Constitutively Activated Jak-STAT Pathway in T Cells Transformed with HTLV-I

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Human T cell lymphotropic virus I (HTLV-I) is the etiological agent for adult T cell leukemia and tropical spastic paraparesis (also termed HTLV-I–associated myelopathy). HTLV-I–infected peripheral blood T cells exhibit an initial phase of interleukin-2 (IL-2)–dependent growth; over time, by an unknown mechanism, the cells become IL-2–independent. Whereas the Jak kinases Jak1 and Jak3 and the signal transducer and activator of transcription proteins Stat3 and Stat5 are activated in normal T cells in response to IL-2, this signaling pathway was constitutively activated in HTLV-I–transformed cells. In HTLV-I–infected cord blood lymphocytes, the transition from IL-2–dependent to IL-2–independent growth correlated with the acquisition of a constitutively activated Jak-STAT pathway, which suggests that this pathway participates in HTLV-I–mediated T cell transformation.

The interaction of IL-2 with its receptor regulates the magnitude and duration of the response of T cells to an antigen (*1*). IL-2 signaling requires the dimerization of the cytoplasmic domains of the β chain (IL-2Rβ) and γ chain (IL-2Rγ) of the IL-2 receptor (*2*). IL-2Rγ is denoted as the common cytokine receptor γ chain γ_c, because it is a component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (*3–7*). IL-2Rβ associates with Jak1 and γ_c associates with Jak3 (*6, 8*), two Janus family tyrosine kinases that are activated in response to IL-2 (*6, 9*). IL-2 rapidly activates Stat5 in resting peripheral blood lymphocytes (PBL) and activates both Stat5 and Stat3 in PBL when they are pre-treated with phytohemagglutinin (PHA) (*10*). The activation of these STAT proteins appears to contribute to the control of cellular proliferation. Cells expressing receptor mutants show diminished responses to IL-2 when tyrosines in the cytoplasmic domain of IL-2Rβ that are essential for STAT protein docking and activation are replaced by phenylalanines (*10, 11*).

HTLV-I is a retrovirus that can immortalize and transform human $CD4^+$ T cells (*12*). After infection of T cells by HTLV-I, there is an initial phase of IL-2–dependent cell growth, followed by an IL-2–independent phase (*13*). To investigate the potential role of the Jak-STAT pathway in HTLV-I–mediated T cell growth, we examined HTLV-I–transformed T cell lines for the presence of nuclear complexes capable of binding to the γ interferon activated sequence (GAS) motif from the FcγRI promoter. The GAS site binds IL-2–induced complexes in normal PHA-activated PBL (*10, 14*). In contrast to PBL treated with PHA, in which complexes were seen only after stimulation of cells with IL-2 (*10, 14*) (Fig. 1A), constitutive complexes were seen in two T cell lines, MT-2 (Fig. 1A) and HUT-102B2 (*15*), that are transformed with HTLV-I. The fact that IL-2 increased the DNA binding activity in these cells indicates that they retain the ability to transduce at least some IL-2 signals, even though these and other HTLV-I–transformed T cell lines do not produce IL-2 nor require IL-2 for survival (*12, 15, 16*). Similar constitutive nuclear complexes were not observed in T cell lines not transformed by HTLV-I, such as Jurkat and HUT-78 T cells (*15*), nor in the absence of IL-2 stimulation in IL-2–dependent CTLL-2 (*10*) and Kit-225 (*14*) T cell lines. Of the two complexes seen in PHA-treated PBL, cells transformed with HTLV-I contained more of the complex with slower mobility (Fig. 1A); nevertheless, cross-linking mediated with ultraviolet (UV) light revealed the presence of the same adducts in both cell types (Fig. 1B). Both Stat3 and Stat5 are tyrosine-phosphorylated in response to IL-2 in PHA-treated PBL (*10*) (Fig. 1C). These same STAT proteins were constitutively tyrosine-phosphorylated in MT-2 cells (Fig. 1C), and their phospho-

