RAPID COMMUNICATION

Constitutive Activation of STAT Proteins in Primary Lymphoid and Myeloid Leukemia Cells and in Epstein-Barr Virus (EBV)–Related Lymphoma Cell Lines

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Although various molecular mechanisms of STAT protein (signal transducers and activators of transcription) activation have been identified, little is known about the functional role of STAT-dependent transcriptional activation. Herein we report the constitutive nuclear localization, phosphorylation, and DNA-binding activity of STAT proteins in leukemia cells and lymphoma cell lines. With the use of oligonucleotide probes derived from the FcγRI promoter, the β-casein promoter and a STAT-binding element in the promoter of the *Bcl-2* gene constitutive activation of STAT proteins was detected in untreated acute T- and C/B-leukemia cells (3 of 5 and 12 of 19 patients, respectively). Supershift analyses using Stats 1-6 specific antisera showed the constitutive DNA binding activity of Stat5 in these cells. Confocal microscopy revealed the nuclear localization of Stat5 and Western blot analyses showed tyrosine phosphorylation of Stat5 in nuclear extracts of acute leukemia cells. In contrast, peripheral blood mononuclear cells did not display constitutive STAT-DNA interaction. Further studies were performed on freshly isolated acute myeloid leukemia cells as well as on cell line derived K562, lymphoblastoid cells (LCL), and Burkitt's lymphoma cells (BL). Fluorescence microscopy, gelshift, and supershift experiments showed the nuclear localization and constitutive DNA-binding activity of Stat5 in K562 cells. Stat1 and Stat3 were constitutively activated in freshly isolated AML cells (10 of 14 patients) and in Epstein-Barr virus–positive or interleukin-10 expressing permanent LCL and BL cells. Thus, these data indicate a differential pattern of STAT protein activation in lymphoid or myeloid leukemia and in lymphoma cells.

STAT PROTEINS (signal transducers and activators of transcription) are latent cytoplasmic transcription factors that become activated by tyrosine phosphorylation in response to cytokines. The cDNAs for six STAT-family members have been cloned and expressed identifying proteins in the 90-kD range that share low overall sequence homology.[1-4] STAT phosphorylation occurs in response to the activation of receptor-tyrosine kinases such as epidermal-growth factor (EGF) as well as in response to the activation of cytokine receptors that lack intrinsic tyrosine kinases but have Janus-Kinases associated with their intracellular domains.[5-9] STAT proteins become activated by various extracellular and intracellular stimuli. Cytokine-receptor interaction that is followed by the oligomerization of the receptor chains and the activation of Janus-Kinases (JAK) results in the phosphorylation of specific tyrosine residues within the intracellular part of a cytokine receptor which then serve as docking sites for STAT proteins.[10] Phosphorylation of receptor-associated STAT proteins by JAK affects the dissociation of the STAT proteins from the receptor, as well as STAT dimerization, entry into the nucleus and STAT-DNA binding activity.[1,5,11] Thus, STAT proteins recruit a family of cytoplasmic proteins that directly transduce signals from the cytoplasma to the nucleus. In addition to the activation of STAT proteins through extracellular cytokines, intracellular events have been identified that activate the JAK/STAT pathway.[12] Thus, Stat5 activation not only occurs upon treatment of a cell with interleukin-3 (IL-3), IL-2, prolactin, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, or thrombopoietin, but Stat5 activation results from human T-cell leukemia virus type I (HTLV-I) infection in T cells.[7,13-17] Furthermore, Stat3 becomes activated in early events of liver cell regeneration, which may indicate that STAT proteins are involved in mediating proliferative signals.[18] Examination of the protein sequences showed a single, highly conserved MAPK phosphorylation site (PXS/TP) in Stat1, Stat3, Stat4, and Stat5 indicating a functional role of MAPK-dependent STAT activation.[19] Recent data indicate that serine/threonine phosphorylation is necessary to fully restore STAT activity.[19,20] The mitogen-activated-kinase (MAPK; ERK2) has been identified to physically associate to the α-chain of the interferon (IFN)-α/-β receptor as well as to Stat1, indicating a link between the JAK/STAT and the MAPK pathways.[19,21,22] Furthermore, the *src*-kinase as well as v-*abl* have been shown to activate STAT proteins.[12,23,24] Thus, STAT activation appears to play a central role in cellular signaling integrating distinct signaling pathways. Although the molecular mechanisms of STAT protein activation become more obvious, little information is currently available defining the functional role of STAT-mediated transcriptional activation.[25] Recent reports have shown that the inhibition of JAK-2 phosphorylation in primary pre-B leukemia could inhibit cell proliferation, indicating a link of cell proliferation and JAK2 phosphorylation in acute leukemia cells.[26] However, virtually nothing is known about the role of STAT protein activation in the process of leukemogenesis. The purpose of this report is to characterize STAT activation in primary human leukemic cells obtained

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from peripheral blood (PB) of untreated patients with acute lymphoid or acute myeloid leukemia (AML). Moreover, we show constitutive STAT activation in unstimulated Burkitt's lymphoma (BL) or lymphoblastoid (LCL) and K562 cells, whereas no such activation is observed in mononuclear cells isolated from healthy individuals. These data show the constitutive STAT activation in acute leukemia cells, suggesting a role of STAT activation in the leukemogenesis of acute lymphoid, acute and chronic myeloid leukemia, as well as in Epstein-Barr virus (EBV)–transformed lymphoblastoid cells.

