

A Novel Serine-dependent Proteolytic Activity Is Responsible for Truncated Signal Transducer and Activator of Transcription Proteins in Acute Myeloid Leukemia Blasts¹

Zheng Xia, Robert R. Salzler, Daniel P. Kunz, Maria R. Baer, Latif Kazim, Heinz Baumann, and Meir Wetzler²

Leukemia Section, Department of Medicine, Biopolymer Facility and Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT

Hematopoietic cytokine receptor signaling involves activation of signal transducer and activator of transcription (STAT) proteins that are thought to control cellular differentiation. Truncated STAT isoforms (β forms, rather than the normal α forms) have been described and found to block the normal signaling function of the α isoforms. We recently demonstrated STAT β isoforms in bone marrow samples from 21 of 27 (78%) acute myeloid leukemia (AML) patients. We sought to determine the mechanism by which the STAT β forms were generated. Samples from eight newly diagnosed AML patients were studied; four expressed predominantly STAT α , and four expressed predominantly STAT β . The reverse transcription-PCR generated identical products in the two groups, suggesting that alternate mRNA splicing is not responsible for the genesis of STAT β . Extracts from cells expressing predominantly STAT β incubated with cell extracts from the MO7E cell line, which expresses predominantly STAT α , caused a decrease of the α isoforms and an increase of the β isoforms, suggesting the presence of proteolytic activity. This proteolytic activity was: (a) specific for STAT3 and STAT5, but not for STAT6; (b) serine dependent; (c) equally present in nuclear and cytoplasmic fractions of the leukemic blasts; and (d) different than the activity detected in a murine hematopoietic cell line. The cleaved β isoforms retained their DNA-binding activity. Because expression of truncated STATs may be involved in blocking differentiation of AML blasts, elucidation of the regulation of the proteolytic activity may contribute to our understanding of leukemogenesis.

INTRODUCTION

Members of the JAK³ family of protein kinases and the STAT DNA-binding proteins are activated by tyrosine phosphorylation and involved in signal transduction by many hematopoietic cytokine receptors (reviewed in Refs. 1 and 2). It is postulated that ligand binding results in aggregation of the receptor subunits and the associated JAKs, allowing transphosphorylation and activation of the JAKs. The JAKs subsequently phosphorylate the receptors and various cellular substrates (e.g., STATs) that are recruited to the activated receptor complexes. Subsequently, the STAT molecules undergo tyrosine phosphorylation and dimerization. They then translocate to the nucleus, where they bind to DNA, alone or in conjunction with other proteins, and direct specific transcriptional responses. The current notion is that STAT proteins are involved in the regulation of cellular differentiation, whereas other pathways (e.g., mitogen-activated protein kinase) are involved in cellular proliferation.

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² To whom requests for reprints should be addressed, at Leukemia Section, Department of Medicine, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-8447; Fax: (716) 845-2343; E-mail: meir.wetzler@roswellpark.org.

³ The abbreviations used are: JAK, Janus Kinase; STAT, signal transducer and activator of transcription; AML, acute myeloid leukemia; RT-PCR, reverse transcription-PCR; TPO, thrombopoietin; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; ALLN, *N*-acetyl-Leu-Leu-norleucinal; SE-FPLC, size exclusion-fast protein liquid chromatography.

The existence of different functional isoforms of several STAT proteins has been documented. Full-length STAT3 (referred to here as STAT3 α) has been described as having an isoform (STAT3 β) that is truncated by 55 amino acid residues at the COOH-terminal end (3). STAT3 β has been found in AML blasts in some patients (4, 5). Similarly, STAT5A and STAT5B, originating from two separate genes, are described to have at least two isoforms (α/β) each (6–8). The COOH-terminal sequence that is missing in the β -forms of both STAT3 and STAT5 encodes the transcriptional activation domain (7, 9, 10). The β isoforms were shown to have a transdominant negative effect on gene induction in the STAT pathway (9–12). If the alternate STAT β forms are expressed, they may inhibit differentiation, which is normally induced by the α forms.

The short forms of STAT proteins (termed β) are believed to be derived from either alternatively spliced mRNA or proteolytic cleavage. Alternative splicing has been suggested for STAT1 (13), STAT3 (3), and STAT5 (14). However, truncations do not arise only by alternative splicing. Azam *et al.* (15) were the first to suggest that truncated STAT5 isoforms can be generated by proteolysis. Recently, two groups studying a murine multipotent hematopoietic cell line showed that STAT5 β is produced by a nuclear serine protease (16, 17) with a molecular weight of 25,000 (17). To the best of our knowledge, no information exists about a similar activity for STAT3. We sought to determine how the β isoforms are generated in AML cells. We have previously demonstrated that the majority of AML samples express the β isoforms, that AML samples containing the STAT3 β isoform also contain the STAT5 β isoform, and that samples with the β isoforms maintain the same forms on exposure to exogenous cytokines (18). These data suggest that a common mechanism is responsible for the appearance of the truncated forms in AML blasts. We demonstrate here that the STAT β forms in AML cells are generated by a specific serine-dependent proteolytic activity that is different from the activity demonstrated in the murine hematopoietic cell line. This activity has the following characteristics: (a) it modifies both STAT3 and STAT5; (b) it is present in both the cytoplasm and the nucleus; and (c) it migrates with a molecular weight of approximately 40,000.

