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# Cutting Edge: Constitutive Activation of TCR Signaling Molecules in IL-2-Independent *Herpesvirus saimiri*-Transformed T Cells[1]

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**Both human T cell leukemia virus type I and simian *Herpesvirus saimiri* (HVS) transform human T cells in vitro. Although IL-2-independent growth in human T cell leukemia virus type I-transformed T cells is associated with constitutive phosphorylation of JAK/STAT kinases, we now demonstrate that different mechanisms may be responsible for the ability of HVS-transformed T cells to proliferate in the absence of exogenous cytokines. The IL-2 independence of an HVS-transformed cell line correlated with constitutive activation of protein tyrosine kinases known to be induced following TCR engagement. Thus, in these cells we observed increased phosphotransferase activity of Lck as well as constitutive tyrosine phosphorylation of the TCR-associated ZAP-70 kinase and expression of the related Syk protein tyrosine kinase. While Syk is generally not expressed in activated T cells, its introduction has been shown to enhance TCR responsiveness. These results suggest that distinct signal transduction cascades can participate in the transition of T cells to IL-2 independence.** ***The Journal of Immunology,*** **1998, 160: 2042–2045.**

Human T cells can be transformed following infection with either the human T cell leukemia retrovirus type I (HTLV-I)[3] or the simian *Herpesvirus saimiri* (HVS) (1, 2). HVS persists without apparent infection in squirrel monkeys, its natural host. However, HVS is oncogenic when transferred to other primate species, resulting in lymphomas, leukemias, and acute lymphoproliferative syndromes (3, 4).

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[3] Abbreviations used in this paper: HTLV-1, human T cell leukemia retrovirus type I; HVS, *Herpesvirus saimiri*.

T cells transformed by HTLV-I are initially IL-2 dependent, but can transition to an IL-2-independent stage (5, 6). The acquisition of IL-2 independence has recently been shown to be correlated with activation of a group of JAK/STAT kinases (7, 8). Constitutive activation of JAK-STATs in IL-2-independent HTLV-I-transformed cells as well as in acute B cell leukemia cells, chronic myelogenous leukemia cells, and B cells transformed with the Abelson virus (9–11) led to the hypothesis that stimulation of this pathway is a general strategy enabling an abnormal regulation of lymphoid cell growth. However, the mechanism by which human T cells transformed with HVS proliferate in the absence of IL-2 has not been elucidated. Although a majority of T cells transformed with HVS require exogenous IL-2 to support long term cell growth (2), a small percentage of HVS-transformed lines can be maintained in the absence of exogenous cytokines (2, 12, 13).

In this study we show that IL-2-independent growth of an HVS-transformed T cell line is associated with constitutive activation of Lck and ZAP-70, kinases required for propagation of the TCR signaling cascade. Moreover, Syk, a protein tyrosine kinase not normally expressed in activated T cells but whose presence augments TCR responsiveness (14), is only expressed in the IL-2-independent HVS-transformed cell line. Thus, it appears that activation of distinct pathways can result in dysregulated cell growth and transformation of lymphoid cells.

## Materials and Methods

### *Cytokines and Abs*

Recombinant human IL-2 was obtained from Chiron Corp. (Paris, France). Abs against ZAP-70, CD3 (UCHT1), JAK3, Syk, and Lck were gifts from A. Weiss, K. Soo, J. O'Shea, and B. Sefton, respectively. The 4G10 monoclonal anti-phosphotyrosine Ab was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

### *Cell lines*

Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS. The IL-2-independent human leukemia T cell line, Jurkat and the HTLV-I-transformed human T cell line, MT-2, were obtained from the American Type Culture Collection (Rockville, MD). Establishment of HTLV-I- and HVS-transformed $CD4^+$ T cell lines has been previously described (13, 15, 16). Briefly, the MB-HTLV-I cell line was maintained in medium containing IL-2 (50 U/ml), and after 6 mo in culture, this cell line expressed TCR-$\alpha/\beta$ as well as CD4. No cell growth was observed in the absence of IL-2, confirming the IL-2-dependent nature of these cells. The CH-HVS and MH-HVS cell lines were established after infection with HVS, group C, strain 488-77 (13). MH-HVS cells were maintained in culture for >1 yr in the absence of IL-2. Although these cells expressed CD2, CD3, CD4, TCR-$\alpha\beta$, HLA-DR, and CD69, expression of the IL-2R $\alpha$-chain was not detectable by FACS analysis. In contrast, the CH-HVS cell line required the addition of exogenous IL-2 (50 U/ml) for continued