MATERIALS AND METHODS

Reagents and cytokines. Cell culture medium (RPMI) was purchased from GIBCO Life Technologies (Paisley, Scotland) and PolydIdC was obtained from Pharmacia LKB Biotechnology, Inc (Piscataway, NJ). HPLC purified oligonucleotides were purchased from Birsner & Grob Biotech GmbH (Freiburg, Germany). Cytokines were purchased from Genzyme (Cambridge, MA).

Cells. PB from healthy volunteers or from patients suffering from common (c)/B-T-acute lymphoid leukemia (ALL) or AML was obtained after informed consent. The disease classification was established according the French-American-British (FAB) classification. PB was diluted 1:2 in phosphate-buffered saline (PBS), pH 7.5, and mononuclear cells were prepared by Ficoll-Hypaque centrifugation for 30 minutes at 1,000*g* (Pharmacia LKB Biotechnology Inc). Cells were isolated from the interphase, washed twice in PBS, and nuclear extracts were prepared. Alternatively, acute lymphoid leukemia cells that were frozen for up to 6 years in 10% dimethyl sulfoxide (DMSO) in fetal calf serum (FCS) were thawed, DMSO was washed away, and nuclear extracts were prepared. Cells of the P12/ICHIKAWA and K652 lines were purchased from DSM Collection of human and animal cell lines (Braunschweig, Germany). LCL cells (Cherry) and BL cells (E95/Ramos, Namalva, Akata, Daudi, Raji, DG75, BL41) were kindly provided by Dr George Klein (Karolinska-Institute, Stockholm, Sweden). Cells were maintained in RPMI medium, 10% FCS, 1% penicillin-streptomycin.

Electrophoretic mobility shift assay (EMSA). Two million cells were left untreated or were treated with 100 ng/mL IFN-γ for 10 minutes at 37°C. Nuclear extracts and EMSA were prepared as described.[27] In brief, cells were washed once in ice-cold PBS and incubated with ice-cold cell lysis buffer (10 mmol/L HEPES, pH 7.9; 10 mmol/L KCL; 0.1 mmol/L EDTA; 0.1 mmol/L EGTA; 1 mmol/L dithiothreitol [DTT]; and 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF]) containing Nonidet P-40 (Sigma, St Louis, MO) at a final concentration of 0.05% vol/vol. Incubation was continued for 30 minutes, nuclei were pelleted from the lysed cells at 1,000*g* for 5 minutes at 4°C, and nuclei were resuspended in 50 μL of ice-cold extraction buffer (20 mmol/L HEPES, pH 7.9; 0.4 mol/L NaCl; 1 mmol/L EDTA; 25% glycerol; 1 mmol/L DTT; 0.5 mmol/L PMSF). Extraction was performed on ice for 2 hours, nuclei were centrifuged (10,000*g*) for 5 minutes, and nuclear proteins were transferred. The protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). Electrophoretic mobility shift assay was performed by incubating 3 μg of the nuclear extracts with 25,000 cpm (1 ng) of a double-stranded ^{32}P-labeled oligonucleotide probe. Sequences were based (1) on the GRR of the FcγRI gene promoter (top strand: 5′ ATGTATTTC-CCAGAAA 3′), (2) on the PRL of the β-casein promoter (top strand: 5′ AGATTTCTAGGAATTCAAATCCAC 3′), and (3) a STAT consensus element in the *Bcl*-2 promoter (top strand: 5′ AGGACTTCT-GCGAATACCGG 3′).

Incubation was performed for 20 minutes at room temperature in 15 μL of binding buffer (10 mmol/L Tris-HCL, pH 7.5; 100 mmol/L KCL; 5 mmol/L $MgCl_2$; 1 mmol/L DTT, and 10% glycerol) in the presence of 2 μg of Poly (dIdC). The reaction mixtures were separated by electrophoresis on a 6% polyacrylamide gel in 0.25 × TBE for 3.5 hours at 150 V. Gels were dried and analyzed by autoradiography. Supershift experiments were performed to determine the nature of the DNA-binding STAT transcription factors present in the nuclear proteins using specific antisera for STATs(1-6) or normal rabbit serum at a dilution of 1:100. STATs(1-4)-specific antisera were provided by Dr J.E. Darnell (Rockefeller University, New York, NY), Stat5-specific antibody was provided by Dr B. Groner (Tumor Biology Center, Freiburg, Germany), and Stat6-specific antisera was provided by Dr S. McKnight (Tularik, San Francisco, CA, and Dr B. Groner).

