Inhibition of T Cell Receptor Signal Transduction by Tyrosine Kinase–interacting Protein of Herpesvirus saimiri

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Abstract

T cells play a central role in orchestrating immunity against pathogens, particularly viruses. Thus, impairing T cell activation is an important strategy employed by viruses to escape host immune control. The tyrosine kinase–interacting protein (Tip) of the T lymphotropic Herpesvirus saimiri (HVS) is constitutively present in lipid rafts and interacts with cellular Lck tyrosine kinase and p80 endosomal protein. Here we demonstrate that, due to the sequestration of Lck by HVS Tip, T cell receptor (TCR) stimulation fails to activate ZAP70 tyrosine kinase and to initiate downstream signaling events. TCR γ chains in Tip-expressing T cells were initially phosphorylated to recruit ZAP70 molecule upon TCR stimulation, but the recruited ZAP70 kinase was not subsequently phosphorylated, resulting in TCR complexes that were stably associated with inactive ZAP70 kinase. Consequently, Tip expression not only markedly inhibited TCR-mediated intracellular signal transduction but also blocked TCR engagement with major histocompatibility complexes on the antigen-presenting cells and immunological synapse formation. These results demonstrate that a lymphotropic herpesvirus has evolved a novel mechanism to deregulate T cell activation to disarm host immune surveillance. This process contributes to the establishment and maintenance of viral latency.

Key words: Lck • ZAP70 • immunological synapse • tyrosine phosphorylation • CD3γ

Introduction

Activation of T cells is initiated when TCRs bind stimulatory peptides in the context of self-MHC molecules. Engagement of TCR triggers an intracellular signaling cascade that culminates in cytokine gene expression, proliferation, and the execution of T cell effector functions (1, 2). Signal transmission from TCR is mediated by sequential activation of Src family kinases, Lck, and Fyn, which phosphorylate tyrosine residues within the immunoreceptor tyrosine-based activation motifs of the CD3 and ζ subunits of TCR (3). The phosphorylation of immunoreceptor tyrosine-based activation motifs allows the tyrosine kinase ZAP70 to be recruited to the receptor complexes where it is phosphorylated and activated. Upon activation, Lck and ZAP70 presumably act in concert to propagate signals from TCR and lead to Ca²⁺ mobilization and the transcription of gene products for T cell activation and proliferation.

Several recent papers have described the spatial and temporal recruitment of signaling molecules to an organized contact zone known as the immunological synapse (2). The mature immunological synapse is a highly organized interaction site at the interface between a T cell and an APC. At the immunological synapse, TCRs, coreceptors, adhesion molecules, and signaling molecules are assembled. An elaborately choreographed series of movements of signaling molecules including Lck, ZAP70, LAT, and PKCθ to the immunological synapse has been shown upon TCR stimulation (4–6). Particularly, ZAP70 has been shown to be necessary to supply the synapse with LAT and PKCθ, indicating that ZAP70-dependent signaling is required for the formation of a functional immune synapse (5). Furthermore, recent studies have demonstrated that TCR signaling precedes immunological synapse formation and that the immunological synapse acts as a type of adaptive controller that not only boosts TCR signaling but also attenuates strong signals (7, 8).

Herpesvirus saimiri (HVS), an oncogenic γ2 herpesvirus, naturally infects squirrel monkeys of South America. HVS persists in T lymphocytes of the natural host without any
were mixed at a 1:1 ratio, placed on poly-
were stained and analyzed by confocal microscopy.
were mapped to 37 amino acid residues of Tip, termed the Lck-binding domain (LBD). The LBD consists of two motifs: a COOH-terminal Src family kinase homology motif and an SH3-binding motif (12). Tip has been also shown to interact with a novel cellular endosomal protein, p80, that contains an NH2-terminal WD (tryptophan-aspartic acid) repeat domain and a COOH-terminal coiled-coil domain (13). Interaction of Tip with p80 facilitates endosomal vesicle formation (13). Our recent study has shown that the Tip–Lck interaction recruits TCR complex to lipid rafts, and the Tip–p80 interaction subsequently induces the aggregation and internalization of lipid rafts and thereby down-regulates the TCR complex (14). Thus, the signaling and targeting functions of HVS Tip rely on two functionally and genetically separable mechanisms that independently target cellular Lck tyrosine kinase and p80 endosomal protein.

