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D A Frank, … , S Mahajan, J Ritz

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Research Article

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# B Lymphocytes from Patients with Chronic Lymphocytic Leukemia Contain Signal Transducer and Activator of Transcription (STAT) 1 and STAT3 Constitutively Phosphorylated on Serine Residues

**David A. Frank, Sudipta Mahajan, and Jerome Ritz**

*Department of Adult Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115*

## Abstract

**To explore the pathogenesis of chronic lymphocytic leukemia (CLL), we examined whether phosphorylation of one or more signal transducer and activator of transcription (STAT) factors was abnormal in cells from CLL patients. No constitutive tyrosine phosphorylation was detected on any STAT in CLL cells. To assess the phosphorylation of serine residues of STAT1 and STAT3 in CLL cells, we raised antibodies that specifically recognize the form of STAT1 phosphorylated on ser-727 and the form of STAT3 phosphorylated on ser-727. We found that in 100% of patients with CLL (*n* = 32), STAT1 and STAT3 were constitutively phosphorylated on serine. This was in contrast to normal peripheral blood B lymphocytes or CD5$^+$ B cells isolated from tonsils, in which this phosphorylation was absent. Serine phosphorylation of STAT1 and STAT3 was seen occasionally in other leukemias, but it was a universal finding only in CLL. The serine phosphorylation of these STATs was a continuous process, as incubation of CLL cells with the kinase inhibitor H7 led to the dephosphorylation of these serine residues. The STAT serine kinase in CLL cells has not been identified, and appears to be neither mitogen-activated protein kinase nor pp70$^{s6k}$. In summary, the constitutive serine phosphorylation of STAT1 and STAT3 is present in all CLL samples tested to date, although the physiologic significance of this modification remains to be determined. (*J. Clin. Invest.* 1997. 100:3140–3148.) Key words: transcription factor • cancer • kinase • phosphatase • H7**

## Introduction

Chronic lymphocytic leukemia (CLL)[1] is characterized by the proliferation and accumulation of a clonal population of B lymphocytes distinguished by the expression of the CD5 cell surface antigen (1). Although the true normal counterpart to the CLL B cell is in dispute (2), CD5$^+$ B cells are normally found in greatest numbers in the mantle zone of lymph nodes. Despite the fact that CLL is the most common form of leukemia in Western societies, its pathogenesis is unknown. Several cytogenetic abnormalities have been reported in small numbers of patients, but no consistent molecular defect has been identified (3). Perhaps related to this lack of understanding of its pathogenesis, CLL remains an incurable disease. The absence of a single diagnostic abnormality in CLL raises the possibility that multiple distinct defects might feed into a common pathway responsible for inappropriate proliferation and/or protection from apoptosis. It is known that a variety of cytokines can have important effects in supporting the growth and survival of lymphocytes, and that many forms of leukemia are characterized by cell growth that is no longer dependent on the presence of cytokines (4, 5). Most hematopoietic cytokines exert some or all of their effects by activating one or more of a group of transcription factors termed STATs (for signal transducer and activator of transcription) (6, 7). When a cytokine binds to its cell surface receptor, it triggers the activation of a family of tyrosine kinases termed Janus kinases (JAKs), which then lead to the phosphorylation of one or more members of the STAT family on single specific tyrosine residues. The tyrosine phosphorylation of the STATs then allows these proteins to dimerize, translocate to the nucleus, and bind specific DNA sequences in the promoters of target genes, where they are able to activate transcription (8). Some STATs may be phosphorylated on individual serine residues as well (9, 10). Although this serine phosphorylation is not sufficient to activate a STAT, serine phosphorylation appears to increase the magnitude of gene transcription induced by tyrosine-phosphorylated STATs. Thus, serine phosphorylation of STATs allows the integration of signals from multiple pathways, and provides a degree of modulation of the gene activation induced by the tyrosine phosphorylation of the STATs.

Given that cytokines may promote the proliferation and survival of hematopoietic cells, and that leukemias are characterized by the unbalanced growth of these cells, it was hypothesized that constitutive activation of STATs might be found in cells from patients with leukemias. In fact, such STAT activation has been found in acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) (11, 12), and in cells

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Address correspondence to David A. Frank, M.D., Ph.D., Department of Adult Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: 617-632-4714; FAX: 617-632-5167; E-mail: david_frank@dfci.harvard.edu

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1. *Abbreviations used in this paper:* ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; CREB, cAMP response element–binding protein; MAP, mitogen-activated protein; OA, okadaic acid; P-ser-STAT1, STAT1 phosphorylated on ser-727; Per-ser-STAT3, STAT3 phosphorylated on ser-727; STAT, signal transducer and activator of transcription.

transformed with the leukemogenic retrovirus human T lymphotropic virus 1 (13) or with BCR/abl, the hallmark of chronic myelogenous leukemia (14, 15). Although it has not been demonstrated that such STAT activation is causal in the development of these leukemias, the consistent finding of abnormal STAT activation in these cells suggests that STATs may play a role in the development of hematologic malignancies.

As STATs appear to play a role in the genesis of a variety of neoplasms, and as the pathogenesis of CLL remains obscure, we tested the hypothesis that abnormal STAT activation was a consistent feature of CLL. Although no constitutive tyrosine phosphorylation of STATs was found in CLL cells, we did find that the lymphocytes from all of the CLL patients we examined displayed phosphorylation of ser-727 of both STAT1 and STAT3. STAT serine phosphorylation was found occasionally in other leukemias; however, this finding was seen universally only in CLL. The kinase(s) responsible for these phosphorylation events was sensitive to the kinase inhibitor H7, and does not appear to be mitogen-activated protein (MAP) kinase or pp70$^{s6k}$. The finding of serine phosphorylation of STAT1 and STAT3 in all cases of CLL examined to date may indicate a potential role for this modification in the pathogenesis of CLL, and may suggest targets for rationally designed therapies.

## Methods

*Reagents.* H7, staurosporine, phorbol 12-myristate 13-acetate (PMA), forskolin, and okadaic acid (OA) were purchased from Sigma Chemical Co. (St. Louis, MO). Ionomycin was obtained from Calbiochem Corp. (La Jolla, CA). Rapamycin was a gift from Dr. Barbara Bierer (Dana-Farber Cancer Institute, Boston, MA). Antibodies to STAT1 and STAT3 were purchased from Transduction Laboratories (Lexington, KY). Calf intestine phosphatase, PD 098059, and antibodies to pp70$^{s6k}$ and the phosphorylated form of MAP kinase were purchased from New England Biolabs Inc. (Beverly, MA).