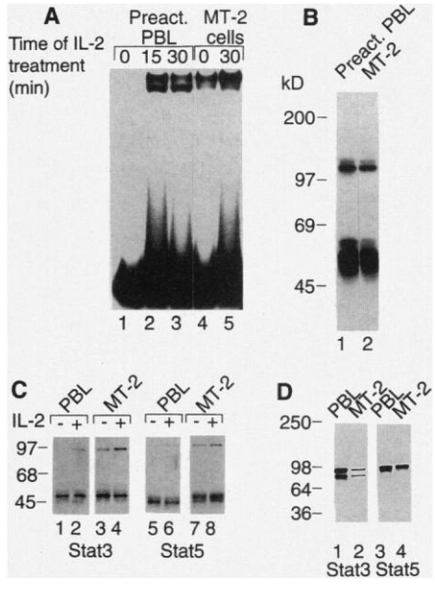

Fig. 1. Constitutive but inducible Stat3 and Stat5 activation in HTLV-I–transformed T cell lines. (**A**) PBL preactivated by treatment with PHA (preact. PBL) (lanes 1 to 3) or HTLV-I–transformed MT-2 cells (lanes 4 and 5) were not stimulated (lanes 1 and 4) or were stimulated with 2 nM IL-2 for the indicated times (lanes 2, 3, and 5). Nuclear extracts were prepared, and electrophoretic mobility-shift assays (EMSAs) were done as described (*10*) with the FcγRI probe (5′-AGCTTGTATTTCCCAGAAAAGGGATC-3′, GAS motif underlined) from the FcγRI promoter. We prepared PHA-treated PBL by isolating PBL using Lymphocyte Separation Medium (Organon Teknika Durham, North Carolina), culturing them for 72 hours in RPMI 1640 medium containing fetal bovine serum (FBS) (10%) and PHA (1 μg/ml, Boehringer Mannheim), and resting them overnight in RPMI 1640 containing FBS (10%). Cells were washed and resuspended in fresh medium and stimulated for the indicated times with 0 or 2 nM IL-2. MT-2 cells were grown in RPMI 1640 containing FBS (10%), washed, cultured for 4 hours in RPMI 1640 containing 1% FBS, and then resuspended in RPMI 1640 containing 10% FBS for 1 hour before treatment with 0 or 2 nM IL-2. (**B**) UV cross-linking of DNA protein complexes from PHA-treated PBL (preact. PBL) stimulated with IL-2 (lane 1) or from MT-2 cells (lane 2). UV cross-linking and analysis on SDS gels were done as described (*10*). (**C**) PHA-treated PBL and MT-2 cells (5×10^6 to 10×10^6) were not stimulated (lanes 1, 3, 5, and 7) or were stimulated with 2 nM IL-2 (lanes 2, 4, 6, and 8) for 10 min at 37°C, washed twice with phosphate-buffered saline (PBS), and lysed in 10 mM tris (pH 7.5) containing 2 mM EDTA, 0.15 M NaCl, 0.875% Brij 96, 0.125% Nonidet P40, 0.4 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine (ICN, Aurora, Ohio), 2.5 mM leupeptin, and 2.5 mM aprotinin. Immunoprecipitations were done with antiserum to Stat3 (Santa Cruz, Santa Cruz, California) (lanes 1 to 4) or R1146 antiserum to Stat5 (lanes 5 to 8) (*10*), separated by SDS-PAGE (8% gel), transferred to Immobilon P membranes (Millipore, Bedford, Massachusetts), immunoblotted with antibody to phosphotyrosine 4G10 (UBI, Lake Placid, New York), and developed with enhanced chemiluminescence (ECL; Amersham). Molecular masses are given at left in kilodaltons. (**D**) Affinity purification of nuclear complexes with a multimerized GAS motif and protein immunoblotting were done as described (*10*). Nuclear extracts were from PHA-treated PBL treated with IL-2 (lanes 1 and 3) or from MT-2 cells (lanes 2 and 4). Protein immunoblotting was done with antisera to Stat3 (lanes 1 and 2) or Stat5 (lanes 3 and 4). Molecular masses are given at left in kilodaltons.

