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Activation of the STAT6 transcription factor in Jurkat T-cells by the herpesvirus saimiri Tip protein

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Herpesvirus saimiri (HVS), a T-lymphotropic monkey herpesvirus, induces fulminant T-cell lymphoma in non-natural primate hosts. In addition, it can immortalize human T-cells *in vitro*. HVS tyrosine kinase-interacting protein (Tip) is an essential viral gene required for T-cell transformation both *in vitro* and *in vivo*. In this study, we found that Tip interacts with the STAT6 transcription factor and induces phosphorylation of STAT6 in T-cells. The interaction with STAT6 requires the Tyr^{127} residue and Lck-binding domain of Tip, which are indispensable for interleukin (IL)-2-independent T-cell transformation by HVS. It was also demonstrated that Tip induces nuclear translocation of STAT6, as well as activation of STAT6-dependent transcription in Jurkat T-cells. Interestingly, the phosphorylated STAT6 mainly colocalized with vesicles containing Tip within T-cells, but was barely detectable in the nucleus. However, nuclear translocation of phospho-STAT6 and transcriptional activation of STAT6 by IL-4 stimulation were not affected significantly in T-cells expressing Tip. Collectively, these findings suggest that the constitutive activation of STAT6 by Tip in T-cells may contribute to IL-2-independent T-cell transformation by HVS.

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INTRODUCTION

Herpesviruses persist in their hosts by entering a latent state and reactivating periodically to produce infectious virus particles. Herpesvirus saimiri (HVS), an oncogenic $\gamma 2$ herpesvirus, persists in the T-lymphocytes of its natural host, squirrel monkey, without any apparent disease symptoms, but infection of other New World and Old World primate species results in fulminant T-cell lymphoma (Jung *et al.*, 1999). In addition, when HVS infects the primary T-lymphocytes of humans, Old World primates, New World primates or rabbits *in vitro*, it can immortalize infected T-cells, allowing them to grow independently of IL-2 (Biesinger *et al.*, 1992).

Tyrosine kinase-interacting protein (Tip) is encoded in the first ORF at the left end of the highly oncogenic strains of HVS. Although it is not required for virus replication, Tip is required for T-cell transformation in cultures and for lymphoma induction in primates (Jung *et al.*, 1999). Tip has multiple binding sites for cellular proteins. The interaction of Tip with Lck kinase, which is mediated by the Src homology 3-binding (SH3B) motif and C-terminal Src-related kinase homology (CSKH) domain of Tip (Hartley *et al.*, 2000; Jung *et al.*, 1995), interferes with early events in the T-cell receptor (TCR) signal-transduction pathway, resulting in inhibition of immunological-synapse formation (Cho *et al.*, 2004). Tip also interacts with p80, a cellular endosomal protein that contains an N-terminal WD-repeat domain and a C-terminal coiled-coil domain (Park *et al.*, 2002). The interaction of Tip with p80, which is mediated by a region containing a serine-rich (SR) motif, facilitates the formation of enlarged lysosomal vesicles and results in the targeting of Lck and TCR–CD3 complexes for lysosomal degradation. It has been demonstrated previously that Tip

constitutively localizes in lipid rafts and exploits Lck and p80 to recruit TCR–CD3 complexes, leading to lipid-raft aggregation and internalization (Cho *et al.*, 2006; Park *et al.*, 2003). Constitutive localization of Tip in lipid rafts depends on its C-terminal transmembrane (TM) domain, but not Lck and p80 interaction, and is also necessary for the efficient downregulation of TCR–CD3 and CD4 surface expression without affecting the inhibition of TCR signal transduction (Cho *et al.*, 2006). Recently, it was also reported that the membrane-proximal amphipathic helix preceding Tip's TM domain mediates lipid-raft localization and membrane deformation (Min *et al.*, 2008). In turn, this motif directs Tip's lysosomal trafficking and selective TCR downregulation. The amphipathic helix of Tip binds to the negatively charged lipids and induces liposome tabulation, whilst its TM domain mediates oligomerization. Moreover, cooperation of the membrane-proximal helix with the TM domain is sufficient for the localization of Tip to lipid rafts and lysosomal compartments, especially the mutivesicular bodies.

In addition to Lck and p80, Tip has been shown to interact with signal transducers and activators of transcription (STATs) and to bind to phosphorylated STAT1 and STAT3 together with Lck (Heck *et al.*, 2005; Lund *et al.*, 1997). A YXPQ motif of Tip conforms to a putative binding site for STAT factors (Shao *et al.*, 2004), and phosphorylation at Tyr^{114} in the C488 strain, equivalent to Tyr^{72} in the C484 strain, is required for STAT binding and transcriptional activation (Cho *et al.*, 2006; Hartley & Cooper, 2000). STATs are key mediators involved in signalling by various cytokines, including the interleukin (IL)-6 and IL-2 family cytokines, as well as numerous growth factors (Levy & Darnell, 2002). STATs reside in the cytoplasm during unstimulated conditions and, upon stimulation, are recruited to a cytokine receptor where they are tyrosine-phosphorylated. Active STAT dimers are then formed via the reciprocal interaction between their SH2 domains. Subsequently, they translocate to the nucleus, where they bind to specific DNA-response elements in the promoters of target genes to activate transcription. Furthermore, various oncoproteins can activate specific STAT molecules, and inappropriate STAT activation contributes directly to oncogenesis by stimulating cell proliferation and preventing apoptosis (Haura *et al.*, 2005). Constitutive activation of several STATs was detected frequently in a wide range of human cell lines and primary tumours, including lymphoid malignancies (Haura *et al.*, 2005; Yu *et al.*, 2009). Recently, however, HVS carrying a mutant Tip in which Tyr^{114} was changed into phenylalanine was still able to transform human T-lymphocytes, while losing its capability to activate STAT3 as well as STAT1 (Heck *et al.*, 2005). This result demonstrated that growth transformation by HVS is independent of STAT3 activation. Interestingly, recombinant virus expressing Tip with a mutation at Tyr^{127} was still capable of transforming human T-lymphocytes but, in contrast to the wild type (wt), was strictly dependent on exogenous IL-2 (Heck *et al.*, 2006). The Tyr^{127} of Tip was particularly required for transformation in the absence of exogenous IL-2, suggesting its involvement in cytokine signalling pathways.

Based on the significance of Tyr^{127} of Tip on IL-2-independent transformation, we aimed to search for a novel binding target of the tyrosine residue to reveal the basic mechanisms by which HVS Tip can contribute to IL-2-independent transformation of human T-cells. Here, we show that the Tyr^{127} residue of Tip is required for the interaction with STAT6 after phosphorylation, leading to nuclear translocation and transcriptional activation of STAT6 and, ultimately, IL-2-independent transformation of T-cells by HVS.