Although the interaction of HVS Tip with Lck significantly down-regulates TCR signal transduction, the detailed mechanism is mostly unknown. To further delineate the effect of HVS Tip on TCR signal transduction, we examined the effect of Tip expression on T cells upon stimulation. In this report, we demonstrate that Tip expression inhibits the activation of T cells through the precise interruption of the phosphorylation and activation of ZAP70 kinase upon stimulation. This inhibitory activity of Tip ultimately blocks inophosphorylation and activation of ZAP70 kinase upon stimulation. This inhibitory activity of Tip ultimately blocks immunological synapse formation. This inhibitory activity of Tip ultimately blocks inophosphorylation and activation of ZAP70 kinase upon stimulation. This inhibitory activity of Tip ultimately blocks immunological synapse formation. This inhibitory activity of Tip ultimately blocks immunological synapse formation. 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induced intracellular calcium mobilization, we included numerous Tip mutants in the same assay. These mutants were: TipmLBD, which does not interact with Lck; Tip LBD, which contains only 37 amino acids of the Lck-binding domain and is able to bind to Lck; Tip H9004, which does not interact with p80 (13); Tip Y114F, which does not interact with STAT3 (17); Tip Y127F, which lacks tyrosine phosphorylation at Y127; and Tip H9004TM, which has a deletion of the COOH-terminal transmembrane region. Flow cytometry showed that Tip H9004, Tip H9004TM, Tip Y114F, and Tip Y127F were capable of inhibiting the elevation of intracellular calcium concentration upon anti-CD3 stimulation as efficiently as WT Tip. Thus, the interaction with p80 and STAT, the tyrosine phosphorylation at Y114 or Y127 residues, and the COOH-terminal transmembrane region are not necessary for Tip to down-regulate TCR signal transduction (Fig. 1 B). By contrast, both TipmLBD and Tip LBD were incapable of inhibiting the increase of intracellular calcium concentration, indicating that Lck interaction is necessary but not sufficient for Tip to down-regulate TCR signal transduction (Fig. 1 B).

Furthermore, the surface expression of CD69 early lymphocyte activation marker, which is the consequence of TCR signal transduction, was measured on CD2+ primary T cells infected with lentivirus containing GFP, Tip, or TipmLBD and on Jurkat T cells electrooporated with pTracer, pTracer/Tip, or pTracer/Tip H9004 expression vector upon TCR stimulation. PMA stimulation was included as a control. As seen with IL-2 production and intracellular calcium mobilization, T cells expressing GFP or the TipmLBD mutant up-regulated the surface expression of CD69 upon TCR stimulation, whereas T cells expressing WT Tip or Tip H9004 mutant showed little or no increase of CD69 surface expression (Fig. 1 C). CD3 and/or CD4 surface expression was detected on CD2+ primary T cells and Jurkat T cells at equivalent levels before anti-CD3 stimulation (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20040924/DC1). These results indicated that Tip expression effectively inhibits TCR signal transduction, evidenced by the suppression of IL-2 production, intracellular calcium mobilization, and CD69 surface expression, and that Lck interaction is required but not sufficient for Tip to exhibit such activity.

**Selective Inhibition of ZAP70 Activation by Tip Expression.**

The phosphorylation of CD3z by Lck and the subsequent recruitment and activation of ZAP70 kinase are proximal signaling events of TCR signal transduction. Particularly, the phosphorylation of the Y319 residue of ZAP70 has been
shown to be a critical event for T cell activation (18). To delineate the molecular mechanism of Tip-mediated inhibition of TCR signal transduction, Jurkat T cells were infected with lentivirus carrying GFP or WT Tip, stimulated with anti-CD3 antibody–coated beads for the indicated times, and then examined for the tyrosine phosphorylation of CD3ζ and ZAP70. Phospho-specific CD3ζ (pCD3ζ) and ZAP70 Y319 [pZAP70 Y319]) antibodies were used for immunoblot assay. The result showed that upon anti-CD3 stimulation GFP-expressing Jurkat cells showed robust tyrosine phosphorylation of the CD3ζ chain and ZAP70 Y319 residue (Fig. 2 A). Despite the strong tyrosine phosphorylation of CD3ζ, however, Jurkat T cells expressing Tip displayed little or no phosphorylation of ZAP70 at Y319 (Fig. 2 A). By striking contrast, Jurkat T cells expressing TipmLBD showed robust phosphorylation of both CD3ζ and ZAP70 Y319 upon TCR stimulation, as seen in GFP-expressing Jurkat T cells (Fig. 2 B). Both CD3ζ and ZAP70 expression levels were equivalent in Jurkat-GFP, Jurkat-Tip, and Jurkat-TipmLBD cells (Fig. 2, A and B).

