Identification of CRKL as the Constitutively Phosphorylated 39-kD Tyrosine Phosphoprotein in Chronic Myelogenous Leukemia Cells

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Chronic myelogenous leukemia (CML) is characterized by the presence of the Philadelphia (Ph) chromosome in clonally derived hematopoietic precursors and their progeny. The Ph chromosome arises from a translocation that deregulates the c-ABL protein tyrosine kinase, giving it transforming potential and increased kinase activity. We observed a unique 39-kD tyrosine phosphoprotein (pp39), previously reported in blastic CML cell lines, in neutrophils from 50 cases of chronic phase CML. This protein was prominently and constitutively tyrosine-phosphorylated in CML neutrophils and was not phosphorylated in normal neutrophils. Stimulation of normal neutrophils with cytokines and agonists did not induce tyrosine phosphorylation of proteins migrating in the region of pp39, and the phosphorylation state of pp39 in CML neutrophils was not affected by kinase inhibitors known to downregulate the ABL kinase. The pp39 was not phosphorylated in hematopoietic cells from healthy donors or from patients with Ph chromosome-negative myeloproliferative disorders. Using micro amino acid sequencing of purified preparations of pp39, we identified pp39 as CRKL protein, which is consistent with recent immunologic studies in the blastic K562 cell line. Immunoblotting with anti-CRKL antibodies showed the presence of CRKL protein in CML cells and cell lines as well as in antiphosphotyrosine immunoprecipitates from CML cells. Our results suggest that pp39 CRKL in CML neutrophils may be stably tyrosine-phosphorylated by the BCR/ABL kinase at an early stage of myeloid differentiation when the ABL kinase is active. CRK, CRKL, and other SH2 (SRC homology domain)/SH3-containing protein functions as adaptor molecules in nonreceptor tyrosine kinase signalling pathways. Although the CRKL protein is present in normal neutrophils, it is not tyrosine-phosphorylated, and the inability to induce such phosphorylation in normal neutrophils suggests a special role of this phospho-protein in the pathogenesis of CML. Constitutive phosphorylation of CRKL is unique to CML, indicating that it may be a useful target for therapeutic intervention.

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when the BCR/ABL kinase activity is downregulated as measured by autophosphorylation.

MATERIALS AND METHODS

Cells. Human blood and BM samples were obtained from healthy donors and patients with written informed consent. Neutrophils and lymphocyte/monocyte layers were isolated using neutrophil isolation media (Cardinal Products, Santa Fe, NM). For larger samples (leucopacs), neutrophils were isolated by centrifugation through Ficoll-Hypaque gradient, followed by dextran sedimentation as previously described.26 Under these conditions, populations of neutrophils were obtained of greater than 95% purity as assessed by Wright-Giemsa staining. Contaminating erythrocytes were removed by hypotonic solution with sterile water. Cell lines were maintained in Iscove’s medium supplemented with 10% fetal bovine serum (FBS). Monocytes were separated by plastic adherence, and macrophages were purified by plating in media supplemented with 15% FBS and 5% normal human serum for 72 to 96 hours. The Epstein-Barr virus (EBV)-transformed B-cell lines were a gift from Dr Bayard Clarkson (Memorial Sloan-Kettering, New York, NY). The Mo7 line transfected with P210 was a gift from Dr Brian Drucker (Oregon Health Sciences University, Portland). The RW Leu4 line was a gift from Dr Richard Huhn (University of Medicine & Dentistry of New Jersey, New Brunswick).

Lysis. Purified cells were lysed in buffer (20 mMol/L Tris, pH 7.4; 1 mMol/L EGTA; 1 mMol/L phenylmethylsulfonyl fluoride; 50 mMol/L sodium vanadate; 50 mMol/L sodium fluoride; 10 mg/mL aprotinin; 40 µg/mL leupeptin) and disrupted by sonication. In experiments requiring stimulants, cells were treated with 500 mMol/L granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), or 100 mMol/L 12-0-tetradecanoylphorbol-13-acetate (TPA) at 37°C for 5 minutes to 4 hours. For experiments with inhibitors, cells were treated with 50 µMol/L erbstatin analog (methyl 2,5-dihydroxy cinnamate; BioMol, Plymouth Meeting, PA), 100 µMol/L genistein (4',5,7-trihydroxy isoflavone; BioMol), 1 µMol/L okadac acid, 100 µMol/L herbimycin A, and 1 µMol/L staurosporin (BioMol), for the times indicated. Cells were also treated with 5 × 10^(-5) mol/L herbimycin A for 24 hours, followed by 3 days of culture in Iscove’s modified Dulbecco’s medium.