RESULTS

HVS Tip interacts with the STAT6 transcription factor

In order to identify host proteins interacting with Tip, a glutathione *S*-transferase (GST) pull-down assay was performed using bacterially produced GST-fusion proteins. Unphosphorylated GST–Tip containing the cytoplasmic region of Tip was produced from *Escherichia coli* strain BL21(DE3), and tyrosine-phosphorylated GST–Tip(pY) was purified from *E. coli* strain TKX1, containing the elk tyrosine kinase, which has broad specificity and phosphorylates mammalian proteins efficiently in *E. coli*. We found that the GST–Tip protein purified from *E. coli* TKX1 was tyrosine-phosphorylated efficiently by elk kinase (Fig. 1a). To identify cellular proteins interacting with Tip in a phosphorylation-dependent manner, the bacterially purified GST–Tip and GST–Tip(pY) fusion proteins were used on an affinity column for ^{35}S-labelled lysates of Jurkat cells. Polypeptides with apparent molecular masses of 56 and 80 kDa interacted specifically with GST–Tip, whereas they did not interact with the GST protein (Fig. 1a). Furthermore, polypeptides with a molecular mass of 90 kDa interacted specifically with the GST–Tip(pY) fusion protein, but did not interact with the GST and GST–Tip fusion proteins (Fig. 1a). To characterize these cellular proteins further, they were analysed by mass spectrometry and matched with known sequences. The two cellular proteins that interacted with both GST–Tip and GST–Tip(pY) were the Lck and p80 proteins, which are the known cellular targets of Tip (Jung *et al.*, 1995; Park *et al.*, 2002). The cellular proteins with a molecular mass of 90 kDa that interacted only with the tyrosine-phosphorylated GST–Tip(pY) protein but not with the unphosphorylated GST–Tip protein were identified as the STAT3 and STAT6 transcription factors (Fig. 1a), indicating that these transcription factors interact with Tip in a phosphorylation-dependent manner.

Previous reports have shown that Tip binds directly to STAT3 and activates the transcription factor through tyrosine phosphorylation in the presence of Lck (Hartley

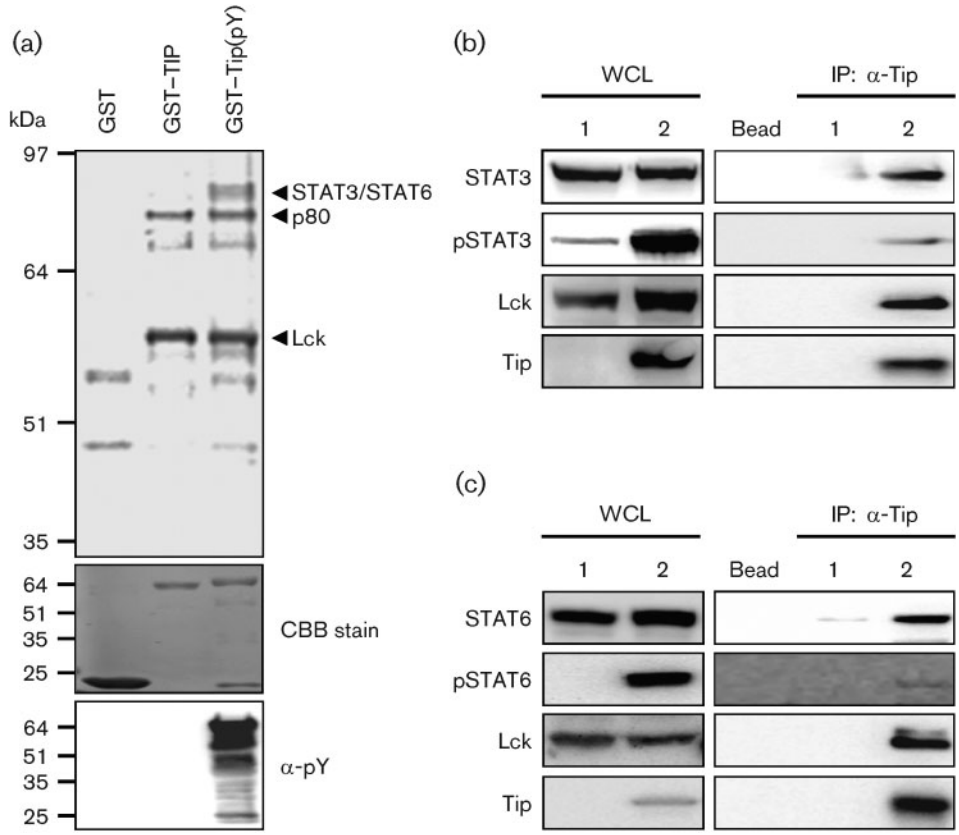

Fig. 1. Tip interacts with STAT6. (a) To identify the proteins binding to Tip, a GST pull-down assay was performed with GST-fused phosphorylated Tip purified from *E. coli* strain TKX1 and Jurkat T-cell lysate. Expression of GST-tagged proteins and formation of phosphorylated protein were visualized by Coomassie brilliant blue (CBB) staining and immunoblotting using an anti-phosphotyrosine antibody (α-pY), respectively. Binding proteins were analysed by mass spectrometry. To confirm the interaction between Tip and STAT proteins, HEK293T cells were transfected with Lck and (b) STAT3 or (c) STAT6, together with (lane 2) or without (lane 1) Tip. Lysates were immunoprecipitated using anti-Tip antibody and immunocomplexes were analysed by immunoblotting using the indicated antibodies. Protein A/G beads without antibody were used to exclude non-specific binding (Bead). IP, Immunoprecipitation; WCL, whole-cell lysate.

& Cooper, 2000; Lund *et al.*, 1997). To confirm the functional interaction of Tip with STAT6 in addition to STAT3, 293T cells were electroporated with plasmids encoding Tip and its cellular partners. Tip was precipitated from these cells and an immunoblot analysis was performed to test the complex formation and tyrosine phosphorylation of the STAT transcription factors. As shown in Fig. 1(b, c), rigorous phosphorylation of STAT6 as well as STAT3 was detected in the presence of Tip, and both transcription factors were present in Tip–Lck complexes.