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cell growth (data not shown). This is comparable to the growth characteristics observed for other IL-2-dependent cell lines transformed with HVS strain 488-77 (2, 12). In addition to high levels of expression of the IL-2R $\alpha$-chain, CH-HVS cells expressed CD2, CD3, CD4, TCR-$\alpha\beta$, HLA-DR, and CD69 as assessed by FACS analysis.

### *Immunoprecipitation and Western blotting analysis*

Cells were stimulated with either IL-2 ($10^3$ U/ml) for 10 min or the anti-CD3 UCHT1 Ab (10 $\mu$g) for 3 min. Cells were lysed in a 1% Nonidet P-40 lysis buffer, and proteins were immunoprecipitated with the specified Ab at 4°C. Following separation on SDS-polyacrylamide gels, membranes were probed with the 4G10 anti-phosphotyrosine mAb as previously described (17). Blots probed with polyclonal anti-JAK3, Lck, and Syk Ab or with ZAP-70 mAb were blocked in 150 mM NaCl and 20 mM Tris, pH 7.5, containing 5% BSA and 0.1% Tween-20, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Les Ulis, France), and visualized using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

### *In vitro kinase assays*

Immunoprecipitates were washed twice in kinase buffer (20 mM Tris-HCl, pH 7.5, and 10 mM $MnCl_2$) and then resuspended in 25 $\mu$l of kinase buffer containing 10 $\mu$Ci of [$\gamma^{32}$-P]ATP (Amersham) and 1 $\mu$M ATP (Boehringer Mannheim, Meylan, France) for 15 min at 25°C. Alternatively, immunoprecipitates were resuspended in kinase buffer containing 2.5 $\mu$g of enolase (Sigma Chemical Co., St. Louis, MO) as a substrate. Precipitates were resolved on SDS-PAGE and visualized by autoradiography. Quantitative analysis was performed on a PhosphorImager (model 445SI, Molecular Dynamics, Sunnyvale, CA).

## Results and Discussion

### *IL-2-independent cell growth of HVS-transformed T cells is not associated with constitutive JAK3 phosphorylation*

The JAK3 kinase associates directly with the common $\gamma$-chain of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, and its phosphorylation is a marker of receptor stimulation (18, 19). As previously reported, we found that the extent of constitutive JAK3 phosphorylation correlated with the acquisition of IL-2-independence in HTLV-I-transformed cells (7, 8) (Fig. 1*A*). Additionally, both IL-2-dependent and -independent HTLV-I cell lines maintained their ability to respond to IL-2, as demonstrated by increased tyrosine phosphorylation of JAK3 upon IL-2 stimulation (Fig. 1*A*). To address the question of whether the IL-2R pathway is constitutively activated in HVS-transformed T cells and whether constitutive activation correlates with IL-2 independence, the tyrosine phosphorylation status of JAK3 was assessed in HVS-transformed T cells. Interestingly, no constitutive tyrosine phosphorylation of JAK3 was observed in either IL-2-dependent or -independent HVS-transformed cells (Fig. 1*A*). This was not due to an intrinsic defect in JAK3, since it was phosphorylated following IL-2 stimulation in both the IL-2-dependent and the IL-2-independent HVS cell lines (Fig. 1*A*). Nevertheless, significantly lower levels of phosphorylated JAK3 were detected in the IL-2-independent HVS cells following stimulation, probably due to very low levels of IL-2R $\alpha$-chain on the cell surface (as assessed by FACS analysis, data not shown) and consistently lower levels of JAK3 protein in these cells (Fig. 1*B*). The absence of IL-2R on the cell surface, the lack of modulation of cell growth in response to either exogenous IL-2 or anti-IL-2R Abs (CD25), and the inability to detect endogenous IL-2 secretion from these cells strongly suggest that there is no autocrine IL-2 activation loop (data not shown). Additionally, neither STAT3 nor STAT5, proteins activated downstream of JAK3 in the IL-2R signaling cascade, were constitutively phosphorylated in the IL-2-independent HVS-transformed cells (data not shown). These data indicate that the JAK/STAT kinases, normally involved in the IL-2R signaling cascade, do not play a role in the IL-2-independent cell growth of HVS-transformed T cells.