MATERIALS AND METHODS

Patient Population. Pretreatment cells from eight newly diagnosed AML patients were studied. The patients' clinical characteristics are shown in Table 1. Studies were approved by the Roswell Park Cancer Institute Institutional Review Board. Informed consent was obtained from all patients.

Materials. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified, and all cytokines and growth factors were kindly provided by Amgen (Thousand Oaks, CA) unless otherwise specified.

Cell Collection. Light-density bone marrow cells were isolated by 1.077 gram/mm³ Ficoll-Hypaque density gradient centrifugation and either used immediately or cryopreserved under viable conditions, using standard methodology. Cell viability was verified by trypan blue dye exclusion.

RT-PCR. Total RNA was extracted from the cells by RNeasy Minikit (Qiagen Inc., Valencia, CA) as recommended by the supplier. The presence of STAT3, STAT5A, and STAT5B mRNA was determined by RT-PCR using 5' primers extending from position 2310 to 2336, 3123 to 3142, and 2246 to 2265

Table 1 Patient characteristics

UPN ^a	Age (yrs)/sex	FAB	BM blasts (%)	Karyotype	STAT α/β
960421	77/M	M1	84.0	41,X,-Y,?del(5)(p14), ?dup(6)(p21.3p25),-7,-7, der(8)t(7;8)(q11;q24),+del(11)(p13),-17,-17,-18,del(20)(q11)	α
980083	65/F	M0	88.4	46,XX,add(20)(q13.3)	α
970256	81/M	M1	88.0	45,XY,del(7)(p11),-13 45,XY,del(7)(p11),-13,dmin	α
970173	54/M	M4	80.2	47,XY,+8	α
970271	61/M	M4	36.6	46,XY	β
980079	56/F	M2	54.0	46,XX	β
970158	58/M	M1	81.6	47,XY,+8	β
970316	26/M	M1	87.0	46,XY	β

^a UPN, unique patient number; BM, bone marrow; FAB, French-American-British classification.

and 3' primers extending from position 2508 to 2533, 3621 to 3647, and 2641 to 2650 (5, 19, 20). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard for mRNA semiquantitation (21). One μ g of total RNA was reverse-transcribed to cDNA using 20 pM random hexamers (Perkin-Elmer, Branchburg, NJ) in a mixture of 25 mM MgCl₂; 10 \times PCR buffer II; and 1 mM deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxyribosyl thymine triphosphate (Pharmacia Biotech, Piscataway, NJ); 20 units of RNasin (Promega, Madison, WI); and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The total volume of each reaction was 20 μ l. The reverse transcription reaction was first incubated for 10 min at room temperature, reverse transcribed for 15 min at 42°C, and then denatured for 5 min at 99°C. Each reaction was placed on ice for 5 min.

PCR was performed in a final volume of 100 μ l using 20 μ l of the reverse transcriptase mixture containing 20 pM each of the 3' and the 5' PCR primers and 2.5 units of Taq Gold Polymerase (Perkin-Elmer) in its buffer. The amplification was performed for 34 cycles in a Hybaid temperature cycler (National Labnet, Woodbridge, NJ) with initial hot start at 94°C for 5 min, followed by 1 min of denaturation at 94°C, 1 min of annealing at 59°C, 1 min of extension at 74°C, and a final extension period of 7 min. Ten- μ l aliquots of this final reaction were analyzed on a 2% agarose gel (FMC Bioproducts, Rockland, ME) that contained 0.01% ethidium bromide in Tris-borate-EDTA electrophoresis buffer.

To determine whether alternatively spliced STAT3 messages are present in samples expressing the full-length and the truncated proteins, RT-PCR reactions were performed with serially diluted RNA preparations initially diluted 1:10 and then further diluted 1:50 and 1:250. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The STAT PCR products were extracted from the gel with the QIAquick gel extraction kit (Qiagen Inc., Santa Clarita, CA) as recommended by the supplier. The purified DNAs were sequenced with an Applied Biosystems model 373 stretch DNA sequencer (Perkin-Elmer). The data were analyzed with GeneWorks computer program (IntelliGenetics, Campell, CA).

MYC-STAT3 α Constructs. MYC-STAT3 α constructs were generated by inserting the rat STAT3 cDNA into the pSVSPORT1 plasmid (Clontech, Palo Alto, CA; Ref. 22). The NH₂ terminus of STAT3 α was modified by inserting six tandem copies of the MYC epitope in-frame (construct generously provided by Dr. T. Kordula, University of Georgia, Atlanta, GA).

Cell Lines. COS-1 cells were maintained in MEM supplemented with 5% FCS. FDCP-1 and NIH 3T3 cells (obtained from American Type Culture Collection, Manassas, VA) were maintained in DMEM with 4 mM L-glutamine and 4.5 grams/liter glucose. Medium for FDCP-1 cells was supplemented with mouse interleukin 3 as recommended by the supplier. MO7E cells were maintained in RPMI 1640 with 10% FCS. Activation of STAT3 and STAT5 was achieved by treating MO7E cells with TPO (40 ng/ml) for 15 min (23).

Transfection. COS-1 cells were transfected by the DEAE-dextran method (9). After a 36-h recovery, whole cell extracts were prepared.

Western Blotting. Western blot analysis was performed as described previously (18). Briefly, whole cell extracts (50–100 μ g of protein) were separated on 7.5% SDS polyacrylamide gels. The proteins were transferred onto a 0.1 μ m nitrocellulose membrane and incubated with antibodies against the NH₂-terminal portion of STAT3, STAT5, STAT6 (obtained from Transduction

Laboratories, Lexington, KY), STAT3 COOH-terminal-specific antibody (C-20), or MYC (SC-40; Santa Cruz Biotechnology). The immune complexes were detected by an enhanced chemiluminescence reaction (Amersham Life Science, Arlington Heights, IL).

