our results indicate that StpC activates NF-κB in T and non-T cells by triggering the consensual TRAF/NIK/IκB kinase (IKK) pathway leading to IκB phosphorylation and degradation. These results argue that the effect of StpC on NF-κB is similar to those of Tax of human T cell lymphotropic virus-I and LMP1 of Epstein-Barr virus (see Refs. 45–47) and reinforce the notion that the role of StpC in cell transformation by \textit{H. satmiri} may be mediated by signaling that results in NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human renal embryocarcinoma 293T cells were obtained from ATCC. Stable 293T-derived cell lines were generated by transducing them with a retroviral vector carrying StpC- and/or Tip-coding DNA. The pGEX-based plasmid for the repressor (SR)) were kindly provided by Dr. A. Baldwin (University of North Carolina, Chapel Hill, NC). The pGEX-based plasmid for the repressor (SR)) were kindly provided by Dr. A. Baldwin (University of North Carolina, Chapel Hill, NC). The pGEX-based plasmid for the repressor (SR)) were kindly provided by Dr. A. Baldwin (University of North Carolina, Chapel Hill, NC).

**DNA Constructs**—Mammalian expression plasmids encoding for wild-type IκBα and non-degradable S23A/S66A IκBα (IκBα super-repressor (SR)) were kindly provided by Dr. A. Baldwin (University of North Carolina, Chapel Hill, NC). The pGEX-based plasmid for the expression of GST-IκBα (1–54) was kindly provided by Dr. M. Karin (University of California, San Diego, CA). Mammalian expression plasmids for Src family PTKs were described previously (49). The full-length FLAG-tagged human TRAF2 and Myc-tagged NIK expression plasmids were kindly provided by Dr. D. Baldwin (Tularak Inc., South San Francisco, CA). TRAF2 deletion mutants were constructed using Pfu DNA polymerase as described previously (50). The primer pairs were 5'-AGGGAGGTGGAGCCCCTCCTGGCCGCTGCTGTTCAAGCAGATCCGCGGCTGCTTCCAG-3' and 5'-CATGGATCCCTTGTCATCGTCGTCCTTGTAGTCCA-3' for TRAF2-1 (1–500) and 5'-AGGGAGGTGGAGCCCCTCCTGGCCGCTGCTGTTCAAGCAGATCCGCGGCTGCTTCCAG-3' and 5'-CATGGATCCCTTGTCATCGTCGTCCTTGTAGTCCA-3' for TRAF2-2 (1–500). The pGEX-based plasmid, if required, to the total amount of 6 μg using 10 μl of LipofectAMINE-2000 (Invitrogen)/well for 6 h according to the manufacturer's protocol. Transfected cells were activated, where indicated, with 1 ng/ml TNF-α (Calbiochem) 24 h after transfection.

**Reporter Assays**—The NF-κB and AP-1 reporter constructs containning firefly luciferase cDNA and the pRL-null normalization construct containing \textit{Renilla reniformis} luciferase cDNA have been described previously (40). To measure reporter activity, 293T cells (95% confluent in a 6-well plate) were transfected with 3 μg of the NF-κB or AP-1 reporter, 0.4 μg of pRL-null and 10 μl of LipofectAMINE-2000 (Invitrogen)/well for 6 h. The transfected cells were activated or left untreated. The measurement of luciferase activity was carried out using the dual luciferase reporter assay (Promega) 24 h after adding DNA.

**Transcription Factor Binding to DNA**—The NF-κB and AP-1 DNA binding activity assays were performed using Trans-AM ELISA-based kits from Active Motif (Carlsbad, CA) according to the manufacturer's protocol. Briefly, cell extracts were incubated in a 96-well plate coated with an oligonucleotide containing the NF-κB or AP-1 consensus binding site. Activated transcription factors from extracts specifically bound to the respective immobilized oligonucleotides were detected using the antibodies to NF-κB p65 and p50 (in NF-κB assays) or those to c-Fos and c-Jun (in AP-1 assays) followed by a secondary antibody conjugated to horseradish peroxidase in an ELISA-like assay.

