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Enhanced Downregulation of Lck-Mediated Signal Transduction by a Y_{114} Mutation of Herpesvirus Saimiri Tip

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Tip of herpesvirus saimiri associates with Lck and downregulates Lck function in cellular signal transduction. In this report, we demonstrate that mutation of tyrosine 114 of Tip significantly increases Lck-binding activity. This mutant exhibits a dramatic increase in the suppression of cellular tyrosine phosphorylation and surface expression of lymphocyte antigens in comparison with wild-type Tip. In addition, the expression of $TipY_{114}$ converted the transforming morphology of fibroblasts induced by oncogenic F505 Lck to a normal cellular morphology. These results further support a mechanism by which the association of Tip with Lck negatively regulates Lck-mediated signal transduction.

Herpesvirus saimiri (HVS), a member of the gamma-2 subgroup of herpesviruses, naturally infects squirrel monkeys (*Saimiri sciureus*) of South America. Recently, Kaposi's sarcoma-associated herpesvirus has been shown to be a close relative to HVS (6). HVS persists in T lymphocytes of the natural host without any apparent disease, but infection of other species of New World primates results in fulminant lymphomas, lymphosarcomas, and leukemias of T-cell origin (12). Nucleotide sequence analysis of the entire HVS genome has revealed a number of genes with homology to cellular genes, some of which could possibly contribute to T-cell transformation (2). These include the STP oncogene (7, 8, 14), superantigen gene homolog (27, 31), interleukin 8 receptor gene homolog (1), CD59 gene homolog (3, 25), bcl-2 gene homolog (23), and genes for virus-encoded interleukin 17 (30) and virus-encoded cyclin (17, 24).

A protein called Tip (tyrosine kinase interacting protein) encoded by the gene adjacent to *STP-C488* at the left end of the viral genome was previously identified in virus-transformed T cells (4, 5). Tip did not show transforming activity in rodent fibroblast cells (18), but the protein was found to be associated with the major T-cell tyrosine kinase Lck and phosphorylated on tyrosine residues by purified Lck in several cell-free assay systems (5). Mutational analysis of a glutathione *S*-transferase-Tip fusion protein revealed that binding to Lck requires a putative SH3 binding sequence and a sequence homologous to the carboxy terminus of Src-related kinases (15). These sequences are referred to as SH3-binding (SH3B) and C-terminal Src-related kinase homology (CSKH) elements. Peptide fragments as short as 37 amino acids containing CSKH, a spacer sequence, and SH3B motifs are sufficient to form a stable complex with Lck in vitro. Expression of Tip in Jurkat T cells dramatically suppressed cellular tyrosine phosphorylation and surface expression of lymphocyte antigens (16). Expression of Tip also blocked the induction of tyrosine phosphorylation by anti-CD3 stimulation. Furthermore, the expression of Tip in fibroblast cells partially suppressed the transforming activity of oncogenic F505 Lck (16). Therefore, Tip can act at an early stage of the T-cell signal transduction cascade by associating with Lck and downregulating Lck-mediated activation. Inhibition of Lck-mediated signal transduction by Tip in T cells appears to be analogous to the inhibition of Lyn/Syk-mediated B-cell activation by Epstein-Barr virus (EBV) LMP2A (20, 21).

While LMP2A of EBV is not required for B-cell transformation, we have found that Tip is essential for primary T-cell immortalization and for lymphoma induction in vivo (10). These data suggest that Tip may have additional functions other than blocking the Lck-mediated signal transduction. In order to study the events elicited by Tip other than the association with Lck, we have exploited a yeast system for detecting protein interactions and identified a novel cellular protein, designated Tap (tip-associated protein) (32). Expression of Tip and Tap in Jurkat T cells induced dramatic cell aggregation. This phenotype was likely caused by the upregulated surface expression of lymphocyte adhesion molecules, including integrin α (CD11a), L-selectin (CD62L), ICAM-3 (CD50), and H-CAM (CD44) (32). In fact, the surface expression of integrin α was induced more than 100-fold in these cells. Furthermore, the NF-κB transcriptional factor of these aggregated cells had approximately 40-fold higher activity than that of the parental Jurkat T cells. Thus, the Tap protein is likely to be an important cellular mediator of Tip function in T-cell transformation by HVS.