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nuclear extracts (50 μg protein per lane) were separated by electrophoresis on an 8% SDS-polyacrylamid gel, transferred to nitrocellulose, and probed with HRPO conjugated PY20 antiphosphotyrosine-specific antibody (1:2,500) (Signal Transduction Laboratories, Lexington, KY) in PBS. Blots were washed in TBST (50 mmol/L Tris-HCL, pH 7.5; 150 mmol/L NaCL; 0.5% Tween-20) and developed using the ECL system (Amersham Corp, Arlington Heights, IL). Blots were then stripped as described[27] and reprobed with anti-Stat5 antisera (1:3,000 in TBST).

Alternatively, nuclear extracts from 1 to 2 × 10^7 acute leukemia cells were precipitated using 3 μg of phosphotyrosine-specific antibody or 3 μL of normal rabbit serum for 1 hour at 4°C followed by the addition of 100 μL of Protein-A coupled agarose (50% vol/vol) (Sigma, St Louis, MO) for 30 minutes at 4°C. Proteins were eluted in Laemmli-Buffer and resolved by SDS-PAGE. After transfer to nitrocellulose, blots were probed with anti-Stat5 antisera (1:3,000 in TBST).

Confocal microscopy. Ten thousand cells in 10 μL PBS were added on cell adhesion slides (Bio-Rad Laboratories). Cells were fixed in 1% paraformaldehyde in PBS and stained by sequential incubation with anti-STAT antisera 1:50 in PBS and goat-antirabbit Ig-CY3-conjugate from Amersham (1:500 in PBS) for 30 minutes at room temperature, respectively. Slides were washed for 15 minutes in PBS on a shaker and flourescence was analyzed by fluorescence microscopy using a laser confocal microscope (LSM 410 invert; Zeiss, Jena, Germany).

RESULTS

STAT-related transcription factors with binding affinity to promoters of multiple genes are constitutively activated in primary ALL blasts. To study the activation state of STAT proteins in primary cells derived from untreated ALL patients, leukemic cells were isolated from peripheral blood of ALL patients (c-ALL, B-ALL, T-ALL) before therapy. Nuclear extracts were prepared and STAT-binding activity was tested using a gel-shift assay that employed distinct double-stranded oligonucleotide probes representing STAT-binding consensus sequences from the promoters of selected genes. In the nuclear extracts of the untreated ALL blasts constitutive DNA binding activity was observed to the GRR probe of the FcγRI promoter, the PRL probe of the β-Casein gene promoter, as well as to a STAT consensus element at position −822 to −805 upstream of the transcriptional startpoint in the promoter of the *Bcl*-2 gene[28] (Fig 1A). To investigate whether constitutive activation of the STAT proteins was an artefact caused by the isolation procedure, nuclear extracts were prepared directly after cell harvest or

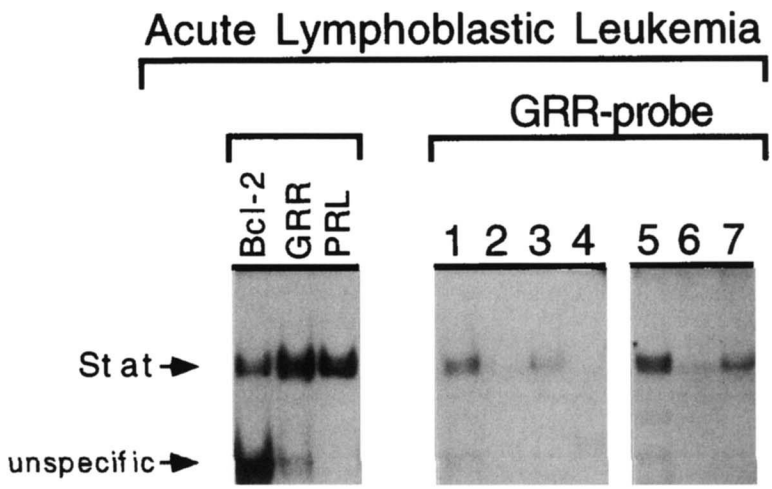

Fig 1. STAT-related proteins display constitutive DNA binding activity in the nuclear extracts derived from untreated cells of acute leukemia patients. Three micrograms of nuclear protein derived from ALL cells was analyzed by EMSA for DNA-binding activity to Stat consensus sequences from promoter regions of the FcγRI gene (lane GRR), the β-casein gene (lane PRL), and the *Bcl*-2 gene (lane Bcl-2). Nuclear extracts from ALL cells were analyzed in EMSA using a ^{32}P-labeled GRR probe in the absence (lane 1) or presence of 100-fold excess of unlabeled GRR (lane 4), *Bcl*-2 (lane 3), or PRL double-stranded oligonucleotides (lane 2). Further competition experiments of STAT protein-GRR probe interaction were performed in the absence (lane 5) or presence of 1,000- (lane 6) or 100-fold (lane 7) excess of unlabeled *Bcl*-2 probe.