In Vitro Proteolysis of STATs. MO7E cells containing predominantly the STAT α isoforms were lysed (5 \times 10⁶ cells in 45 μ l of lysis buffer). Aliquots of 2.5 μ l were mixed with equal volumes of the patients' cell extracts and then incubated at 25°C for 0, 15, and 30 min. Sensitivity of the proteolysis to inhibitors was tested as follows: apyrase (10 units) and calf intestinal alkaline phosphatase (4 units), both of which are known to hydrolyze ATP, were used to determine whether the proteolytic activity is ATP dependent (24); PMSF was used at 2–4 mmol/liter, to inhibit serine protease activity (16); calpain I and II inhibitor peptide (BioSource International, Camarillo, CA) were used at 10 μ M and 1 mM to inhibit calpain activity (25); and ALLN (at 12 and 60 μ M) and latacystin (at 2 and 10 μ M; both from Clabiochem, La Jolla, CA) were used to inhibit proteasome activity (26). Reaction mixtures were then separated on SDS-polyacrylamide gel and subjected to Western blotting as described above.

EMSA. Whole cell extracts were prepared as described previously (18) and incubated with ³²P-labeled oligomers corresponding to the binding element for STAT1 and STAT3 (SIE; Ref. 27) and STAT5 (TB2; Ref. 28). The complexes were analyzed by 5% PAGE and autoradiography. Extracts of TPO-treated MO7E cells served as standard for activated STAT3 α and STAT5 α . The identity of the STAT proteins contributing to the gel-shifted bands was determined by antibody supershift with the anti-STAT3 (C-20) or anti-STAT5 (C-17) monoclonal antibodies (Santa Cruz Biotechnology).

Cell Fractionation. To initiate preparation of nuclear and cytoplasmic fractions (16), cells were washed once with PBS containing 1 mmol/liter orthovanadate and then resuspended in a hypotonic buffer containing 20 mmol/liter HEPES (pH 7.6), 10 mmol/liter KCl, 1 mmol/liter MgCl₂, 0.5 mmol/liter DTT, 0.1% Triton X-100, and 20% glycerol and protease inhibitors (5 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 200 KIU/ml aprotinin, and 1 mmol/liter sodium orthovanadate). The cells were then homogenized by 20 strokes in a glass Dounce homogenizer. The homogenate was centrifuged at 2,000 \times g for 5 min. The supernatant (cytoplasmic fraction) was centrifuged again for 15 min at 14,000 rpm. The clear lysate was flash frozen in liquid nitrogen. The pelleted nuclei were resuspended in a hypertonic extraction buffer [20 mmol/liter HEPES (pH 7.9), 0.4 mol/liter NaCl, 1 mmol/liter EDTA, 0.5 mmol/liter DTT, 0.1% Triton-X 100, and 20% glycerol] containing protease inhibitors (as listed above) for 15 min at 4°C. The extracts were recovered by centrifugation for 5 min at 14,000 rpm. Aliquots were frozen in liquid nitrogen and stored at -70°C. The protein concentrations of nuclear and cytoplasmic extracts were adjusted with buffer to reflect their cellular ratios. The purity of the cell fractions was confirmed by Western blot analysis using antiserum against c-src (Santa Cruz Biotechnology) that is specific for the cytoplasm.

SE-FPLC. Two hundred μ l of cell extract were loaded onto a superose 6 prep grade column (Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with eluent buffer (HEPES, 40 mM; NaF, 40 mM; Na₃VO₄, 2 mM; Na₄P₂O₇, 2 mM; EDTA, 2 mM; EGTA, 2 mM; NaCl, 420 mM) and calibrated with the following standard proteins: cytochrome c (M_r 12,500), ovalbumin (M_r 45,000), monomeric and dimeric bovine serum albumin (M_r 67,000 and 120,000), and catalase (M_r 240,000). The eluates were collected in 500- μ l fractions, and each fraction was concentrated 10-fold with Centricon centrifugal filter devices (YM-10; Millipore, Bedford, MA).

RESULTS

STAT β Isoforms Are Generated by Proteolytic Cleavage. To study the mechanism by which STAT β isoforms are generated, samples from AML patients were analyzed by RT-PCR and Western blotting. Representative data are shown in Fig. 1. Sample from patient 960421 showed predominantly the α isoforms by Western blot analysis, whereas the sample from patient 970271 expressed predominantly the β isoforms (Fig. 1, A and B, bottom panels). The samples had the same mRNA species as demonstrated by RT-PCR, whether they expressed the α or the β proteins (Fig. 1, A and B, top panels). Serial mRNA dilutions did not detect any difference between the two