**Immunoblotting, Immunoprecipitation, and Immune Complex Kinase Assays**—Immunoblotting and immunoprecipitation were performed essentially as described previously (49). Briefly, cells were lysed in 1% Nonidet P-40 in Tris/NaCl/EDTA buffer with NaF, aprotinin, leupeptin, and sodium vanadate (Sigma). Equal amounts of total protein were treated with SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to nitrocellulose (Amersham Biosciences), and probed with the appropriate antibodies. Rabbit polyclonal antibodies to Tip and StpC were described previously (48). Rabbit polyclonal antibodies to IκBα (C-23), IκBβ (C-20), NF-κB p65 (H-86), and IκBκ (M-280) and mouse monoclonal antibody to phospho-IκBα (B-9) were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-FLAG was purchased from Sigma. To verify equal loading, immunoblotting with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (Research Diagnostic Inc., Flanders, NJ) or mouse monoclonal anti-actin (Santa Cruz Biotechnology) was carried out. Protein bands were then visualized by chemiluminescence using an ECL Plus kit (Amersham Biosciences).

When immunoprecipitation preceded immunoblotting, equal amounts of total protein were mixed with the appropriate antibody followed by Pansorbin (heat- and formalin-treated \textit{Staphylococcus aureus} cells from Calbiochem), which was added to bind the immune complexes formed in solution. The pellet was extensively washed with lysis buffer, and the components of the precipitated immune complexes were extracted with SDS-PAGE sample buffer.

For IKK kinase assays, cells were lysed in the buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NaF, 1 mM sodium vanadate, and 1 mM dithiothreitol. IKKα was immunoprecipitated from cell lysates with mouse monoclonal anti-IKKα (B-8) antibody (Santa Cruz Biotechnology) (1 μg of the antibody/1 mg of total protein), and the obtained immune complexes were extensively washed in lysis buffer containing 1% Triton X-100. The immunoprecipitates were then treated with 20 μM HEPES (pH 7.6), 20 mM β-glycerophosphate, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 0.1 μg GST-IκBα-(1–54) and 30 μCi of [γ-32P]ATP (7000Ci/mmol, ICN, Irvine, CA). The samples were incubated on a shaker at 30 °C for 30 min. The reaction was stopped by adding SDS-PAGE sample buffer, and the kinase reactions were separated using SDS-PAGE. The gels were dried, and the phosphoprotein bands were visualized using autoradiography.

**Subcellular Fractionation**—Nuclear/cytoplasmic fractionation of 293T cells was performed using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) according to the manufacturer's protocol. Briefly 293T cells were scraped off the 100-mm plates, washed in phosphate-buffered saline, and pelleted by centrifugation. NE-PER Nuclear and Cytoplasmic Extraction Reagents were used to lyse harvested cells stepwise to generate functional cytoplasmic and nuclear protein fractions. Nuclear and cytoplasmic fractions of MOLT4 cells were obtained using hypotonic lysis of cells followed by high salt extraction of nuclear and cytoplasmic fractions as described previously (40).

**Protein Labeling**—293T cells were washed twice in cysteine- and methionine-deficient DMEM and incubated in cysteine- and methionine-deficient DMEM supplemented with 1% dialyzed fetal bovine serum for 30 min at 37 °C. Then cells were incubated with 5μS-Trans-label reagent (1000 Ci/mmol, ICN) at a concentration of 200 μCi/ml for 6 h at 37 °C. Cells were washed twice with phosphate-buffered saline, and complete DMEM supplemented with 10% fetal bovine serum was added to the cells. TNF-α was added to the cell culture when needed. Following treatments, cells were washed and lysed. The protein of interest was immunoprecipitated as described previously (49). Proteins were separated by SDS-PAGE, and the fluorography with sodium salicylate was performed.

**RESULTS**

We previously demonstrated that expression of StpC substantially facilitated NF-κB activity in MOLT4 T lymphoblastoid cells and that this effect was enhanced by Tip expression. Furthermore co-expression of StpC and Tip caused a dramatic increase in interleukin-2 production by MOLT4 cells (40). To elucidate signaling pathways causing these effects and to evaluate their cell type specificity, we assessed the influence of StpC and Tip on NF-κB activity in both MOLT4 lymphoblastoid and 293T epithelial cells. As expected, StpC increased the basal NF-κB activity in MOLT4 cells; Tip showed no effect on NF-κB activity, while expressed alone, but somewhat enhanced the effect of StpC (Fig. 1A). Likewise StpC activated NF-κB activity in 293T cells, and Tip enhanced the effect of StpC,
StpC Activates NF-κB

We next examined whether Tip and/or StpC modifies the effect of TNF-α, a consensus NF-κB-activating stimulus. TNF-α was shown to activate NF-κB in both MOLT4 and 293T cells if they were Tip StpC or Tip StpC. In contrast, Tip StpC and Tip StpC cells, both MOLT4 and 293T, exhibited a dramatic increase in the basal NF-κB activity but were unresponsive to TNF-α (Fig. 1A and B). Extending incubation times with TNF-α did not abolish this difference between Tip StpC and Tip StpC cells on the one hand and Tip StpC and Tip StpC cells on the other hand (data not shown). Taken together, these results indicated that StpC greatly increases the basal activity of NF-κB while uncoupling it from TNF-α stimulation.