after a 3-day culture in medium supplemented with 10% FCS. Equal constitutive STAT-DNA–binding activity was observed in ALL cells independently from the use of these cells directly after cell harvest or after an in vitro culture period (data not shown). To control for the specificity of the constitutive STAT-DNA interaction in these cells competition studies were performed to further characterize the STAT-related DNA binding activity in the different promoter sequences. In these competition experiments different STAT consensus probes were used to address whether the binding of the constitutively activated STAT proteins was specific and whether similar STAT factors were involved in the complexes obtained with the different probes. A 100-fold excess of unlabeled GRR probe completely competed the amount of STAT proteins that bound to 1 ng labeled GRR probe, indicating the specificity of the STAT-DNA interaction (Fig 1, lanes 1 and 4). Furthermore, similar results were obtained when nuclear proteins were incubated with phosphotyrosine-specific antibody (data not shown). A 100-fold excess of unlabeled PRL or *Bcl*-2 probe competed 90% or 50% of the GRR-STAT interaction, respectively, as determined by densitometry (Fig 1, lanes 1 through 3). However, a 1,000-fold excess of unlabeled *Bcl*-2 probe was needed to compete 95% of the GRR-STAT interaction, thus indicating that the GRR sequence comprises a comparatively high-affinity binding of the STAT proteins (Fig 1, lanes 5 through 7). Further control experiments were performed using primary human mononuclear cells derived from three healthy individuals. No constitutive STAT-DNA binding activity was detected in the nuclei from mononuclear cells of healthy individuals (see Fig 3). However, these cells were viable and could respond to IFN-γ by STAT activation (see Fig 3). Thus, STAT activation specifically occured in PB ALL blasts but was undetectable in mononuclear cells derived from healthy individuals. To test for the generality of constitutive STAT activation in acute leukemia cells, independent samples derived from patients with acute lymphoid or myeloid leukemia were obtained and tested by gel-shift analyses.

Stat5 is constitutively activated in primary cells from ALL patients as well as in K562 cells and Stat1/Stat3 DNA binding activity is found in primary AML cells. To determine the nature of the STAT proteins that were constitutively activated in ALL blasts, supershift experiments were performed using Stat1 through Stat6 specific antisera. These experiments showed the basal activation and DNA binding activity of Stat5 in ALL (Fig 2). No supershift was obtained using Stat1-, Stat2-, Stat3-, Stat4-, or Stat6-specific antisera. Thus, Stat5 was shown to interact with the GRR, PRL, and *Bcl*-2 probe in acute lymphoid leukemia (Fig 2). To test for

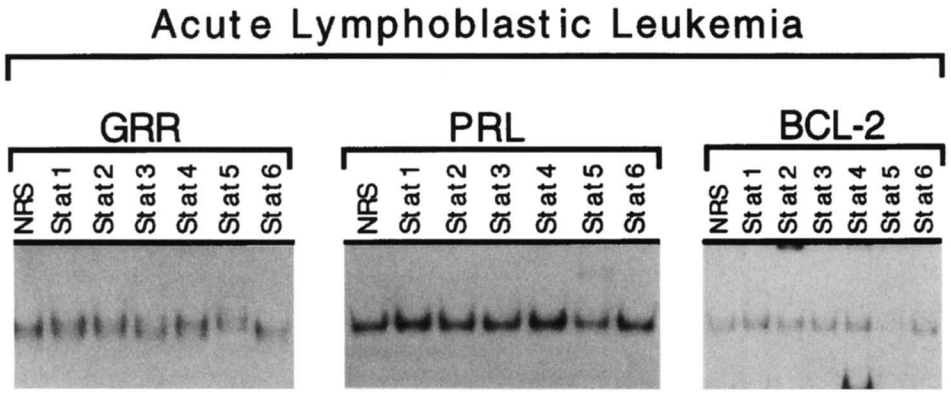

Fig 2. Stat5 is constitutively activated in the nuclei of ALL cells. Supershift experiments were performed using Stat1- through Stat6-specific antisera in the presence of 3 μg of nuclear proteins derived from freshly isolated ALL cells. Nuclear extracts were incubated with 1 ng of radiolabeled GRR probe, PRL probe, or *Bcl*-2 probe in the presence of normal rabbit sera (NRS) or Stats 1-6 specific antisera and protein-DNA complexes were separated by PAGE.

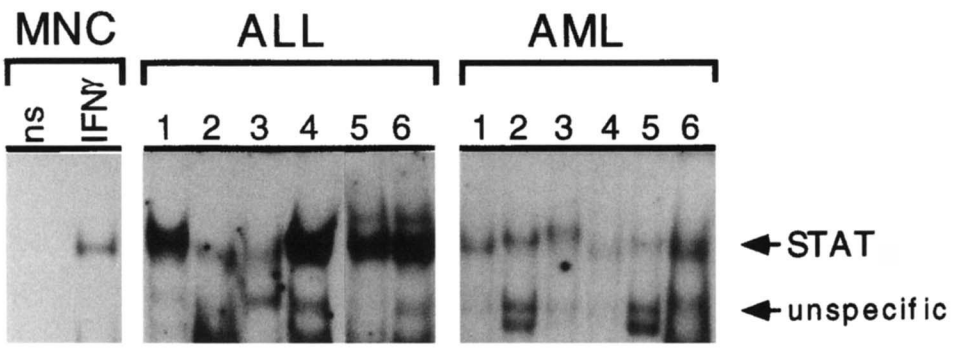

Fig 3. STAT proteins are constitutively activated in acute lymphoid and myeloid leukemia cells but not in mononuclear cells of healthy individuals. Mononuclear cells from healthy individuals were analyzed for constitutive Stat protein activation (lane 1) or were treated with 100 ng/mL of IFN-γ (lane 2) and nuclear extracts were used in EMSA experiments employing the 18-bp GRR oligonucleotide probe. Further nuclear extracts of acute lymphoid or myeloid leukemia cells were tested for constitutive STAT activation by gel-shift analyses.