*Cells.* NKL cells (16) were grown in RPMI 1640 medium supplemented with 15% FBS, 1 mM sodium pyruvate, 100 U/ml IL-2, 250 U/ml penicillin, and 250 μg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% $CO_2$.

PBMC were isolated by density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). B lymphocytes from normal donors were purified by two different techniques. Cells were stained with an FITC-conjugated antibody to CD20 (Coulter Corp., Miami, FL), and then purified by sorting on a flow cytometer (EPICS Elite ESP; Coulter Corp.). Alternatively, a column purification system was used with antibodies to CD19 (CellPro, Inc., Bothell, WA). In either case, > 95% of cells recovered expressed κ or λ light chains. CD5$^+$ B cells were isolated from tonsils. Tonsils were disaggregated by passage through a 70-μm filter, followed by density gradient centrifugation using Ficoll-Paque. Cells were stained with an anti-CD5 antibody conjugated to phycoerythrin and antibodies to CD19 and CD20 conjugated to FITC, and sorted on the EPICS Elite ESP flow cytometer. Cells expressing both CD5 and CD19 or CD20 were collected. Archival cells were obtained from the tumor bank of the Division of Hematologic Malignancies, Dana-Farber Cancer Institute.

*Antibody generation.* Antibodies specific for STAT1 phosphorylated on ser-727 (P-ser-STAT1) and for STAT3 phosphorylated on ser-727 (P-ser-STAT3) were generated in rabbits. Briefly, peptides representing amino acids 720–730 of STAT1 with phosphoserine in position 727, and amino acids 720–730 of human STAT3 with phosphoserine in position 727 were synthesized, with a cysteine residue placed in the amino terminus of each peptide. The peptides were conjugated to BSA with a succinimide ester, and used for immunizations.

*Cell extracts.* After appropriate stimulation, cells were placed on ice, washed once with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4), and extracted in buffer containing 10 mM Tris, pH 8.0, 0.5% NP-40, 250 mM NaCl, 10 mM sodium orthovanadate, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation at 12,000 *g* for 1 min.

*Western blots.* Protein was resolved by SDS-PAGE, transferred to nitrocellulose, and blocked with 5% nonfat dry milk (for antiphospho-STAT Western blots) or 5% BSA (for antiphosphotyrosine Western blots) in TBST (100 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). Primary antibody was diluted 1:10,000 in TBST containing 3% BSA and incubated for 1 h at room temperature. After extensive washing, the blot was incubated with horseradish peroxidase–conjugated secondary antibody (Calbiochem Corp.; anti–rabbit for the phospho-STAT Western blots and anti–mouse for the phosphotyrosine Western blots) diluted 1:10,000 in TBST containing 1% BSA for 1 h at room temperature. After extensive washing, bound antibody was detected by chemiluminescence (Renaissance kit; New England Nuclear, Boston, MA).

*Immune complex phosphatase treatment.* Whole cell extracts were prepared from $2 \times 10^7$ NKL cells, and antibodies to STAT1 or STAT3 were added. After 1 h at 4°C, 25 μl of protein A–Sepharose (50% slurry) was added, and the extracts were incubated for an additional 1 h at 4°C with rotation. The immune complex was washed three times with ice-cold NETN (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and once with ice-cold PBS, then divided into two. One half was left untreated, and the other was washed twice with phosphatase buffer (100 mM Tris, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl) and treated with 100 U calf intestine phosphatase in 50 μl of phosphatase buffer at 37°C for 1 h. The protein A–Sepharose was then washed twice in NETN and once in PBS. 40 μl of 2× SDS loading buffer was added to both the untreated and phosphatase-treated immune complexes, and the mixtures were boiled and centrifuged. Proteins were resolved by SDS-PAGE on a 7% gel, and separate Western blots were performed with antibodies to STAT1 and P-ser-STAT1 (for the STAT1 immunoprecipitation) and STAT3 and P-ser-STAT3 (for the STAT3 immunoprecipitation).

*Statistical analysis.* Differences in the frequency of STAT1 and STAT3 serine phosphorylation between CLL and other leukemias were assessed using a test for homogeneity of binomial proportions. The reported *P* values are two-sided.

## Results

*Absence of constitutive tyrosine phosphorylation of STATs in CLL.* Given the evidence for constitutive tyrosine phosphorylation of STATs in a variety of leukemic cells, we tested the hypothesis that tyrosine phosphorylation of STATs might be present in lymphocytes from patients with CLL. Whole cell extracts were prepared from untreated or IFN-α–treated PBMC isolated from untreated patients with CLL. Western blots were performed on these extracts using antibodies that specifically recognize the tyrosine-phosphorylated forms of STAT1 and STAT5 (17, 18) (Fig. 1). No tyrosine phosphorylation of these STATs was detected in untreated cells (Fig. 1, lanes *1*, *3*, *5*, and *7*). That the cells were viable, and could respond with tyrosine phosphorylation of STAT1 was demonstrated by treating the cells with IFN-α. In each case, IFN-α treatment led to the induction of tyrosine-phosphorylated STAT1 (Fig. 1, lanes *2*, *4*, *6*, and *8*). As a control, the NKL cell line was also shown to display IFN-α–induced phosphorylation of STAT1 (Fig. 1, lanes *9* and *10*). Antibodies that specifically recognize the tyrosine-phosphorylated forms of STAT3, STAT4, STAT5, and STAT6

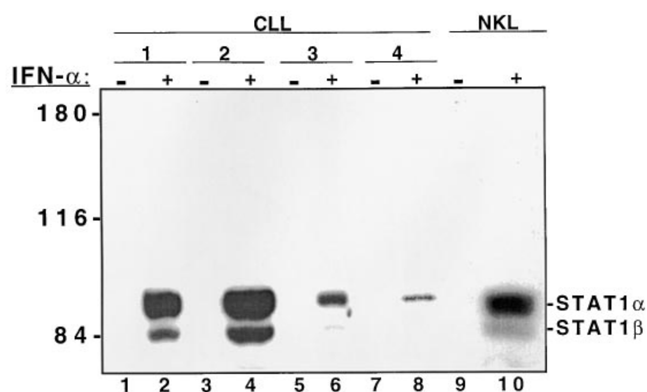


*Figure 1.* STAT1 is not constitutively phosphorylated on tyrosine in CLL cells. A Western blot specific for the tyrosine-phosphorylated form of STAT1 was performed on whole cell extracts of PBMC from four representative patients with CLL, that had either been untreated (−, lanes *1*, *3*, *5*, and *7*) or treated with IFN-α (500 U/ml) for 10 min (+, lanes *2*, *4*, *6*, and *8*). As a control, extracts from untreated (lane *9*) or IFN-α–treated NKL cells (lane *10*) were included.

