Identification of Molecular Variants of p210bcr-abl in Chronic Myelogenous Leukemia

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The aberrant abl protein product of a chronic myelogenous leukemia (CML) blast crisis cell line (K562) and of five Philadelphia chromosome-positive CML patients in blast crisis were analyzed by an immune complex kinase assay using two antipeptide sera generated against the hydrophilic domain of v-abl and a region within the third exon of the breakpoint cluster region (bcr) respectively. Both the anti-abl and anti-bcr sera detected a 210 kd band in extracts derived from K562 cells and from two CML patients with myeloid blast crisis. p210 was detected by the anti-abl but not the anti-bcr sera in three CML patients with myeloid (one patient) and lymphoid (two patients) blast crisis, indicating the absence of bcr exon 3 in this protein. Southern blot analysis on DNA derived from one of the patients in the latter group was consistent with the break on chromosome 22 occurring 5' to bcr exon 3. Our observations demonstrate that the Philadelphia translocation results in the generation of a chimeric bcr-abl protein with at least two molecular variants, both of which are enzymatically active as protein kinases.

A SHORTENED chromosome 22, designated the Philadelphia (Ph) chromosome, is found in the majority of patients with chronic myelogenous leukemia (CML) and is considered the cytogenetic hallmark of the disease. This chromosomal anomaly is usually the result of a reciprocal translocation between chromosomes 9 and 22, (t(9;22) [q34;q11]). The breakpoints on chromosome 22 occur within a 5.8 kb segment of DNA, termed the breakpoint cluster region or bcr. A crucial event in the Ph translocation is the transfer of the c-abl oncogene from its normal residence on chromosome 9 to chromosome 22 so that bcr and c-abl sequences are juxtaposed in a head-to-tail configuration with bcr closer to the centromere. The resulting messenger RNA (mRNA) is a hybrid of 5' bcr sequences and a truncated c-abl gene lacking the first exon. This chimeric bcr-abl mRNA encodes a novel 210,000 MW protein, whereas the normal c-abl gene is expressed as a 145,000 MW protein. Therefore p210bcr-abl represents a unique cancer-specific marker.

The Abelson murine leukemia virus encoded p160 is a tyrosine-specific protein kinase activity that is closely associated with its ability to transform cells. Although both the aberrant p210bcr-abl and the normal p145c-abl also have an associated tyrosine-protein-kinase activity, p 160v. and p210abI also have an enzymatic activity as protein kinases.

In this report we have used anti-abl and anti-bcr (to exon 3) peptide sera in an immune complex kinase assay (ICKA) to investigate p210bcr-abl expression in Ph-positive CML. We demonstrate that a variable number of bcr exons may be incorporated into p210bcr-abl and that the resulting heterogeneous products retain enzymatic activity as protein kinases.

MATERIALS AND METHODS

Antiserum. Antiserum was made against a peptide representing the predicted hydrophilic v-abl sequence 389-403 (DEVE-KELKGRGTRGG-C) and a region within bcr exon 3 (DEDESGLYFLNV-C), as described by Kloetzer et al. The peptide was synthesized using solid-phase synthesis. Cysteine residues were added to the carboxyl termini during synthesis for MBS crosslinking 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 phosphate-buffered saline (PBS) with 1.5 mL of complete Freund's adjuvant. Subcutaneous (SC) injections of rabbits were administered at multiple sites. Three booster injections of antigen suspended in incomplete Freund's adjuvant were given at 2-week intervals.

Immune complex kinase assays (ICKAs). Blood samples were obtained from patients after informed consent. The blood was prepared for ICKAs by treating with 5 volumes of 1.22% ammonium oxalate to lyse red blood cells (RBCs) and Ficol-Hypaque (FH) gradient centrifugation to enrich for immature cells. K562 cells (Ph-positive CML erythroid blast crisis cell line) were also assayed. Each sample was divided into four aliquots of 5 x 10^6 cells. Each aliquot was disrupted with 1 mL of lysis buffer (1% Triton X-100, 5 mmol/L EDTA, 100 mmol/L NaCl, 5 mmol/L phenylmethylsulfonylfluoride, 100 KIU/mL Trasylol, in 10 mmol/L sodium phosphate, pH 7.5) with 20 strokes in a tight-fitting Wheaton homogenizer. Lysates were subjected to a 10,000 g centrifugation for ten minutes at 4°C. The supernatant from the four aliquots was then incubated with (1) 5 µL of anti-abl389-403 serum, (2) 5 µL of anti-abl389-403 serum and 5 µL of abl389-403 peptide (3) 5 µL of anti-bcr3 serum, or (4) 5 µL of anti-bcr3 serum and 5 µL of bcr3 peptide. Concentration of the peptide solution was 1 mg/mL H₂O. Incubation was carried out for one hour on ice. The resulting immune complexes were then precipitated by incubation with 10 µL of Pansorbin for 20 minutes on ice. Immunoprecipitates were washed twice with wash buffer (0.1% Triton X-100, 150 mmol/L NaCl, in 10 mmol/L sodium phosphate, pH 7.5). The pellets were drained, resuspended in 50 µL of 20 mmol/L HEPES (pH 7.0), containing 0.1% Triton X-100, and 150 mmol/L NaCl, and then reacted with...
RESULTS

A 210,000 MW abl protein was detected in extracts of K562 cells using anti-abi389-403 serum in an ICKA (Fig 1, lane 1), a result consistent with previous reports. The 190,000 MW protein seen on the gels is thought to be either a proteolytic fragment of p210 or a second translation product of the bcr-abl gene. The 210,000 and 190,000 MW proteins were determined to be abl specific, since these proteins were not detected in assays in which the reactivity of the anti-abi389-403 serum was blocked by prior addition of excess abi389-403 peptide (Fig 1, lane 2). Addition of an unrelated peptide did not change the reactivity of anti-abi389-403 serum with p210 or p190 (Fig 1, lane 3), demonstrating that the blocking observed after addition of cognate peptide is not due to nonspecific competition. Using identical assay conditions, p210 was also detected in K562 cell extracts when the anti-bcr3 serum was used (Fig 1, lane 3), indicating that p210 consists of both bcr and c-abl sequences. The incorporation of bcr exon 3 in K562 p210bcr-abl is supported by the detailed investigations of Shtivelman et al, indicating that the RNA junction point in the bcr-abl transcript derived from K562 cells includes the third bcr exon.

