Analysis of P210<sup>bcr-abl</sup> Tyrosine Protein Kinase Activity in Various Subtypes of Philadelphia Chromosome-positive Cells from Chronic Myelogenous Leukemia Patients

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ABSTRACT

An altered c-abl gene product (P210<sup>bcr-abl</sup>) possessing associated tyrosine protein kinase activity has recently been reported in several blast chronic myelogenous leukemia (CML) cell lines. We have examined different morphological types of leukocytes directly obtained from patients at the blast crisis stage of CML for expression of P210<sup>bcr-abl</sup> tyrosine protein kinase activity. Phosphorylation of P210<sup>bcr-abl</sup> in an immune complex kinase assay using an anti-c-ABL peptide serum was observed in blast cells from four Philadelphia chromosome (Ph<sup>1</sup>)-positive CML patients in blast crisis. P210<sup>bcr-abl</sup> protein kinase activity was detected regardless of whether the blast cells were of myeloid, lymphoid, or undifferentiated morphology. P210<sup>bcr-abl</sup> protein kinase activity was not detected in immune complexes either from leukocytes of four Ph<sup>1</sup>-negative CML patients in blast crisis, or five acute myelogenous leukemia patients, or in the promyelocytic cell line HL-60. Mature myeloid cells are associated with an inhibitory factor for not only P210<sup>bcr-abl</sup> protein kinase activity, but also protein kinases in general. Therefore, analyses of Ph<sup>1</sup>-positive blast crisis CML myeloid cells, the majority of which are well differentiated, could not be successfully performed. The inhibition of P210<sup>bcr-abl</sup> protein kinase activity is not a specific property of mature cells from CML patients since granulocytes from a normal volunteer also demonstrated a similar effect. However, extracts of Ph<sup>1</sup>-positive cultured B-lymphocytes from a patient in benign phase demonstrated active P210<sup>bcr-abl</sup> protein indicating that the P210<sup>bcr-abl</sup> protein is expressed in an enzymatically active form in the earlier phases of CML.

INTRODUCTION

In approximately 90% of patients with CML, a characteristic...
but not of lymphoid cells. In contrast, both myeloid and lymphoid lineages may be involved in the terminal blast transformation phase. P210<sup>bcR/MY</sup> has been detected in several CML cell lines by metabolic labeling experiments and by immune complex kinase assays (9, 10) and thus has been implicated in the pathogenesis of CML. However, phenomena observed in cell lines may not always reflect events in the original cells. Although this protein has also been detected with v-abl-specific antiserum in primary cultures of CML leukocytes which had been metabolically labeled with <sup>32</sup>P (25), an active P210<sup>bcR/MY</sup> tyrosine kinase has not yet been demonstrated in fresh CML cells. Furthermore, it is not known whether or not differential expression of P210<sup>bcR/MY</sup> could account for the variation in behavior of the different Ph<sup>1</sup>-positive leukocyte subtypes involved in CML. Therefore, we have undertaken a study to examine P210<sup>bcR/MY</sup> kinase activity in fresh Ph<sup>1</sup> containing cells from CML patients. We demonstrate for the first time that P210<sup>bcR/MY</sup> is expressed as an active protein kinase in fresh Ph<sup>1</sup>-positive cells from CML patients, in Ph<sup>1</sup>-positive B-lymphocytes from the benign phase, and in the lymphoid, the myeloid, as well as the undifferentiated blast crises of CML.

**MATERIALS AND METHODS**

Preparation of Cell Lines and Fresh Cells from CML Patients. K562 and EM2 cell lines are established CML blast crisis cell lines. The EM2 cell line was kindly donated by Aramand Keating. HL-60 is a myeloid cell line derived from a patient with acute promyelocytic leukemia. Cell lines were washed twice with cold PBS and immediately frozen and stored in liquid nitrogen. In some cases the frozen cell pellets were lyophilized and stored at -20°C. Blood samples were obtained from patients with leukemia by peripheral venipuncture according to institutional guidelines after patient consent was obtained. Leukocytes were isolated from blood samples of CML patients by adding 5 volumes of 1.22% ammonium oxalate (210 mOsM/liter). WBC were then pelleted at 2000 × g for 5 min and treated with ammonium oxalate to obtain an additional one to two times. Ficoll-Hypaque or Percoll gradient centrifugation was used in further purification of immature cells from ammonium oxalate-treated CML blood and bone marrow samples. Purified leukocyte populations were then washed once with cold PBS and frozen and stored in liquid nitrogen. Ammonium oxalate-purified cells which were not subjected to blast enrichment rapidly lost P210<sup>bcR/MY</sup> kinase activity.