*Table I. Tyrosine and Serine Phosphorylation of STAT1 and STAT3 Was Determined by Preparing Whole Cell Lysates of Tumor Cells, and Performing Western Blots with the Appropriate Phosphorylation-specific Antibodies*

| | Tyrosine phosphorylation | | Serine phosphorylation | |
|---|---|---|---|---|
| Tumor | STAT1 | STAT3 | STAT1 | STAT3 |
| CLL | 0/13 (0%) | 0/13 (0%) | 32/32 (100%)* | 32/32 (100%)* |
| AML | 8/16 (50%) | 3/16 (19%) | 5/10 (50%) | 5/10 (50%) |
| ALL–B cell | 6/10 (60%) | 9/10 (0%) | 5/10 (50%) | 5/10 (50%) |
| ALL–T cell | 6/10 (60%) | 2/10 (20%) | 2/10 (20%) | 4/10 (40%) |

*$P < 0.001$, when CLL is compared with each other leukemia.

failed to detect the tyrosine-phosphorylated forms of these STATs in CLL cells (data not shown). The absence of tyrosine phosphorylation of these STATs has been confirmed in all of 13 CLL patient samples examined. By contrast, constitutive tyrosine phosphorylation of STAT1 and STAT3 was seen in leukemic cells from patients with AML, and tyrosine phosphorylation of STAT1 was seen in cells from patients with ALL of both B and T cell lineage (Table I). These results are similar to previously reported findings (11, 12).

*Generation of antibodies to P-ser-STAT1 and P-ser-STAT3.* In contrast to the acute leukemias, CLL is characterized by the slow accumulation of relatively well-differentiated lymphocytes. Thus, the lack of tyrosine phosphorylation of STATs in CLL may reflect the absence of rapid growth of these cells. It was hypothesized that CLL cells might possess another modification of STATs, namely serine phosphorylation, which could underlie their pathobiology. We chose to examine the serine phosphorylation of STAT1 and STAT3 for two reasons. First, these STATs are activated in response to IL-2 and IL-6, two cytokines which have major effects on lymphocyte growth and differentiation (19, 20). Second, STAT1 and STAT3 (as well as STAT4) share a motif, leu-pro-met-ser-pro, that is a consensus site for phosphorylation by a number of serine/threonine kinases (21, 22). STAT4 was excluded from analysis because Western blots failed to detect STAT4 in any CLL samples tested (data not shown).

Although the tyrosine phosphorylation of STATs can be assessed in unperturbed cells by electrophoretic mobility shift assays or immunoprecipitation of the STAT followed by Western blot with antibodies to phosphotyrosine, the detection of serine phosphorylation of these proteins involves metabolic labeling of cells with [$^{32}$P]orthophosphate. Given the difficulty of doing this without inducing artifactual findings in primary CLL cells, we chose instead to generate antibodies that could recognize specifically only the forms of STAT1 and STAT3 phosphorylated on their critical serine residues, ser-727 in each protein. Rabbits were immunized with phosphopeptides mimicking the sites of serine phosphorylation of STAT1 or STAT3, and antisera were obtained. To determine the specificity of the P-ser-STAT1 antiserum, immunoprecipitation of STAT1 was performed on extracts of untreated NKL cells, which contain constitutively serine-phosphorylated STAT1 and STAT3, but little tyrosine phosphorylation of these STATs. The immunoprecipitated protein was divided into two aliquots, one of which was treated in vitro with calf intestine phosphatase to remove any phosphate groups on ser-727. The proteins were resolved by SDS-PAGE, and Western blots were performed with antibodies to STAT1 or P-ser-STAT1 (Fig. 2 *A*). The phosphatase treatment had no effect on the ability of the antibody to STAT1 to detect this protein (Fig. 2 *A*, lanes *1* and *2*). The P-ser-STAT1 antibody, by contrast, only recognized the protein that had not been enzymatically dephosphorylated, indicating that it specifically recognizes the form of STAT1 phosphorylated on ser-727 (Fig. 2 *A*, lanes *3* and *4*). Similarly, immunoprecipitation of STAT3 was performed on NKL cell extracts, followed by no treatment or calf intestine phosphatase treatment (Fig. 2 *B*). Like the P-ser-STAT1 antibody, the P-ser-STAT3 antibody recognized only the serine-phosphorylated form of STAT3 (Fig. 2 *B*, lanes *3* and *4*), although STAT3 could be detected in the phosphatase-treated samples (Fig. 2 *B*, lanes *1* and *2*). It should also be noted that in the untreated STAT3 immunoprecipitate, two protein bands of very similar mobility can be detected (Fig. 2 *B*, lane *1*). It has been shown previously that the slower migrating form represents the serine-phosphorylated form of STAT3 (10). After phosphatase treatment, the slower migrating form is lost (Fig. 2 *B*, lane *2*). In the P-ser-STAT3 Western blot, the single protein seen in the untreated extracts (Fig. *2*, lane *3*) corresponds to the upper band of the two seen in the anti-STAT3 blot (Fig. 2, lane *1*). This provides additional evidence that the P-ser-STAT3 antibody is specific for the form of STAT3 phosphorylated on ser-727.