To elucidate the mechanism of StpC-induced activation of NF-κB, we first examined whether the activation of NF-κB by StpC is linked to an increase in NF-κB DNA binding activity in 293T cells. Therefore, we analyzed binding of NF-κB p65 to the corresponding DNA sequence using an ELISA-based technique. These experiments showed that StpC expression substantially increased this binding in the absence of any stimulation (Fig. 2A). In contrast, Tip had no effect on basal NF-κB/DNA binding, although the analysis of multiple clones indicated that simultaneous expression of Tip and StpC enhanced this binding to some extent as compared with that in the presence of StpC alone (data not shown). Similar experiments carried out with NF-κB p50 indicated that the effects of Tip and StpC on NF-κB p65 and p50 subunits were essentially identical (data not shown). Overall these results were in agreement with those obtained using NF-κB-specific reporters and described above. Likewise the effects of StpC and Tip on the response of the DNA binding activity of NF-κB to TNF-α were similar to those on the activity of NF-κB reporters; TNF-α elevated DNA binding activity of NF-κB p65 and p50 in 293T cells expressing resistance markers or Tip alone but did so only modestly in StpC Tip or StpC Tip cells (Fig. 2A and data not shown). Specificity of NF-κB/DNA interactions was verified using a soluble NF-κB-binding oligonucleotide, which is capable of disrupting the interactions between NF-κB and a microtiter plate-immobilized NF-κB-binding oligonucleotide, and a mutant form of this oligonucleotide, which cannot interfere with NF-κB/DNA interactions. These experiments showed that both basal and TNF-α-induced DNA binding activity of NF-κB was specific (Fig. 2A). The results obtained with 293T cells were consistent with those obtained previously in MOLT4 cells using electrophoretic mobility shift assays; StpC significantly up-regulated the basal DNA binding activity of NF-κB in MOLT4 cells as well (40).

To further analyze the effect of StpC on NF-κB activity, we measured the levels of NF-κB p65 protein in the cytosol and the nucleus since the translocation of p65 to the nucleus is a crucial step of NF-κB activation. These experiments indicated that a substantial fraction of NF-κB p65 is constitutively localized to the nucleus in StpC Tip or StpC Tip cells in contrast to the situation in vector control and StpC Tip cells (Fig. 2B). Furthermore the simultaneous expression of Tip and StpC in these experiments resulted in a higher level of the nuclear translocation of NF-κB p65 than did the expression of StpC alone. Similar findings were made in MOLT4 cells (data not shown).
immunoblotting. I-κBα cell clones expressing Tip and/or StpC was determined using DNA binding activity is typically preceded by I-κBα degradation. NF-κB activity is also abrogated by cycloheximide, a translation inhibitor, on the level of I-κBα in cells expressing Tip and/or StpC. These results demonstrated that blocking protein synthesis de novo resulted in a rapid decline of the I-κBα protein level in cells expressing StpC but not in those expressing Tip alone or in vector control cells (Fig. 3D). This result argued that StpC constitutively induces a rapid degradation of I-κBα, which is countered by an active I-κBα production de novo. To further analyze the effect of StpC on I-κBα stability, we 32P-labeled proteins of 293T cells in vivo, immunoprecipitated I-κBα, and compared the amount of radioactivity in I-κBα immediately after labeling and after a 1-h chase. Like the experiments with cycloheximide, this approach demonstrated that the turnover of I-κBα in non-stimulated cells was significantly increased by StpC expression while remaining unaffected by the expression of Tip alone (Fig. 3E).