the generality of this observation and to confirm the result of basal STAT activation in acute lymphoid leukemia, cells from three more patients presenting with acute B-ALL were freshly isolated (Fig 3, ALL panel, lanes 1 through 4). In all of these B-ALL cells basal DNA binding activity of the Stat5 transcription factor was detected. In one of the ALL patients Stat1 was activated in addition to Stat5 (Table 1). To increase patient numbers, samples of acute leukemia patients were analyzed that were stored frozen in liquid nitrogen for up to 7 years. Using the frozen leukemia specimens, constitutive STAT activation was detected in 8 of 15 patients with B-ALL and in 3 of 5 patients with T-ALL (Fig 3, ALL panel lanes 5 and 6; Table 1). To study whether constitutive activation of STAT proteins was a phenomenon that was common in blasts derived from PB of acute leukemia patients we assayed primary blasts from patients suffering from AML. In 4 of 14 AML patients tested we did not find basal STAT activity. However, in 10 of the 14 AML patients we identified basal activation of Stat1 and Stat3 (Figs 3 and 4) and in 1 case we found activated Stat1, Stat3, and Stat5 in the nuclear extracts (Table 1; Fig 3, AML panel, lane 3). Figure 4 shows a representative supershift obtained with the AML-derived nuclear extracts in which DNA binding of Stat1 and Stat3 could be identified. To address the question whether basal STAT activity was restricted to patient-derived freshly isolated acute lymphoid and myeloid leukemias, we probed nuclear extracts of K562 permanent cells. Constitutive DNA binding activity was present in the nuclei of the permanent K562 CML-based cells (Fig 4). In supershift experiments this DNA binding complex was identified to consist of Stat5. Thus, basal STAT-DNA binding activity presented as a phenomenon that was observed in freshly isolated cells from acute lymphoid and myeloid leukemia patients and in K562 cells.

Table 1. Basal Activation of STAT Proteins in Primary Leukemic Cells of Acute Lymphoid and Myeloid Leukemia Patients

Leukemia	Stat1	Stat2	Stat3	Stat4	Stat5	Stat6
T-ALL					(3/5*)	
cALL					1/1	
B-ALL	1/3				3/3	
					(8/15)	
AML	10/14		10/14		1/14	

* No. of positive samples/samples tested. Frozen samples are indicated in parentheses.

Activation of STAT proteins involves tyrosine phosphorylation, entry of the latent cytosolic STAT proteins into the nucleus, and subsequent DNA binding activity. To further test for STAT activation in the leukemia cells single-cell staining was performed employing STAT 1 to 6 specific antisera. STAT-antibody interaction was detected by fluorescein-conjugated secondary antibodies and STAT proteins were visualized by fluorescence microscopy. In acute leukemia cells as well as in K562 cells, Stat5 could be located to the nucleus of the cells (Fig 5). In contrast, the mass of Stat2 (Fig 5) as well as of Stat1, Stat3, Stat4, and Stat6 was located in the cytoplasm of these leukemic cells (data not shown). Moreover, PB cells from healthy donors was tested for the expression of STAT proteins. All of the Stats including Stat5 were located to the cytoplasm of the healthy mononuclear cells indicating the functional inactivity of the STAT proteins in these cells (Fig 5). These data thus demonstrate the nuclear localization and the activation of Stat5 but not Stats 1-4 or Stat6 in freshly isolated acute leukemia cells as well as in K562 cells. No constitutive nuclear localization of the STAT proteins was found in mononuclear cells from healthy individuals. Furthermore, Western Blot analyses were performed using the nuclear extracts of freshly isolated acute leukemia cells or control cells. Multiple tyrosine phosphorylated proteins were detected in the nuclei of leukemic cells. One phosphotyrosine containing band at approximately 92 kD was identified to consist of Stat5 by Stat5 reblotting and specific precipitation experiments (Fig 6). These experiments further confirmed the presence of tyrosine-phosphorylated Stat5 in the nuclei of the untreated ALL cells but not in untreated mononuclear cells from healthy individuals (Fig 6).