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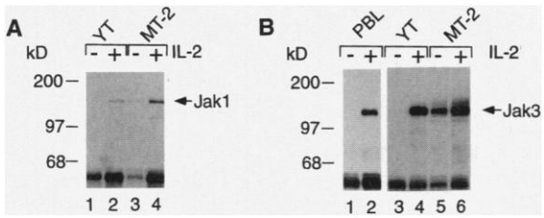

Fig. 2. Constitutive but inducible Jak1 (**A**) and Jak3 (**B**) tyrosine phosphorylation in HTLV-I–transformed T cell lines. (A) YT cells (lanes 1 and 2) or MT-2 cells (lanes 3 and 4) were not stimulated (lanes 1 and 3) or were stimulated with 2 nM IL-2 for 10 min (lanes 2 and 4). Proteins from cell lysates were immunoprecipitated with antiserum to Jak1 (UBI) and blotted with 4G10. (B) PHA-treated PBL (lanes 1 and 2), YT cells (lanes 3 and 4), and MT-2 cells (lanes 5 and 6) were not stimulated (lanes 1, 3, and 5) or were stimulated with 2 nM IL-2 for 10 min (lanes 2, 4, and 6). Proteins from cell lysates were immunoprecipitated with antiserum to Jak3 (*25*) and blotted with 4G10.

rylation was increased in cells treated with IL-2. To confirm the presence of Stat3 and Stat5 in the nuclear complexes formed from MT-2 cells, we purified complexes that bound to the GAS probe. Proteins in the complexes were separated by SDS–polyacrylamide gel electrophoresis and detected by protein immunoblotting with antisera to Stat3 and Stat5 (Fig. 1D).

HTLV-I does not encode any proteins with intrinsic tyrosine kinase domains (*12*); therefore, we assumed that cellular tyrosine kinases mediated the activation of Stat3 and Stat5 in MT-2 cells. Because Jak1 and Jak3 are activated in cells treated with IL-2 (*6*, *8*), we investigated the tyrosine phosphorylation status of these kinases in MT-2 cells. Whereas Jak1 and Jak3 were not tyrosine-phosphorylated in PHA-treated PBL or in natural killer–like YT cells in the absence of IL-2 (*6*, *8*), they were constitutively phosphorylated in MT-2 cells (Fig. 2), which suggests that Jak1 and Jak3 mediated the constitutive STAT activation in these cells. Corresponding to the IL-2–mediated augmentation of STAT protein activation in MT-2 cells, tyrosine phosphorylation of both Jak1 and Jak3 was increased by IL-2 in these cells (Fig. 2) (*17*).

Because the association of Jak3 with γ_c in YT cells (*6*) and PHA-treated PBL (*15*) is only very weakly detected before stimulation with IL-2 and is potently increased after IL-2 stimulation, we investigated whether Jak3 and γ_c were constitutively associated in MT-2 cells. A monoclonal antibody (mAb) to γ_c coprecipitated Jak3 in MT-2 cells even when these cells were not stimulated with IL-2, whereas in YT cells stimulation with IL-2 was required for substantial Jak3 association with γ_c (Fig. 3A). Thus, at least some of the IL-2 receptor complexes appear to be constitutively assembled in cell lines transformed with HTLV-I. Indeed, both γ_c (*15*) and Jak3 (Fig. 3B) were constitutively coprecipitated with IL-2Rβ in these cells; γ_c and Jak3 normally associate with IL-2Rβ only in cells stimulated with IL-2 (*3*, *6*). Jak3 and Stat5 were also coprecipitated from HTLV-I–transformed cells (Fig. 3C). When proteins from cell lysates were immunoprecipitated with antiserum to Jak3 and the immunoprecipitates were blotted with antibody to phosphotyrosine (4G10) (Fig. 3C), Jak3 and a minor smaller band were detected. The smaller band comigrated with Stat5. Moreover, an antiserum to Stat5 coprecipitated Jak3 (Fig. 3D), which suggests the possibility of a direct Jak-STAT interaction. In contrast to our ability to coprecipitate Jak3 and Stat5, we have not been able to coprecipitate Jak3 and Stat3. Although none of the HTLV-I–transformed T cell lines we have studied produced IL-2 (*15*, *16*), some, such as HUT-102B2, can produce IL-15 (*18*), a T cell growth factor that also activates Stat3 and Stat5 in PHA-treated PBL (*10*) and whose receptor contains IL-2Rβ and γ_c (*7*). The possible role of IL-15 in STAT protein activation in T cells transformed with HTLV-I was suggested by the ability of a mAb to IL-2Rβ (R&D Systems, number 27302.1) that blocks IL-2 and IL-15 binding to diminish (by approximately 30%) but not abrogate the constitutive nuclear complexes detected in MT-2 cells (*15*).