Tip induces nuclear translocation and activation of STAT6 in Jurkat T-cells

As STAT transcription factors are known to be translocated into the nucleus after being activated by phosphorylation, we next examined the effect of Tip on STAT6 localization. Jurkat T-cells electroporated with a plasmid encoding GFP (vector) or GFP–Tip were monitored using confocal immunofluorescence. As reported previously (Cho *et al.*, 2006), STAT3 was translocated efficiently into the nucleus in cells expressing GFP–Tip, whereas this transcription factor was detected throughout cells expressing GFP (Fig. 2a). In addition, phosphorylated STAT3 was primarily present in the nucleus, indicating that the induced phosphorylation of STAT3 by Tip enhanced nuclear translocation of the transcription factor. When we examined STAT6 in Jurkat T-cells expressing Tip, it also showed an increase in nuclear translocation compared with control cells (Fig. 2b, upper panels). Unexpectedly, however, phosphorylated STAT6 colocalized primarily with Tip complexes located within the cytoplasm rather than the nucleus. To investigate further the role of Tip in the localization of STAT6, especially its phosphorylated form, we treated Jurkat T-cells with IL-4, which is a well-known cytokine inducing activation of STAT6 (Yu *et al.*, 2009). Fifteen minutes after IL-4 treatment, STAT6 was

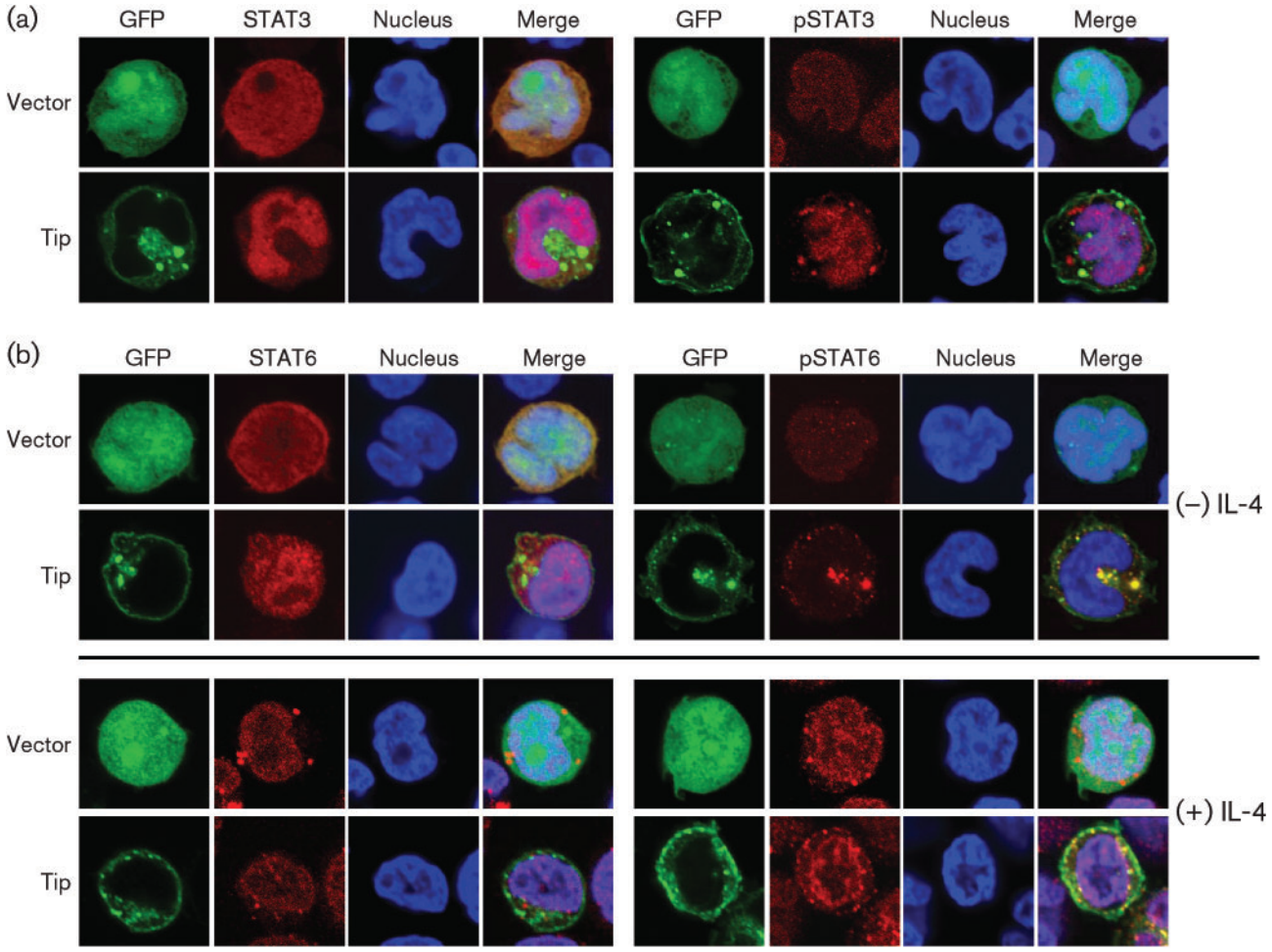

Fig. 2. Tip induces nuclear translocation of STAT6. Jurkat T-cells were electroporated with plasmids encoding GFP (vector) or GFP–Tip together with pVR/STAT3 or STAT6. Twenty-four hours after electroporation, localization of STAT3 (a) and STAT6 (b) was examined by confocal microscopy after staining with anti-STAT or anti-phosphoSTAT antibodes (red). TO-PRO-3 staining was used to visualize the nucleus (blue). Nuclear translocation of STAT6 was also examined in the absence (–) or presence (+) of IL-4 treatment (100 ng ml^{-1}, 15 min).

translocated efficiently into the nucleus in Jurkat T-cells expressing GFP or GFP–Tip (Fig. 2b, lower panels). Phosphorylated STAT6 was also detected primarily in the nucleus in IL-4-treated cells, regardless of Tip expression, indicating that Tip does not significantly affect the nuclear translocation of STAT6 in response to IL-4. To demonstrate further that the Tip-mediated phosphorylation and nuclear translocation of STAT6 lead to transcriptional activity of STAT6, Jurkat T-cells were electroporated with a STAT6-responsive luciferase reporter plasmid together with GFP vector or GFP–Tip plasmid. As shown in Fig. 3, Tip expression enhanced STAT6 transcriptional activity strongly in Jurkat T-cells compared with control cells expressing GFP (vector). The level of induction of STAT6 activity by Tip was comparable to that of control cells stimulated by IL-4, and there was only a slight increase in STAT6 activity in cells expressing Tip upon IL-4 treatment. The results further showed that Tip could dramatically induce the activation of STAT6 without exogenous stimulation in Jurkat T-cells.