Western blot analysis. One-hundred-microgram samples of cell extracts were loaded on 10% acrylamide gels and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with specific antibodies. Immunoblotting. After transfer to polyvinylidene difluoride (PVDF) membranes, the nitrocellulose was treated with 5% non-fat milk for 1 hour before blocking. Competitive inhibition of antibody binding was performed using native CRKL and anti-CRKL antibody R62 recognizing the second SH3 region of CRKL (gift of Drs Feller and H. Hanafusa, Rockefeller University, New York, NY) or anti-CRKL antipeptide antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein purification and amino acid sequence analysis. Lysates of large-scale preparations of K562 cells were prepared. Approximately 100 mg of total lysate was subjected to ion-exchange purification over mono-Q columns (Bio-Rad, Richmond, CA) using 20 mMol/L Tris and 0.25 mMol/L NaCl as a solvent. This step was followed by size-selection on a Superdex 75 HiLoad 16/60 column (Pharmacia, Uppsala, Sweden) according to the manufacturer’s directions. Presence of pp39 in the aliquots was tested by blotting and antiphosphotyrosine staining. Partially purified fractions were concentrated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), electroblotted onto a nitrocellulose membrane, and visualized by Ponceau-S staining. After transfer, the 39-kDa band was excised and processed for internal amino acid sequence analysis, as described.27 Removal of the phosphorylated pp39 band was assessed by subsequent antiphosphotyrosine staining of the nitrocellulose.

Membrane-bound protein was subjected to in situ proteolytic cleavage with 0.7 µg trypsin (Promega, Madison, WI) in 25 µL of 100 mMol/L NH₄HCO₃ (supplemented with 10% acetonitrile and 3% Tween-80) at 37°C for 3 hours. The resulting peptide mixture was reduced and S-alkylated with 0.1% β-mercaptoethanol and 0.3% 4-vinyl pyridine, and fractionated by microbore reversed-phase high-performance liquid chromatography (HPLC). System assembly and chromatography solvents were as described28 with a 1.0-mm SGE ODS-2 C18 column installed. Fractions were collected by hand, kept on ice for the duration of the run, and then stored at ~70°C before analysis. An enzyme blank was run on an equally sized strip of nitrocellulose cut from a blank area of the same blot.

Peak fractions over background were analyzed by a combination of matrix-assisted laser-desorption (MALDI-TOF) mass spectrometry and automated Edman degradation.28 After storage, column fractions were reconstituted with neat trifluoroacetic acid to give a final concentration of 10%. Mass analysis of several peptides (on 2% aliquots) was then performed using a model LaserFlec Research MALDI-TOF instrument (Vestec), with a 337-nm output nitrogen laser and 1.2-m flight tube. The matrix was α-cyano-4-hydroxycinnamic acid, and a 28-kV ion acceleration and 4.3-kV multiplier voltage were used. Laser power and number of acquisitions were adjusted as judged from optimal deflections of specific maxima, monitored on a Tektronix TDS 520 digitizing oscilloscope. MS spectra were generated from the time files using the GRAMS data analysis software. Chemical sequencing of selected peptides (on 95% of the sample) was performed with the aid of a model 477A instrument from Applied Biosystems (AB; Foster City, CA). Stepwise-liberated phenylthio-hydantoin-amino acids were identified using an on-line 120A HPLC system (AB) equipped with a PTH C18 column. Instruments and procedures were optimized for femtomole level analysis as described.30,31 Theoretical average isozopic masses of predicted tryptic peptides in the CRKL sequence were calculated using ProComp version 1.2 software (obtained from Dr P.C. Andrews, University of Michigan, Ann Arbor, MI).

RESULTS

Presence of a hyperphosphorylated pp39 protein in CML neutrophils and Ph-positive cell lines. The tyrosine phosphorylation patterns of CML neutrophils were examined using an antiphosphotyrosine antibody and Western blot analysis. In comparison with normal neutrophils, chronic phase CML neutrophils had a novel phosphoprotein migrating at approximately 39 kDa (pp39; see Fig 1A). This protein was one of the most heavily phosphorylated in CML neutrophils and showed little, if any, phosphorylation in neutrophils from healthy donors. Pp39 was observed in neutrophils from 50 chronic phase CML patients and was not found in any of an
Fig 1. Pp39 is associated with CML. Western blot analysis of 100 μg neutrophil lysates with antiphosphotyrosine antibody is shown. Size markers are noted. Arrows indicate pp39. (A) Normal neutrophils, lanes 1 through 5 and CML neutrophils, lanes 6 through 9. (B) Lane 1, normal neutrophils; lane 2, normal lymphocytes/monocytes; lane 3, normal BM; lane 4, CML chronic phase neutrophils; lane 5, CML lymphocytes; and lane 6, CML BM. (C) Lysates from cell lines are shown. Lane 1, Mo7; lane 2, KG-1; lane 3, 3T3; lane 4, K562; lane 5, HL-60; and lane 6, CML chronic phase neutrophils. (D) Anti-CRKL reacts with pp39. Antiphosphotyrosine immunoprecipitation followed by anti-CRKL antibody staining is shown. Lane 1, K562 lysate; lane 2, K562 antiphosphotyrosine immunoprecipitate followed by anti-CRKL staining. Pp39 band is shown at arrow. (E) Anti-CRKL Western blot is shown. Lane 1, normal neutrophils; lane 2, CML chronic phase neutrophils; and lane 3, K562 lysate. (F) Antiphosphotyrosine antibody restaining of 6E is shown. Pp39 is marked.