Constitutive activation of STAT-related proteins in lymphoblastoid cells and Burkitt's lymphoma (BL) appears to be related to the presence of EBV and cellular IL-10. We next sought to investigate constitutive STAT activation in EBV^+ versus EBV^- cell lines. Therefore, nuclear extracts

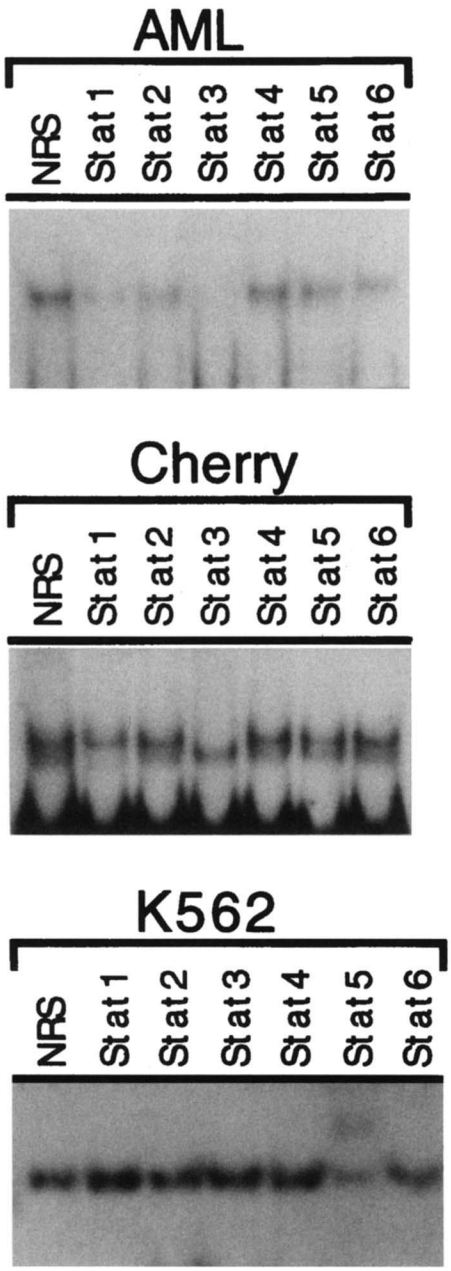

Fig 4. Stat1 and Stat3 are constitutively activated in cells from AML as well as in lymphoblastoid cells and Stat5 shows constitutive DNA binding activity in K562 cells. Three micrograms of nuclear proteins derived from freshly isolated AML, permanent lymphoblastoid cells (Cherry), and permanent *bcr-abl*$^+$ K562 cells were incubated with STAT protein-specific antisera and separated by gel-shift analysis.

were prepared from cultured lymphoblastoid cells and BL cells (Table 2). Cells were further characterized for the presence of the EBV DNA, for the presence of *Bcl*-2 and IL-10 expression using reverse-transcriptase polymerase chain reaction. These experiments showed constitutively activated Stat1 and Stat3 in cells that were positive for EBV and/or cellular IL-10 (Fig 4 and Table 2). Figure 4, middle panel, shows the results of basal STAT activation obtained in the Cherry lymphoblastoid cells (Fig 4 and Table 2). In the proliferating Cherry cells as well as in the EBV$^+$ BL cells, Stat1 and Stat3 showed constitutive DNA binding activity (Fig 4 and Table 2). Cells that were negative for EBV or IL-10 expression appeared not to display constitutive activation of STAT-related proteins (Table 2). Thus, these data suggested that activation of Stat1 and Stat3 transcription factors coincide with the presence of the EBV. Moreover, Stat1 and Stat3 activation in lymphoblastoid cells coincided with cellular IL-10 expression and high expression levels of the Bcl-2 protein in these cells. In contrast, cells derived from a T-cell leukemia cell line that was negative for the presence of HTL-I/II virus and human immunodeficiency virus type I (HIV-I) did not display basal STAT activation. Thus, constitutive activation of STAT proteins in lymphoma cells appears to be linked to the presence of transforming viruses and/or cellular IL-10 expression, thus coinciding with increased *Bcl*-2 expression in the lymphoblastoid and lymphoma cells.

DISCUSSION

The activation of Stat proteins by the action of cytokines is known to link receptor-ligand interaction at the cell surface to specific gene activation in the nucleus.[2,29,30] Besides this extracellular mechanism of Stat activation, intracellular events influence the activation state of STAT transcription factors. Thus, HTL-I virus has been shown to activate Stat5 upon infection of T lymphocytes.[31] Proliferative responses in liver cell regeneration have been connected to Stat3 activation.[18] Furthermore, activated Stat3 has been found in the nucleus of mammary carcinoma cells but not in cells derived from normal mammary gland.[27] In the current report we now extend the concept of basal activation of Stat proteins in malignant cells.

As shown herein, DNA binding activity of Stat proteins was found in freshly isolated leukemic blasts from acute lymphoid and myeloid leukemia. Supershift experiments revealed that different Stat transcription factors were activated in different leukemias. We found Stat5 to be predominantly activated in acute lymphoid leukemia and Stat1 and Stat3 to be predominantly activated in AML as well as in EBV$^+$ and IL-10–producing lymphoblastoid and BL cells. We and others have shown that these Stat proteins bind to Stat consensus elements derived from selected gene promoters with distinct affinities.[32,33] Activated Stat5 protein in the nucleus of ALL cells bound strongly to the GRR of the FcγRI gene promoter, to the PRL of the β-casein promoter, and displayed low-affinity binding to a STAT consensus element identified in the *Bcl*-2 promoter. In contrast, basal Stat1 and Stat3 DNA-binding activity was observed to the GRR sequence in the nuclei of AML cells. Thus, the nature of the STAT proteins that become constitutively activated and the affinities of the DNA-transcription factor interaction may play a critical role in determining the on/off rates of selected transcriptional activation in leukemic cells. The presence of a STAT consensus element in the promoter of the *Bcl*-2 gene as well as basal DNA-binding activity of STAT proteins in the *Bcl*-2 promoter in leukemic cells might predict a role of STAT activation in the context of *Bcl*-2 expression and

