ANTIBODY RECOGNITION OF THE TUMOR-SPECIFIC bcr-abl JOINING REGION IN CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a pluripotent stem cell disorder characterized by the presence of the Philadelphia (Ph') chromosome in the leukemic cells of 96% of all CML patients (1). The Ph' chromosome is formed by a reciprocal translocation between chromosomes 22 and 9 (2, 3). In this translocation, the c-abl oncogene has moved from chromosome 9 into the breakpoint cluster region (bcr), within the bcr gene on chromosome 22, resulting in a chimeric bcr-c-abl gene (3, 4). The fused gene encodes an 8.5-kb chimeric mRNA (5, 6), which is translated into a 210-kD protein (7). This P210bcr-abl protein shows tyrosine kinase activity and is uniquely present in the leukemic cells of CML (and a number of Ph'+ ALL) patients (8–12).

The breakpoint in the bcr gene occurs either between bcr exon 2 (b2) and 3 (b3), or alternatively between bcr exon 3 (b3) and 4 (b4). Therefore, in the mature bcr-abl mRNA, either b2 or b3 is spliced to abl exon a2, which results in two alternative chimeric P210bcr-abl proteins, comprising either the b2-a2 or b3-a2 junction (13). As such, the two different amino acid sequences at the point of the junction represent unique tumor-specific determinants.

In this study we investigate whether these joining determinants are exposed on the P210bcr-abl molecule in an immunogenic fashion. Our data indicate that the joining determinants b2-a2 can indeed be recognized by antibodies. The strategy we used to generate and characterize the anti-b2-a2 antiserum has potential for the further development of antibodies detecting tumor-specific proteins resulting from chromosomal rearrangements.

Materials and Methods

Cell Lines. K562, LAMA-84, and BV173 are Ph' cell lines derived from patients during the blast crisis phase of CML. Cells were cultured in RPMI medium supplemented with 7.5% FCS, 100 µg/ml penicillin, and 60 µg/ml streptomycin.

Peptide Synthesis, Purification, and Conjugation. Peptides were synthesized according to the
solid phase method developed by Merrifield (14) on polystyrene resin (1% crosslinking), using an automated peptide synthesizer (SAM-2; Biosearch, San Rafael, CA). The reaction sequence was performed according to the standard protocol using tertiary-butyl-oxy carbonyl amino acids with the following side chain protection: lys-2-chlorocarbobenzoxy, glu-benzyl, gly-benzyl, cys-tertiary-butylmercapto, all commercially available (Fluka A. G. Buchs, Switzerland and Bachem A. G. Bubendorf, Switzerland). Boc-arg-4-methoxybenzenesulfonyl was synthesized from Boc-Arg (Fluka A. G. Buchs) and MBS-Cl (Aldrich Chemical Co., Milwaukee, WI) according to Nishimura and Fujino (15). Final deblocking and cleavage from the resin was performed by treatment with trifluoromethanesulfonic acid/Thioanisol/m-Cresol in trifluoro acetic acid (TFA) for 1 h at 0°C (16), followed by filtration and precipitation from ether/n-pentane. Cys-containing peptides were treated with 10 equivalent threo-1,4-dimercapto-2,3-butan e n eol, pH 8, for 1 h and lyophilized. Gelfiltration of the crude peptide was performed on Sephadex-G15 (Pharmacia Fine Chemicals, Piscataway, NJ) using 5% (vol/vol) acetic acid as the eluent. For purification by HPLC (Pharmacia Fine Chemicals) a reverse-phase ultrasphere C18 column, 10 x 250 mm (Beckman Instruments, Inc., Palo Alto, CA), was used applying a linear gradient from 15% to 40% solution B (0.1% TFA in acetonitrile) into solution A (0.1% TFA in water), in 15 min at 2.5 ml/min. Amino acid analysis was performed on the hydrolyzed peptide using precolumn derivatisation of the amino acids according to Janssen et al. (17), confirming the expected composition. Peptides were coupled to a carrier protein (chicken gamma globulin, and BSA) via the terminal cystein residue using m-male imido benzoxy-sulfo succinimide-ester (18) (MBS) as a bifunctional coupling agent.

**Immunizations and Ig Purification.** Flemish rabbits were primed intracutanously with 250 µg protein complex consisting of SP b2-a2 coupled via MBS to CGG, emulsified in an equal volume of CFA. Rabbits were boosted twice after intervals of 4 wk; the first time with 250 µg of protein in CFA, the second time with the same dose in IFA. 14 d after the last boost rabbits were bled and sera were collected. The Igs were purified from the serum by precipitation with 16% Na2SO4.