Antiserum. Antiserum was made against a peptide representing the predicted hydrophilic v-abl sequence 389-403 (DEVEKELKGRTR- GG-C) as described by Kloetzer et al. (9). The peptide was synthesized using solid phase synthesis. Cysteine residues were added to the carboxyl termini during synthesis for MBJ cross-linking through free sulfhydryl groups to the carrier protein keyhole limpet hemocyanin.

Antigen was prepared by emulsifying at 1:1 ratios 0.2 mg of cross-linked peptide in Dulbecco’s PBS with 1.5 ml of complete Freund’s adjuvant. Injections s.c. of rabbits were administered at multiple sites. Three booster injections of antigen suspended in incomplete Freund’s adjuvant were given at 2-week intervals.

Monoclonal antiserum (GD-11) specific for the cellular src protein was prepared as previously described (26). The particular domain on the c-src protein which is recognized by GD-11 (amino acids 92–128) shows no homology to the v-abl sequence 389-403.

Immune Complex Kinase Assays. Cell lines were assayed essentially as described by Konopka et al. (10) with modifications. Cells were washed twice with cold phosphate-buffered saline and then stored frozen at -70°C. In some cases, the washed cell pellet was lyophilized to a powder. Lyophilized or frozen cell pellets were disrupted in RIPA buffer containing 5 mM EDTA and 5 mM phenylmethylsulfonylfluoride with 10-20 strokes in a tight-fitting Wheaton homogenizer. Cell lysates were clarified at 100,000 × g for 60 min at 4°C, and 1 ml of the clarified extract was then incubated on ice with 5 μl of anti-v-abl peptide serum for approximately 1 h. The immune complexes were then precipitated with 10 μl of pansorbin (Sigma) and the resulting pellets were washed twice with RIPA followed by one wash in 50 mM Tris-HCl, pH 7.5.

Phosphoamino Acid Analysis. Phosphoamino acids were analyzed by the procedure of Hunter and Sefton (30). Proteins were eluted from gel slices in 0.5% SDS and 10 mM sodium phosphate, pH 7.5, at 37°C with shaking for 16 h. Eluted proteins were trichloroacetic acid precipitated, suspended in 0.5 ml 6 N HCl, and hydrolyzed at 110°C for 90 min. Hydrolysates were diluted 10-fold with water and lyophilized to a powder. The residue was then mixed with cold phosphoamino acid buffer and the mixture was fractionated by electrophoresis on thin-layer cellulose plates at pH 3.5 at 500 constant V for 2 h.

Preparation of B-Lymphocytes. Peripheral blood was obtained from CML patients by venipuncture. Peripheral blood mononuclear cells were obtained from Ficoll-Hypaque gradients (28). Macrophages were removed by adherence to glass. Subsequently, the nonadherent population was removed, mixed with neurominidase-treated sheep erythrocytes and incubated for 1 h at 4°C. Following this incubation period, the B-cells and rosetted T-cells were separated on Ficoll-Hypaque gradients. Cells obtained from the buffy coat containing the B-cells were initially resuspended at 1 × 10<sup>6</sup> cells/ml and incubated with 15 μg/ml soluble [F(ab')<sub>2</sub> fragment] of anti-human IgM, heavy chain specific (anti-μ), 10% partially purified B-cell growth factor (Cellular Products, Buffalo, NY), and 10% heat-inactivated fetal calf serum. Both B-cell growth factor and inactivated fetal calf serum were replenished every 4 days. In addition, the cells were stimulated with anti-μ at 4-day intervals. After 7 days and at 4-day intervals thereafter, the cells were counted, their viability was determined by trypan blue exclusion, and they were adjusted to 0.5 × 10<sup>6</sup> cells/ml. Furthermore, the phenotype of the cells was checked weekly using fluorescein-labeled anti-B1 (Coulter Corp., Hialeah, FL) and anti-poly immunoglobulin (Cooper Biomedical, Malvern, PA). Within 2-3 weeks following this treatment, 95–98% of the cells stained positively for B1 and poly immunoglobulin antigens. These cells were used as the B-cell population and were prepared in the same manner as the CML cell lines for the immune complex kinase assay.