*Serine phosphorylation of STAT1 and STAT3 in CLL.* To assess the serine phosphorylation of STAT1 and STAT3 in CLL, PBMC were isolated from patients with untreated disease. Flow cytometry revealed that $\geq$ 90% of the cells were $CD19^{+}CD20^{+}CD5^{+}$ (data not shown). Western blot analysis revealed that 100% of the samples analyzed contained STAT1 and STAT3 that was phosphorylated on ser-727. Western blots of the first five samples analyzed are shown using P-ser-STAT1 (Fig. 3 *A*) and P-ser-STAT3 (Fig. 3 *B*). By contrast, PBMC from normal donors had no serine-phosphorylated STAT1 or STAT3, although they could become phosphory-

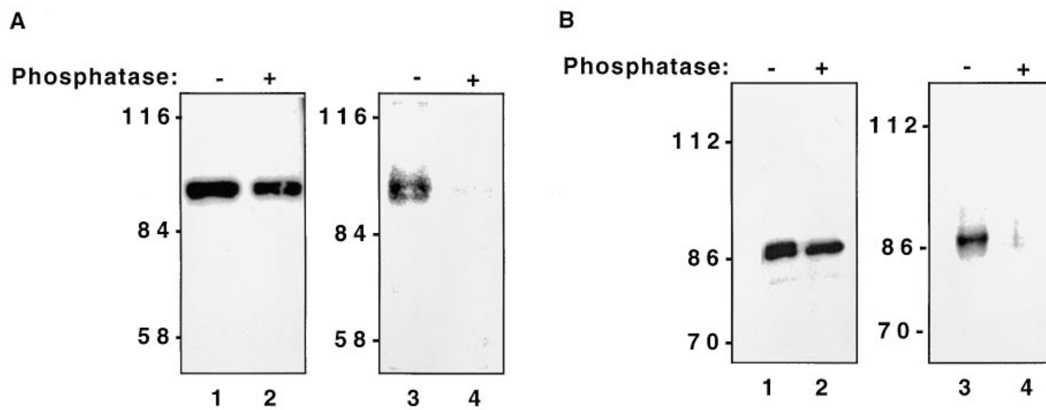


*Figure 2.* P-ser-STAT1 and P-ser-STAT3 antibodies specifically recognize the ser-727–phosphorylated forms of STAT1 and STAT3, respectively. Immunoprecipitations were performed on whole cell extracts from untreated NKL cells with antibodies to STAT1 (*A*) or STAT3 (*B*). Immunoprecipitated proteins were left untreated (−, lanes *1* and *3*) or treated with calf intestine phosphatase (+, lanes *2* and *4*), and Western blots were performed with antibodies to STAT1 (*A*, lanes *1* and *2*), P-ser-STAT1 (*A*, lanes *3* and *4*), STAT3 (*B*, lanes *1* and *2*), and P-ser-STAT3 (*B*, lanes *3* and *4*).

lated if treated with PMA (data not shown). NKL cells, which show constitutive phosphorylation of ser-727 of STAT1 and STAT3, were used as a positive control (Fig. 3, *A* and *B*, lane *7*). PBMC isolated from normal donors were largely T lymphocytes. To compare CLL cells with circulating B lymphocytes in normal donors, B cells were purified using two techniques. PBMC were isolated, and then B lymphocytes were purified either by flow cytometry using an antibody directed to CD20, or by column purification using an antibody to CD19. In either case, B lymphocytes from normal donors revealed little to no serine phosphorylation of STAT1 or STAT3 (Fig. 4 *A*). Thus, normal circulating peripheral blood B lymphocytes do not contain constitutively serine-phosphorylated STAT1 or STAT3. Treatment of the B cells with PMA did lead to phosphorylation of both of these sites, indicating that both STAT1 and STAT3 are present in the cells, as well as a kinase(s) that can phosphorylate ser-727 of each protein (Fig. 4 *A*, lanes *3* and *7*).

A characteristic of CLL cells is the expression of CD5 in addition to B cell markers. $CD5^+$ B cells are rare in peripheral blood, and sufficient numbers of $CD5^+$ B cells to perform biochemical analyses could not be obtained from the peripheral blood of normal donors. Thus, tonsils from two different patients were obtained, and $CD5^+$ B cells were purified by fluorescence-activated cell sorting. The number of cells isolated from patient 2 was sufficient only for analysis of P-ser-STAT1. No serine phosphorylation of STAT1 (Fig. 4 *B*, lanes *1* and *3*) or STAT3 (Fig. 4 *B*, lane *5*) could be detected in untreated $CD5^+$ B cells. However, treating the cells with PMA led to the prominent phosphorylation of both of these sites, indicating that the cells are viable and can phosphorylate these serine residues. Thus, $CD5^+$ B cells from normal patients do not display constitutive serine phosphorylation of STAT1 and STAT3, and the phosphorylation of these proteins seen in CLL cells does not represent the expansion of a population in which these proteins are already phosphorylated.

Given the finding of constitutive serine phosphorylation of STAT1 and STAT3 in CLL cells, we wished to determine whether such phosphorylation was common to other lymphocytic and myeloid leukemias. Extracts were prepared from PBMC of untreated patients with a variety of hematologic malignancies, and Western blots were performed with the P-ser-STAT1 and P-ser-STAT3 antibodies. Phosphorylation of these sites was seen occasionally in other forms of leukemia; however, it was a consistent finding only in CLL (Table I).

*Dynamics of serine phosphorylation of STAT1 and STAT3 in CLL.* The finding that serine phosphorylation of STAT1 and STAT3 is found in all CLL cells examined to date sug-

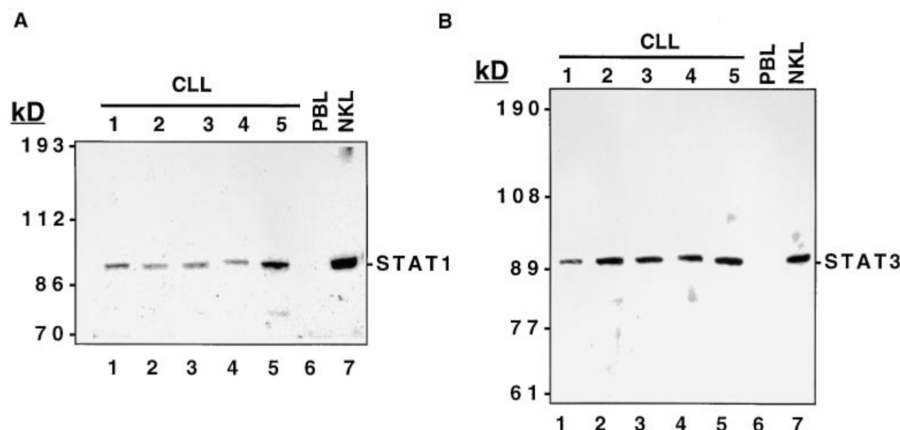


*Figure 3.* CLL cells contain STAT1 and STAT3 constitutively phosphorylated on ser-727. Western blots were performed with antibodies P-ser-STAT1 (*A*) or P-ser-STAT3 (*B*) on whole cell extracts from five representative patients with CLL (lanes *1–5*), PBL from normal donors (lane *6*), and untreated NKL cells (lane *7*).