equal number of normal control samples. Pp39 was also seen in BM, lymphocytes, and monocytes from CML patients (Fig 1B). Maturation of CML monocytes to macrophages in vitro did not alter expression of this phosphoprotein (data not shown). Pp39 was not observed in BM, lymphocyte, or monocyte samples from 3 healthy donors.

Evaluation of a variety of cell lines for the presence of pp39 by Western blotting was undertaken to determine the specificity of its phosphorylation to the presence of the BCR/ABL fusion gene. The protein was tyrosine phosphorylated in the blastic CML cell lines K562 and RW leu 4. Daudi, HL-60, and KG-1 hematologic cell lines did not have a protein phosphorylated in this region (Fig 1C). A p190 Ph-positive acute lymphoblastic leukemia cell line also contained heavily phosphorylated pp39 (data not shown).

Further evidence that phosphorylation of pp39 is correlated to the presence of the Ph chromosome comes from data obtained from two matched pairs of EBV-transformed B-cell lines generated from CML patients, in which one set of the pair contains the BCR/ABL and the other from the same patient does not. Only the lines containing the BCR/ABL gene show the pp39 protein phosphorylation. Similarly, the Mo7 cell line transfected with the p210 BCR/ABL showed pp39 phosphorylation, whereas sham-transfected Mo7 did not (data not shown).

Pp39 is constitutively phosphorylated on tyrosine. To prove that pp39 was phosphorylated on tyrosine, membranes containing transferred proteins were pretreated with phosphoserine, phosphothreonine, or phosphotyrosine and then exposed to primary antiphosphotyrosine antibody for Western blotting. Only incubation with phosphotyrosine decreased the reactivity of antiphosphotyrosine antibodies with pp39. Treating the membranes with PTPase, a specific phosphotyrosine phosphatase before immunodetection also eliminated pp39 phosphorylation (data not shown).

Regulation of pp39 phosphorylation. Tyrosine phosphorylation of pp39 was not inducible in normal neutrophils. Exposure to GM-CSF and TPA, stimuli known to enhance protein tyrosine phosphorylation and to activate neutrophils, did not increase phosphorylation of pp39 in normal neutro-
CML-RELATED TYROSINE PHOSPHOPROTEIN, CRKL

pp39 in normal neutrophils. The same cytokines had no observable effect on pp39 in CML neutrophils. These findings are in contrast to a number of other phosphoproteins whose phosphorylation is readily induced by stimulation with cytokines. Experiments using interleukin-1, fibroblast growth factor, epidermal growth factor, insulin, and platelet-derived growth factor showed no induction of pp39 phosphorylation in normal neutrophils. Two samples from patients with Ph-negative myeloproliferative syndromes showed no induction of pp39 phosphorylation in normal neutrophils. pp39 is not MAP kinase. MAP kinases are a family of serine/threonine kinases involved in signal transduction and amplification. MAP kinases are known to be activated by a variety of stimuli that induce protein phosphorylation in neutrophils. Because of its association with neutrophil activation and cellular signalling processes, MAP kinase was a potential candidate for pp39. We examined CML lysates with antibodies having affinity for the two common forms of human MAP kinase, pp42 and p42. Western blots with anti-MAP kinase antibody and subsequent reblopping of the

Phosphatase inhibition does not activate phosphorylation of pp39 in normal neutrophils. A possible explanation for the presence of pp39 phosphorylation in CML and not normal cells might be decreased phosphatase activity in CML neutrophils. These effects can be mimicked in vitro using okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A that markedly increases tyrosine phosphorylation of a number of cellular proteins. Treatment of freshly prepared normal neutrophils with okadaic acid did not lead to phosphorylation of pp39 (Fig 3); however, it increased the phosphorylation of pp42 microtubule-associated protein (MAP) kinase. Treatment of normal neutrophils with vanadate (2 mmol/L), a potent phosphatase inhibitor, also did not induce phosphorylation of pp39 (data not shown).

