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Phosphoproteomic analysis of AML cell lines identifies leukemic oncogenes

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Abstract

STAT5 is constitutively phosphorylated in leukemic cells in approximately 70% of acute myeloid leukemia (AML) patients. To identify kinase candidates potentially responsible for STAT5 phosphorylation, we used liquid chromatography–tandem mass spectrometry (LC–MS/MS) mass spectrometry to detect phosphoproteins in AML cell lines. We established TEL-ARG and BCR-ABL fusion proteins as the mechanism underlying STAT5 phosphorylation in HT-93 and KBM-3 cells, respectively. In addition, we identified a JAK2 pseudokinase domain mutation in HEL cells and using siRNA downregulation, established JAK2 as the kinase responsible for phosphorylating STAT5. This study illustrates the benefit of LC–MS/MS mass spectrometry and siRNA for the identification of novel targets and mutations.

Keywords: AML; Phosphopeptide; Tyrosine kinases; Leukemia

1. Introduction

Acute myeloid leukemia (AML) is an aggressive hematological malignancy that results from the acquisition of multiple genetic defects including translocations and/or mutations in proto-oncogenes. These defects compromise the regulation of critical signaling components involved in proliferation and apoptosis. For example, constitutive phosphorylation of STAT5 is observed in approximately 70% of AML patient samples [1,2]. STAT5 is a member of the signal transducer and activator of transcription (STAT) family of transcription factors. STAT proteins are phosphorylated and activated by janus kinases (JAKs), a family of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3 and TYK2) that provide kinase activity for a variety of cytokine receptors that lack intrinsic kinase activity. Ligand binding to cytokine receptors induces receptor dimerization, which allows specific associated JAK proteins to undergo transphosphorylation and activation (reviewed in ref. [3]). Activated JAK proteins then rapidly phosphorylate specific tyrosine residues on the receptor subunits. These phosphorylated residues serve as docking sites for proteins such as the STAT proteins, which are also phosphorylated by the activated JAKs. The phosphorylated STATs then dimerize via SH2-phosphotyrosyl interactions and in this activated form, translocate to the nucleus, where they regulate the transcription of several genes by interacting with specific DNA promoter sequences.

The mechanism of constitutive tyrosine phosphorylation of STAT5 in AML patient samples can be attributed to activating mutations of FLT3 [4] or KIT [5] in up to 35% of patients. However, an additional 35% of patients have STAT5 tyrosine phosphorylation in their leukemic cells in the absence

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of such mutations. Consequently, the exact mechanism of STAT5 phosphorylation remains unclear for these patients. Because STAT5 is constitutively tyrosine phosphorylated in AML patient samples, we hypothesized that the upstream activator of STAT5 is an unregulated tyrosine kinase. However, currently over 90 members belong to the tyrosine kinase family. To facilitate the identification of potential candidates, we employed a phosphoproteomic approach in which phosphopeptide immunoprecipitation in conjunction with liquid chromatography–tandem mass spectrometry (LC–MS/MS) mass spectrometry was used to identify activated tyrosine kinases in AML cell lines with constitutive STAT5 phosphorylation but without FLT3 or KIT mutations. Subsequent siRNA-induced downregulation of potential candidates provided validation for their role as STAT5 activators. Using this methodology, we were able to rapidly identify a JAK2 pseudokinase domain mutation in the HEL cell line and establish JAK2 as the upstream kinase responsible for phosphorylating STAT5.

2. Materials and methods

2.1. Cell lines and reagents

HEL cells were obtained from the German National Resource Centre for Biological Material (DSMZ). HT-93, HU-3 and KBM-3 were generously provided by Dr. Kenji Kishi, Dr. Doris Morgan and Dr. Borje Andersson, respectively. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 1 unit/mL penicillin G and 1 μg/mL streptomycin at 37 °C and 5% CO_2. The pan JAK inhibitor, JAK I, was purchased from Calbiochem, San Diego, CA.