Since Tip is associated with Lck and is phosphorylated at tyrosine residues in vivo, we studied the importance of tyrosine residues of Tip for its binding activity to Lck and for its ability to downregulate the Lck-mediated signal transduction. We found that a substitution mutation at tyrosine 114 significantly enhances the Lck-binding activity of Tip. The tyrosine residue at amino acid 114 was replaced with a serine (S) or glycine (G) residue by oligonucleotide-directed mutagenesis using PCR (9). To facilitate the mutagenesis, the Tip gene was subcloned into the pSP72 vector (Promega Biotec, Madison, Wis.). PCR cycling for mutagenesis was accomplished with a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, Conn.) under the following conditions: 30 cycles of 2 min at 50°C for annealing, 5 min at 72°C for polymerization, and 1 min at 94°C for denaturation. *tip* mutants were completely sequenced to verify the presence of the desired mutations and the absence of any other changes, and they were recloned into *Eco*RI and

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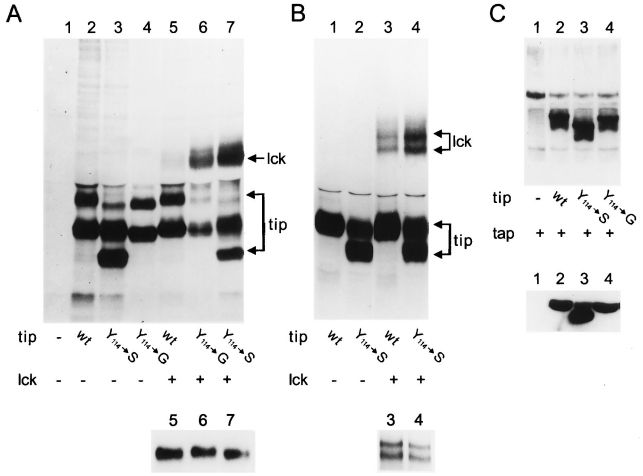

FIG. 1. Enhanced Lck-binding activity of the $TipY_{114}$ mutant. (A) COS-1 cells were transfected with pFJ-AU1-Tip, pFJ-AU1-$TipY_{114}$→S, or pFJ-AU1-$TipY_{114}$→G alone or together with pFJ-Lck as indicated below the gel. After 48 h of transfection, cells were labeled with [^{35}S]methionine and [^{35}S]cysteine. Radioactively labeled cell lysates were used for immunoprecipitation with anti-AU1 antibody. Untransfected COS-1 cells (lane 1) were used as a control. Labeled proteins were fractionated by SDS-PAGE and detected by autoradiography. The equivalent level of Lck expression was detected by radioactive immunoprecipitation with anti-Lck antibody in cotransfected COS-1 cells (bottom panel). (B) Sf9 insect cells were infected with recombinant baculoviruses expressing Tip or $TipY_{114}$→S with or without Lck as indicated below the gel. After 48 h of infection, cells were labeled with [^{35}S]methionine. Cell lysates were used for precipitations with anti-AU1 antibody. After immunoprecipitation, proteins were separated by SDS-PAGE and detected by autoradiography. The equivalent level of Lck expression was detected by radioactive immunoprecipitation with anti-Lck antibody in coinfected Sf9 cells (bottom panel). (C) No change of Tap-binding activity of $TipY_{114}$ mutants. COS-1 cells were transfected with pFJ-Tip, pFJ-$TipY_{114}$→S, or pFJ-$TipY_{114}$→G together with pcDNA-HA-Tap as indicated below the gel. After 48 h of transfection, cell lysates were used for immunoprecipitation with anti-HA antibody. Polypeptides associated with HA immune complexes were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with anti-Tip antibody. The levels of wt Tip and Tip mutant expression were determined by immunoblotting with anti-Tip antibody (bottom panel). Reactivity was detected by enhanced chemiluminescence (ECL; Amersham Life Science).