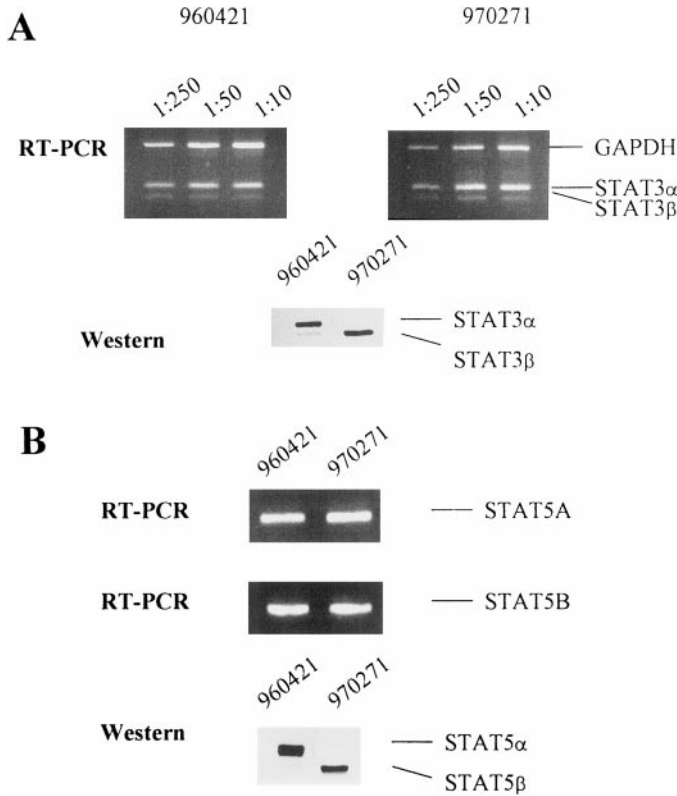


Fig. 1. Expression of STAT α versus STAT β mRNA and protein in AML blasts. *A*, STAT3; *B*, STAT5. *Top part of each panel*, RT-PCR products from representative patients; *bottom part of each panel*, Western blot analysis of STAT expression in cells from the same patients. The RT-PCR products were verified by sequencing. For STAT3, a serial dilution of 1:10, 1:50, and 1:250 is presented. The position of the STAT α and STAT β bands is indicated. Note that the sample from the patient that expressed primarily STAT β protein had mRNA products similar to those of the sample expressing primarily STAT α .

messages for STAT3 (Fig. 1A). STAT5A and STAT5B each have a single form of message (Fig. 1B), and therefore no analysis of dilutions was performed. Findings were similar for all other patient samples (data not shown). These results suggest that the STAT β isoforms were generated by proteolytic cleavage.

We reason that patient cells with STAT α have low STAT proteolytic activity, and patient cells with STAT β have high proteolytic activity. We also considered it conceivable that the samples have a differential expression of factors that inhibit the constitutively expressed proteolytic activity. To detect proteolysis, we combined whole cell extracts from patient samples expressing predominantly either the α or the β STAT isoforms with extracts from TPO-treated MO7E cells containing primarily activated α isoforms. The reaction mixtures, after 15 and 30 min of incubation, were analyzed by immunoblotting for the pattern of STAT proteins (Fig. 2). When using an extract from patient cells with α isoform, there was no change in the α/β pattern (Fig. 2, A and B). However, with patient samples containing predominantly the β isoforms, there was a decrease in the substrate of MO7E α isoforms and a concomitant increase in the intensity of the β isoforms (Fig. 2, C and D). The pattern was similar for STAT3 and STAT5. The proteolytic activity was specific for STAT3 and STAT5 because the extracts from patient samples with β isoforms had no effect on STAT6 of MO7E cells (Fig. 3). Essentially identical results regarding changes in STAT protein patterns were obtained when extracts from MO7E cells exposed to medium alone (predominantly the latent inactive STAT proteins) or with TPO (predominantly the activated dimerized STAT proteins) were used; data not shown.

A similar type of proteolytic activity was detected in samples from patients 970158 and 970316, in which there were >80% leukemic blasts, or in samples from patients 970271 or 980079, in which the leukemic blasts constituted only 36.6% and 54.0% of the cells. Because we did not detect additional, nonspecific bands even after incubation for 30 min, as one would expect in the presence of proteases from phagocytes, we ruled out an effect of contaminating neutrophils and monocytes, which could be present in samples with lower percentages of blasts.

To study the effect of the proteolytic activity on STAT DNA binding, EMSA assays were performed as shown in Fig. 4, A and B. These assays used only TPO-activated STAT proteins because the nonactivated proteins do not bind to DNA. The presence of STAT α or β forms in the extracts was identified by distinct electrophoretic mobility of the DNA-STAT complexes. Combination of patient cell extract with MO7E extract and incubation for 30 min produced characteristic modification of EMSA patterns. Cell extracts from patient samples expressing predominantly the β isoforms digested the MO7E-derived α isoforms without compromising their DNA binding activity. For STAT3, the mixture of cell extracts induced the conversion of the MO7E α pattern to the STAT3 β protein that gave rise to the slower migrating complex (Fig. 4A). The slower migration results from the loss of the acidic COOH-terminal domain of STAT3. To confirm that the slower migrating band did indeed contain a COOH-

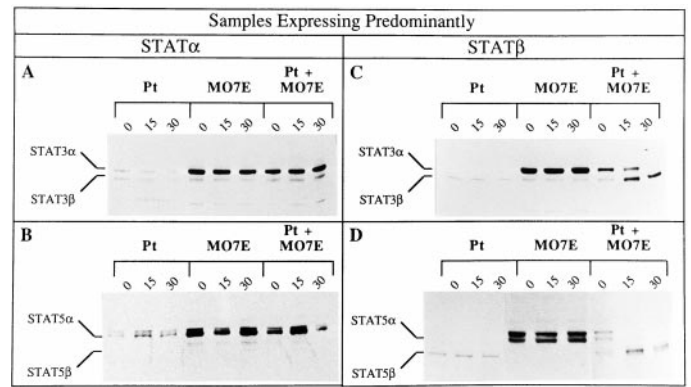


Fig. 2. *In vitro* peptide digestion of STAT α . Cell extracts from patients expressing primarily STAT α (A and B) or primarily STAT β (C and D) were incubated with or without MO7E cell extracts for 0, 15, and 30 min. MO7E cell extracts maintained for the same time periods were used as controls. The location of the α and β isoforms is indicated. A and C were analyzed for STAT3, and B and D were analyzed for STAT5 (A and B together). When using an antibody directed against the COOH-terminal domain, we did not detect the STAT β forms (data not shown). The pattern was similar for STAT3 and STAT5.