Patient Data. Data pertaining to the disease characteristics of the patients were obtained by review of hospital medical records. The accelerated stage was defined as evidence of extramedullary tissue infiltration, basophilia ≥15%, clonal evolution, or marrow blasts ≥15%. Blastic transformation was defined by the presence of ≥30% bone marrow blasts. The blast morphology was determined on the basis of standard cytochemical stains and surface markers (31). All patients had been removed from chemotherapy at least 3 weeks prior to assay.
Partial Peptide Mapping. Peptide mapping was performed as described by Cleveland et al. (32).

Glycerol Gradient Fractionation of Cellular Lysates. Fractionation of K562 cell lysates on continuous 10-30% glycerol gradients was performed by modification of the method of Brugge et al. (33). K562 cells were washed twice in cold PBS and lysed by homogenization in cold cell lysis buffer containing 1% Triton X-100, 5 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1% aprotinin, 5 mM phenylmethylsulfonyl fluoride, and 20 mM sodium phosphate, pH 7.2. Lysates were clarified by centrifugation at 100,000 x g for 1 h. Clarified cell lysates were then layered on 10-30% glycerol gradients in cell lysis buffer and sedimented for 16 h at 49,000 rpm in an SW50.1 rotor. The gradient was then fractionated into 16 fractions and each was diluted with 1 ml cell lysis buffer. Odd-numbered fractions were immunoprecipitated with anti-src serum and even-numbered fractions were precipitated with anti-aabl serum. Immune complex kinase assays were then performed and the phosphorylated proteins were visualized by SDS-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Analysis of P210abl Kinase Activity in Blast Crisis CML Cell Lines. To examine the ability of the anti-aabl antiserum to function in immune complex kinase assays, the Philadelphia chromosome-positive blast crisis CML cell lines K562 and EM2 were analyzed. The results are shown in Fig. 1. In each cell line, phosphorylation of a protein with a molecular weight of 210,000 was observed (Fig. 1, Lanes 1 and 3) in agreement with published observations (9, 10). Furthermore, a protein with a molecular weight of 190,000 was phosphorylated. The M190,000 protein (P210abl) and M210,000, 190,000 proteins were determined to be a,abl specific since these proteins were not detected in assays in which the reactivity of the antisera was blocked by prior addition of an excess of the 389-403 a,abl peptide (Fig. 1, Lanes 2 and 4). P190 has been previously reported to be a partially degraded P210abl fragment (10), as suggested by its variable presence in the immune complex kinase assay. As the a,abl peptide does not contain sequences related to the bcr gene sequence (9), P190 is unlikely to represent a product of the normal bcr gene. In addition, partial peptide mapping by the Cleveland method demonstrated that P190 is homologous to P210abl (data not shown). A third protein with molecular weight of M, 53,000 was found to be specifically phosphorylated only in the presence of P210abl (Fig. 1, Lanes 1 and 3). Specificity is indicated by the absence of this protein (as well as P190 and P210abl) in immune complex kinase assays in which reactivity of the antiserum was inhibited by prior incubation with excess free peptide (Fig. 1, Lanes 2 and 4).