**Preparation of Anti-bcr Antiserum.** A polyclonal antiserum directed against the NH2 terminus of the bcr protein was generated using the bacterial expression vector pEX (19). A 513-bp Bam HI–Pst I cDNA fragment, derived from the first exon of the bcr gene, was cloned in the Bam HI and Pst I site of pEX2, thus maintaining the translational reading frame of the normal bcr protein. The resulting hybrid β-galactosidase bcr protein was expressed according to standard procedures (7) and isolated from the bacteria as follows. The bacteria were collected and sonicated for 6 min in PBS on ice. After a 5-min centrifugation in an Eppendorf centrifuge, the pellet was resuspended in PBS and emulsified in an equal volume of Freund adjuvant. Immunization procedures and Ig purification were as described above.

**ELISA.** Binding of antiserum to the synthetic peptides was investigated in a micro ELISA system as described previously (20). Terasaki trays were coated with 0.1 µg antigen/well. Sera were diluted in PBS supplemented with 0.05% Tween-20. An optimal dilution of donkey anti-rabbit F(ab)`2 fragments conjugated to β-galactosidase (Amersham Corp., Arlington Heights, IL) was used as detecting reagent. Binding of antibodies was visualized by incubation with the fluorogenic substrate 4-methyl-umbelliferyl-β-galactopyranoside. Binding is expressed as arbitrary fluorescence units.

**Immunoprecipitation and Protein Kinase Reaction.** Immunoprecipitation and kinase reaction were carried out according to Chan et al. (21) with some minor modifications. Cells (5 x 10⁶) were washed once in PBS, then lysed for 5 min in 0.5 ml ice-cold lysis buffer (1% Triton X-100. 0.05% SDS, 150 mM NaCl, 5 mM EDTA in 10 mM sodium phosphate, pH 7.0), supplemented with 0.6 mg/ml gelatine, 4 mM PMSF, and 0.3 mg/ml of each of the following protease inhibitors: aprotinin, trypsin inhibitor, leupeptin, and bestatin (Sigma Chemical Co., St. Louis, MO). After centrifugation of the lysate in an Eppendorf centrifuge, 5–50 µl of Na2SO4-precipitated Iggs were added. Antigen-antibody interaction was allowed for 2 h at 4°C. Next, 40 µl of a 1:7 dilution of packed protein A-Sepharose beads (Pharmacia Fine Chemicals) was added. Beads were collected after a 30-min incubation at 4°C, and washed twice in lysis buffer without SDS and subsequently washed with 50 mM Tris-HCl, pH 7.0. The beads were resuspended in 20 µl 20 mM Pipes buffer, pH 7.0 (Sigma Chemical Co.), supplemented with 20 mM MnCl2 and 20 µCi (γ-32P) ATP was added (Amersham Corp.).
Incubation was allowed for 10 min at 37°C. Beads were then washed twice with lysis buffer without SDS and boiled in 100 μl sample buffer. Samples were run at 6% polyacrylamide gels. Gels were washed in distilled water, dried, and autoradiographed for 30 min, using Fuji (RX-NIF) films.

**RNA Analysis.** RNA was extracted according to the Li/Cl method (22). Samples were subjected to electrophoresis on formaldehyde 1% agarose gels, transferred to nitrocellulose, and after hybridization, exposed for 3 d to Kodak XAR 5 films using intensifying screens. The oligonucleotides covering the bcr-abl junctions were composed as follows: (b3-a2)5' TGGATTTAAGCCAGATTCCAA AAGCCCTTCAAGCGGAGG 3'; (b2-a2)5' GCTGAC-CATCAATAAGGAAG AAGCCCTTCAAGCGGAGG 3'; (primer)5' TACTGGCC 3. 32P-labeled probes were synthesized by extension of the 8-mer primer hybridized to one of the 40mers. The primer extension mixture contained 6 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 6 mM β-mercaptoethanol, 50 mM NaCl, 30 μCi (α-[32P]) dATP, 30 μCi (α-[32P]) dCTP, 100 mM dGTP, 100 mM dTTP, 8 ng of the 40-mer, 4 ng of the primer, and 5 U Klenow polymerase in a total volume of 10 μl. The reaction was performed at room temperature for at least 1 h and stopped by the addition of 90 μl TES (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.1% SDS) followed by removal of free nucleotides on a Sephadex-G50 column.