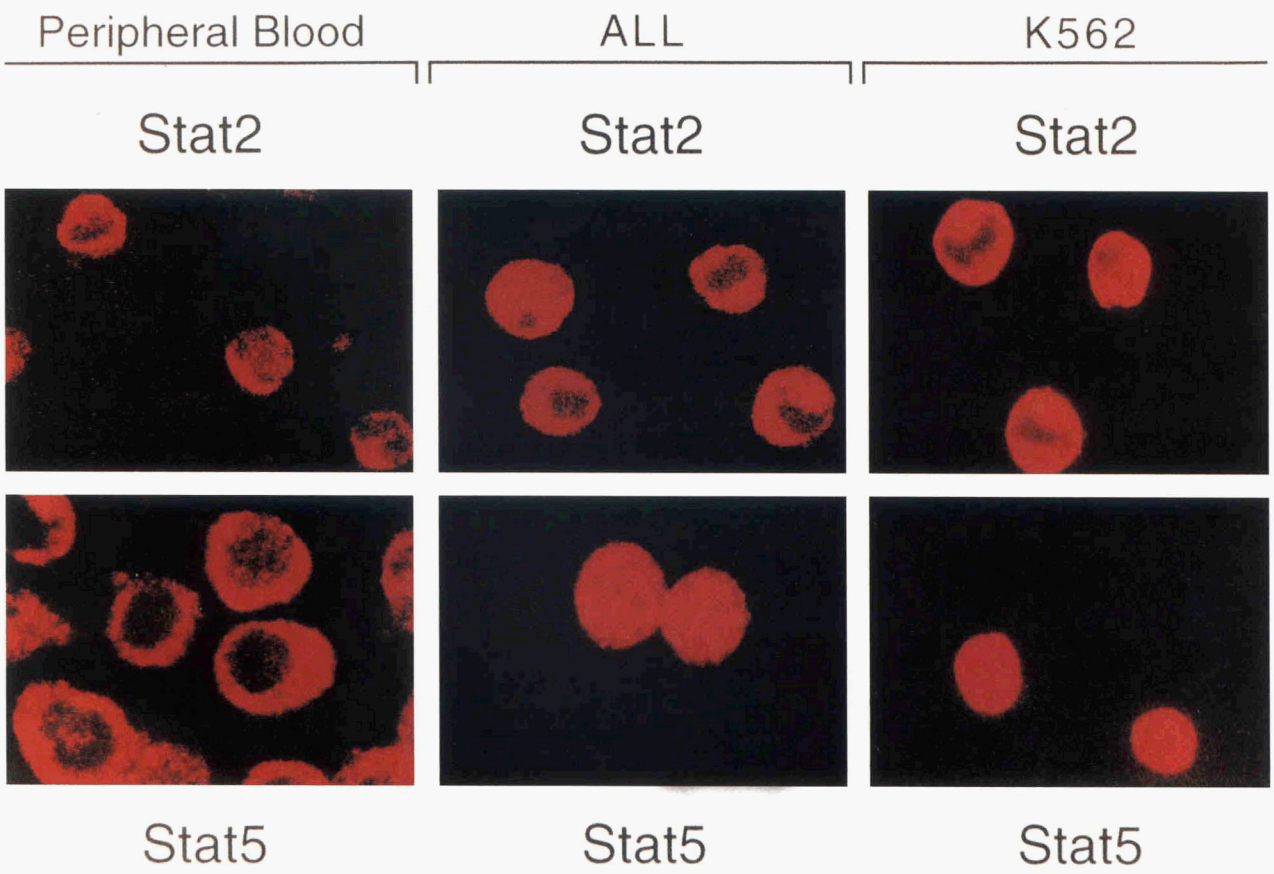

Fig 5. Stat5 is located in the nucleus of acute leukemic cells. Mononuclear cells of healthy donors, acute leukemic blasts, and K562 cells were added to adhesive slides and stained by sequential incubation with anti-STAT specific antisera (anti-Stat2, top panel; anti-Stat5, bottom panel, respectively) followed by incubation with goat-antirabbit antisera coupled to fluorescein (Cy3). Antibody binding was detected by fluorescence microscopy using a laser confocal microscope.

programmed cell death.[34] Current studies are in progress to define the role of activated STAT-DNA binding in the promoter of the *Bcl*-2 gene. However, molecular mechanisms leading to constitutive STAT activation, nuclear translocation, and DNA-binding activity in leukemic cells are virtually unknown. Recent data suggested a functional role of JAK activation in the proliferative response and the survival of leukemic cells in vitro.[26] Whereas this report demonstrates the coincidence of cell death and the inhibition of JAK activity, the biologic importance of constitutive STAT phosphor-