Tyr^{127} of Tip is required for STAT6 interaction and activation

Previously, it has been demonstrated that Tip becomes tyrosine-phosphorylated by Lck at two sites and that one (Y^{72} of Tip-C484 and Y^{114} of Tip-C488) of the tyrosine residues embedded in a STAT SH2-binding motif (YXPQ) is required for direct interaction with, and thus activation of, STAT1 and STAT3 in the presence of Lck (Hartley & Cooper, 2000; Kjellen *et al.*, 2002; Lund *et al.*, 1997, 1999). In order to examine whether tyrosine phosphorylation of Tip is required for the binding of STAT6 and induction of STAT6 phosphorylation, Jurkat T-cells were electroporated

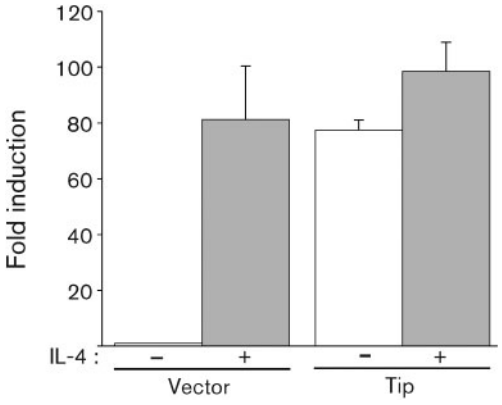

Fig. 3. Tip induces transcriptional activation of STAT6. Jurkat T-cells were electroporated with plasmids encoding Tip, STAT6 and 3×STAT6-luc plasmid and cultured in the presence (grey) or absence (white) of IL-4 (20 ng ml^{-1}) for 18 h. To normalize transfection efficiency, pGK-βgal vector was included in the transfection mixture and fold induction of luciferase activity was determined after normalization with β-galactosidase activity. Each bar represents data from triplicate assays; error bars indicate SD.

with plasmids encoding Tip wt or its mutants and subjected to immunoprecipitation. As seen in Fig. 4, STAT3 was tyrosine-phosphorylated and co-precipitated with Tip in cells expressing Tip wt and the Y127F mutant, whereas expression of the Y114F or Lck-binding (mLBD) mutants of Tip failed to induce tyrosine phosphorylation of STAT3 and the inclusion of STAT3 into a Tip–Lck complex, as expected. However, when the Y114F mutant was expressed, there was an increase in the tyrosine phosphorylation of STAT6, which correlated with the immune-complex formation with Tip and Lck. In contrast, the Y127F and mLBD mutants did not induce phosphorylation of STAT6 and failed to interact with STAT6. These results indicate that Tyr^{127} in Tip is required for STAT6 binding and phosphorylation, whereas Tyr^{114} serves as a docking site for STAT3 for its phosphorylation.

We have demonstrated previously that Tip is targeted constitutively to lipid rafts, membrane microdomains that function as platforms for modulating both TCR signalling and trafficking (Cho *et al.*, 2004, 2006; Min *et al.*, 2008; Park *et al.*, 2002). This functional association of Tip with lipid rafts is dependent on multiple sequence motifs, including the p80-binding domain (Park *et al.*, 2003), transmembrane domain (Cho *et al.*, 2006) and membrane-proximal amphipathic helix (Min *et al.*, 2008). To test whether the association of Tip with lipid rafts affects the activation of STAT6, tyrosine phosphorylation of STAT6 and its complex formation with Tip–Lck were examined in Jurkat T-cells expressing Tip wt or its mutants that fail to associate with lipid rafts. As shown in Fig. 5, all tested mutants induced tyrosine phosphorylation of STAT6 and interacted with the transcription factor as efficiently as Tip wt. Even the 97–211 fragment mutant, which has extensive

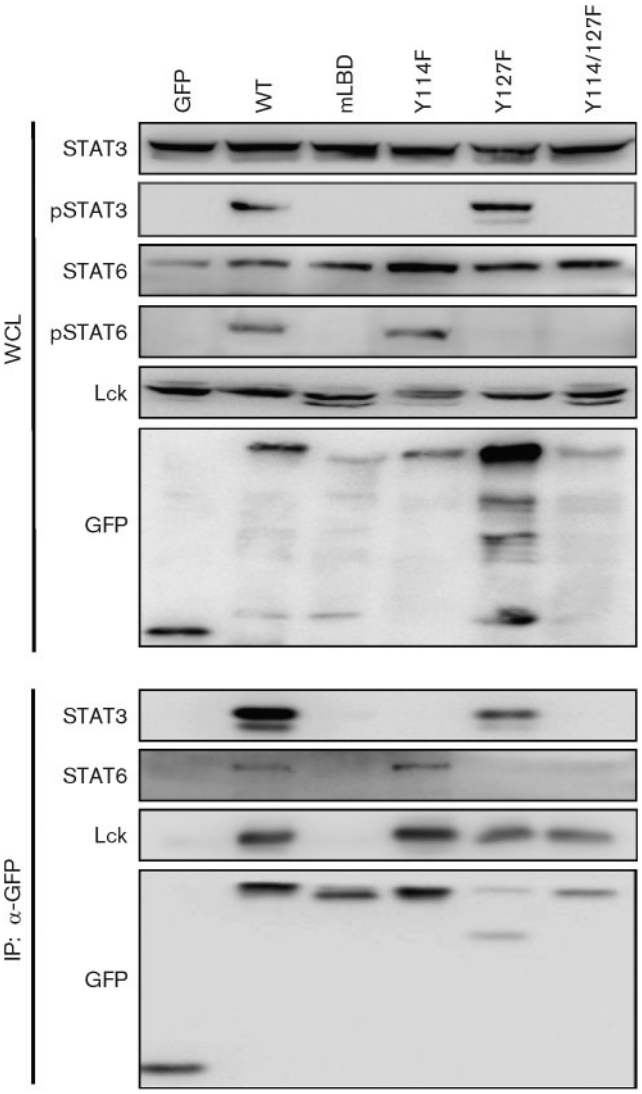

Fig. 4. The Tyr^{127} residue of Tip is required for the interaction with and phosphorylation of STAT6. Jurkat T-cells were electroporated with GFP, GFP–Tip or GFP–Tip mutant expression constructs. Twenty-four hours after electroporation, cells were lysed and used for immunoprecipitation (IP) with an antibody against GFP, followed by immunoblotting with the indicated antibodies. Whole-cell lysates (WCL) were used to detect the expression level of STATs and Lck.

deletions in both the N and C termini, induced STAT6 phosphorylation sufficiently and interacted with the transcription factor.