To further elucidate the effect of StpC on I-κB, we analyzed phosphorylation of I-κBα in cells expressing Tip and/or StpC in various combinations. First we determined the level of Ser-32 phosphorylation of I-κBα in 293T and MOLT4 cells (Fig. 4, A and B, respectively); StpC caused a detectable increase in I-κBα serine phosphorylation in both cell types, and the simultaneous expression of StpC and Tip increased this phosphorylation further, which was especially well seen in 293T cells. Since the serine phosphorylation of I-κBα is attributed to IKK, we next examined the complex kinase assays with a substrate portion of I-κBα and IKK and compared the amount of radioactive I-κBα used as a control for the inhibitory potential of NIKK429A/K430A; these experiments showed a dramatic up-regulation of IKK activity by StpC, which was evident from both IKK autoprophorylation and phosphorylation of GST-I-κBα in both 293T (Fig. 4C) and MOLT4 (data not shown) cells.

To further elucidate the mechanisms controlling the effects of StpC on NF-κB activity, we examined the contribution of NIK, a protein kinase located upstream of IKK in the NF-κB signaling pathway, to these effects. The experiments indicated that NIK(K429A/K430A), an inactive form of this kinase, completely abolished StpC-dependent constitutive activation of NF-κB in both 293T and MOLT4 cells (Fig. 4, D and E, respectively). NF-κB degradation-induced activation of NF-κB in vector control cells was used as a control for the inhibitory potential of NIK(K429A/K430A) as expected, NIK(K429A/K430A) abrogated this activity. In contrast, wild-type NIK activated NF-κB to levels that were comparable in cells expressing StpC and/or Tip in various combinations (Fig. 4, D and E). Expression of NIK(K429A/K430A) did not vary in individual transfections as determined using immuno-
Fig. 3. Role of I-κBα degradation in the effect of StpC on NF-κB activity. A, I-κBα was determined, using immunoblotting of whole cell lysates, in individual stable 293T cell clones expressing StpC and/or Tip or drug resistance markers alone (neopac) in the absence of stimulation. B, individual stable 293T cell clones expressing StpC and/or Tip or drug resistance markers alone transfected with NF-κB reporter plasmids were treated with 25 μM MG132 immediately after transfection or left untreated, and NF-κB activity in these cells was determined using the dual luciferase assay. C, 293T cells were transiently co-transfected with vectors encoding for StpC and/or Tip or drug resistance markers alone, NF-κB reporter plasmids, and the expression plasmid for I-κB SR and stimulated with TNF-α for 3 h or left unstimulated as indicated. The level of NF-κB activity was determined using the dual luciferase assay; its level in unstimulated vector control cells was assigned the value of 1.0 in B and C. D, protein levels of I-κBα were determined, using immunoblotting of whole cell lysates, in pooled stable 293T cell clones expressing StpC and/or Tip or drug resistance markers alone following treatment of unstimulated cells with cycloheximide (CHX) for the time indicated. The levels of I-κBα in the absence of cycloheximide treatment were assigned the value of 1.0 for each type of cells. E, levels of I-κBα-GFP, an I-κBα-bait, were determined in pooled stable 293T cell clones expressing StpC and/or Tip or drug resistance markers alone using metabolically labeled cells with l-[35S]methionine/S-(35S) cysteine. 35S-Labeled cells were lysed immediately or after a 1-h chase during which they were left unstimulated or stimulated with TNF-α as indicated. Radioactivity of I-κBα was determined using fluorography following its immunoprecipitation from cell lysates. WB, Western blot; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

 blotting (data not shown) and, thus, could not account for the observed effects.

Next we analyzed the role of TRAF2, a crucial upstream element of NF-κB signaling, that is known to be involved in the activation of NIK. TRAF2-(249–501), a truncated dominant-inhibitory form of TRAF2, effectively suppressed both TNFα-induced and StpC-induced NF-κB activation in 293T cells, whereas wild-type TRAF2 enhanced NF-κB activity in these cells whether or not they expressed StpC (Fig. 5A). Another dominant-inhibitory form of TRAF2, TRAF2-(A101–249), also suppressed the effect of StpC on NF-κB but to a lower extent (data not shown). To rule out a possible contribution of the differential expression of the dominant-inhibitory forms of TRAF2 in individual transfections to the observed effects, we compared levels of TRAF2-(249–501) and TRAF2-(A101–249) in various samples using immunoblotting and demonstrated that they did not differ (Fig. 5B and data not shown).

To further analyze the role of TRAF2 and NIK in the effects of StpC, we examined co-immunoprecipitation of TRAF2 with either TRAF2 or NIK from the lysates of pooled stable 293T cell clones expressing StpC and/or Tip, which were transiently transfected to overexpress TRAF2 or NIK. No co-precipitation was detected for NIK and StpC (data not shown), but TRAF2 co-precipitated with StpC from both StpC + Tip− and StpC + Tip+ 293T cells (Fig. 5B).