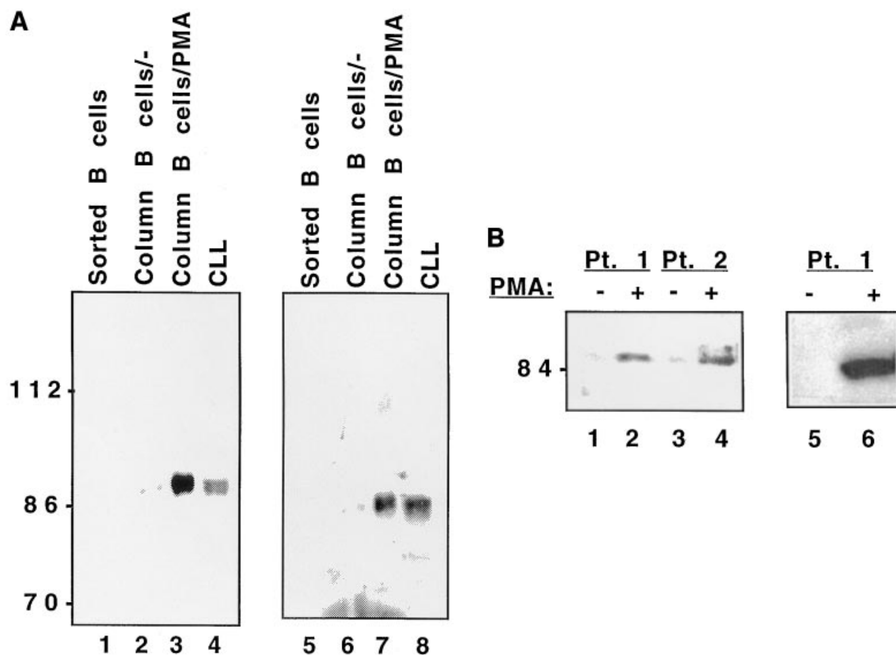


*Figure 4.* Normal B lymphocytes do not contain ser-727–phosphorylated STAT1 or STAT3. (*A*) B lymphocytes from normal donors were isolated using fluorescence-activated cell sorting with a fluorescein-conjugated antibody to CD20 (lanes *1* and *5*) or column purification using an antibody to CD19 (lanes *2*, *3*, *6*, and *7*), and left untreated (lanes *1*, *2*, *5*, and *6*) or treated with PMA (100 nM) for 10 min (lanes *3* and *7*). Untreated CLL cells from a representative patient were included as controls (lanes *4* and *8*). Whole cell extracts were prepared, and Western blots were performed using antibodies to P-ser-STAT1 (lanes *1–4*) or to P-ser-STAT3 (lanes *5–8*). (*B*) $CD5^+$ B cells were isolated from tonsils from two patients using fluorescence-activated cell sorting, and left untreated (−, lanes *1*, *3*, and *5*) or treated with PMA for 10 min (+, lanes *2*, *4*, and *6*). Whole cell extracts were prepared, and Western blots were performed using antibodies to P-ser-STAT1 (lanes *1–4*) or P-ser-STAT3 (lanes *5* and *6*).

gested that this modification might be important in the pathogenesis of this leukemia. If so, then identification of the kinase(s) involved in phosphorylation of these sites might provide important information about the steps leading to the development of CLL. The phosphorylation of STAT1 and STAT3 could potentially occur early in the differentiation of these lymphocytes, and the STATs could remain phosphorylated indefinitely thereafter. If that were the case, the kinase(s) responsible for phosphorylation of these sites might not be present or activated in the CLL cells in the peripheral blood. Alternatively, the phosphorylation of these sites could be a continuous process mediated by a kinase active in the CLL cells. To distinguish these possibilities, CLL cells were treated with the serine/threonine kinase inhibitor H7 (23). Within 30 min of treatment with H7, ser-727 of STAT1 and STAT3 became dephosphorylated (Fig. 5, *A* and *B*). The tyrosine kinase inhibitor staurosporine, which could block the tyrosine phosphorylation of STAT1 induced by IFN-α in CLL cells, had no effect on phosphorylation of these sites (data not shown). That the kinase inhibitor H7 could lead to the rapid dephosphorylation of STAT1 and STAT3 suggested that there was a rapid turnover of phosphate groups at these sites, and that one or more STAT serine kinases must be active in CLL cells. By extension, this result also suggested that one or more phosphatases that could dephosphorylate these sites must be active in CLL cells as well. To examine this possibility, we treated CLL cells with OA, an inhibitor of protein phosphatases 1 and 2A (24). Treatment with OA alone led to an increase in the phosphorylation of ser-727 of STAT1 and STAT3 in CLL cells, indicating that a phosphatase sensitive to OA must be active in CLL cells, and is able to dephosphorylate ser-727 of STAT1 and STAT3 (Fig. 6, lanes *3* and *7*). Treating cells with OA before treatment with H7 blocked the dephosphorylation of STAT1 and STAT3, further confirming that an active cycle of phosphorylation and dephosphorylation of these sites is present in CLL cells (Fig. 6, lanes *4* and *8*).