**Amplification of cDNA.** The cDNA amplification was performed using the polymerase chain reaction. 10 μl of total RNA was ethanol precipitated; washed with 70% ethanol, and dried in a desiccator. The pellet was dissolved in 9 μl of annealing buffer (250 mM KCl, 10 mM Tris-HCl, pH 6.3 at 42°C, 1 mM EDTA) and 1 μg of the c-abl primer 5’ GAGCTCGGATCATCGAAGAAGA TCCGAGGCAAGT 3’ was added. The sample was heated for 3 min at 80°C and transferred to a 65°C waterbath to allow annealing of the primer for 1 h. 15 μl of cDNA buffer (24 mM Tris-HCL, pH 8.3 at 42°C, 16 mM MgCl2, 8 mM DDT, 0.4 mM dNTP) and 5 U of avian myeloblastosis virus reverse transcriptase were added. This sample was incubated for 1 h at 42°C. After this step, 1 μg of a bcr primer 5’ GAAGAAGTGTCTT CAGAAGCTTCTCC 3’ (from exon b2) was added and 26 cycles of the polymerase chain reactions were performed directly in a volume of 100 μl, using Taq polymerase.

**Results**

**Synthesis of Peptides Corresponding the bcr-abl Junction.** Two peptides were produced by solid phase synthesis according to Merrifield (14) based on the previously published (6) nucleotide sequence of the chimeric bcr-abl gene. The peptides were prolonged with an NH2-terminal cysteine providing a specific coupling site to carrier molecules.

(a) Synthetic peptide b2-a2 (SP b2-a2) represents the fusion part of b2-a2 P210bcr-abl and consists of the amino acids:

<table>
<thead>
<tr>
<th>cys</th>
<th>-ile-asn-lys-glu-g</th>
<th>lu-al-a-leu-gln-arg-pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcr2</td>
<td>abl2</td>
<td></td>
</tr>
</tbody>
</table>

(b) Synthetic peptide b3-a2 (SP b3-a2) represents the b3-a2 fusion part of b3-a2 P210bcr-abl and consists of the amino acids:

<table>
<thead>
<tr>
<th>cys</th>
<th>-lys-gln-ser-ser-l</th>
<th>ys-al-a-leu-gln-arg-pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcr3</td>
<td>abl2</td>
<td></td>
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</table>

Since the fusion of the bcr and abl genes occurs within a coding triplet (6), the second glu in SP b2-a2 and the second lys in SP b3-a2 are newly generated by the translocation process. Thus, both peptides contain newly formed amino acids, share the c-abl COOH-terminal amino acids and differ at the NH2-bcr terminus.
Antibody Binding to Synthetic Peptides. Antiserum BP-1 was raised against SP b2-a2. Here, we show the binding of BP-1 to the peptide in a micro ELISA system. Terasaki trays were coated with SP b2-a2 conjugated to BSA. As a control, Terasaki plates were coated with BSA-MBS or BSA-MBS-SP b3-a2. As shown in Fig. 1a, BP-1 bound to BSA-MBS-SP b2-a2 and, to an equal extent, to BSA-MBS-SP b3-a2 (Fig. 1b). However, binding to BSA-MBS occurred to a much lower extent (Fig. 1c). This indicates that antiserum BP-1 contains antibody molecules directed against MBS and against determinants on both synthetic peptides, most probably on the shared a2 part of the peptides.

We then investigated the presence of antibodies in the serum directed against the b2 side of SP b2-a2 by absorption studies (Fig. 1d). As expected, preincubation of antiserum BP-1 with BSA-MBS-SP b2-a2 completely abrogated binding to BSA-MBS-SP b2-a2. However, absorption with BSA-MBS maintained binding of BP-1 to BSA-MBS-SP b2-a2, suggesting a predominance of anti-SP b2-a2 antibodies in the serum. Absorption of BP-1 to BSA-MBS-SP b3-a2 showed only partial inhibition of antibody binding to BSA-MBS-SP b2-a2. This indicates that the majority...
of the anti-SP b2-a2 antibodies in serum BP-1 is directed against the b2 side and/or the b2-a2 joining region of the peptide.