Overall the results of the above experiments indicated that StpC induced constitutive TRAF2/NIK-dependent activation of IKK in 293T cells that resulted in I-κB phosphorylation and led to an increase in I-κB degradation rate and NF-κB activation. Undoubtedly NF-κB activation in our experimental system is caused primarily by StpC. However, Tip enhances the effect of StpC in both 293T and MOLT4 cells albeit to a varying degree. In the course of our previous study conducted in MOLT4 cells, we speculated that this effect of Tip might be dependent on Lck (40). However, 293T cells lack Lck, and therefore Lck cannot be involved in the enhancing effect of Tip in 293T cells. This consideration compelled us to examine the dependence of this effect of Tip in 293T cells on PTKs. To do so, we treated 293T cells with herbimycin, a PTK inhibitor of wide specificity, and PP1, an inhibitor of Src family PTKs (55). Neither compound affected NF-κB activity in 293T cells in the same dramatic manner as observed previously in MOLT4 cells (40). Although
herbimycin modestly inhibited NF-κB in 293T cells, its effect was not nearly as profound as that of MG132 (Fig. 6A). Furthermore PP1 slightly activated NF-κB in 293T cells (Fig. 6A). These findings argued that the effect of StpC or Tip/StpC was independent of herbimycin- or PP1-sensitive PTKs.

To further evaluate the contribution of PTKs, we transfected 293T cells with expression vectors carrying cDNA of wild-type c-Src, wild-type Lck, or inactive Lck. Unexpectedly wild-type Lck induced a significant increase in NF-κB activity in vector control and all Tip- and/or StpC-expressing cells. This effect was specific and dependent on the enzymatic activity of Lck since it was demonstrated neither by wild-type c-Src nor by inactivated Lck (Fig. 6B). The differences in the effects of various PTKs were not due to their differential expression as determined by immunoblotting (data not shown).

Since the effect of Lck on NF-κB activity was irrelevant for the actions of Tip and/or StpC in 293T cells, which express no Lck, we were compelled to search for another reason(s) for the enhancing effect of Tip on StpC-induced activation of NF-κB. Considering that neither I-κBα degradation nor IKK activity was enhanced by Tip in 293T cells (see above), we surmised that Tip might up-regulate degradation of I-κBα species other than I-κBα.

Using immunoblotting of I-κBα, we showed that Tip or StpC alone exerted only a modest effect on the steady-state level of I-κBα, whereas their simultaneous expression reduced this level ∼3-fold in non-stimulated cells (Fig. 7). This result argues that although an increase in constitutive NF-κB activity caused by StpC expression is not mediated by I-κBα degradation, the ability of Tip to enhance this effect of StpC may well result from the observed cooperative effect of Tip and StpC on I-κBα.

**DISCUSSION**

This study provides additional insight into the mechanism of StpC-mediated activation of NF-κB. First, StpC activates NF-κB via the consensus pathway involving activation of IKK and subsequent phosphorylation and degradation of I-κB in both T lymphoid and epithelial cells, thus demonstrating the lack of strict cell type specificity. Second, triggering of this pathway by StpC in both T lymphoid and epithelial cells is independent on the presence of functional, wild-type NIK. Third, our results confirm that StpC physically interacts with TRAF in epithelial cells and that the effect of StpC on NF-κB activity in these cells requires functional TRAF. Finally the effect of StpC is completely independent of TNF-α, a well described stimulus that induces NF-κB activity in a variety of cell types. Moreover StpC uncouples stimulation of NF-κB activity from TNF-α stimulation. This uncoupling effect may be caused simply by the sequestration of TRAF(s) by StpC. It is also conceivable that the StpC-induced activation reaches the limit of signaling flow through the NIK/IKK-mediated pathway so no further increase of this flow is possible.