P210abl Kinase Activity from CML Patients in Blast Crisis. Initial attempts to detect P210abl kinase activity in fresh Ph1-positive CML leukocytes were largely unsuccessful when the conditions of Konopka et al. (10) were used. However, when (a) the cells were immediately ammonium oxalate treated (to lyse RBC); (b) the resulting leukocyte population was enriched for blasts by either Ficoll-Hypaque or Percoll gradient centrifugation; (c) SDS and DOC were omitted from the lysis buffer; (d) the extract was subjected to only a low speed spin for clarification; and (e) the kinase assay was performed on ice, P210abl kinase activity was observed in cells from four Ph1-positive CML patients in blast crisis (Table 1, Patients A, B, C, and D). Fig. 2A displays results obtained from samples of two blast crisis patients. The results are similar to those obtained from cell lines (Fig. 1). P210abl, P190, and P53 were specifically observed in each sample from Ph1-positive patients in the blast crisis of the disease (Fig. 2A, Lanes 3 and 5). As with the cell lines, specificity of the reaction was indicated by the absence of these proteins in assays performed with antisera blocked with excess free peptide (Fig. 2A, Lanes 4 and 6). In Patient C, peripheral blood cells expressed common acute lymphoblastic leukemia antigen and were positive for terminal deoxynucleotidyl transferase, indicating that the patient was in lymphoid blast crisis. In Patients A and B, the majority of the cells were positive for myeloperoxidase and Sudan black histochemical stains but negative for common acute lymphoblastic leukemia antigen and terminal deoxynucleotidyl transferase. These results indicate that these patients had myeloid blast crisis. These results demonstrate the presence of P210abl kinase activity in each of the CML patients with the Ph1 chromosome regardless of whether the blasts were of the myeloid, lymphoid, or the undifferentiated morphology (Table 1, Patients A, B, C, and D). Lack of specific detection of any protein of the size of the normal M145,000 a,abl gene...
leukocytes purified from peripheral blood by Ficoll-Hypaque centrifugation were composed of greater than 70% blast cells. P210<sup>cr/-</sup> kinase activity was also not detected in blast cells from an acute myelogenous leukemia patient in whom a 9q-cytogenetic abnormality was present (Table 1, Patient M), in a myeloid cell line derived from an AML patient (data not shown), or in the promyelocytic cell line HL-60 (data not shown). The P53 protein detectable in assays containing P210<sup>cr/-</sup> activity (Figs. 1 and 2; Fig. 3, Lane 1) is not present in assays of Ph<sup>1</sup>-negative cells which lack P210<sup>cr/-</sup>.

Analyses of Cells Isolated at the Early Stages of CML. Ficoll-Hypaque or Percoll gradient-purified peripheral blood and bone marrow cell populations from two Ph<sup>1</sup>-positive CML patients in the accelerated stage and of six Ph<sup>1</sup>-positive CML patients in the benign stage could not be successfully examined for P210<sup>cr/-</sup> protein kinase activity due to the presence of large numbers of mature myeloid cells (blast cells comprised less than 30% of leukocytes) which are associated with an inhibitor for not only the P210<sup>cr/-</sup> protein kinase activity but also protein kinase in general. As a result, no P210<sup>cr/-</sup> kinase activity or background kinase activity was detected either in immune complexes from accelerated CML patients (Fig. 4A, Lane 3) or in immune complexes from benign patients (Fig. 4A, Lanes 2 and 5).

To attempt to identify the source and nature of the inhibitor, several experiments were performed. Inhibition was detected by mixing an extract of K562 cells with an extract of either benign or accelerated CML samples, harvesting immune complexes from the lysate mixture, and then performing the kinase assay. Complete inhibition of P210<sup>cr/-</sup> protein kinase activity resulted in each mixing experiment when extracts of early stage CML samples which contained fewer than 30% blasts were tested (Fig. 4B, Lanes 2 and 3). Addition of Ph<sup>1</sup>-negative blast crisis Patient F lysates did not inhibit P210<sup>cr/-</sup> kinase activity (Fig. 4B, Lane 1). The inhibitor is not a specific characteristic of mature myeloid cells from CML patients since an extract of granulocytes from a normal volunteer also was inhibitory (Fig. 4B, Lane 5). This also indicates that the lack of P210<sup>cr/-</sup> protein kinase activity in these samples is the result of an inhibitory activity released by granulocytes and/or mature cells. Lane 3 of Fig. 4A and Lane 3 of Fig. 2 represent samples from the same patient (Patient A) in the accelerated phase and blast crisis stage, respectively. When this patient entered blast crisis, P210<sup>cr/-</sup> kinase activity was present, suggesting that lack of detection of activity at early stages was not the result of differences among patients. The inhibitor was resistant to a variety of protease inhibitors (phenylmethylsulfonylfluoride, aprotinin, and leupeptin) or phosphatase inhibitors (NaF, vanadate, ZnCl<sub>2</sub>, and sodium pyrophosphate). P210<sup>cr/-</sup> activity could only be detected in Ph<sup>1</sup> chromosome-positive blast crisis CML patient samples in which the majority of cells were blasts (Table 1).