Relationship of the phosphorylated p39 to an activated BCR/ABL kinase. pp39 appears to be almost exclusively associated with BCR/ABL-containing cells, suggesting that pp39 may be a substrate for the BCR/ABL kinase. BCR/ABL kinase activity can be evaluated indirectly by measuring ABL autophosphorylation. Using an immune complex kinase assay, we found no autophosphorylation of the BCR/ABL kinase in CML neutrophils as has been reported previously. In contrast, both K562 and the EBV-transformed cell lines containing the BCR/ABL showed autokinase activity. Activity of the ABL kinase appears to be regulated by cellular inhibitors, however, CML neutrophils with low levels of kinase activity and blasts with high levels both have increased phosphorylation of pp39.

Phosphorylation of pp39 was evaluated after incubation of cells with a variety of inhibitors known to inhibit tyrosine kinases including BCR/ABL. CML neutrophils cultured in the presence of genistein or erbstatin analog, in doses known to inhibit the ABL kinase, showed no decrease in pp39 phosphorylation after treatment (Fig 3B). Longer incubations (24 hours to 4 days) at lower levels of genistein (1 x 10^-7 mol/L) showed no change in pp39 phosphorylation, whereas other phosphoproteins appeared to be decreased. Both TPA and herbimycin A can be used to differentiate the K562 cell line toward the erythroid series, with concomitant decrease in BCR/ABL kinase activity and tyrosine phosphorylation. Using these differentiating conditions and 4-day exposures to herbimycin (1 x 10^-7 mol/L), no downregulation of pp39 was observed, whereas ABL tyrosine phosphorylation, as measured by Western blotting, was decreased (data not shown). Similarly, staurosporin, another potent inhibitor of protein kinases, showed marked inhibition of pp42 MAP kinase phosphorylation in response to GM-CSF in normal and CML neutrophils but had no effect on pp39 (Fig 4).

**Fig 2.** Phosphorylation state of pp39 is not affected by neutrophil stimulation. Western blot analysis of 100-μg samples of neutrophil lysates from normal and CML samples with antiphosphotyrosine antibody is shown. (A), normal neutrophils; (B), CML chronic phase neutrophils. Lane 1, nonstimulated; lane 2, GM-CSF (500 pmol/L); lane 3, G-CSF (500 pmol/L); and lane 4, TPA (100 nmol/L). All experiments were performed at 37°C for 5 minutes. No changes are observed with longer exposures. Size markers are indicated. Arrows indicate pp42 (MAP kinase) and pp39.

**Fig 3.** Phosphorylation state of pp39 is not affected by kinase or phosphatase inhibitors. Western blot analysis, 100-μg samples, antiphosphotyrosine antibody. (A) Normal neutrophils cultured with media alone (lane 1) and okadaic acid for 4 hours (lane 2). (B) CML neutrophils cultured with media alone (lane 1), genistein for 30 minutes (lane 2), and erbstatin for 30 minutes (lane 3).
Fig 4. Effect of staurosporin on normal and CML neutrophils is shown. Lane 1, normal neutrophils; lane 2, normal after 30-minute incubation with staurosporin; lane 3, normal plus GM-CSF for 5 minutes; lane 4, staurosporin for 30 minutes and GM-CSF for 5 minutes; lanes 5 through 8, identical conditions in CML chronic phase neutrophils (note the persistence of p39 phosphorylation); lane 9, K562; and lane 10, K562 plus staurosporin. Size markers and P42 and P39 are indicated.

same gel with antiphosphotyrosine antibody showed that pp39 did not comigrate with MAP kinase (data not shown). A parallel study with antiphosphotyrosine-immunoprecipitated protein confirmed that pp39 was distinct from MAP kinase, because the immunoprecipitated sample did not react with anti-MAP kinase antibody (data not shown). Tyrosine phosphorylation of pp42 MAP kinase was inducible in CML and normal neutrophils with GM-CSF and TPA and was clearly distinct from pp39 (Fig 2).