Based on these and previously reported (42) data, the event that results in StpC-dependent triggering of the NF-κB-activating pathway is likely to be binding of StpC to TRAF(s), which initiate this pathway in multiple cell types (56, 57). The next step of StpC-induced signaling leading to NF-κB activation appears to be mediated by NIK. It is unclear whether activation of NIK is required for the StpC-mediated activation of NF-κB, but it is clear that the presence of active NIK is essential since kinase-dead NIK acts as a potent dominant-negative form of this protein. The contributions of TRAF2 and NIK to the NF-κB activating effect of StpC out-
line some differences between StpC-dependent events in T and non-T cells. In 293T cells, both NIK and TRAF2 dominant-negative mutant forms abrogated activation of NF-κB by StpC. In MOLT4 cells, kinase-dead NIK abrogated StpC-dependent activation of NF-κB, whereas defective TRAF2 was not effective (data not shown). These results are likely to reflect differential expression of TRAF proteins and/or cell-specific nuances of NF-κB activation pathways in epithelial and lymphoid cells.

Most results obtained in this study are entirely consistent with the notion that StpC activates NF-κB by triggering the consensus NIK/IKK/IκB pathway, which leads to degradation of IκB and release and derepression of NF-κB followed by dimerization of the NF-κB and binding of the active dimers to the specific DNA sequences. Indeed expression of StpC activates IKK and phosphorylation of IκB followed by nuclear translocation of NF-κB, its binding to DNA, and the induction of NF-κB-driven transcription. Furthermore dominant-negative forms of TRAF, NIK, and IκB significantly inhibit the effect of StpC on NF-κB activity. However, StpC-expressing cells, which exhibit a significant increase in constitutive NF-κB activity, demonstrate no substantial decrease in the steady-state IκBα level. The experiments carried out to explain this paradoxical result have indicated that the rate of constitutive degradation of IκBα in StpC-expressing cells is dramatically increased most likely due to the constitutive activation of IKK. However, the resultant IκBα degradation appears to be balanced by a matching increase in IκBα production, which is consistent with activation of IκBα gene transcription by NF-κB (53, 54). Therefore, the steady-state level of IκBα in
StpC-positive cells remains essentially unchanged. However, it is possible that some time is required for the newly synthesized molecules of I-κBα to form inhibitory complexes with active NF-κB dimers at the relevant locations to return NF-κB to the inactive state. As a result, the fraction of active NF-κB proteins would be higher in StpC-expressing cells, which rapidly exchange I-κBα, than that in StpC-negative cells with the slow I-κBα turnover. To ascertain the conclusion that StpC activates NF-κB via the consensus TRAF/NIK/IKK pathway, we considered a likely alternative hypothesis. It has been shown that some signals, such as oxidative stress, induce activation of NF-κB via an atypical pathway that involves tyrosine phosphorylation of I-κB, which appears to cause dissociation of NF-κB and I-κBα without inducing degradation of I-κBα (58–60). Using I-κBα immunoprecipitation followed by anti-phosphotyrosine immunoblotting, we have found that no I-κBα tyrosine phosphorylation occurred in either 293T or MOLT4 cells expressing StpC with or without stimulation (data not shown). Furthermore PTK inhibitors have failed to reduce the effect of StpC on NF-κB activity in 293T cells, thus indicating that tyrosine phosphorylation is not essential for this effect.

Overall our data strongly argue that StpC activates NF-κB via the TRAF/NIK/IKK consensus signaling pathway. They do not indicate, however, whether or not StpC affects additional signaling pathways resulting in the activation of NF-κB. Nor do they rule out the effect of StpC on other, NF-κB-unrelated signaling pathways. However, our experiments using reporter constructs and DNA binding activity assays have demonstrated that StpC, while activating NF-κB, does not exert a significant effect on AP-1 activity in either stimulated or non-stimulated 293T cells (data not shown). Therefore, if StpC indeed triggers NF-κB-unrelated pathways, these pathways do not result in the activation of AP-1. Undoubtedly potential effects of StpC on NF-κB-unrelated signaling pathways should be investigated further.