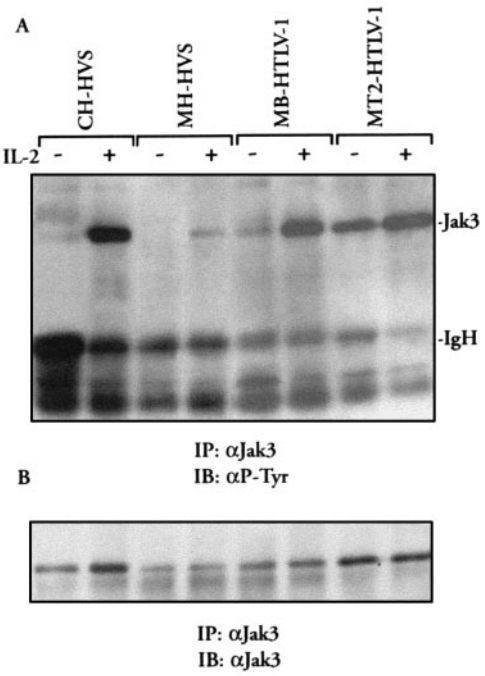


**FIGURE 1.** Constitutive tyrosine phosphorylation of JAK3 in IL-2-independent HTLV-I-transformed, but not in HVS-transformed, cell lines. HTLV-I- and HVS-transformed cell lines were stimulated with IL-2, and lysates from $3 \times 10^6$ IL-2-dependent CH-HVS and MB-HTLV-I cells as well as IL-2-independent MH-HVS and MT-2-HTLV-I cells were immunoprecipitated (IP) with a rabbit polyclonal anti-JAK3 Ab, fractionated on a 7.5% polyacrylamide gel, immunoblotted (IB) with an anti-phosphotyrosine mAb, and developed with enhanced chemiluminescence to assess the phosphorylation status of JAK3 (*A*). Blots were then stripped and reprobed with the same anti-JAK3 Ab to assess the levels of JAK3 protein in each lane (*B*). The positions of JAK3 and the Ig heavy chain (IgH) are indicated.

### *Lck kinase activity is increased in IL-2 independent HVS-transformed cells*

As constitutive activation of the IL-2R pathway was not observed in the HVS-transformed T cells, we assessed whether an alternative signaling cascade might be associated with the IL-2-independent growth of these cells. The TCR signaling pathway was monitored, since short term proliferation can be induced in primary human T cells upon stimulation of this receptor. Following TCR engagement, the Lck tyrosine kinase is one of the first molecules to be activated and is essential for appropriate TCR signal transduction (20, 21). Thus, the kinase activity of Lck was measured in HVS- as well as HTLV-I-transformed T cells.

As demonstrated by the level of in vitro phosphorylated Lck and the ability of Lck immunoprecipitates to phosphorylate the enolase substrate in an in vitro kinase assay, the enzymatic activity of Lck was significantly higher in the IL-2-independent HVS cell line than in the IL-2-dependent HVS line (Fig. 2, *A* and *B*). The phosphorylation of enolase was 5.0-fold higher in Lck immunoprecipitates from the IL-2-independent HVS cell line (Fig. 2*B*) despite equivalent levels of Lck protein in the IL-2-independent and -dependent cell lines (Fig. 2*C*). Both 56- and 59-kDa forms of Lck were observed in these cells, with the higher molecular mass form reported to represent a more highly phosphorylated active state (22).