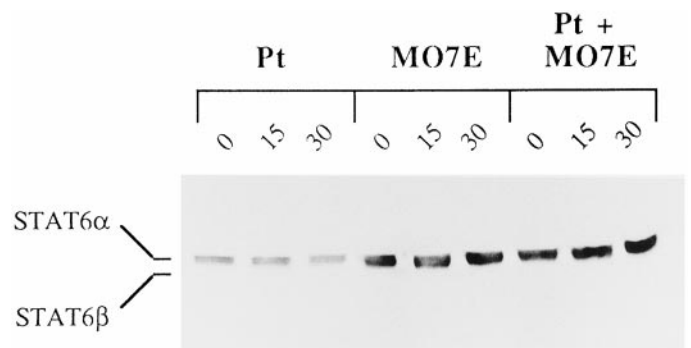


Fig. 3. *In vitro* peptide digestion of STAT6 α . Cell extracts from a representative patient expressing primarily STAT β were incubated with or without MO7E cell extracts for 0, 15, and 30 min. MO7E cell extracts maintained for the same time periods were used as controls. The patient sample did not express STAT6 (either α or β). There was no proteolytic activity of STAT6 α from MO7E in the patient's cell extract.

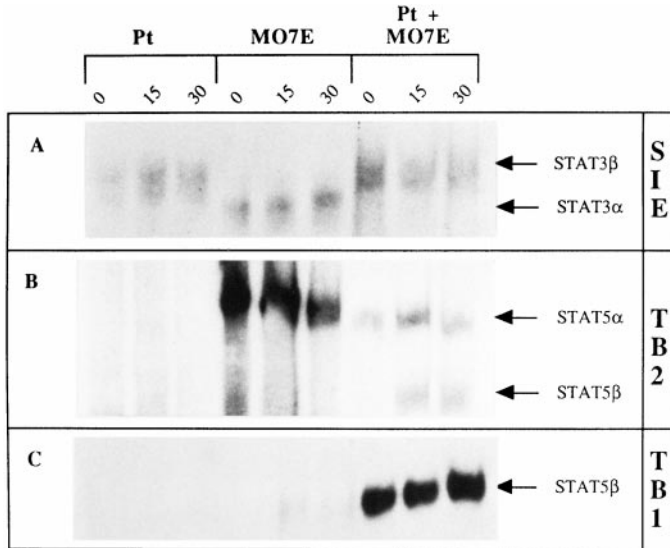


Fig. 4. Conservation of DNA binding activity of the digested STAT3 and STAT5. DNA binding activity of STAT proteins in extracts from AML blasts incubated with or without MO7E cell extracts for 0, 15, and 30 min is shown. MO7E cell extracts maintained for the same time periods were used as controls. The location of the α and β isoforms is indicated. A, whole cell extracts were prepared and analyzed by EMSA using radiolabeled SIE-duplex oligonucleotides. B, whole cell extracts were prepared and analyzed by EMSA using radiolabeled TB2-duplex oligonucleotides. C, whole cell extracts were prepared and analyzed by EMSA using radiolabeled TB1-duplex oligonucleotides. The appearance of TB1-specific DNA binding activity at time 0 suggests that some proteolytic activity occurred at this time point. The addition of an antibody directed against the COOH-terminal domain supershifted the SIE or TB2 bands only in samples expressing STAT α and not in samples expressing STAT β (data not shown). The DNA binding activity was conserved for STAT3 and STAT5.

terminal truncated STAT form, an antibody against the COOH-terminal domain was added to the mixture. No supershift was detected in any of the MO7E samples incubated with whole cell extracts from samples expressing predominantly the β isoforms (data not shown). In contrast, the antibody shifted the majority of the STAT3 DNA band when MO7E extract was not treated with leukemic cell extract. For STAT5, the same mixture showed an additional faster migrating band (Fig. 4B). This faster migrating band was found to contain STAT5 β that could only form a dimer bound to TB2 element, whereas a tetramer of STAT5 α bound to the same probe was thus responsible for the slower migrating complex (29). As with STAT3, no supershift was detected with STAT5 when using MO7E samples incubated with whole cell extracts from samples expressing predominantly the β isoforms (data not shown). The appearance of STAT5 β -specific DNA binding action could be independently confirmed by the binding to the TB1 oligomer (18),⁴ an oligonucleotide developed as a substrate specific for the β forms of STAT5A and STAT5B (29). As shown in Fig. 4C, the MO7E-derived activated STAT5 α isoforms, when incubated with extracts from patient samples expressing predominantly the β isoforms, were processed to a TB1 complex on EMSA. Control MO7E extracts treated with whole cell extracts from patients expressing predominantly the α isoforms did not alter the size of STAT3 and STAT5 or their DNA binding activity (data not shown). Thus, the data indicate that cells from AML patients expressing predominantly STAT β isoforms contain a proteolytic activity that cleaves STAT α forms and that these proteolytic products retain their DNA binding activity. In the case of STAT5 β , the truncation may also modify the binding specificity.