To further detail the Tip-mediated inhibition of ZAP70 activation, we investigated whether Tip expression affected the recruitment of ZAP70 into the tyrosine phosphorylated CD3ζ chain upon TCR stimulation. Jurkat T cells infected with GFP or Tip lentivirus were treated with or without anti-CD3 antibody–coated beads, and their lysates were used for immunoprecipitation with anti-CD3ζ or anti-ZAP70 antibody, followed by immunoblotting with anti-CD3ζ, anti-phospho CD3ζ, anti-phospho CD3ζ, or anti-Tip antibody. (B) Requirement of Lck interaction for Tip to inhibit the activation of ZAP70 kinase. (Left) At 24 h postinfection with GFP lentivirus (lanes 1 and 2), Tip lentivirus (lanes 3 and 4), or TipmLBD lentivirus (lanes 5 and 6), Jurkat T cells were unstimulated (lanes 1, 3, and 5) or stimulated (lanes 2, 4, and 6) with an anti–CD3 antibody–coated beads for 5 mn. WCL were used for immunoblotting (IB) with anti-ZAP70, anti-phospho ZAP70 Y319, anti-CD3ζ, anti-phospho CD3ζ, or anti-Tip antibody. (Right) Lysates of Jurkat T cells infected with GFP lentivirus (lanes 1 and 2) or Tip lentivirus (lanes 3 and 4), Jurkat T cells were unstimulated (lanes 1 and 3) or stimulated (lanes 2 and 4) with an anti–CD3 antibody–coated beads for 5 mn. WCL were used for immunoprecipitation (IP) with anti-ZAP70, anti-CD3ζ, or anti-PLCγ1 antibody. Each immunoprecipitate was IB with anti-phospho PLCγ1, anti-PLCγ1, anti-phospho ZAP70 Y191, anti-ZAP70, anti-phospho CD3ζ, or anti-CD3ζ antibody. Cell lysates were also used for IB with anti-phospho-LAT Y191 and anti-LAT antibodies.

Inhibition of Immunological Synapse Formation by Tip. One of the first phenomena described during TCR activation by APCs is the clustering of the TCR–CD3 complex in the zone of contact, resulting in the formation of an immunological synapse (2). Several recent papers have shown that this clustering of receptors, adhesion molecules, and signaling molecules is dependent on signaling events mediated by Lck and ZAP70 (5, 19). Since Tip and Lck interaction effectively inhibited ZAP70 activation induced by TCR stimulation, we investigated whether Tip affected the formation of the immunological synapse. To do this, Jurkat T cells electroporated with vector or GFP–Tip fusion vector were engaged with Raji B cells primed with SEE, and the localization of CD3ζ, LFA-1, Lck, and cellular tyrosine phosphorylation was then detected by the corresponding antibodies and examined by confocal microscopy. Jurkat T cells in the control set showed well-organized recruitment of CD3ζ, LFA-1, and Lck to the zone of contact between T cells and B cells in the presence of SEE (Fig. 3 A). In ad-
dition, the intracellular localized appearance of tyrosine-phosphorylated proteins was strongly detected at the T cell–APC contact area in control Jurkat T cells (Fig. 3). By striking contrast, the recruitment of CD3ζ, LFA-1, Lck, and tyrosine phosphorylated proteins to the T cell–APC contact area was remarkably impaired in Jurkat T cells expressing Tip–GFP under the same conditions (Fig. 3). Specifically, instead of being recruited to the immunological synapse area, these proteins accumulated in the intracellular enlarged endosomal compartments of the cells; this localization has been shown to be induced by Tip and p80 interaction (13) (Fig. 3). However, Jurkat T cells expressing TipmLBD showed concentrations of CD3ζ, LFA-1, Lck, and tyrosine phosphorylated proteins at the contact areas between T cells and APCs as strongly as control Jurkat cells (Fig. 3). This indicates that Tip expression efficiently inhibits immunological synapse formation and that this activity of Tip requires Lck interaction.