Both current and previous (40, 42) results indicate that in both T and non-T cell systems Tip facilitates the effect of StpC on NF-κB while not activating NF-κB in the absence of StpC. The lack of effect of Tip on NF-κB in the absence of StpC is entirely consistent with the inability of Tip alone to activate IKK or to increase I-κBα phosphorylation or degradation. Moreover Tip does not augment the effect of StpC on IKK activity or the constitutive degradation rate of I-κBα. By contrast, Tip facilitates the reduction of the steady-state level of I-κBβ by StpC. These findings suggest that Tip-dependent facilitation of StpC-induced NF-κB activity is mediated not by I-κBα degradation but by that of I-κBβ. It is important to note that the observed decrease in the I-κBβ steady-state level cannot explain StpC-dependent activation of NF-κB since the decrease in the I-κBβ steady-state level caused by StpC alone is similar to that caused by Tip alone, whereas the partial effects of StpC and Tip on NF-κB are dramatically different. Instead the cooperative effect of Tip and StpC on I-κBβ provides a possible explanation for the modest, but appreciable, facilitation of StpC-dependent activation of NF-κB by Tip. Although the molecular mechanism by which Tip affects the level of I-κBβ requires further analysis, it is possible that Tip acts as an adaptor protein linking IKK and I-κBβ, thus promoting phosphorylation of the I-κBβ. Our finding that Tip facilitates the effect of StpC on I-κBα phosphorylation without affecting StpC-dependent activation of IKK favors this possibility. It is clear, however, that the effect of Tip does not require the presence of Lck, a Src family protein-tyrosine kinase specifically interacting with Tip (27, 28) since Tip augments the effect of StpC on NF-κB in 293T cells, which, along with other epithelial cells, do not express Lck.

Interestingly, although the ability of Tip to facilitate StpC-induced NF-κB activation is independent of Lck, this kinase greatly enhances the effect of StpC on NF-κB in 293T cells. The two characteristics of the molecular basis of this effect of Lck are now apparent. First, the effect of Lck is dependent on its kinase activity since the inactive mutant form of Lck does not affect the StpC-induced NF-κB activation. Second, this effect of Lck is specific since fully functional Src cannot substitute for Lck. Clearly, in the absence of forced expression of Lck, this kinase does not contribute to the effect of StpC on NF-κB activity in 293T cells due to the lack of its expression in these and other epithelial cells. However, the previously reported effect of StpC on NF-κB activity in MOLT4 cells is likely to be influenced by endogenous Lck (40). This notion is in agreement with the observed differential sensitivity of the StpC-induced NF-κB activity in 293T and MOLT4 cells to PTK inhibitors. Whereas the effect of StpC on NF-κB in 293T cells is insensitive to either herbimycin, a wide specificity PTK inhibitor, or PP1, a Src family-specific inhibitor, it is significantly reduced by herbimycin in MOLT4 cells (40).

Although our results, both current and previous (40), indicate that Tip facilitates the StpC-dependent activation of NF-κB in both non-lymphoid and T lymphoid cells, the involvement of this effect of Tip in its biological function remains uncertain. Indeed the effect of StpC alone on constitutive NF-κB activity is quite close to that of StpC co-expressed with Tip, although Tip is capable of increasing it further. It is possible that the role of Tip in H. saimiri-mediated transformation of T cells is related instead to the activation of Stat1, Stat3, and/or nuclear factor of activated T cells transcription factors (33, 36, 38, 39) and/or the induction of latent infection of H. saimiri via the mechanism involving down-regulation of CD4 and/or Lck in infected T cells (61). Thus, the mechanisms by which Tip exerts its biological effects, including its possible contribution to the StpC-mediated NF-κB activation, require further evaluation.

Overall the effect of StpC on NF-κB is similar to the effects of other viral proteins “usurping” the signaling pathway resulting in NF-κB activation, including Tax of human T cell lymphotrophic virus-I, LMP1 of Epstein-Barr virus, and HBx of hepatitis B virus (for reviews, see Refs. 45–47). Specifically the molecular mechanism by which StpC triggers this pathway are reminiscent of those mediating the effects of LMP1, which activates NF-κB by physically and functionally interacting with TRAF components of this pathway (Refs. 62 and 63, and for a review, see Ref. 46). Considering that NF-κB activation by Tax and LMP1 is believed to be essential for cell transformation by human T cell lymphotrophic virus-I and Epstein-Barr virus, respectively (64–70), and the results implicating NF-κB activation in cell transformation by H. saimiri (42, 71), our study reinforces the notion that the role of StpC in cell transformation by H. saimiri may be mediated by its effect on the NF-κB signaling. It is clear, however, that the contribution of the TRAF/NIK/IKK pathway resulting in the activation of NF-κB to the transformation potential of StpC needs further elucidation since other effects of StpC, such as its reported association with Ras (43), may also be involved. To ultimately define the role of the TRAF/NIK/IKK pathway in the biological function of StpC, studies in peripheral blood T cells expressing StpC, especially in those transformed by H. saimiri, are needed. Despite some associated technical difficulties, these cells represent the only system that may allow the researchers to conclusively determine the involvement of a particular signaling pathway in the biological function of StpC.

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