The Proteolytic Cleavage Is Serine Dependent. To assess whether the proteolytic cleavage is serine dependent, PMSF (2–4

mmol/liter) was added to the cell extracts during the *in vitro* proteolysis reaction. As shown in Fig. 5, PMSF blocked the proteolytic activity from leukemic samples and prevented the appearance of the STAT β isoforms in a dose-dependent manner. This proteolytic activity was minimally inhibited by high concentration of calpain I and II peptide inhibitor and was not dependent on ATP because apyrase or calf intestine alkaline phosphatase did not have any effect. Also, there was no evidence for a functional relationship with proteasome activity, because ALLN (Fig. 5) and latacystin (data not shown) did not affect STAT conversion. These data suggest that the proteolytic activity in AML samples is mainly serine dependent.

The Proteolytic Activity Is Found in the Cytoplasmic and Nuclear Fractions. To assess the subcellular localization of the proteolytic activity, patient samples with predominantly STAT β form were fractionated into cytoplasmic and nuclear fractions. As shown in Fig. 6, STAT3 β -generating activity was found in the cytoplasmic and nuclear fractions of the cells in similar amounts. Similar data were obtained for STAT5 β (data not shown).

The Proteolytic Activity in Human AML Blasts Differs from the Proteolytic Activity in the Murine FDCP-1 Cells. To date, two groups have shown that FDCP-1 cells express a serine-dependent nuclear proteolytic activity that cleaves STAT5 (16, 17). Because our assays show that AML blasts express a proteolytic activity that is able to digest both STAT3 and STAT5 and is present in both the cytoplasm and the nucleus, we compared the proteolytic activity between human AML blasts and murine FDCP-1 cells by using the *in vitro* STAT α digestion assay. Our results demonstrate that whole cell (Fig. 7, A and B) and nuclear (Fig. 7, C and D) extracts of FDCP-1 cells had no significant proteolytic activity on either STAT3 α or STAT5 α of murine (NIH 3T3 cells) or human (MO7E cells; data not shown)

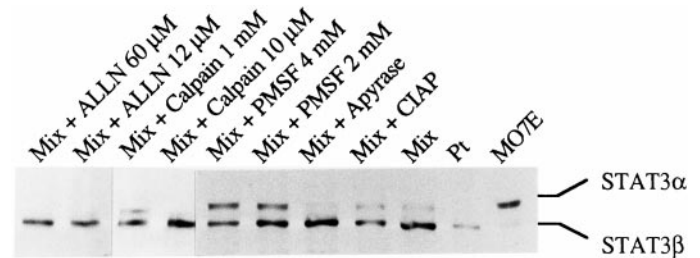


Fig. 5. The proteolytic activity is serine dependent. Cell extracts from a representative patient (*Pt*) expressing primarily STAT β were incubated with (*Mix*) or without MO7E cell extracts for 30 min. MO7E cell extracts maintained for 30 min were used as controls. Calf intestine alkaline phosphatase (*CIAP*) and apyrase, which are used to block ATP-dependent reactions, were added to the mixture without any effect. PMSF, which is used to block serine-dependent reactions, was added to the mixture at a concentration of 2 and 4 mM. There was a dose-dependent inhibition of the appearance of STAT β isoforms. Calpain I and II inhibitor peptide, which are used to block a cytoplasmic cysteine protease that requires Ca²⁺, were added to the mixture at a concentration of 1 mM and 10 μ M. ALLN, a proteasome inhibitor, was added at a concentration of 12 and 60 μ M. The location of the STAT3 α and STAT3 β isoforms is indicated.

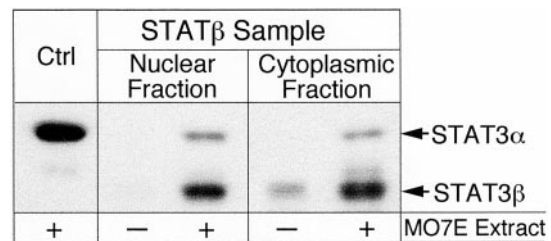


Fig. 6. The proteolytic activity is found in the cytoplasmic and nuclear moieties. Nuclear and cytoplasmic extracts from a patient expressing STAT β were incubated with or without MO7E cell extracts for 30 min. MO7E cell extracts incubated for 30 min were used as control. The location of the STAT3 α and STAT3 β isoforms is indicated.

⁴ C. F. Lai and H. Baumann, unpublished data.