*Bgl*II cloning sites of the pFJ vector for gene expression. To facilitate the Lck-binding assay of Tip, the amino termini of wild-type (wt) Tip and the $TipY_{114}$ mutants were tagged with AU-1 epitope (16). COS-1 cells were transfected with pFJ-AU1-Tip, pFJ-AU1-$TipY_{114}$→S, or pFJ-AU1-$TipY_{114}$→G. Forty-eight hours after transfection, cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 8 h. ^{35}S-labeled proteins from transfected COS-1 cells were used for immunoprecipitation with anti-AU1 antibody. wt Tip protein migrated with an apparent molecular size of 40 to 45 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15), whereas $TipY_{114}$→S and $TipY_{114}$→G had apparent molecular sizes of 35 to 40 and 38 to 43 kDa in SDS-PAGE, respectively (Fig. 1A). As with Tip, point mutations of the STP-C488 oncoprotein of HVS have been shown to significantly change the migration rate in SDS-PAGE (13).

In order to examine whether mutations at Y_{114} affect the Lck-binding activity of Tip, wt Tip and mutants $TipY_{114}$→S and $TipY_{114}$→G were coexpressed with Lck in COS-1 cells. ^{35}S radioactively labeled 55-kDa Lck was detected in wt Tip immune complexes in transfected COS-1 cells, while a greater amount of Lck was detected in $TipY_{114}$→S or $TipY_{114}$→G immune complexes (Fig. 1A). The amount of radioactivity of coprecipitated Lck was quantitated with a Bio-Rad molecular imager, model GS-250. It showed that Lck was associated with $TipY_{114}$→S at levels 8 times greater, and with $TipY_{114}$→G at levels 5 times greater, than those with wt Tip in COS-1 cells (Fig. 1A). Under these conditions, similar amounts of Lck were expressed in cotransfected COS-1 cells (Fig. 1A, bottom). To confirm this result, recombinant baculoviruses were employed for Lck-binding assays. Sf9 insect cells were coinfected with Tip baculovirus or $TipY_{114}$→S baculovirus together with Lck baculovirus as described previously (15). While coinfection of insect cells with wt Tip or $TipY_{114}$→S and Lck recombinant baculoviruses resulted in Tip-Lck complex formation, a greater amount of Lck was also detected in $TipY_{114}$→S complexes than in wt Tip complexes (Fig. 1B). Recently, we have described a novel cellular protein, Tap, which was identified as Tip-associated protein by a yeast two-hybrid system (32). To examine whether Y_{114} mutation of Tip altered its Tap-binding activity, COS-1 cells were cotransfected with a hemagglutinin (HA)-tagged Tap gene together with pFJ-Tip, pFJ-$TipY_{114}$→S, or pFJ-$TipY_{114}$→G. After transfection, cell lysates were used for immunoprecipitation with anti-HA antibody. Polypeptides associated with anti-HA immune complexes were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with rabbit anti-Tip antibody. Similar amounts of wt Tip, $TipY_{114}$→S, and $TipY_{114}$→G were detected in anti-HA immune complexes, showing that Y_{114} mutation did not alter the Tap-binding activity of Tip in co-transfected COS-1 cells (Fig. 1C). Under these conditions, similar amounts of wt Tip, $TipY_{114}$→S, and $TipY_{114}$→G were expressed in COS-1 cells (Fig. 1C, bottom). These results thus indicated that the Y_{114} mutation specifically altered the Lck-binding activity of Tip.