Using confocal microscopy, we also estimated the percentages of T cell–APC conjugates that showed the enhanced recruitment of CD3ζ and Lck to the contacting synaptic area (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20040924/DC1). Approximately 65% of conjugates between Jurkat T cells and Raji B cells showed the cluster of CD3ζ and Lck at the synaptic area in the presence of SEE (Fig. S2 A). By contrast, only 15% of conjugates between Jurkat-Tip–GFP T cells and Raji B cells showed the cluster of CD3ζ and Lck at the synaptic area in

Figure 3. Inhibition of immunological synapse formation by Tip. Jurkat T cells (T) electroporated with vector (vec), GFP-Tip, or GFP-TipmLBD fusion vector where Tip was in-frame fused into the COOH terminus of GFP were mixed with SEE-primed Raji B cells (B) for 15 min. Cells were permeabilized, reacted with the indicated antibodies, and subjected to confocal microscopy. P-Y indicates tyrosine-phosphorylated proteins. Cells were also visualized with Nomarski optics.
the presence of SEE (Fig. S2 A). This result was almost comparable to that observed when Jurkat T cells were incubated with unprimed APCs. In addition, ~70% of conjugates between Jurkat-TipmLBD-GFP T cells and SEE-primed Raji B cells also showed the cluster of CD3ζ and Lck in the synapatic area (Fig. S1 A).

Finally, we investigated whether, besides blocking immunological synapse formation, Tip also inhibited the conjugation between T cell and APC. To do this, we used a flow cytometry-based assay as described previously (15). Raji B cells were stained with HE, incubated with or without SEE, and mixed with Jurkat T cells expressing GFP, Tip-GFP, or TipmLBD-GFP at a 1:1 ratio. Although a low level of conjugation between Jurkat-GFP or Jurkat-TipmLBD T cells and Raji B cells was detected in the absence of SEE, the percentage of conjugation between these cells increased by approximately three- to fivefold in the presence of SEE (Fig. S2 B). However, Jurkat T cells expressing WT Tip-GFP fusion showed little or no increase of conjugation between T and B cells induced by SEE treatment (Fig. S2 B). These results collectively indicate that Tip inhibits the movement of TCR and Lck to the site of contact with SEE-primed APC, suppresses the formation of the immunological synapse, and ultimately abrogates the activation of TCR stimulation.

Because of its biological significance in orchestrating immunity against pathogenic challenges, particularly viral infections, TCR signal transduction is a common target of immunosuppressive drugs. We demonstrated that, due to the sequestration of Lck by HVS Tip, TCR stimulation fails to activate ZAP70 tyrosine kinase and to initiate downstream signaling events. CD3ζ chains in Tip expressing T cells were initially phosphorylated to recruit ZAP70 kinase upon TCR stimulation, but the recruited ZAP70 molecule was not subsequently phosphorylated, resulting in TCR complexes that were stably associated with inactive ZAP70 kinase. This phenomenon is strikingly similar to that in CD4+ and CD8+ thymocytes whose TCR signaling has been shown to be blocked due to the occupation of TCR ζ chains with inactive ZAP70 kinase (22). Furthermore, TCR signaling in CD4+CD8+ thymocytes is impaired because the number of available Lck molecules is diminished by intrathymic CD4–MHC II interactions that initially activate Lck molecules, which are subsequently degraded (22). We have also shown that Tip recruits Lck to the lysosomal compartments where it likely undergoes degradation (13). This suggests that, as seen in thymocytes (22), HVS utilizes Tip–Lck interaction to block TCR signal transduction, which renders virus-infected cells inert and unresponsive to stimulus. Thus, impairment of the TCR signal transduction pathway, in particular Lck and ZAP70 function, may be a vital strategy employed by T lymphotrophic HVS to escape host immune control and maintain latency.

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