*Induction of phosphorylation of ser-727 of STAT1 and STAT3 in normal PBMC.* The finding of phosphorylation of ser-727 of STAT1 and STAT3 in all CLL samples examined to date suggests that this modification may be important in the pathogenesis of this disease. Thus, determining the kinase(s) responsible for these events might shed light on the transformation events occurring in these B lymphocytes. To give an indication of the kinase(s) in lymphocytes that could phosphory-

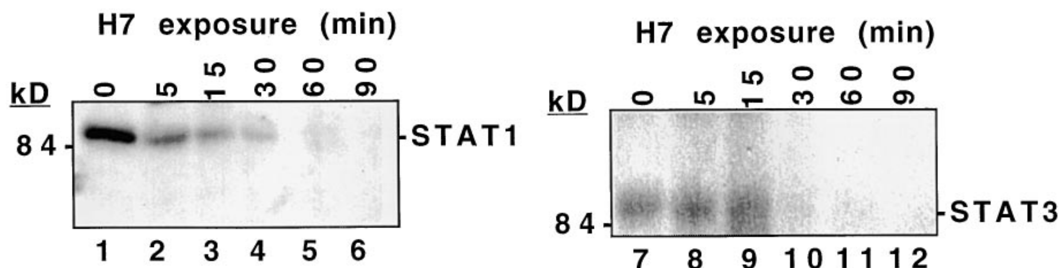


*Figure 5.* The ser-727 phosphorylation of STAT1 and STAT3 is inhibited by H7. Cells from a patient with CLL were left untreated (lanes *1* and *7*) or treated with 50 μM H7 for the indicated times. Whole cell extracts were prepared, and Western blots were performed using antibodies to P-ser-STAT1 (lanes *1–6*) or P-ser-STAT3 (lanes *7–12*). The experiment shown is representative of experiments performed with cells from four different patients.

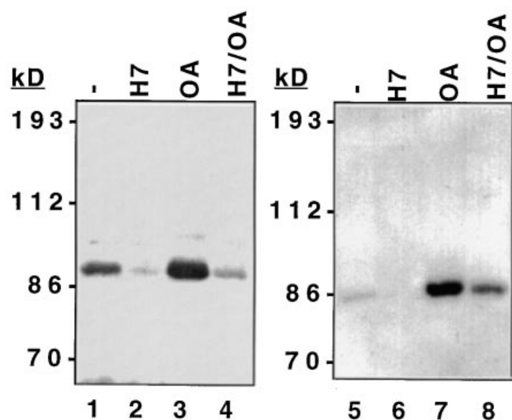


*Figure 6.* The dephosphorylation of ser-727 of STAT1 and STAT3 is inhibited by OA. Cells from a patient with CLL were left untreated (−, lanes *1* and *5*), treated with H7 (50 μM) for 30 min (lanes *2* and *6*), treated with OA (1 μM) for 30 min (lanes *3* and *7*), or treated with OA for 30 min followed by the addition of H7 for 30 min (lanes *4* and *8*). Whole cell extracts were prepared, and Western blots were performed with antibodies to P-ser-STAT1 (lanes *1–4*) or P-ser-STAT3 (lanes *5–8*).

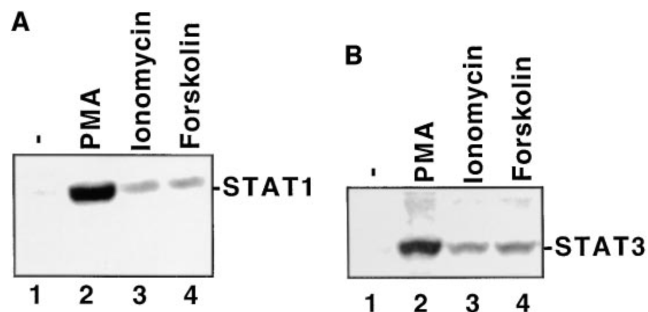


*Figure 7.* Multiple stimuli can induce the phosphorylation of ser-727 of STAT1 and STAT3 in normal PBMC. PBMC obtained from a normal donor were left untreated (−, lane *1*), or treated for 15 min with 100 nM PMA (lane *2*), 1 μM ionomycin (lane *3*), or 1 μM forskolin (lane *4*). Whole cell extracts were prepared, and Western blots were performed with antibodies to P-ser-STAT1 (*A*) or to P-ser-STAT3 (*B*).

late these serine residues of STAT1 and STAT3, we treated normal PBMC with a number of stimuli known to activate a variety of serine/threonine kinases. Specifically, we employed forskolin (an activator of the cAMP-dependent kinase protein kinase A), the calcium ionophore ionomycin (an activator of calcium, calmodulin-dependent protein kinases), and PMA (an activator of protein kinase C). We found that each of these agents was able to induce the rapid phosphorylation of ser-727 of STAT1 and STAT3 in normal PBMC (Fig. 7, *A* and *B*). That multiple agents can induce these phosphorylation events suggests that serine phosphorylation of STATs is an important point of convergence of signaling pathways. However, this finding did not restrict the search for the STAT serine kinase(s) in CLL cells.

*Activation of MAP kinase in CLL cells.* It had been suggested that the sites surrounding ser-727 of STAT1 and STAT3 are potential sites of phosphorylation by MAP kinase (21, 22). Furthermore, MAP kinase appears to be important in the STAT-mediated transcriptional activation induced by IFN-α (25). To investigate the activation state of MAP kinase in CLL cells, Western blots were performed using antibodies that specifically recognize the phosphorylated, activated form of MAP kinase. Using these antibodies, no activation of MAP kinase could be found in resting CLL cells. However, treating the cells with PMA led to a rapid and prominent phosphorylation of MAP kinase (Fig. 8). This activation of MAP kinase had no additional effect on the serine phosphorylation of STAT1 or STAT3 (data not shown). Taken together, these results indicate that although MAP kinase is present in CLL cells and can be stimulated by pharmacological agents, it does not appear to be the STAT serine kinase in CLL cells. In addition, the kinase inhibitor PD 098059, which inhibits MEK (26), MEK kinase, and raf (27), had no effect on the serine phosphorylation of STAT1 and STAT3 in CLL cells (data not shown). This suggests further that the MAP kinase pathway is unlikely to be playing a role in STAT serine phosphorylation in CLL.