Previously, we have shown that the expression of Tip in Jurkat T cells dramatically suppressed cellular tyrosine phosphorylation and surface expression of lymphocyte antigens (16). To investigate the effect of the increased Lck-binding activity of $TipY_{114}$→S on these phenotypes, we established a stable Jurkat T cell line that expressed $TipY_{114}$→S. The full length of $TipY_{114}$→S tagged with an AU-1 epitope at the amino terminus was cloned into the retroviral vector pBabe-puro as described previously (16). pBabe-$TipY_{114}$→S was introduced into Jurkat T cells by electroporation (Bio-Rad) at 200 V and 960 μF in serum-free RPMI 1640 medium. After incubation for 48 h, the cells were cultured with selective medium containing 5 μg of puromycin per ml for the next 5 weeks. To demonstrate expression of the Tip_{Y114}→$_S$ gene in puromycin-resistant cells, lysates from Jurkat-$TipY_{114}$→S cells were immunoblotted with anti-AU1 antibody along with control lysates from Jurkat-babe and Jurkat-Tip cells. Forty-kilodalton wt Tip and 35-kDa $TipY_{114}$→S were detected from their respective cell lysates (Fig. 2A). We compared the levels of tyrosine phosphorylation of these three Jurkat T-cell lines. While overall cellular tyrosine phosphorylation was decreased in both Jurkat-Tip and Jurkat-$TipY_{114}$→S cells compared to control Jurkat-babe cells, it was more dramatically decreased in Jurkat-$TipY_{114}$→S cells (Fig. 2B). Control Jurkat-babe cells contained many tyrosine-phosphorylated proteins, whereas tyrosine phosphorylation of these proteins was drastically decreased in Jurkat-Tip and Jurkat-$TipY_{114}$→S cells. In fact, only a few tyrosine-phosphorylated proteins were detected in Jurkat-$TipY_{114}$→S cells. Thus, expression of the $TipY_{114}$→S mutant induced a more dramatic decrease of cellular tyrosine phosphorylation in Jurkat T cells than expression of wt Tip.

The expression of wt Tip in Jurkat T cells has also been shown to drastically affect the surface expression of lymphocyte antigens, including CD2 and CD4 (16). Since the overall cel-

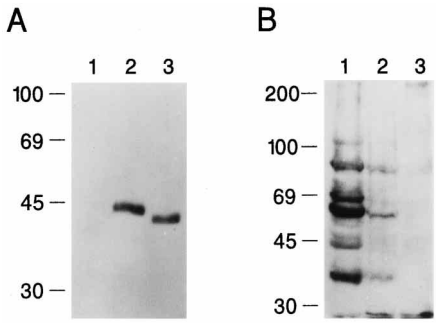

FIG. 2. Downregulation of tyrosine phosphorylation by expression of TipY_{114}→S in Jurkat T cells. (A) Expression of TipY_{114}→S in Jurkat T cells. Tip protein in T-cell lines was detected by immunoblotting with anti-AU1 antibody; 2×10^6 cells were used for preparation of extracts. Lane 1, Jurkat-babe; lane 2, Jurkat-Tip; lane 3, Jurkat-TipY_{114}→S. (B) Downregulation of tyrosine phosphorylation by expression of TipY_{114}→S in Jurkat T cells. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-phosphotyrosine antibody. Reactivity was detected by ECL. Lane 1, Jurkat-babe; lane 2, Jurkat-Tip; lane 3, Jurkat-TipY_{114}→S.