The inhibition by normal granulocytes suggests that the absence of kinase activity at early CML stages is due to the large numbers of mature cells as compared to those found in blast crisis samples and that it is not a property characteristic of the disease. In order to remove the inhibitory factor from early stage CML samples, attempts were made to enrich the leukocyte populations for blast cells by Percoll gradient centrifugation. Percoll gradient-purified cell populations of peripheral blood and bone marrow from CML benign phase patients could only be enriched to approximately 11% blasts and consisted of mainly mature myeloid cells. A representative kinase assay of a Percoll gradient-purified benign cell population is shown in Fig. 2. P210<sup>cr/-</sup> kinase activity isolated by immunoprecipitation from cells of CML patients. Cells 10<sup>6</sup> were lysed in 2 ml of lysis buffer and analyzed for kinase activity as described in “Materials and Methods.” A. Lane 1, K562; Lane 2, K562 assayed with v-abl peptide-blocked antisera; Lane 3, Patient A in myeloid blast crisis; Lane 4, Patient A in myeloid blast crisis phase using blocked serum; Lane 5, Patient C in lymphoid blast crisis; Lane 6, Patient C in lymphoid blast crisis using blocked serum. B. Lane 1, assay of Ph<sup>1</sup>-positive B-cells cultured from a patient in benign phase; Lane 2, assay of Ph<sup>1</sup>-positive B-cells from benign stage patient using peptide-blocked serum.

Analyses of Ph<sup>1</sup>-negative Patients and Cell Lines. P210<sup>cr/-</sup> kinase activity is intrinsic specifically to blasts with the Ph<sup>1</sup> chromosome. As shown in Fig. 3 and described in Table 1, no kinase activity was detected in four Ph<sup>1</sup>-negative CML patients (Table 1, Patients E–H; and Fig. 3, Lane 3) or in five AML patients (Table 1, Patients I–M; Fig. 3, Lane 5) although product is apparently due to inhibition of P145<sup>cr/-</sup> kinase activity by the Staphylococcus aureus used to precipitate the immune complexes (11).

Analyses of Ph<sup>1</sup>-negative Patients and Cell Lines. P210<sup>cr/-</sup> kinase activity is intrinsic specifically to blasts with the Ph<sup>1</sup> chromosome. As shown in Fig. 3 and described in Table 1, no kinase activity was detected in four Ph<sup>1</sup>-negative CML patients (Table 1, Patients E–H; and Fig. 3, Lane 3) or in five AML patients (Table 1, Patients I–M; Fig. 3, Lane 5) although product is apparently due to inhibition of P145<sup>cr/-</sup> kinase activity by the Staphylococcus aureus used to precipitate the immune complexes (11).
Table 1 Characteristics of leukemia patients tested for P210* activity in immune complex kinase activity