*pp70$^{s6k}$ activation in CLL.* pp70$^{s6k}$ is activated by a variety of stimuli that promote lymphocyte activation (28). Thus, we considered the possibility that pp70$^{s6k}$ is active in CLL cells and could be involved in the phosphorylation of ser-727 of STAT1 and STAT3. To assess the activation state of pp70$^{s6k}$ in CLL cells, we performed Western blots with an antibody that specifically recognizes the 70- and 85-kD forms of this kinase. In the phosphorylated, activated state, each of these proteins migrates with a slightly slower mobility when subjected to SDS-PAGE (29). We found that pp70$^{s6k}$ was activated in all of seven CLL samples analyzed (a representative experiment is shown in Fig. 9). To determine whether this activation was related to the serine phosphorylation of STAT1 and STAT3, we used rapamycin, a specific inhibitor of pp70$^{s6k}$ activation (29, 30). Treatment of CLL cells with rapamycin led to the rapid dephosphorylation of pp70$^{s6k}$ (Fig. 9, lanes *1* and *2*). However, rapamycin had no effect on the phosphorylation of ser-727 of

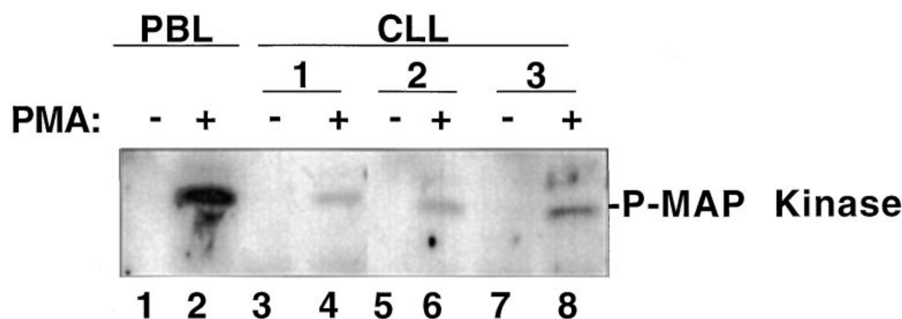


*Figure 8.* MAP kinase is not activated constitutively in CLL cells. Normal PBL (lanes *1* and *2*) and cells from three patients with CLL (lanes *3–8*) were left untreated (−, lanes *1*, *3*, *5*, and *7*) or treated with 100 nM PMA for 15 min (+, lanes *2*, *4*, *6*, and *8*). Whole cell extracts were obtained, and Western blots were performed using an antibody to the activated form of MAP (*P-MAP*) kinase.

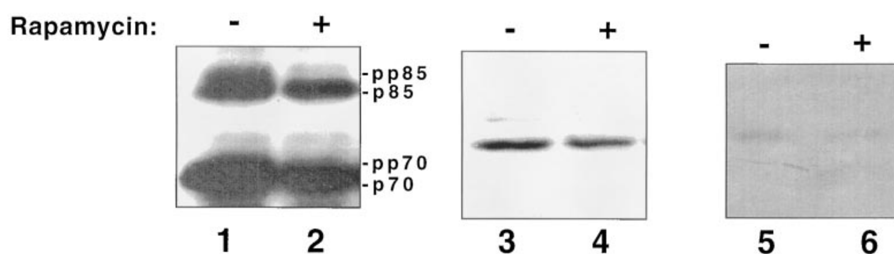


*Figure 9.* Rapamycin does not inhibit the phosphorylation of ser-727 of STAT1 and STAT3 in CLL cells. Cells from a patient with CLL were left untreated (−, lanes *1*, *3*, and *5*) or treated with 10 μM rapamycin for 30 min (+, lanes *2*, *4*, and *6*). Whole cell extracts were obtained, and Western blots were performed with antibodies to $p70^{s6k}$ (lanes *1* and *2*), to P-ser-STAT1 (lanes *3* and *4*), and to P-ser-STAT3 (lanes *5* and *6*). The results are representative of experiments performed on five patients. *pp70*, Phosphorylated p70. *pp85*, Phosphorylated p85.

STAT1 (Fig. 9, lanes *3* and *4*) or STAT3 (Fig. 9, lanes *5* and *6*) in these cells. Thus, although $pp70^{s6k}$ is present and activated in CLL cells, it does not appear to contribute to the serine phosphorylation of STAT1 and STAT3.

## Discussion

In this report, we define a consistent biochemical abnormality, the constitutive phosphorylation of STAT1 and STAT3 each on ser-727, that is common to all CLL samples examined to date. The finding that ser-727 phosphorylation of STAT1 and STAT3 is found only sporadically in other hematologic malignancies suggests that this is not merely a marker of transformed or rapidly proliferating cells. Although constitutive tyrosine phosphorylation of STAT1 and STAT3 is present in AML and ALL, the serine phosphorylation of these STATs is not found uniformly (Table I). By contrast, tyrosine phosphorylation of STAT1 and STAT3 is never seen in freshly isolated CLL cells, whereas the serine phosphorylation of these STATs is present consistently. Thus, the pattern of phosphorylation of STAT1 and STAT3 is clearly distinct between CLL cells and AML or ALL cells.

In attempting to understand the importance of STAT serine phosphorylation in the pathogenesis of CLL, several factors need to be considered. One critical issue is how these modifications of STAT1 and STAT3 compare with those found in the normal counterpart to the cell from which CLL arises. The serine phosphorylation of STAT1 and STAT3 could represent an acquired alteration necessary for the generation of the malignant phenotype. Alternatively, it could reflect the proliferation of a population of cells in which STAT serine phosphorylation is found normally. We compared the serine phosphorylation of STAT1 and STAT3 demonstrated in peripheral blood CLL cells with that of B lymphocytes purified from the peripheral blood of normal donors. Although no serine phosphorylation was found basally in the normal cells, treatment of these cells with PMA did induce this phosphorylation (Fig. 4 *A*). This indicated that these cells were viable, contained STAT1 and STAT3, and could undergo phosphorylation of ser-727 of each of these STATs with appropriate stimulation. Thus, the constitutive serine phosphorylation of STAT1 and STAT3 detected in circulating CLL cells is not found in normal circulating B cells. Since CLL cells are characterized by the presence of CD5 on the cell surface (2), we analyzed STAT phosphorylation in $CD5^+$ B lymphocytes isolated from tonsil. Here again, basal phosphorylation of STAT1 and STAT3 on ser-727 was not seen (Fig. 4 *B*). Thus, the serine phosphorylation of STAT1 and STAT3 seen in CLL does not appear to represent the expansion of a normal B cell population in which this phosphorylation occurs normally.