To further confirm the role of Tyr^{127} of Tip in STAT6 activation, we examined nuclear translocation of the transcription factors using the Tip mutants. Jurkat T-cells were electroporated with plasmids encoding GFP–Tip wt or its mutants for confocal microscopy analysis. STAT6 was translocated efficiently into the nucleus upon expression of Tip wt and the Y114F and 92–211 mutants, whereas

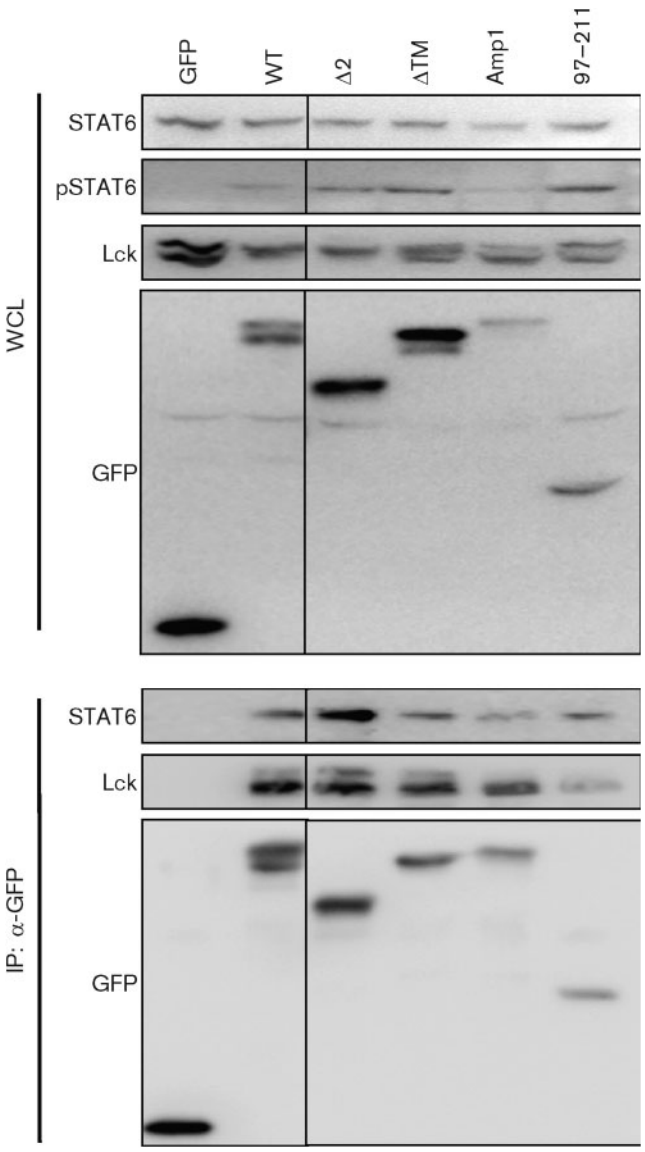

Fig. 5. Association of Tip with membrane lipid rafts is not required for its interaction with and phosphorylation of STAT6. Jurkat T-cells were electroporated with plasmids encoding Tip or its mutants that are defective in lipid-raft association or membrane trafficking. Twenty-four hours after electroporation, cells were lysed and used for immunoprecipitation (IP) with an antibody against GFP. Whole-cell lysates (WCL) and immunocomplexes were subjected to immunoblot assay using the indicated antibodies.

expression of Y127F or mLBD did not induce nuclear localization of STAT6 under the same conditions (Fig. 6). Consistently, phosphorylated STAT6 was detected primarily in association with the Tip complex within the cytosol in cells expressing Tip wt or Y114F. In contrast, it was barely detected in Jurkat T-cells expressing the mLBD or Y127F mutants. Interestingly, phosphorylated STAT6 was observed within the nucleus in cells expressing the 97–211 mutant, incapable of associating with lipid rafts or membranous vesicles (Fig. 6). The role of the Tyr^{127} residue in the activation of STAT6 transcription-factor activity was also tested using a STAT6-responsive reporter system. The Tip Y127F mutant exhibited no effect on STAT6 transcriptional activity, whereas the Y114F mutant induced STAT6 activation as proficiently as Tip wt (Fig. 7). In addition, the 97–211 deletion mutant showed a remarkable increase in STAT6 transcriptional activity. Taken together, these results indicate clearly that the Tyr^{127} residue is essential for the interaction of Tip with STAT6 and is required for the activation of STAT6 transcriptional activity.

DISCUSSION

The genome of the highly oncogenic HVS subgroup C strain contains one bicistronic gene with two ORFs, *tip* and *stpC*, which are essential for T-cell transformation. Deletions affecting the ORFs for StpC and/or Tip within the viral genome of subgroup C strains 484 or 488 abolished transformation *in vitro* and pathogenicity *in vivo* (Duboise *et al.*, 1998; Heck *et al.*, 2006). Independent of the viral context, both viral oncoproteins induced tumours in transgenic mice: expression of StpC resulted in epithelial hyperplasia, whilst Tip caused peripheral T-cell lymphoma closely resembling the tumours in susceptible New World primates (Heck *et al.*, 2006; Murphy *et al.*, 1994; Wehner *et al.*, 2001). The effects of StpC are assigned to its interaction with the ubiquitous cellular oncoprotein Ras and to its ability to activate the transcription factor NF-κB (nuclear factor kappa B) (Choi *et al.*, 2000; Lee *et al.*, 1999). In accordance with the phenotype of T-cell tumours in transgenic mice, Tip binds directly to the Src family tyrosine kinase Lck, a key regulator for T-cell activation (Biesinger *et al.*, 1995; Heck *et al.*, 2006). The recruitment by Tip and subsequent phosphorylation by Lck provide an explanation for the observed activation of STAT1 and STAT3 in the presence of Tip and Lck (Hartley & Cooper, 2000; Lund *et al.*, 1997, 1999). The implication of constitutively active STATs, especially STAT3, in growth regulation and oncogenesis in multiple cell types (Bowman *et al.*, 2000; Bromberg & Darnell, 2000) suggested a central role for Tip-induced STAT activity in viral T-cell transformation. However, a recombinant HVS C488 strain expressing Tip with a tyrosine-to-phenylalanine substitution at position 114 was able to transform primary human T-lymphocytes in the absence of STAT1 or STAT3 activation (Heck *et al.*, 2005, 2006). Thus, the essential function of Tip in lymphocyte transformation does not rely on Lck-mediated STAT1/3 phosphorylation. Previously, however, it was reported that a recombinant HVS strain C488 lacking LBD motifs of Tip lost its transforming potential on human cord-blood lymphocytes (Heck *et al.*, 2006). In addition, a recombinant virus expressing Tip with a mutation at position Tyr^{127} was still able to transform human T-lymphocytes but, in contrast to wt virus, was strictly dependent on exogenous IL-2 (Heck