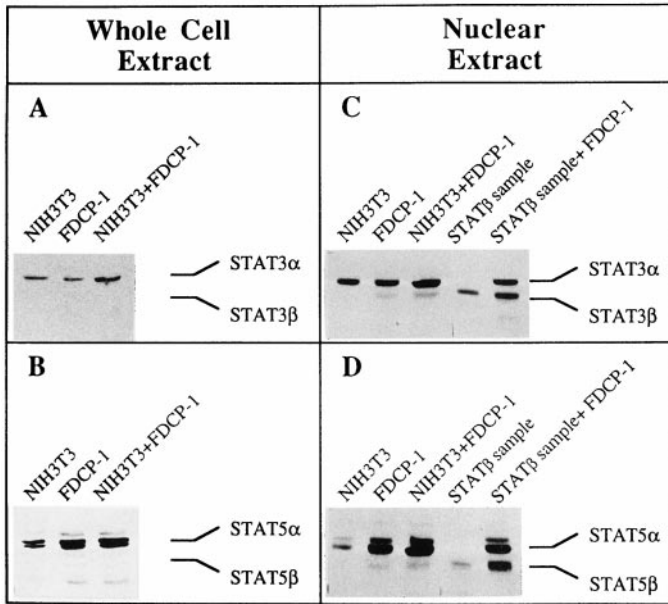


Fig. 7. The proteolytic activity in samples from AML patients is different than the activity from FDCP-1 cells (whole cell or nuclear extracts). Whole cell (A and B) and nuclear (C and D) extracts of FDCP-1 cells were tested against NIH 3T3 cell extracts. Extracts were tested for STAT3 (A and C) and STAT5 (B and D) digestion. The last lanes in C and D represent the mixture of a STAT β -expressing cell extract with FDCP-1. Note that there is no difference between FDCP-1 (whole cell or nuclear) and NIH 3T3 extracts.

origin. However, AML blasts expressing the STAT β isoforms and therefore carrying the proteolytic activity had a prominent effect (Fig. 7, C and D) on STAT3 α and STAT5 α .

To further characterize the murine and the human proteolytic activities, we analyzed these for their ability to modify the MYC-STAT3 α construct. Due to the MYC epitope added to the NH₂ terminus, this tagged STAT3 can readily be distinguished from normal proteolysis by physicochemical and immunological procedures (Fig. 8). MYC-STAT3 was expressed in COS-1 cells. Extracts from these COS-1 cells transfected with MYC-STAT3 construct were then incubated with extracts from either an AML patient or FDCP-1 cells. No change in MYC-STAT pattern occurred with extracts from AML patients expressing the STAT α form or with extracts from FDCP-1 cells. However, treatment of COS-1 cell extract with extract from an AML patient expressing STAT β caused a complete conversion of MYC-STAT to STAT3 β (Fig. 8, Lane 5). This suggests that the assay conditions identified a proteolytic activity in AML cells distinct from that reported for FDCP-1 cells.

Because the STAT5-degrading protease in FDCP-1 cells was reported to have a molecular weight of 25,000 (17), we determined the molecular weight of the proteolytic activity in AML cells by SE-FLPC separation. The activity was assessed in the MYC-STAT3 degradative assay (Fig. 9). Extracts from AML patients expressing STAT β resulted in the separation of STAT3 β that was collected at a molecular weight between 120,000 and 240,000, and the proteolytic activity converting MYC-STAT3 α to MYC-STAT3 β was collected at a molecular weight of approximately 40,000. These results support the conclusion that the proteolytic activity in AML cells is distinct from that in FDCP-1 cells.

DISCUSSION

This study shows that STAT β in AML blasts is generated by proteolytic cleavage. This activity appears to affect STAT3 and STAT5, but not STAT6. Although an equivalent STAT5-sensitive

mechanism has previously been suggested for cell lines (15–17), to the best of our knowledge, this is the first demonstration of such a process in fresh AML blasts.

The serine-dependent protease described in cell line models was nuclear, had a molecular weight of 25,000, and was tested only against STAT5 (16, 17). If one considers nucleus-localized STAT5 to represent activated STAT dimers (30), then the nuclear protease would have to act on dimerized STAT. In contrast, a cytoplasmic protease would have the ability to cleave STAT proteins in a different form, mainly as a “monomeric” (*i.e.*, latent) STAT. Moreover, in view of our data on AML blasts and the cell lines (15–17), it is still unclear whether one enzyme or a family of enzymes with conserved proteolytic activity but differences in substrate specificity accounts for the production of STAT β proteins. The minimal inhibition of proteolytic activity by the calpain inhibitor in AML cells suggests the presence of more than one activity. Taken together, one can conclude that the AML-derived protease is different from the one described in the murine hematopoietic cell line.

The role of the STAT β proteins and hence the functional consequence of STAT α proteolysis in AML blasts remain unknown. Recent work by Bromberg *et al.* (31) suggests that introducing a cysteine at the COOH-terminal domain of STAT3 α causes the molecule to dimerize, promote transcription, and induce cell transformation. STAT3-responsive elements have been identified primarily in cytokine-responsive genes, including acute phase genes or immediate early genes (*c-fos* and *JunB*). Similarly, STAT5A and STAT5B, which originate from two separate genes, are described to have at least two isoforms (α/β) each (6–8). The β forms were shown to have a competitive or even transdominant negative effect on gene induction mediated by the STAT pathway (9, 10). This is supported by an experimental model in tissue culture (32). In this system, using a murine myeloid hematopoietic cytokine-dependent cell line, a COOH-terminal truncation of STAT5A resulted in inhibition of both interleukin 3-dependent proliferation and granulocyte colony-stimulating factor-dependent differentiation, without induction of apoptosis. These data suggest that the truncated STATs may have a role in blocking differentiation and hence promoting survival.