**DNA and RNA Analysis of bcr-abl Joining Regions in CML Cell Lines.** As a source for native P210bcr-abl, we used three cell lines derived from CML patients in blast crisis: K562 (23), BV173 (24), and LAMA-84 (25). To determine whether the cell lines contain b3-a2 P210bcr-abl or b2-a2 P210bcr-abl, we first localized at the DNA and RNA level the bcr breakpoints and bcr-abl junctions of the BV173 and LAMA-84 cells. As previously published, K562 cells contain a breakpoint 3' of exon b3, and express a hybrid mRNA in which exon b3 is spliced to exon a2 (6). Southern blot analysis was performed using Bam HI and Bgl II restriction enzyme digests to pinpoint to genomic breakpoints on chromosome 22 in BV173 and LAMA-84 (Fig. 2). One extra band was visible using 5' bcr probes (probes a and b), while two rearranged bands were visible in both digests of LAMA-84 DNA, using a 3' bcr probe. This indicates that the 3' bcr probe (probe c) detects both the 22q- and the 9q+ hybrid fragments in LAMA-84 DNA, which maps the breakpoint within this 1.2-kb 3' Hind III–Bgl II fragment. Thus, LAMA-84 contains a breakpoint in the intron between exon b3 and b4. In BV173 DNA, only in the Bgl II digest was an extra band visible, using the 5' bcr probe. However, using another 5' bcr Ava I probe (probe b), two extra bands were seen in the Bam HI digest, apart from the two normal fragments. Thus, the breakpoint in BV173 is located just 5' of exon b3 within the Bam HI–Ava I fragment.

To confirm the expected RNA structure surrounding the bcr-abl junction, we hybridized specific oligonucleotides (40-mers) comprising the b3-a2 and b2-a2 junction sequences to RNA preparations of the CML cell lines on Northern blots (Fig. 3). After hybridization, stringent washings were performed, i.e., above the Tm of the three different 20-mers (b2, b3, and a2). Thus, a signal could only be obtained when a hybrid bcr-abl mRNA was present. As shown in Fig. 3, the b3-a2 oligomer hybridizes exclusively to the 8.5-kb hybrid bcr-abl mRNA of the K562 and LAMA-84 cell lines. The b2-a2 oligomer however, only hybridizes to the bcr-abl mRNA of BV173. These results are in concordance with the DNA breakpoints in the bcr gene of these cell lines, and strongly indicate that BV173 cells contain b2-a2 P210bcr-abl, whereas K562 and LAMA-84 cells both contain b3-a2 P210bcr-abl. Surprisingly, we do not detect a b2-a2 alternative splice product in K562 RNA, as was previously found by others (13).
To confirm these observations we decided to use a highly sensitive assay based on the polymerase chain reaction (PCR) (26). A cDNA synthesis of K562 and BV173 RNA was performed, using a c-abl primer from exon a2. This cDNA was amplified using the PCR with bcr and c-abl primers mapping 345 and 270 bp apart in the b3-a2 and b2-a2 cDNAs, respectively. As is shown in Fig. 4, the amplified fragment from K562 RNA is 345 bp long and hybridizes to the b3-a2 oligonucleotide, while no 270-bp fragment is detected after hybridization with the b2-a2 oligonucleotide. In contrast, in BV173 the 270-bp fragment is amplified and hybridizes to the b2-a2 oligonucleotide. These findings strongly argue against a b2-a2 alternative splice in the K562 cell line we used for the present experiments.

Immunoprecipitation of P210bcr-abl from CML Cell Lines. Binding of antiserum BP-1 to both b2-a2 P210bcr-abl and b3-a2 P210bcr-abl was tested by immunoprecipitation and autophosphorylation of the proteins. Fig. 5 shows a clear precipitation of b2-a2 P210bcr-abl from BV173 cells (lane 2). Strikingly, b3-a2 P210bcr-abl from LAMA-84 and K562 cells was not precipitated by BP-1 (Fig. 5, lanes 4 and 6). To show that b3-a2 P210bcr-abl indeed is present in our K562 and LAMA-84 cells, we precipitated these molecules with a polyclonal antiserum directed against the NH2 terminus of the bcr protein (Fig. 6, lanes 2 and 4). Since P210bcr-abl is clearly precipitated, we conclude that antiserum BP-1 recognizes an antigenic determinant on b2-a2 P210bcr-abl, which is not expressed on b3-a2 P210bcr-abl.
To analyze the specificity of the binding of BP-1 to P210\textsuperscript{bcr-abl} in more detail, peptide blocking studies were performed, either with unconjugated SP b2-a2 or with SP b3-a2. Fig. 3 shows that immunoprecipitation of b2-a2 P210 was prevented after incubation of BP-1 with the cognate peptide b2-a2 (Fig. 5, lane 7). In contrast, incubation with SP b3-a2 (Fig. 5, lane 8) had no effect on immunoprecipitation. This indicates that precipitation of b2-a2 P210\textsuperscript{bcr-abl} could not be caused by antibodies directed against the a2 part of P210\textsuperscript{bcr-abl}, since those antibodies were removed by absorption with the b3-a2 peptide.