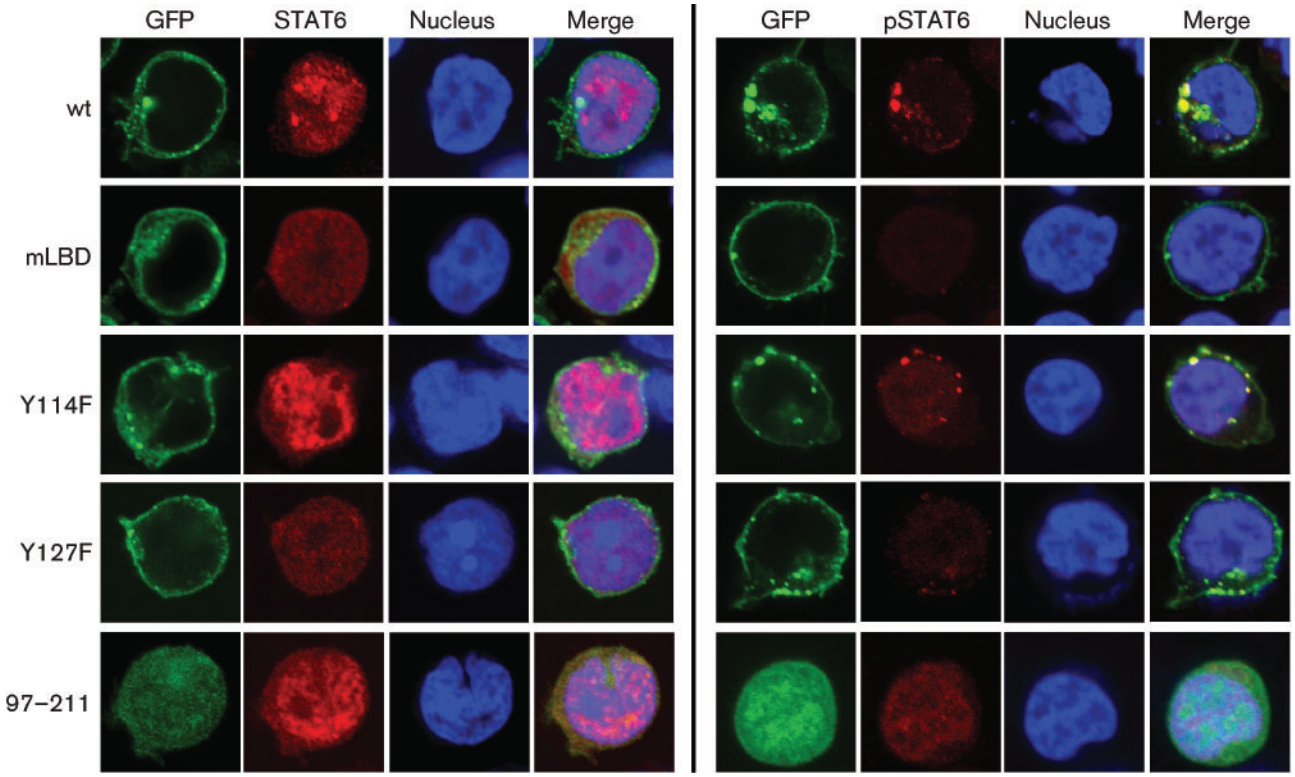

Fig. 6. The Tyr[127] residue of Tip is required for nuclear translocation of STAT6 by Tip. Jurkat T-cells were electroporated with plasmids encoding GFP-fused Tip wt or its mutants (green) together with pVR/STAT6. Twenty-four hours after electroporation, localization of STAT6 (left panels) and phosphoSTAT6 (right panels) was visualized by confocal microscopy after staining with anti-STAT6 or anti-phosphoSTAT6 antibodies (red). TO-PRO-3 staining was used to visualize the nucleus (blue).

et al., 2006). Thus, the major tyrosine-phosphorylation site Tyr[127] of Tip might be required for compensating the requirement of exogenous IL-2 by constitutively activating STAT6, as shown in this study.

STATs possess SH2 domains required for interaction with proteins containing phosphotyrosine (pY). SH2 domains specifically recognize protein modules composed of pY and three to five carboxyl-terminal residues. Although each protein interacting with SH2 domain-containing proteins has a different docking-site sequence, they commonly have the motif pY-X-X-hydrophobic residue (Zhou *et al.*, 1993). Tip has a STAT3-binding motif, Tyr[114]-Arg-Pro-Gln, which aligns well with the known docking sequence, Tyr-X-Pro-Gln, for STAT3 (Hartley & Cooper, 2000). Thus, phosphorylation of Tyr[114] by Lck makes Tip capable of binding to STAT3 for activation (Lund *et al.*, 1997, 1999). STAT6 also has an SH2 domain and interacts with its natural docking protein, the IL-4 receptor (IL-4R) (Hou *et al.*, 1994). When IL-4 binds to its receptor, activated JAKs phosphorylate five tyrosine residues at Y497, Y575, Y603, Y631 and Y713 in IL-4R (Hartley & Cooper, 2000; Johnston *et al.*, 1994; Keegan *et al.*, 1994; Murata *et al.*, 1996; Witthuhn *et al.*, 1994). In particular, three conserved tyrosine residues of IL-4R, Y575, Y603 and Y631, that are critical for STAT6 binding and IL-4 signal transduction (Reichel *et al.*, 1997; Ryan *et al.*, 1996) are surrounded by a conserved motif, Gly-Tyr-Lys/Gln-X-Phe. In addition, Y497 of IL-4R, which is required for STAT6 binding (Ryan *et al.*, 1996) and IL-4-mediated proliferation (Deutsch *et al.*, 1995; Keegan *et al.*, 1994), is also surrounded by a sequence motif, Ala-Tyr-Arg-Ser-Phe. Taken together, the common sequence, Tyr-X-X-Phe, contributes to the interaction with the SH2 domain of STAT6 after tyrosine phosphorylation. In case of Tip, the Tyr[127] residue is surrounded by Tyr[127]-Thr-Thr-Phe, which is consistent with the general SH2 domain-binding motif in IL-4R for STAT6 binding. Furthermore, the residues (Tyr-Thr-Thr/Ser-Phe) surrounding Tip Y127 are highly conserved among different HVS subgroup C isolates (Ensser *et al.*, 2003). Therefore, Tyr[127] of Tip in the context of Tyr-X-X-Phe forms an authentic docking site for the SH2 domain of STAT6 after tyrosine phosphorylation by Lck, as shown in this study.