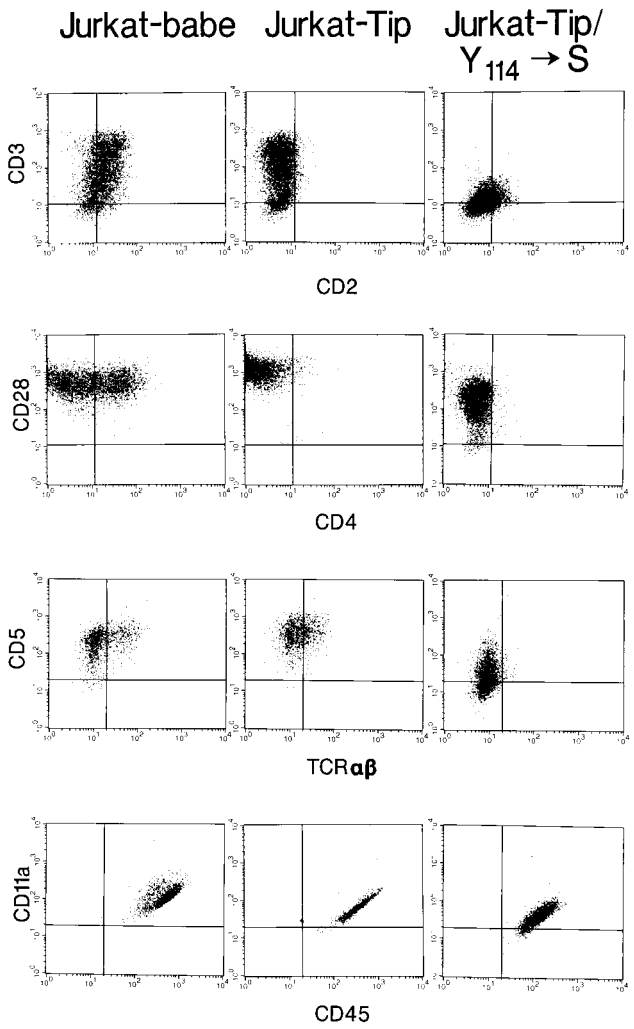

FIG. 3. Suppression of surface expression of lymphocyte antigens by expression of TipY_{114}→S. Five hundred thousand Jurkat-babe, Jurkat-Tip, and Jurkat-TipY_{114}→S cells were washed with RPMI medium containing 10% fetal calf serum and incubated with fluorescein isothiocyanate-conjugated monoclonal antibody (horizontal axis) or phycoerythrin-conjugated monoclonal antibody (vertical axis) for 30 min at room temperature. After washing, cytofluorometric analysis of cell populations was performed with a FACScan.

lular tyrosine phosphorylation was more dramatically suppressed by the expression of TipY_{114}→S in Jurkat T cells than by the expression of wt Tip, we compared the levels of surface expression of lymphocyte antigens on Jurkat-TipY_{114}→S cells to those on Jurkat-babe and Jurkat-Tip cells by fluorescence-activated cell sorter (FACS) analysis. Five hundred thousand cells were washed with RPMI medium containing 10% fetal calf serum and incubated with fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated monoclonal antibody for 30 min at room temperature. After washing, each sample was fixed with 1% formalin solution and cytofluorographic analysis of cell populations was performed with a FACS-1 (Becton Dickinson and Co., Mountain View, Calif.). Antibodies for CD2 (RPA-2.10), CD3 (UCHT1), CD4 (RPA-T4), CD5 (UCHT2), CD28 (CD28.2), CD11a (HI111), and CD45 (HI30) were purchased from PharMingen (San Diego, Calif.), and antibody for T-cell receptor (TCR) αβ (BW242/412) was purchased from T Cell Diagnostics (Cambridge, Mass.). While both Jurkat-Tip cells and Jurkat-TipY_{114}→S cells displayed dramatic reductions in the surface expression of CD2 and CD4 compared to control Jurkat-babe cells, Jurkat-TipY_{114}→S cells additionally exhibited reductions in the surface expression of CD3, CD5, CD28, CD45, and TCRαβ (Fig. 3). These results demonstrate that the expression of TipY_{114}→S in Jurkat T cells induces more drastic reductions of the overall tyrosine phosphorylation and of the surface expression of several T-lymphocyte antigens than the expression of wt Tip. This phenotype of Jurkat-TipY_{114}→S cells was found to be very similar to that of JCaM1 cells, which do not express a functional Lck (26).