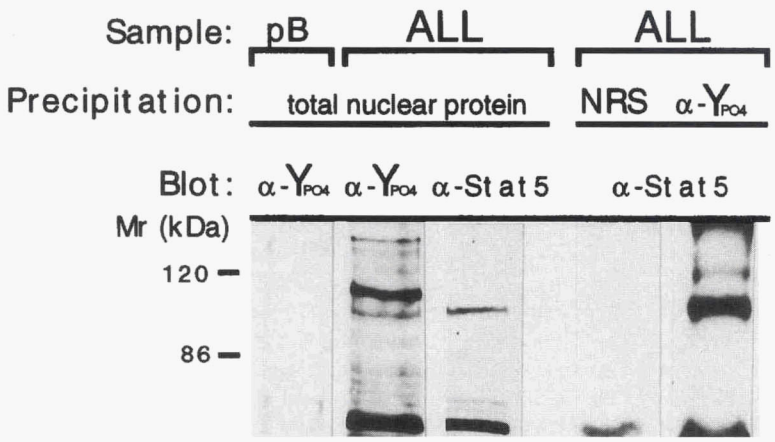

Fig 6. Stat5 is constitutively phosphorylated in nuclear extracts of primary leukemic cells. Fifty micrograms of total nuclear protein of healthy mononuclear cells (lane 1) or acute leukemic blasts (lane 2 and lane 3) were separated by SDS-PAGE and blotted to nitrocellulose. Proteins were detected by blotting with antiphosphotyrosine specific antibody (lanes 1 and 2) or by incubation with Stat5 antisera followed by an incubation step using goat-antirabbit HRPO conjugate. Binding was visualized by the ECL system. Alternatively, nuclear proteins of acute leukemic cells were precipitated with normal rabbit sera (lane 4) or with phosphotyrosine specific antibody (lane 5), and Western blots were developed using the Stat5-specific antisera.

Table 2. Basal Activation of Stat-DNA Binding Activity in Lymphoblastoid and BL Cells as Well as in K652 CML and P12/ICHIKAWA T-ALL Cells

Cell Line	Stat1	Stat3	Stat5	Bcl-2*	IL-10*	EBV*
Cherry (LCL)**	+	+	–	++	++	+
Namalva (BL)**	+	+	–	++	++	+
Akata (BL)	+	+	–	+	+	+
Daudi (BL)	+	+	–	+	+	+
DG75 (BL)	–	–	–	+	–	–
BL41 (BL)	–	–	–	+	–	–
K562	–	–	+	++	–	–
P12/Ichikawa (T-ALL)	–	–	–	ND	ND	ND

Abbreviations: LCL, lymphoblastoid cells; BL, Burkitt's lymphoma cells; ND, not done.

* Data from Finke et al.[37,38]

ylation as well as the molecular mechanisms leading to basal STAT protein activation in leukemic cells are currently unknown. Likely possibilities are that (1) viral infections of a cell could lead to a constitutive STAT activation through the direct action of viral proteins; (2) chromosomal translocations could be involved in the constitutive activation of STAT transcription factors by effecting expression levels of STAT proteins or kinases; or (3) cytokines may be produced as a consequence of viral infections and/or chromosomal translocations which then may activate STAT proteins in an autocrine or paracrine loop.[35,36] Recent reports support these working hypotheses. HTLV-1 virus that has been associated with T-ALL appeared to induce STAT protein activation in T cells.[31] In our study we show that T-ALL–derived cells lacking the HTLV-1 virus do not have constitutively activated STAT proteins in their nuclei. Moreover, pre-B cells overexpressing the v-*abl* protein display basal IL-4/IL-7–related STAT activity.[23] Herein we demonstrate the constitutive activity of STAT proteins in K562 cells. These cells carry the t(9;22) translocation, resulting in the expression of the *bcr-abl* gene product. The finding of constitutively activated STAT transcription factors in these cells supports the general theme of oncogene-dependent STAT activation. Furthermore, EBV has been related to the proliferative potential of BL cells.[37-39] Experiments presented in this study demonstrate the coincidence of EBV, IL-10 production, and constitutive STAT1 and STAT3 activation in lymphoblastoid and BL cells. Moreover, BL cell lines lacking the EBV and IL-10 did not display constitutively active STAT-DNA binding. IL-10 has been shown to induce the phosphorylation, nuclear translocation, and DNA-binding activity of Stat1, Stat3, and Stat5 in pre-B cells (R.M.W.-N., unpublished results, March 1995). IL-10–dependent phosphorylation of STAT proteins was shown to be mediated by the JAK1 and Tyk2 Kinases (J.W., R.M.W.-N., unpublished results, October 1995). Thus, the possibility must be considered that autocrine IL-10 production leads to the activation of STAT proteins in these cells. Current experiments are underway to test this hypothesis. Thus, the activation of cytokine-related signal transduction pathways by different mechanisms that interfere from inside or outside a cell may lead to transformation and proliferation, and subsequently direct the malignant potential of hematologic disorders.

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