Constitutively active STATs and dysregulation of STAT signalling have been found in many haematocytic tumours (Benekli *et al.*, 2003). The abnormal functions of STAT3 and STAT5 in lymphomas and leukaemias have been studied extensively in the aspect of myelopoiesis regulation in response to cytokines or hormones. Recently, the roles

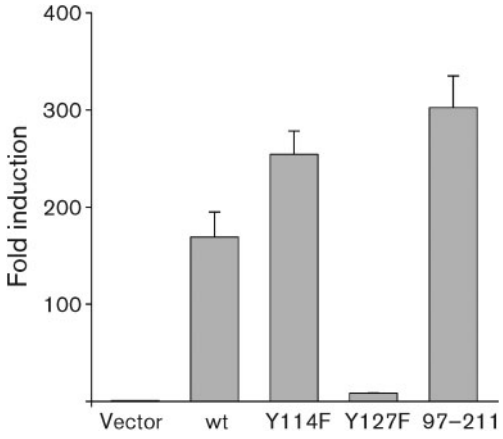

Fig. 7. The Tyr^{127} residue of Tip is required for transcriptional activation of STAT6. Jurkat T-cells were electroporated with plasmids encoding Tip wt or its mutants together with pVR/STAT6 and 3×STAT6-luc plasmid. Twenty-four hours after electroporation, luciferase activities were measured. To normalize transfection efficiency, the pGK-βgal vector was included in the transfection mixture and fold induction of luciferase activity was determined after normalization with β-galactosidase activity. Each bar represents data from triplicate assays; error bars indicate SD.

of other STATs have been gradually emerging for cellular transformation. STAT6 is also overactivated in Hodgkin's lymphoma, primary mediastinal large B-cell lymphomas, cutaneous T-cell lymphomas and acute lymphoblastic leukaemia (Guiter *et al.*, 2004; Ilaria & Van Etten, 1996; Qin *et al.*, 2001; Skinnider *et al.*, 2002). In these tumours, aberrant STAT6 activation occurs because of the high level of cytokines or kinases (except for JAK) or the gain-of-function mutations in STAT6.

Unexpectedly, nuclear translocation of phosphorylated STAT6 was barely detectable in T-cells expressing Tip (Fig. 2). Phosphorylated STAT6 mainly colocalized with Tip-containing vesicles in the cytoplasm. Interestingly, several reports have suggested that cytoplasmic transport of STAT3 is an active process that requires receptor-mediated endocytosis (Bild *et al.*, 2002; Xu *et al.*, 2007). In these reports, phosphorylated STAT3 colocalizes with endocytic vesicles in transit from the cell membrane to the perinuclear region in response to growth-factor stimulation. Consistent with a role for receptor endocytosis in growth-factor signalling, disruption of endocytosis with specific inhibitors blocks STAT3 nuclear translocation and STAT3-dependent gene regulation (Bild *et al.*, 2002). Based on these findings and our current data, it can be suggested that cytoplasmic localization of phosphorylated STAT6 induced by Tip expression in T-cells may result from prolonged association of phosphorylated STAT6 with Tip-containing vesicles in the cytoplasm and that it eventually translocates into the nucleus and rapidly becomes dephosphorylated. Considering that Tip itself has a strong affinity for membrane lipid rafts and interacts with the cellular trafficking protein p80 for lysosomal targeting (Cho *et al.*, 2006; Park *et al.*, 2002, 2003), it is possible that the majority of phosphorylated STAT6 is retained in the Tip-containing trafficking vesicles during the vesicular trafficking in the cytoplasm after its phosphorylation. This hypothesis is consistent with our finding that Tip mutant 97–211, lacking the transmembrane domain and p80-binding motif but still capable of interacting with Lck and STAT6, induces rapid nuclear translocation of phosphorylated STAT6 (Fig. 6). The precise role of vesicular trafficking of the Lck–Tip–phosphoSTAT6 complex in T-cells needs to be characterized further.

Another striking observation was the dramatic increase of unphosphorylated STAT6 in the nucleus. The increased level of STAT6 in the nucleus correlates well with the transcriptional activation of a STAT6-responsive promoter in Jurkat T-cells expressing Tip to a level comparable with that of IL-4-treated cells (Fig. 3). The dramatic increase of unphosphorylated STAT6 in the nucleus might control gene expression by several different mechanisms. First, enhanced nuclear translocation of unphosphorylated STAT6 could affect cellular gene expression directly. Previously, unphosphorylated STAT proteins (U-STATs) have been shown to influence gene transcription through mechanisms distinct from those used by phosphorylated STAT (Yang *et al.*, 2007; Yang & Stark, 2008). For example, U-STAT1 mediates constitutive expression of the low-molecular-mass polypeptide (LMP) 2 gene by collaborating with IRF1 (Chatterjee-Kishore *et al.*, 1998). U-STAT3 also binds to unphosphorylated NF-κB in competition with IκB, and the resulting U-STAT3–NF-κB complex accumulates in the nucleus with help from the nuclear-localization signal of STAT3, eventually activating a subset of NF-κB-dependent genes (Yang *et al.*, 2007). Moreover, U-STAT6 cooperates with p300 and binds to a consensus STAT6-binding site located within the COX-2 promoter to enhance COX-2 expression in human non-small-cell lung cancer (Cui *et al.*, 2007). Constitutively expressed COX-2 produces a high level of PGE2, which increases resistance to apoptosis, promotes angiogenesis and suppresses anti-tumour immunity (Sheng *et al.*, 1998; Stolina *et al.*, 2000). Second, U-STATs may be associated with maintaining the stability of transcriptionally repressed heterochromatin and controlling cellular epigenetic status, which affects expression of genes beyond those under direct STAT transcription control (Li, 2008). It was shown that U-STAT (U-STAT92E) of *Drosophila melanogaster* interacts physically with heterochromatin protein 1 (HP1) to promote heterochromatin stability and that the unphosphorylated or 'transcriptionally inactive' form of STAT92E is required for stabilizing HP1 localization and histone H3 Lys^9 methylation (H3mK9) (Shi *et al.*, 2008). Increased levels of heterochromatin caused by U-STAT in *Drosophila* resulted in diminished DNA damage and increased survival rate under genotoxic stress such as irradiation (Yan *et al.*, 2011). The changes in chromatin structure associated with constitutive STAT activation or the level of U-STATs in the nucleus often found in cancer cells

might be associated with their ability to regenerate a tumour (Brown & Zeidler, 2008). However, when monitored using confocal microscopy, U-STAT6 in T-cells expressing Tip did not colocalize with HP1 protein in the nucleus (data not shown), suggesting that the nuclear U-STAT6 induced by Tip may not be involved in the control of heterochromatin stability.