Although CLL is a common malignancy, no consistent abnormalities have been found to underlie its pathogenesis. It may be that a variety of genetic abnormalities may lead to an imbalance of B lymphocyte proliferation and death, leading to the clinical entity of CLL. If this is the case, then it is likely that these various abnormalities may converge upon certain critical signaling points that are necessary for the development of the malignant phenotype. The finding that ser-727 of STAT1 and STAT3 is phosphorylated in all CLL cells analyzed to date raises the possibility that this phosphorylation may be a central event in the pathogenesis of CLL. Given that tyrosine phosphorylation is necessary for STAT dimerization, nuclear translocation, and DNA binding, the question arises as to how serine phosphorylation of these STATs in the absence of tyrosine phosphorylation might contribute to the biology of CLL. It is known that the phosphorylation of STAT1 and STAT3 on ser-727 in the presence of tyrosine phosphorylation of these STATs leads to an enhancement of transcriptional activation over that seen with the tyrosine-phosphorylated STATs alone (9, 10). Thus, the constitutive serine phosphorylation of these STATs might serve to enhance the transcription of target genes after another stimulus, such as a cytokine, induces their tyrosine phosphorylation. In this way, the signaling events initiated by cytokines that use STAT1 and/or STAT3 would always be maximal, and the ability to modulate the STAT-mediated response would be lost. Furthermore, in some cell lines proliferating in log phase in the presence of a cytokine on which they are dependent, the magnitude of STAT tyrosine phosphorylation is low. However, the tyrosine phosphorylation of these proteins becomes very pronounced if the cells are first starved and then stimulated with that cytokine (our unpublished data). Thus, the absence of prominent tyrosine phosphorylation of STAT1 and STAT3 in CLL cells does not preclude the possibility that such phosphorylation does occur during the physiologic proliferation of these cells. Furthermore, the constitutive serine phosphorylation of these STATs might enhance the magnitude of tyrosine phosphorylation occurring after a cytokine (or another proliferative or antiapoptotic stimulus) interacts with a cell.

Alternatively, the serine phosphorylation of STATs might have an additional function independent of their role in modulating transcriptional activation. Since a hallmark of CLL cells is their long life span and relatively low proliferation, it is possible that STAT serine phosphorylation might in some way directly enhance survival or prevent apoptosis. Finally, the phosphorylation of ser-727 of STAT1 and STAT3 might not have

direct bearing on the biology of CLL cells. It is conceivable that these sites are bystander substrates of one or more serine/threonine kinases that are activated, but whose primary substrate is another protein. The identification of the kinase(s) responsible for STAT1 and STAT3 serine phosphorylation in CLL will likely provide insight into this issue, and into the pathogenesis of CLL as well.

To assess directly the role of the ser-727 phosphorylation of STAT1 and STAT3 in normal and CLL lymphocytes, it will be necessary to alter the phosphorylation of these STATs in these cells. Pharmacological agents that block the STAT serine phosphorylation, such as H7, may be useful in defining the function of these events, although the specificity of these drugs may not be sufficient to draw firm conclusions. Alternatively, the introduction of STAT1 or STAT3 constructs in which ser-727 has been mutated to an alanine, thereby precluding phosphorylation of these sites, might provide insight into the biological effects of disrupting this pathway. Pending the results of such studies, the importance of STAT1 and STAT3 serine phosphorylation in the biology of CLL remains uncertain.

The ability to analyze rapidly and specifically the phosphorylation of ser-727 of STAT1 and STAT3 has been aided by the antibodies that specifically recognize each of these sites. In lieu of such antibodies, it would be necessary to metabolically label cells with [$^{32}$P]orthophosphate, isolate the specific STAT, and perform phospho–amino acid analysis. Aside from being cumbersome and ill-suited to analyzing a large number of samples, this method imposes the stress of phosphate depletion and radioactive labeling at high specific activities, both of which may lead to artifacts, particularly in primary cells such as those from patients with CLL. Furthermore, these antibodies measure phosphorylation of the specific serine residues from the sequences to which they were raised, rather than also measuring additional sites of serine phosphorylation which might not affect the function of these STATs (as occurs with phospho–amino acid analysis).

The phosphorylated serine residues of both STAT 1 and STAT 3 are found in the sequence leu-pro-met-ser-pro, a motif consistent with substrate sites of proline-directed serine/threonine kinases (21, 22). Despite the similarities of these sites, it is notable that the P-ser-STAT1 and P-ser-STAT3 antibodies do not cross-react. This suggests that although the pro-x-ser-pro sequence might be sufficient for recognition by a number of kinases, the specificity for other protein–protein interactions involving these sites may require additional surrounding amino acids. The mechanism by which serine phosphorylation of these STATs leads to enhanced transcriptional activity is not known. The phosphorylation of ser-133 of the transcription factor CREB (for cAMP response element–binding protein) allows CREB to interact with CREB-binding protein, which appears to be critical for the initiation of transcription (31, 32). Whether STAT1 and STAT3 are able to interact with other proteins after their phosphorylation is not known. If STAT1 and STAT3 do interact with coactivators after phosphorylation, it would be important to determine whether they interact with the same or distinct molecules, given the similarity of their sites of serine phosphorylation. Such experiments might also shed light on the determinants of specificity in the cellular response to activated STATs.

Similarly, it will be important to determine whether the phosphorylation of ser-727 of STAT1 and STAT3 is regulated coordinately, and whether their phosphorylation is mediated by the same kinase. Given that ser-727 of both STAT1 and STAT3 are dephosphorylated rapidly in the presence of the kinase inhibitor H7, it is clear that kinase(s) that can phosphorylate these sites are active continuously in CLL cells. It had been suggested that ser-727 of STAT1 and STAT3 might be phosphorylated by MAP kinase (10). In CLL cells, it is clear that MAP kinase is not constitutively activated, and is unlikely to be the STAT serine kinase that maintains phosphorylation of these sites. It is notable that pp70$^{s6k}$ is activated in CLL cells. Although this enzyme also does not appear to be the CLL STAT serine kinase, it may be downstream of a STAT kinase. Alternatively, the activation of pp70$^{s6k}$ may reflect the activation of an independent pathway that contributes to the development of CLL. Ultimately, the identification of the CLL STAT serine kinase is likely to provide important information on the genesis of this malignancy. Furthermore, if the phosphorylation of ser-727 of STAT1 and STAT3 is, in fact, important to the pathogenesis of CLL, then the development of inhibitors of STAT serine kinase(s) might be a fruitful therapeutic strategy.

In conclusion, we have identified constitutive serine phosphorylation of STAT1 and STAT3 as a finding common to all CLL samples examined to date. The role that this phosphorylation plays in the biology of CLL is unknown. However, it is likely that important information on the biology of CLL and of normal lymphocytes will be derived from studying the mechanism of phosphorylation of these STATs, and in determining how these events affect gene transcription and cell growth and survival.

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