The p59 form of Lck was barely detectable in the IL-2-dependent HTLV-I line (Fig. 2, *A* and *C*), and neither form of Lck was

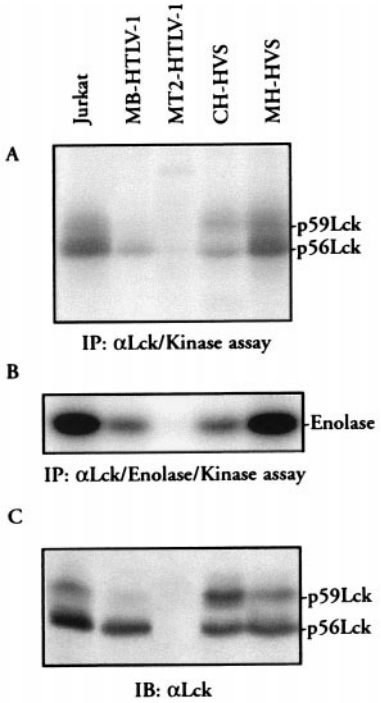


**FIGURE 2.** In vitro kinase activity of Lck is increased in IL-2-independent HVS-transformed cells. Lysates ($3 \times 10^6$ cells) were immunoprecipitated with a polyclonal anti-Lck Ab. Immune complexes were subjected to an in vitro kinase assay with [$\gamma$-$^{32}$P]ATP in the absence (*A*) or the presence (*B*) of enolase as an exogenous substrate. Labeled proteins were separated on a 7.5% polyacrylamide gel and visualized by autoradiography (*A*). Aliquots from each sample were also analyzed concurrently by an anti-Lck immunoblot (*C*). These data are representative of results obtained in three independent experiments.

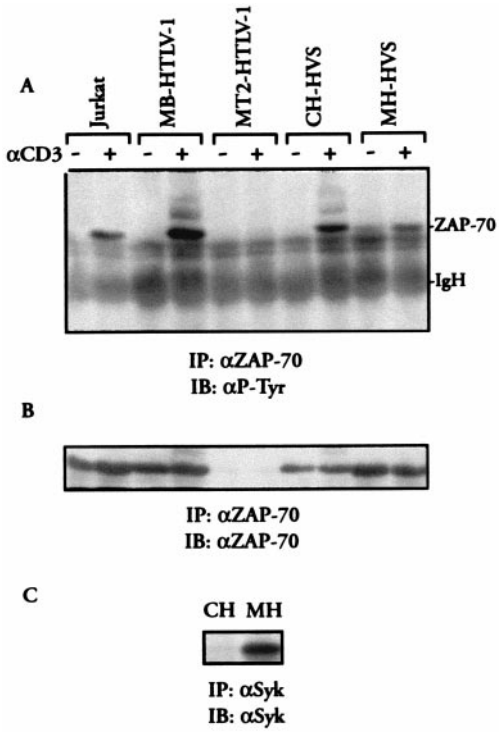


**FIGURE 3.** ZAP-70 is constitutively tyrosine phosphorylated in IL-2-independent HVS-transformed cells. ZAP-70 was immunoprecipitated from either unstimulated (−) or anti-CD3 stimulated (+) cells. Immunoprecipitated complexes were fractionated on a 7.5% polyacrylamide gel and immunoblotted with an anti-phosphotyrosine mAb (*A*). The positions of ZAP-70 and the Ig heavy chain (IgH) are indicated. The blot was stripped and reprobed with an anti-ZAP-70-specific mAb (*B*). Expression of Syk in HVS-transformed T cells was assessed by immunoblotting Syk immunoprecipitates with an anti-Syk polyclonal Ab (*C*).

detected in the IL-2-independent HTLV-I line due to the previously reported inhibition of Lck transcription in these cells (Fig. 2*B*) (23). It is not clear whether higher levels of the p59 form of Lck in HVS-transformed cells compared with those in HTLV-I-transformed cells are directly due to the nature of the transforming virus. However, among the HVS cell lines, IL-2-independent growth was associated with a significantly higher level of Lck kinase activity, a transition distinctly different from that observed in IL-2-independent HTLV-I-transformed cells.