Finally, we investigated the effect of the TipY_{114}→S mutant on Lck transforming activity in fibroblasts. Phosphorylation of tyrosine at amino acid position 505 negatively regulates the enzymatic activity of Lck (28). Thus, the substitution mutation (F505) of this tyrosine to phenylalanine results in a constitutively activated version of Lck which transforms fibroblasts to a round neuron-like shape and increases the level of intracellular tyrosine phosphorylation (28). To investigate whether expression of TipY_{114}→S impeded the transforming activity induced by the oncogenic Lck, NIH 3T3 cells expressing constitutively activated F505 Lck were used for transfection with the recombinant retroviral vector pBabe-TipY_{114}→S and cells were selected by growth in medium containing 5 μg of puromycin/ml. Puromycin-resistant cells were examined for the morphological change. As shown previously, the expression of wt Tip only partially blocked the transforming activity of the constitutively active F505 Lck mutant in NIH 3T3 cells (Fig. 4). In contrast, the expression of the TipY_{114}→S in NIH 3T3 F505 Lck cells resulted in a nearly complete reversion of the transformed neuronal-cell appearance to a normal fibroblast appearance (Fig. 4). Thus, this result further demonstrated that the mutation at Y_{114} augmented the ability of Tip to downregulate Lck-mediated signal transduction through an enhanced association with Lck.

In this report, we have shown that a Y_{114} mutant of HVS Tip has greatly enhanced Lck-binding characteristics in comparison with wt Tip. Although cellular tyrosine phosphorylation and surface expression of lymphocyte antigens are suppressed by expression of wt Tip, these phenotypes are much more dramatic in cells expressing a TipY_{114} mutant. Furthermore, TipY_{114} expression blocks the transforming activity of F505 Lck in mouse fibroblasts more severely than wt Tip expression.