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Stage</th>
<th>WBC × 10^9 (% of blast cells)</th>
<th>Cytogenetics</th>
<th>Blasts morphology</th>
<th>P210 kinase</th>
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<tr>
<td>A</td>
<td>CML</td>
<td>Blast crisis</td>
<td>106 (85%)</td>
<td>Ph^1+ clone, 45,XY t(9q+:22q−)</td>
<td>Robertsonian t(13q,14q)</td>
<td>Myeloid</td>
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<td>B</td>
<td>CML</td>
<td>Blast crisis</td>
<td>64 (38%)</td>
<td>Ph^1+ clone 46,XY t(9q+:22q−)</td>
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<td>Myeloid</td>
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<tr>
<td>C</td>
<td>CML</td>
<td>Blast crisis</td>
<td>43 (93%)</td>
<td>Ph^1+ hypodiploid clone 45,XX, 7q−;9q+:15q−</td>
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<td>Lymphoid</td>
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<td>D</td>
<td>CML</td>
<td>Blast crisis</td>
<td>209 (83%)</td>
<td>Ph^1+ complex clone 46,XX, t(9q+:15q−)</td>
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<td>Undifferentiated</td>
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<td>E</td>
<td>CML</td>
<td>Blast crisis</td>
<td>13 (95%)</td>
<td>Ph^1-negative (46,XX)</td>
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<td>Myeloid</td>
</tr>
<tr>
<td>F</td>
<td>CML</td>
<td>Blast crisis</td>
<td>46 (82%)</td>
<td>Ph^1-negative (46,XX)</td>
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<td>G</td>
<td>CML</td>
<td>Blast crisis</td>
<td>20 (70%)</td>
<td>Ph^1-negative (46,XX)</td>
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<tr>
<td>H</td>
<td>CML</td>
<td>Blast crisis</td>
<td>142 (95%)</td>
<td>Ph^1-negative (46,XX)</td>
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<tr>
<td>I</td>
<td>AML</td>
<td>NA*</td>
<td>3.6 (90%)</td>
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<tr>
<td>J</td>
<td>AML</td>
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<td>25 (85%)</td>
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<td>K</td>
<td>AML</td>
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<td>L</td>
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<td>46,XY,+8−,20,3p−,5q−,7q+,9q−,17p−,22q+</td>
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* NA, not applicable.

In order to address the question of whether or not P210* protein kinase activity is present in cells of Ph^1 chromosome-positive benign phase CML patients, B-lymphocytes cultured from a CML patient in benign phase were analyzed by immune complex kinase assay. Fifty % of these cells were Ph^1 chromsome-positive according to cytogenetic analysis and P210* protein kinase was detected (Fig. 2B, Lane 1), indicating that this protein is present and active at the earlier stages of CML. Phosphoamino Acid Analysis of P210* and P53. To confirm that phosphorylation of P210* occurred at tyrosine and to determine on which amino acids P53 was phosphorylated, phosphoamino acid analysis was performed as described in "Materials and Methods." The results are shown in Fig. 5.
P210<sup>ber-abl</sup> in immune complexes from the K562 cell line was phosphorylated at both tyrosine and serine and contained approximately five times more phosphotyrosine than phosphoserine (Fig. 5, Lane 1) in agreement with published results (9, 10). Phosphoamino acid analysis of P210<sup>ber-abl</sup> phosphorylated in immune complexes from the blast crisis Patient A sample illustrated in Fig. 2, Lane 3, revealed predominantly phosphotyrosine (Fig. 5, Lane 3). Analysis of P53 showed the presence of both phosphotyrosine and phosphoserine in approximately equivalent amounts (Fig. 5, Lane 2). The presence of phosphotyrosine in P53 suggests that it may be either a coprecipitating substrate specifically phosphorylated by the P210<sup>ber-abl</sup> kinase, a degraded P210<sup>ber-abl</sup> fragment, or a serum component phosphorylated on tyrosine and serine in the presence of P210<sup>ber-abl</sup>.

Further Characterization of P53. The apparent molecular weight of P53 and the presence of phosphotyrosine led us to compare it with the cellular src proteins which are frequently found as an intact M, 60,000 src kinase and smaller degradation products with molecular weights of 50,000-56,000 (34, 35). Using an anti-src-monoclonal serum (GD-11), active c-src ki...
P210<sup>onc</sup> PROTEIN KINASE ACTIVITY IN FRESH CELLS FROM CML PATIENTS

Fraction Number

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Fig. 9. Glycerol gradient centrifugation of K562 cell lysates and analysis of even-numbered fractions by immune complex kinase assay with anti-abl serum.

Fraction Number

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Fig. 10. Glycerol gradient centrifugation of K562 cell lysates and analysis of odd-numbered fractions by immune complex kinase assay with anti-src serum.