Because the inappropriate appearance of the β isoforms might have not only a suppressive effect on the signal transduction pathway controlling differentiation but also an enhancing effect on proliferation, controlling β isoform production can contribute to leukemogenesis. These mechanisms do not involve only regulated expression of STAT protease but could conceivably involve the modulating activity of protease inhibitors. For example, possible candidates could be serine protease inhibitors (serpins), which belong to a large family of proteins that include, among others, cytoplasmic antiproteinase (33, 34) and a tumor suppressor called maspin (35) that is presumably related to ovalbumin-type serpin. Recently, bomapin, a novel human

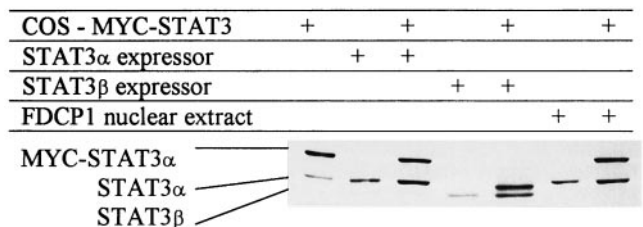


Fig. 8. *In vitro* digestion of recombinant MYC-STAT3 α by cell extracts from AML patients expressing STAT β is different than the activity in FDCP-1 cells. Western blot analysis of COS-1 cells expressing MYC-STAT3 α incubated with cell extract from either an AML patient expressing STAT3 α , an AML patient expressing STAT3 β , or nuclear extract of FDCP-1 is shown. The location of the MYC-STAT3 α construct, STAT3 α , and STAT3 β is indicated.

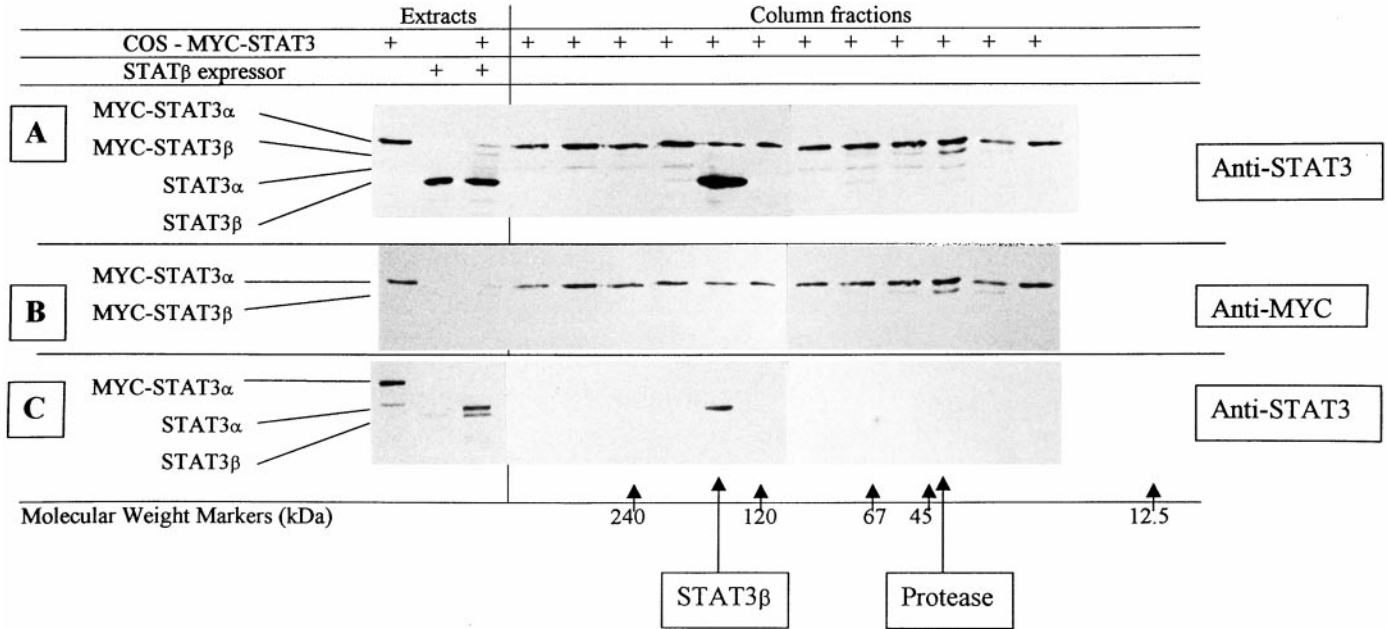


Fig. 9. The proteolytic activity in AML blasts expressing STAT3 β has a molecular weight of approximately 40,000. SE-FPLC separation of cell extract from an AML patient expressing STAT3 β is shown. The MYC-STAT3 α construct was used as a read-out to prevent confounding results due to the migration of resident STAT3 α (A and B). Controls of MYC-STAT3 α , cell extract from a patient expressing STAT3 β , and the combination of the two are shown. Separate fractions of cell extract from a patient expressing STAT3 β were either mixed with MYC-STAT3 α (A and B) or loaded alone (C). Blots were hybridized with anti-STAT3 or anti-MYC antibodies. The eluate of STAT3 β , the protease, and the different standards (by molecular weight) are indicated on the bottom.

serpin, was cloned (36). It was found to be expressed in normal bone marrow cells and in hematopoietic cells of the monocytic lineage (37). We suggest that cells expressing the STAT β isoforms will have increased levels of the protease and/or reduced levels of inhibitors. Conversely, cells that express the STAT α isoform should have either no protease or increased levels of a serpin to prevent the function of the protease. Finally, although it has been noted that cytokines can activate secretory serpins, this is not known for intracellular serpins. Because the STAT patterns are part of the signaling pathways in most hematopoietic and nonhematopoietic cell types but are under the control of distinct sets of regulators, we propose that a protease and a protease inhibitor may establish a yin-yang mechanism in controlling the proliferation and differentiation of their own. The modification of such a mechanism and its role in AML remain to be established.

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