It remains to be determined whether the observed Lck activity in HVS-transformed cells is directly modulated by the expression of viral proteins. The 40-kDa HVS-encoded protein, Tip, is known to interact specifically with Lck and is a substrate for this T cell-specific kinase (24). However, in the presence of Tip, both increases and decreases in Lck activity have been reported (25–27); thus, the functional consequences of this association remain controversial. Since the level of Tip in IL-2-dependent HVS-transformed cells is not detectable by immunoblot analysis, previous analyses were performed in transfected cell lines using high level expression vectors, making it difficult to determine the effect of Tip in the context of HVS transformation (25, 27). In our studies, IL-2-independent growth did not appear to be associated with an increase in the level of Lck-associated Tip in kinase assays. Indeed, preliminary evidence suggests that the level of phosphorylated Tip may be decreased in the IL-2-independent line compared with that in the IL-2-dependent line (N. Noraz, unpublished observations).

### *Differential phosphorylation and expression of ZAP-70 and Syk in IL-2-independent HVS-transformed cells*

To further assess whether the TCR pathway is activated in IL-2-independent HVS-transformed T cells, basal and CD3-stimulated phosphorylation of ZAP-70 were compared in the various HVS- and HTLV-I-transformed cells. Although Lck may be activated by both IL-2R and TCR stimulation (28), ZAP-70 phosphorylation is only observed following TCR engagement. Upon TCR cross-linking, activated Lck phosphorylates the ZAP-70 protein tyrosine kinase, which is recruited to the CD3 $\zeta$-chain (29). These events are among the earliest steps following TCR stimulation and are required for propagation of the signaling cascade (30).

ZAP-70 was phosphorylated following CD3 cross-linking in the IL-2-dependent and -independent HVS cell lines as well as in the IL-2-dependent HTLV-I cell line, but was constitutively phosphorylated only in the IL-2-independent HVS cells (Fig. 3*A*). Interestingly, although ZAP-70 is generally expressed in all T cells, it was not detected in the IL-2-independent-HTLV-I cell line (Fig. 3*B*) (31). The lack of expression of Lck and ZAP-70 in IL-2-independent HTLV-I-transformed cells suggests that the TCR cascade is unlikely to play a role in the IL-2-independent growth and stimulation of these cells.

The Syk protein kinase is closely related to ZAP-70, but while the ZAP-70 protein tyrosine kinase is generally expressed in all T cell subsets, Syk has only been detected in thymocytes, $\gamma\delta$ T cells, and naive, but not proliferating, $\alpha\beta$ T cells (32–34). Latour and co-workers have recently shown that transfection of Syk into a

ZAP-70-expressing Ag-specific murine T cell line greatly enhanced cell responsiveness to TCR stimulation, an effect not mimicked by overexpression of ZAP-70 (14). It was therefore of interest to determine whether Syk was expressed in T cells following HVS transformation. Indeed, we found that Syk was only expressed in the IL-2-independent HVS cells (Fig. 3*C*). Thus, it is likely that expression of Syk in HVS-transformed T cells is associated with a selective growth advantage upon transition to an IL-2-independent stage. Together these results show that IL-2-independent growth of HVS-transformed T cells is correlated with an altered regulation of Lck and ZAP-70 activation as well as Syk expression. Previous studies demonstrating hyper-reactivity of the CD2 signaling pathway in HVS-transformed cells as well as activation of the Ras signaling pathway through interaction of Ras with the HVS-encoded protein STP-C488 (35, 36) suggest that stimulation of one or both of these pathways in conjunction with the CD3 signaling cascade may allow IL-2-independent growth following HVS transformation.

The activation of distinct signaling pathways in IL-2-independent HTLV-I- and HVS-transformed T cells suggests that T cell transformation is preferentially mediated by different mechanisms in the presence of these two viruses. As it is difficult to propagate primary T cells, extensive physiologic studies of human T cells have been performed following their transformation with either HTLV-I or HVS. Further comparisons and identifications of additional downstream targets in HTLV-I- and HVS-infected cells will help to elucidate the mechanisms of T cell transformation.

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