Peptide mapping by the Cleveland technique is a useful method of examining any structural relationship between proteins. Partial digestion with V8 protease and fractionation of phosphopeptides on a 12.5% SDS-polyacrylamide gel indicated that P53 is not similar in structure to P210<sup>onc-abl</sup> in that no common phosphopeptides were generated between the two proteins (Fig. 7, Lanes 4–9). The resolution between the M, 30,000 and 28,000, and the M, 18,000 and 16,000 peptides generated from P53 was greatly improved by using a 10.5% SDS-polyacrylamide gel for the Cleveland analysis as illustrated in Fig. 8. The in vitro phosphorylated P60<sup>src</sup> protein produced the expected 26,000 M, fragment which represents the COOH-terminal one-half of the protein containing the kinase domain (Fig. 7, Lanes 1–3) (35). These peptide mapping data suggest that P53 is not closely related to either P60<sup>src</sup> or P210<sup>onc-abl</sup>. However, P53 did generate a similar peptide profile to P50 in that each protein produced closely migrating M, 30/28,000 and 18/16,000 fragments (Fig. 8, Lanes 4–6 and Lanes 7–9). The M, 30,000 fragment in each case appeared to be more labile to protease digestion and may give rise to the smaller peptides (Fig. 8, Lanes 6 and 9). Thus, the P50 immunoprecipitated and phosphorylated in the anti-src kinase assay appears to be related to the P53 detected in the anti-abl assay.

Since P53 appeared to be a distinct protein related to a P50 protein detected with anti-src serum, we performed experiments to determine if P53 existed in a complex with P210<sup>onc-abl</sup>. Glycerol gradient centrifugation of cellular lysates has been proven successful for analyses of protein complexes (33). Lysates of K562 cells were fractionated on a 10–30% glycerol gradient and fractions of the gradient were analyzed by immune complex kinase assays with either anti-src or anti-abl serum. Analyses of even-numbered fractions of a gradient of fractionated K562 cell lysates with anti-abl serum yielded P210<sup>onc-abl</sup> activity at the M, 200,000 region of the gradient (Fig. 9, Fractions 8 and 10) and a P210/P53 complex at the M, 300,000 portion (Fig. 9, Fractions 12, 14, and 16). Analysis of odd-numbered fractions of the same gradient with GD-11 anti-src serum yielded monomeric M, 60,000 c-src kinase activity (Fig. 10, Fractions 3 and 5) and a P50 size protein at the identical portion of the gradient (Fig. 10, Fractions 11, 13, and 15),
where the P210/P53 complex sedimented (Fig. 9, Fractions 12, 14, and 16). Peptide mapping indicated that the P53 detected in the P210/P53 complex was related to the P50 detected with anti-src serum at the Mr, 300,000 region of the gradient (Fig. 11), in that each protein generated M, 30/28,000 and 18/16,000 phosphopeptides. Thus, P53 appears to bear some structural relationship to pp60<sup>src</sup> in that monoclonal antibody GD-11 is able to recognize this protein. However, the partial mapping studies suggest that this protein is unlikely to be pp60<sup>src</sup> or a known modification of this protein.

**DISCUSSION**

Translocation and subsequent activation of the c-<i>abl</i> oncogene has been implicated as an important event in the pathogenesis of chronic myelogenous leukemia. The formation of the Ph<sup>+</sup> chromosome results in expression of an aberrant 8-kilobase c-<i>abl</i>-specific mRNA which is translated to an Mr, 210,000 c-<i>abl</i> protein containing an associated tyrosine protein kinase as observed in established CML blast crisis cell lines. Because such activity is closely associated with the transforming ability of several oncogene products (19–22), the expression and activity of P210<sup>cr<sup>°</sup></sup> is likely to play an important role in chronic myelogenous leukemia.

To determine if the P210<sup>cr<sup>°</sup></sup> translocation involving chromosomes 9 and 22 as well as in a Ph<sup>+</sup>-positive patient with a rare complex translocation involving 4 chromosomes (Table 1, Patient D). No P210<sup>cr<sup>°</sup></sup> protein kinase activity was detected in Ph<sup>+</sup>-negative CML patients in blast crisis (Table 1, Patients E–H). These observations indicate that the expression of P210<sup>cr<sup>°</sup></sup> kinase activity is specifically dependent on the presence of the Ph<sup>+</sup> chromosome and is unlikely to account for the morphology of the blasts at the acute phase.