Purification of pp39 and demonstration that it is CRKL. Pp39 was partially purified by sequential anion exchange chromatography (mono-Q), gel filtration, SDS-PAGE, and electroblotting onto nitrocellulose. A broad band visualized by Ponceau-S staining in the 39-kD size range was excised from the blot and digested with trypsin. Pp39 was included within this band, because subsequent phosphotyrosine staining of the nitrocellulose showed removal of pp39. The resulting complex peptide mixture was fractionated by microbore reversed-phase HPLC. Preliminary peptide sequencing experiments showed partial sequences matching those reported for the CRKL cDNA; however, the data were not conclusive. We then searched for CRKL-derived tryptic peptides by exhaustive mass spectrometric analysis of peak fractions (2% to 5% aliquots). Experimentally obtained masses (m/z) were compared with theoretical average isotopic masses [MH+] of all predicted tryptic peptides in the CRKL sequence, taking into consideration that tyrosine residues could be phosphorylated (adding 79.98 dalton to the predicted mass). In this way, 2 of 20 peptides were found to have molecular masses closely matching predicted values (m/z = 2035.7 and 2706.3 v [MH+] = 2036.3 and 2706.1). These two peptides were then sequenced to confirm identity, giving the following results: YPSPPMGVSAPNLPTEAE (peptide T54) and IGDOEFDHLPALLEFYK (peptide T62). The sequences are perfect matches to peptides spanning residues 105 through 122 and 73 through 89 in the published CRKL sequence (Fig 5). These sequences vary significantly from human CRK, distinguishing the p39 phosphotyrosine band as CRKL.

Immunoprecipitation with antiphosphotyrosine antibody followed by blotting with anti-CRK antibody showed a band corresponding to pp39 that also reacted with anti-CRK. This antipeptide antibody was raised against the second SH3 domain of c-CRK, which has significant homology to CRKL. Confirmatory studies were performed with anti-CRKL antibody after antiphosphotyrosine immunoprecipitation. The band at 39 kD reacted with anti-CRKL (Fig 1D). Although the CRKL protein band is visible in normal neutrophils, CML chronic phase neutrophils, blasts, and blastic cell lines, it is only phosphorylated in those cells containing the Ph chromosome (Fig 1E and F).

DISCUSSION

CML is a neoplastic process characterized by transformation of a pluripotent hematopoietic stem cell. The disease progresses from the chronic phase in which mature neutrophils predominate to blast crisis phase resembling acute leukemia. Activation of the ABL tyrosine kinase by the BCR/ABL translocation is known to be central to malignant transformation. Tyrosine phosphorylation of cellular proteins plays an important role in signal transduction. A number of oncogenes have tyrosine kinase activity, and dysregulation of the kinase activity may result in neoplastic transformation and abnormal cellular proliferation. Autophosphor-
Tyrosine phosphorylation is required for BCR/ABL transformation, but it is not required for growth factor independence.

CRKL is a major tyrosine phosphoprotein in Ph-positive cells and cell lines. A number of phosphoproteins have been observed in leukemic cells and cell lines, and a phosphoprotein of this size (pp41) has previously been identified in blastic CML cell lines. Our data confirm the findings of ten Hoeve et al that this phosphoprotein is CRKL. CRKL is a recently described SH2/SH3-containing adaptor molecule similar to CRK that may mediate signal transduction from nonreceptor tyrosine kinases such as ABL. We report the unexpected finding that CRKL is constitutively heavily tyrosine phosphorylated in all cells containing the Ph-positive chromosome including neutrophils, even when BCR/ABL kinase activity is low. The specificity and stability of this phosphoprotein are unusual and suggest that pp39 may be an important intermediate in the leukemic process early in CML cellular differentiation.

Pp39 CRKL is specifically associated with the BCR/ABL translocation. It is found in all lineages in CML known to contain the Ph chromosome, as well as in CML lymphocyte lines that contain BCR/ABL and in Mo7 cell lines transfected with p210 cDNA, pointing to the correlation of tyrosine phosphorylation of pp39 with the presence of the p210 BCR/ABL. Phosphorylation of CRKL appears to be either minimal or absent under conditions of normal cellular growth. Although phosphorylation of CRKL is tightly correlated with the presence of the abnormal gene and CRKL is known to bind to ABL, we have shown that CRKL phosphorylation is not regulated in the same fashion as BCR/ABL kinase. CRKL may be phosphorylated earlier in CML myeloid development by the BCR/ABL kinase. The constitutive phosphorylation of CRKL is a novel finding for adaptor molecules. Recent reports have found phosphorylation of c-CRK by c-ABL, altering its ability to complex to other proteins.

It is unknown how constitutive phosphorylation of CRKL may alter protein binding and signal transduction. Pp39 CRKL is a uniquely regulated phosphoprotein found in CML, which suggests a role for this phosphoprotein in differentiating neoplastic from normal growth in CML. Study of the nature of this protein as an adaptor molecule, its phosphorylation, regulation, and role in normal cellular signalling may provide insight into the pathogenesis of CML as well as facilitate approaches to new therapeutic targets in this disease.

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