In vitro, the transition from IL-2 dependence to IL-2 independence spans an average period of 6 to 12 months after HTLV-I infection of normal human cord blood or PBL. We therefore analyzed Jak kinases and STAT proteins in HTLV-I–infected cord blood lymphocytes both in the early IL-2–dependent phase (N1186 cells) and after a period of 11 months (N1186-94 cells) when their dependency on IL-2 had diminished even though the cells were not producing their own IL-2 (*15*). Very

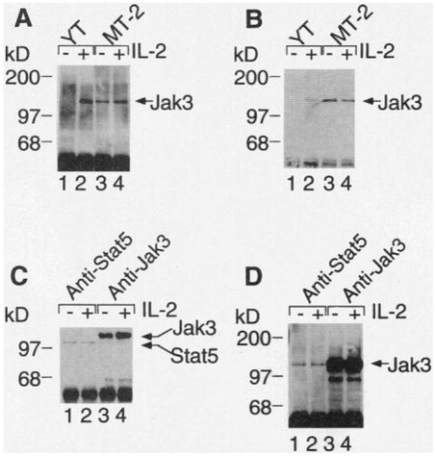

Fig. 3. Constitutive associations of Jak3, Stat5, and IL-2 receptor subunits in HTLV-I–transformed cells. (**A** and **B**) YT (lanes 1 and 2) and MT-2 (lanes 3 and 4) cells were not stimulated (lanes 1 and 3) or were stimulated with 2 nM IL-2 for 10 min (lanes 2 and 4) and proteins from cell lysates were immunoprecipitated with mAb 3B5 to γ_c (A) (*6*) or mAb Mikβ1 to IL-2Rβ (B) (*26*), blotted with antiserum to Jak3, and detected by ECL. (**C**) Proteins from lysates of HUT-102B2 cells not stimulated (lanes 1 and 3) or stimulated with 2 nM IL-2 for 10 min (lanes 2 and 4) were immunoprecipitated with antisera to Stat5 (anti-Stat5) (lanes 1 and 2) or to Jak3 (anti-Jak3) (lanes 3 and 4) and blotted with 4G10. (**D**) Proteins from lysates of MT-2 cells not stimulated (lanes 1 and 3) or stimulated with 2 nM IL-2 for 10 min (lanes 2 and 4) were immunoprecipitated with antiserum to Stat5 (lanes 1 and 2) or antiserum to Jak3 (lanes 3 and 4) and blotted with antiserum to Jak3.

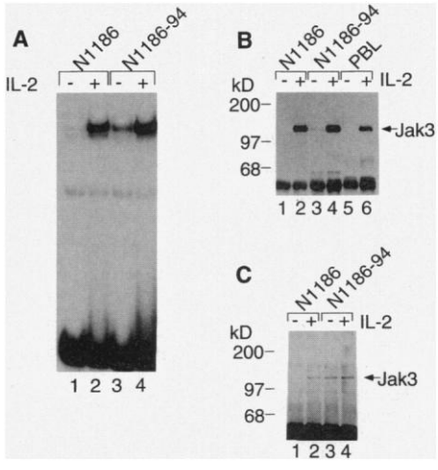

Fig. 4. Time-dependent acquisition of constitutive Jak3 tyrosine phosphorylation, Jak3–IL-2R association, and nuclear STAT protein binding activity in HTLV-I–infected cells. (**A**) Acquisition of constitutive nuclear DNA binding activity to the FcγRI GAS probe in the later phase after HTLV-I transformation. N1186 cells were established by coculture of normal human cord blood lymphocytes with thawed peripheral blood mononuclear cells from a patient with adult T cell leukemia (*27*) and were maintained in medium containing IL-2 (20 U/ml). N1186-94 cells were derived from N1186 cells that were maintained for 11 months in culture, during which time the IL-2 concentration was gradually lowered to 3 U/ml. Cells were washed and treated as described for MT-2 cells (Fig. 1). Nuclear extracts were prepared and EMSAs were done as in Fig. 1. (**B**) N1186 (lanes 1 and 2), N1186-94 (lanes 3 and 4), and PHA-treated PBL (lanes 5 and 6) were not stimulated (lanes 1, 3, and 5) or were stimulated with 2 nM IL-2 for 10 min (lanes 2, 4, and 6). Proteins from cell lysates were immunoprecipitated with antiserum to Jak3 and blotted with 4G10. (**C**) N1186 (lanes 1 and 2) and N1186-94 (lanes 3 and 4) cells were not stimulated (lanes 1 and 3) or were stimulated with 2 nM IL-2 for 10 min (lanes 2 and 4). Proteins were immunoprecipitated with antiserum R878 to γ_c (*4*) and blotted with antiserum to Jak3.