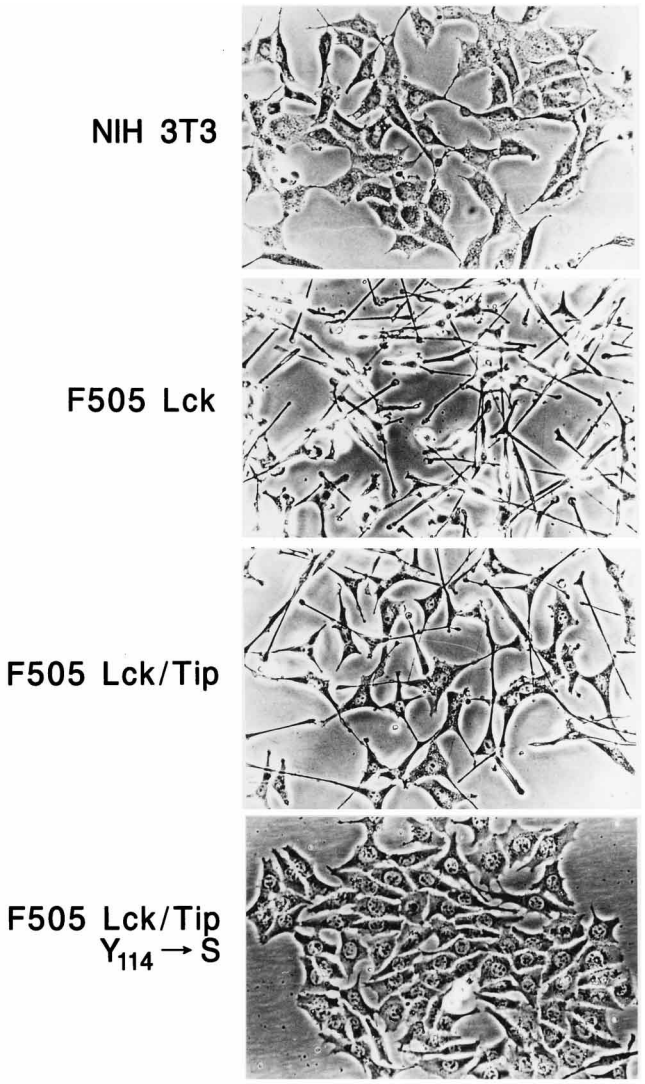

FIG. 4. Suppression of transforming activity of oncogenic F505 Lck by $TipY_{114}\rightarrow S$ in NIH 3T3 cells. NIH 3T3 fibroblasts expressing the F505 mutant form of Lck (F505 Lck) were transfected with retroviral vector pBabe-Tip or pBabe-$TipY_{114}\rightarrow S$. Cells expressing Tip and $TipY_{114}\rightarrow S$ were established by growth in medium containing 5 μg of puromycin/ml. Cells were photographed at a magnification of ×100 with a phase-contrast inverted microscope.

Mutational analysis of Tip shows that binding to Lck requires peptide fragments as short as 37 amino acids containing SH3B and CSKH elements (15). Since Y_{114} does not reside in this region of Tip, the Y_{114} mutation is not likely to directly affect its binding to Lck. Rather, the Y_{114} mutation may cause a conformational change of Tip, which makes the Lck-binding motif more accessible to Lck. Alternatively, the Y_{114} mutation may inhibit the dissociation of Tip from Lck complexes. Further structural analysis of Tip will be required to identify the precise mechanism of action of the Y_{114} mutation.

While Tip is likely to be important for the viral life cycle in T cells in vivo, its specific role has yet to be elucidated. Several reports have demonstrated that Tip associates with Lck (5, 19, 29). While all considered Tip-Lck interaction likely to be important in the biology of HVS, different viewpoints have been expressed regarding whether the interaction results in activation or inactivation of Lck-mediated signal transduction (16, 19, 29). Tip of HVS subgroup C strain 484 (HVS C484) contains a 51-amino-acid deletion at the amino terminus compared to Tip of HVS C488 (19). While Tip of HVS C484 has been shown to activate Lck kinase activity in vitro, its effects on cellular tyrosine phosphorylation and surface expression of lymphocyte antigens have not been demonstrated. Recently, we have shown that the Tip gene is required for T-lymphocyte immortalization in vitro and for lymphoma induction in vivo (10). We have also constructed the recombinant HVS/Tip mSH3B, which contains substitution of alanines for prolines in the SH3B motif of Tip (11). The Tip mSH3B mutant has been shown to be incapable of binding to Lck in vitro and in vivo (15). Analyses of transforming activity of recombinant HVS/Tip mSH3B showed that the interaction of Tip with Lck was not required for in vivo and in vitro oncogenic transformation of HVS. Surprisingly, experimental infection of common marmosets with HVS/Tip mSH3B mutant resulted in more severe fulminant lymphomas than infection with wt HVS (11). The results described here and results with recombinant HVS/tip mSH3B further support the idea that the association of Tip with Lck negatively regulates the cellular signal transduction.

There are intriguing similarities between the Tip gene of HVS and the LMP2A gene of EBV (16). Both genes are similarly located near the ends of their respective genomes. LMP2A is expressed in latently infected B lymphocytes and, like Tip, associates with the major B-cell tyrosine kinases Lyn and Syk. LMP2A blocks reactivation of EBV from latently infected human B lymphocytes by negatively modulating B-cell antigen receptor signaling through its association with Lyn and Syk (20–22). By analogy, Tip-Lck interaction inhibits the T-cell signal cascade by downregulating Lck-mediated activation and it may be important in establishing HVS latency in infected T lymphocytes in vivo. Further analysis of Tip mutants will elucidate the precise roles of Tip-Lck interaction in HVS biology.

J. Guo and M. Duboise contributed equally to this work.

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