2.2. KIT and FLT3 mutational analysis

Mutational analysis was performed as previously described [6,7].

2.3. Phosphopeptide immunoprecipitation

In urea lysis buffer (20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate), 2×10^8 cells were lysed at 1.25×10^8 cells/mL and sonicated. Sonicated lysates were cleared by centrifugation at 10,000 rpm, and proteins reduced and alkylated as described previously [8]. Samples were diluted with 20 mM HEPES pH 8.0 to a final urea concentration of 2 M. Soluble trypsin (1 mg/mL in 0.001 M HCl) or elastase (2 mg/mL in H_2O) was added to the clarified lysate at a 1:100 dilution. Samples were digested overnight at room temperature. Following digestion, lysates were acidified to a final concentration of 1% TFA. Peptide purification was carried out using Sep-Pak C_{18} columns. Following purification, all elutions (8%, 12%, 15%, 18%, 22%, 25%, 30%, 35% and 40% acetonitrile in 0.1% TFA) were combined into one fraction for analysis. Dried peptides were resuspended in 1.4 mL of MOPS immunoprecipitation buffer (50 mM MOPS/NaOH pH 7.2, 10 mM Na_2HPO_4, 50 mM NaCl) and insoluble material removed by centrifugation at 3000 rpm for 10 min. The phosphotyrosine monoclonal antibody P-Tyr-100 from ascites fluid was coupled non-covalently to protein G agarose (Roche) at 4 mg/mL beads overnight at 4 °C. After coupling, antibody-resin was washed twice with PBS and three times with MOPS immunoprecipitation buffer (5–10 bead volumes of buffer for each wash). Immobilized antibody (40 μL, 160 μg) was added as a 1:1 slurry in MOPS immunoprecipitation buffer to the solubilized peptide fraction, and the mixture was incubated overnight at 4 °C. The immobilized antibody beads were washed three times with 1 mL MOPS immunoprecipitation buffer and twice with 1 mL water, all at 4 °C. Peptides were eluted from beads by incubation with 60 μL of 0.1% TFA followed by a second elution with 40 μL of 0.1% TFA.

2.4. Analysis by LC–MS/MS mass spectrometry

Peptides in the immunoprecipitation eluate (53 μL) were concentrated and separated from eluted antibody using ZipTip μC_{18} columns (Millipore). Peptides were eluted from the microcolumns with 1 μL of 60% MeCN, 0.1% TFA into 7.6 μL of 0.4% acetic acid/0.005% heptafluorobutyric acid (HFBA). The sample was loaded onto a 10 cm × 75 μm PicoFrit capillary column (New Objective) packed with Magic C_{18} AQ reversed-phase resin (Michrom Bioresources) using a Famos autosampler with an inert sample injection valve (Dionex). The column was developed with a 45 min linear gradient of acetonitrile in 0.4% acetic acid, 0.005% HFBA delivered at 280 nL/min (Ultimate, Dionex). Tandem mass spectra were collected in a data-dependent manner with an LCQ Deca XP Plus ion trap mass spectrometer (ThermoFinnigan), using a top-four method, a dynamic exclusion repeat count of one, and a repeat duration of 0.5 min. TurboSequest (ThermoFinnigan) searches were done against the NCBI human database released on February 23, 2004 containing 27,175 proteins allowing for oxidized methionine (M + 16) and phosphorylation (Y + 80) as dynamic modifications.

2.5. Sequencing and mutational analysis

Primers for the kinase domain and JH2 pseudokinase domain of JAK2 were as-described [9]. Bidirectional sequence traces were assembled and analyzed for mutations using Mutation Surveyor Version 2.5 (SoftGenetics, State College, PA).

2.6. Source of siRNA

JAK1, JAK2, JAK3 and TYK2 *SMART*pool siRNA duplexes (proprietary target sequences) were purchased from