Southern blot analysis revealed rearrangement in bcr in both BgIII and BamH I digested DNA from patient A (Fig 2, lane 3). These data are consistent with the inclusion of exon 3 of bcr in the expressed p210bcr-abl of this patient (Fig 3). Rearrangement in bcr was observed in BgIII but not BamHI digested DNA from patient B (Fig 2, lane 1). Since the BamHI restriction site is located proximal to exon 3 of bcr (Fig 3), the break on chromosome 22 in patient B must occur 5' to exon 3. Therefore the results obtained from Southern blot analysis are consistent with the absence of exon 3 of bcr in the p210bcr-abl derived from patient B, as demonstrated by ICKA. Differences in the intensity of bands from lane to lane were similar when the filters shown in Fig 2 were reprobed with a beta-actin cDNA probe (data not shown), indicating that these differences reflected variations in the amount of DNA in each lane.

Fig 1. Immune complex kinase assays of CML cells. Lanes 1,6,11,15,19,23, anti-abi389-403 serum; lanes 2,7,12,16,20,24, anti-abi389-403 serum and abi389-403 peptide block; lanes 3,8,13,17,21,25, anti-bcr3 serum; lanes 4,9,14,18,22,26, anti-bcr3 serum and bcr3 peptide block; lane 10, preimmune rabbit serum; lane 5, anti-abi 389-403 serum and unrelated (bcr3) peptide block: Cells were used as follows: lanes 1-5, K562; lanes 6-10, CML patient B; lanes 11-14, CML patient C; lanes 15-18, CML patient A; lanes 19-22, CML patient E; lanes 23-26, CML patient D.
have recently demonstrated that addition, Ben-Neriah et al23 have found that the 210,000 MW protein product of Ph-positive CML patients A and B, Bam HI; Bg, BglII; H, HindIII.

![Diagram](image)

**Fig 3.** Restriction enzyme map of bcr (adapted from Heisterkamp et al14). Exons are indicated by vertical boxes below the map. The horizontal bar above the map shows the 1.2 kb HindIII-BglII bcr probe. Below the map the approximate positions of the breakpoints of Ph-positive CML patients A and B. B, Bam HI; Bg, BglII; H, HindIII.

DISCUSSION

The N-terminal replacement of c-abl by bcr sequences in the 210,000 MW protein product of Ph-positive CML patients has been previously predicted by studies of the aberrant 8 kb bcr-abl mRNA found in these patients.6,19 In addition, Ben-Neriah et al23 have recently demonstrated that p210 contains both bcr and c-abl sequences. Our data indicate that the Ph translocation results in the generation of at least two molecular variants of the bcr-abl protein. These variants differ in that they either contain or lack bcr exon 3—bcr(3):abl v bcr(−3):abl. They are generated as a result of the differing locations of the breaks on chromosome 22, which can occur both proximal and distal to the third exon of bcr. The absence of bcr exon 3 does not appreciably alter the size of the 210-kd bcr-abl protein, probably because this exon is small, consisting of only 25 amino acids.7 Our experiments also demonstrate that both bcr-abl variants are enzymatically active as phosphokinases, a property of potential fundamental importance for CML, since this enzymatic activity has been strongly implicated in the ability of several onco- genes and growth factor receptors to exert their effects.21

The clinical course of CML is characterized by the inevitable progression from an easily controlled benign phase to a terminal blast transformation phase22 in a time period ranging from several weeks to 23 years (median interval = 3.5 years).22,23 The blasts at the time of transformation usually display characteristics of myeloid lineage, although a minority of patients (about 30%) may develop a lymphoid blast crisis.24 The factors influencing the morphology of the blasts and the length of time to the transformation stage remain unknown. Recent work has demonstrated that subtle alterations in oncogene configuration can be associated with phenotypic variations of a tumor, ie, distinct chromosomal 8 breakpoint sites relative to the c-myc oncogene are found in the endemic v sporadic forms of Burkitt’s lymphoma.25 Interestingly, two of three patients with the bcr(−3):abl protein variant demonstrated several unusual clinical features: a survival of 10 and 11 years respectively (seen in less than 5% of patients)26 and a terminal lymphoid blast phase. Both patients with the bcr(3):abl protein showed a more typical clinical course, with a survival of 3 and 3.5 years respectively and a terminal blast crisis of myeloid lineage. Whether the absence of bcr exon 3 in the p210bcr-abl product of some CML patients is related to long survival, and/or the development of a blast crisis of lymphoid lineage, or is merely fortuitous requires further investigation. Studies correlating the exact location of the breakpoint in bcr and the structure of p210bcr-abl with clinical characteristics of a large series of CML patients are in progress and should help elucidate whether or not the generation of molecular variants of p210bcr-abl is important in the malignant evolution of CML.

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