The immune complex kinase assay could not be successfully performed on leukocyte preparations from patients in the benign and the accelerated phases of CML, due to an inhibitor of kinase activity associated with mature myeloid cells. Since granulocytes from normal donors were also inhibitory, the phenomenon is not a specific property for the disease. Attempts to purify the immature cells by Percoll or Ficoll-Hypaque gradients failed to eliminate the inhibition of protein kinase activity. Furthermore, the inhibitory activity was not prevented by various protease and phosphatase inhibitors. However, P210<sup>cr<sup>°</sup></sup> is expressed in Ph<sup>+</sup>-positive cells at the early stages of CML as demonstrated by detection of the activity in a culture of Ph<sup>+</sup>-positive B-lymphocytes from a patient in benign phase. Supporting this observation is a previous report showing the presence of the 8-kilobase c-<i>abl</i> transcript in the early stages of CML (36). The detection of P210<sup>cr<sup>°</sup></sup> protein kinase in B-lymphocytes demonstrates that expression of the protein is not restricted to myeloid cells in <i> vivo</i>, a result consistent with recent work demonstrating the altered c<em>src</em>-<i>abl</i> transcript in somatic cell hybrids between mouse fibroblasts and Ph<sup>+</sup> CML cells (37). In addition, our observations suggest that the proliferative advantage of Ph<sup>+</sup>-positive myeloid cells over Ph<sup>+</sup>-positive lymphoid cells in benign phase CML patients cannot be attributed to the absence of P210<sup>cr<sup>°</sup></sup> protein kinase activity in these lymphocytes.

The presence of P210<sup>cr<sup>°</sup></sup> is not a characteristic of all blast samples. Four blast crisis CML patients whose cells were Ph<sup>+</sup>-negative as well as five AML patients exhibited no P210<sup>cr<sup>°</sup></sup> kinase activity. In addition, no evidence of this activity was found in blasts obtained from an acute myelogenous leukemia patient with a 9q- cytogenetic abnormality (Patient M), in a myeloblast cell line derived from an AML patient, or in the promyelocytic cell line HL-60. The observation of no detectable altered c-<i>abl</i> protein kinase activity in blasts of patient E (9q- cytogenetic abnormality) is consistent with the results of a report of cytogenetic banding studies in the small group of leukemia patients with this aberration which showed that interstitial deletions of the long arm of chromosome 9 occur far upstream of the c-<i>abl</i> gene (38).

The observation of a P53 protein undergoing phosphorylation at both tyrosine and serine in the immune complex kinase assay is of interest because it represents a potential substrate for P210<sup>cr<sup>°</sup></sup>. In support of this possibility is the absence of detectable P53 phosphorylation in immune complexes from either the HL-60 promyelocytic cell line or the PH<sup>+</sup>-negative CML samples. Furthermore, P53 appears to be unrelated to P210<sup>cr<sup>°</sup></sup> based upon partial V8 proteolysis. However, caution should be used in excluding the possibility that P53 contains any structural relationship to P210<sup>cr<sup>°</sup></sup>. Further peptide mapping studies are certainly needed to conclusively determine if P210<sup>cr<sup>°</sup></sup> and P53 contain any structural homology. The Cleveland peptide mapping studies suggest that P53 is related to a P50 size protein detected with anti-src serum. Furthermore, P53 sedimented at an approximately Mr, 300,000 region of the glycerol gradient where the P50 protein was detected with the anti-src serum. From these results it might be inferred that the P53 protein sediments in an Mr, 300,000 complex with P210<sup>cr<sup>°</sup></sup>. However, we cannot rule out the possibility that P53 contains a cross-reactive site to the anti-<i>src</i> serum and that it might form a multimeric complex sedimenting in the heavier portion of the gradient. The reason for the difference in mobility of P53 and P50 is not known, but differences in phosphorylation could account for migration differences. The absence of any detectable P210<sup>cr<sup>°</sup></sup> in anti-src assays could be the result of the anti-src antibody recognizing only the P50 molecules which are not in a complex with P210<sup>cr<sup>°</sup></sup>. In light of these findings, further study into the identity of P53 and the possible interaction of this protein with P210<sup>cr<sup>°</sup></sup> may provide important information about molecular events in the pathogenesis of CML.

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Analysis of P210$^{bcr-abl}$ Tyrosine Protein Kinase Activity in Various Subtypes of Philadelphia Chromosome-positive Cells from Chronic Myelogenous Leukemia Patients

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