little STAT binding activity was detected in unstimulated N1186 cells, whereas N1186-94 cells did contain constitutive binding activity (Fig. 4A). Treatment of both cell populations with IL-2 augmented STAT binding activity. Corresponding to the acquisition of constitutive STAT protein activation in N1186-94 cells, these cells exhibited constitutive tyrosine phosphorylation of Jak3 (Fig. 4B), although less so than that shown by long-term HTLV-I–transformed cell lines such as MT-2 cells (Fig. 2B). In response to IL-2, Jak3 tyrosine phosphorylation was more inducible in N1186-94 cells (Fig. 4B) than in MT-2 cells (Fig. 2B). Like MT-2 cells, N1186-94 cells exhibited constitutive association of γ_c with Jak3 (Fig. 4C) and of IL-2Rβ with both Jak3 and γ_c (*15*).

Although it is conceivable that the HTLV-I transactivator protein Tax (*12*, *19*) might modulate expression of genes that contribute to constitutive activation of the Jak-STAT pathway, it appears not to be a sufficient signal. A T cell line denoted Tax I, in which a herpes saimiri–based vector drives Tax gene expression (*15*, *20*), did not exhibit constitutive STAT binding activity (*15*). Of other recently recognized HTLV-I proteins (p12, p13, and p30) encoded by the pX region (*21*), p12 is weakly oncogenic (*22*) and can associate with both IL-2Rβ and γ_c (*23*), but it remains unknown whether p12 participates in activation of the Jak-STAT pathway. Although the mechanisms by which HTLV-I transformation induces activation of the Jak-STAT pathway are not completely understood, our correlation of the constitutive Jak-STAT pathway with the acquisition of IL-2 independence in HTLV-I–transformed cells provides an additional insight into the mechanisms underlying the molecular basis of HTLV-I–mediated leukemogenesis. Because constitutive Jak-STAT activation also occurs in B cells transformed with Abelson virus (*24*), it is possible that activation of Jaks and STATs may be a strategy used by a number of viruses to mediate cell growth.

Note added in proof: With additional time in continuous cell culture, N1186-94 cells have become completely independent of IL-2. Correspondingly, in these cells the amount of constitutive STAT binding activity has increased as compared with that of the N1186-94 cells that are still partially dependent on IL-2.

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Enhanced DNA-Binding Activity of a Stat3-Related Protein in Cells Transformed by the Src Oncoprotein

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Cytokines and growth factors induce tyrosine phosphorylation of signal transducers and activators of transcription (STATs) that directly activate gene expression. Cells stably transformed by the Src oncogene tyrosine kinase were examined for STAT protein activation. Assays of electrophoretic mobility, DNA-binding specificity, and antigenicity indicated that Stat3 or a closely related STAT family member was constitutively activated by the Src oncoprotein. Induction of this DNA-binding activity was accompanied by tyrosine phosphorylation of Stat3 and correlated with Src transformation. These findings demonstrate that Src can activate STAT signaling pathways and raise the possibility that Stat3 contributes to oncogenesis by Src.

The STATs are latent cytoplasmic transcription factors that, upon activation by tyrosine phosphorylation, translocate to the nucleus and bind to specific regulatory elements that control gene expression (*1*). Members of the cytokine receptor superfamily that lack intrinsic kinase activity recruit Janus kinase (Jak) family tyrosine kinases, which are required for cytokine activation of STAT proteins (*2*). Whether other nonreceptor kinases, such as Src family tyrosine kinases, might also activate STAT proteins has not been explored. Six STAT family members have been molecularly cloned (*3*). Although the roles of STAT proteins in mediating responses to cytokines and growth factors in normal cells are being delineated (*4*), the regulation of STAT signaling pathways by oncoproteins has not been described.

We prepared nuclear extracts from normal 3Y1, NIH 3T3, or BALB/c 3T3 rodent fibroblasts or those cells transformed with the v-Src oncoprotein (*5*). The extracts were incubated with a labeled high-affinity sis-inducible element (SIE) probe (*6*), and the resulting DNA-protein complexes were analyzed by an electrophoretic mobility shift assay (EMSA) for the STAT-related DNA-binding factors sis-inducible factor (SIF)–A, SIF-B,

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