Recently, Cai *et al.* (2010) demonstrated that Kaposi's sarcoma-associated herpesvirus (KSHV) suppresses IL-4-induced signalling by reducing the phosphorylation of STAT6 and its DNA-binding affinity. They showed that KSHV-encoded LANA is essential for viral blocking of IL-4-mediated STAT6 activation. However, it was also observed that knockdown of endogenous STAT6 dramatically increases the sensitivity of KSHV-positive primary effusion lymphoma cells to low-serum stress or chemically mediated cellular apoptosis, and the basal level of constitutive phosphorylation of STAT6 in KSHV-positive cell lines (Cai *et al.*, 2010). In addition, which viral gene is responsible for the constitutive activation of STAT6 in KSHV-infected cells remains to be elucidated. As an oncogenic γ2 herpesvirus homologue of KSHV, we now show that HVS is equipped with a viral protein, Tip, that can activate the STAT6 transcription factor constitutively in Jurkat T-cells and may ultimately contribute to IL-2-independent T-cell transformation. The functional activation and role of STAT6 in T-cell transformation need to be verified further in primary T-cells and HVS-transformed human T-cells in the future.

METHODS

Cell culture and reagents. Jurkat T-cells (clone E6-1; ATCC TIB-152) and 293T cells were grown in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, both supplemented with 10 % FBS and 1 % penicillin/streptomycin (Gibco-BRL, Invitrogen). Jurkat T-cells were electroporated using a Bio-Rad electroporator at 260 V and 975 μF in RPMI 1640 medium in the absence of antibiotics. 293T cells were transiently transfected using calcium phosphate (Clontech). All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Antibodies used were as follows: anti-Tip antibody was generated in a rabbit as described previously (Biesinger *et al.*, 1995). Anti-Lck and anti-GFP antibodies were obtained from Santa Cruz Biotechnology and anti-STAT3, anti-phosphoSTAT3 (Y705), anti-STAT6 and anti-phosphoSTAT6 (Y641) antibodies were purchased from Cell Signaling Technology. For confocal microscopy analysis, anti-STAT6 antibody (BD Transduction Laboratories) was used. Anti-mouse or -rabbit antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) or to Alexa 488 or Alexa 594 (Molecular Probes, Invitrogen) were used for immunoblotting and confocal microscopy, respectively. GFP fusions of Tip or its mutants were made using pEGFP-C2 plasmids (Clontech). The sequences changed in each mutant were as follows: TipmLBD, 150-Ser/Phe/Leu-152 to Ala/Ala/Ala, and prolines at aa 175, 177, 178, 180 and 181 to Ala (Cho *et al.*, 2004, 2006); TipΔTM, deletion from aa 227-Ile to 256-Ser (Cho *et al.*, 2006); TipΔ2, deletion from aa 44-His to 96-Ser (Park *et al.*, 2003); TipAmp1, 216-Ile, 220-Leu, 223-Leu and 227-Ile to Lys (Min *et al.*, 2008).

GST pull-down assay and protein identification by mass spectrometry. GST-fusion proteins were purified from either *E. coli* strain BL21(DE3) or TKX1, which contains a mammalian elk tyrosine kinase expression vector (Stratagene). Jurkat T-cell lysates were incubated with glutathione beads containing GST-fusion protein in binding buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, 1 % NP-40, protease inhibitors] at 4 °C for 2 h. Glutathione beads were then washed four times with binding buffer, and the proteins associated with the beads were analysed by SDS-PAGE (Lee *et al.*, 2005). The purified proteins were visualized by silver staining (Invitrogen) and unique protein bands were cut out and sent to the Taplin Biological Mass Spectrometry facility at the Harvard Medical School (Boston, MA, USA) for mass spectrometry analysis.

Immunoprecipitation and immunoblotting. Cell lysates were prepared as above in 0.5 % NP-40 or RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS] and pre-cleared with protein A/G beads for 2 h before immunoprecipitation. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes (Millipore), blocked in 5 % milk in Tris-buffered saline with 0.05 % Tween 20, and incubated with primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. Immunoblot detection of proteins was performed by using an enhanced chemiluminescence system (Pierce).

Confocal microscopy. Cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized with 0.2 % Triton X-100 for 15 min. After blocking with 1 % BSA in PBS, the cells were reacted with the appropriate primary antibody at 4 °C overnight. Alexa 488- or Alexa 594-conjugated anti-rabbit or anti-mouse antibodies (Molecular Probes) were used as secondary antibodies. Nuclei were stained with TO-PRO-3 iodide (Molecular Probes) at room temperature for 15 min. Confocal microscopy was performed using an Olympus FV1000 laser-scanning microscope (Olympus) with a ×60 Olympus objective. Images were collected at 512 × 512 pixel resolution using Olympus imaging software. The stained cells were sectioned optically in the *z*-axis, and the images in the different channels (photomultiplier tubes) were collected sequentially. The images were rendered using Olympus Fluoview v1.6b or Adobe Photoshop software.

Luciferase reporter assays. Jurkat cells were electroporated with plasmids encoding STAT6 and Tip or its mutant together with the STAT6-responsive luciferase reporter plasmid (3 × STAT6-luc: pTransLucent containing three tandem repeats of pSTAT6-binding sites) provided by Karen Leroy (Université Paris, France) (Ritz *et al.*, 2009). To normalize transfection efficiency, the pGK-βgal vector, which expresses β-galactosidase from a phosphoglucokinase promoter, was included in the transfection mixture. Cells were then sampled at 24 h post-electroporation. For IL-4 (Cell Signaling Technology) treatment, cells were incubated with 20 ng IL-4 ml^{-1} for 18 h prior to cell lysis. Cells were harvested and washed once in cold PBS. The cells were then lysed in 100 μl lysis buffer [0.1 mM potassium phosphate buffer (pH 7.8), 1 % Triton X-100, 1 mM DTT and 2 mM EDTA]. Cells were incubated in lysis buffer for 15–20 min on ice before pelleting in a microcentrifuge for 2 min at high speed (20 000 ***g***) to remove cellular debris. Cell lysate (30 μl) was mixed with 100 μl assay buffer (30 mM Tricine, 3 mM ATP, 15 mM $MgSO_4$, 10 mM DTT, pH 7.8) in a 96-well microtitre plate just prior to measurement. Plates were assayed using the MLX microtitre luminescence detection system (Dynex Technologies), which injects 100 μl of the substrate (1 mM D-luciferin in assay buffer) prior to measuring luminescence. Assays were performed in triplicate.

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