**Discussion**

P210\textsuperscript{bcr-abl} can be considered as a highly tumor-specific protein for CML (8–11). In addition, the molecule is also detected in 10–25\% of Ph\textsuperscript{+} ALL patients (12). It has been suggested that these patients have a CML blast crisis without or with a very short, preceding chronic phase (27).

The tumor-specific character of P210\textsuperscript{bcr-abl} is different from that of other previously described tumor-specific or tumor-associated antigens. A problem occurring for many tumor-associated antigens is that they are not only produced by tumor
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**Figure 5.** Immunoprecipitation analysis of the specificity of antiserum BP-1. BV173, K562, and LAMA-84 cells were lysed and immunoprecipitated with 10 \( \mu l \) \( Na_2SO_4 \) precipitated normal rabbit serum (lanes 1, 3, and 5) and with 10 \( \mu l \) \( Na_2SO_4 \) precipitated serum BP-1 (lanes 2, 4, and 6). (Lane 7 and 8) Immunoprecipitation analysis of BV173 cells with serum BP-1 after addition of 0.25 mM SP b2-a2 (lane 7) and 0.25 mM SP b3-a2 (lane 8). Immunoprecipitation and kinase reaction were carried out according to Chan et al. (21).

**Figure 6.** Immunoprecipitation analysis of K562, LAMA-84, and BV 173 cells with a polyclonal serum directed against the NH \(_2\) terminus of the \( bcr \) protein. The cells were lysed and precipitated with 25 \( \mu l \) \( Na_2SO_4 \) precipitated normal rabbit serum (lanes 1, 3, and 5) and with 25 \( \mu l \) \( Na_2SO_4 \) precipitated anti-bcr antiserum (lanes 2, 4, and 6).
cells, but also, although in a lower degree, by normal cells (28). The P97 antigen, for example, is highly associated with malignant melanoma, but is also expressed on normal tissue (29). Other antigens, such as the TAG-72 antigen (30), are not specific for one particular tumor but are expressed on a variety of tumors. In contrast, P210{bcr-abl} can only be produced by cells carrying the CML-specific bcr-abl translocation and is therefore, by definition, a tumor-specific antigen. P210{bcr-abl}, however, is composed of the normal bcr and abl proteins, which are, as such, non-tumor-specific determinants on the fusion protein. Theoretically, the only tumor-specific determinant on the P210{bcr-abl} molecule is formed just by the joining between bcr and abl.

In this report we show the possibility to produce a polyclonal antiserum recognizing the bcr-abl joining region by using a synthetic peptide corresponding the b2-a2 junction as an immunogen. Molecular studies revealing the nucleotide sequence at this junction (6) made this direct approach possible. As such, our approach differs principally from other strategies meant for the production of tumor-specific antibodies, where tumor cells, tumor cell membranes, or purified proteins were used as immunogens (30–32).

The peptide we synthesized consisted of four amino acids derived from b2 and five amino acids derived from a2. Between these amino acids, one amino acid is located, which is newly generated by the Philadelphia translocation. The polyclonal antiserum we obtained after immunization with the peptide reacted in a very specific way with the native b2-a2 P210{bcr-abl} molecule using the protein kinase assay. In contrast, b3-a2 P210{bcr-abl} was not recognized by BP-1 in this assay. Our ELISA data indicated, however, that BP-1 reacted with different parts of the peptide; the a2 part as well as the b2 and/or b2-a2 junction were recognized by BP-1. Since no precipitation of b3-a2 P210{bcr-abl} was detected, the b2 and a2 amino acids were not recognized in the native b3-a2 P210{bcr-abl} molecules. We conclude, therefore, that the individual b2 and a2 determinants, as exposed on the peptide, are hidden within the tertiary structure of the native b3-a2 P210{bcr-abl} and are inaccessible for anti-b2 and anti-a2 antibodies.

We also showed that b2-a2 P210{bcr-abl} was not precipitated by antibody molecules directed against a2 sequences, because after removing anti-a2 reactivity from BP-1 by absorption, b2-a2 P210{bcr-abl} was still precipitated. This leaves us with the conclusion that under the present experimental conditions, b2-a2 P210{bcr-abl} from BV173 cells is precipitated by antibodies recognizing the b2-a2 joining region itself or by antibodies that recognize newly created tertiary b2 or a2 determinants introduced by the bcr-abl joining region.

Furthermore, the observation that no b2-a2 P210{bcr-abl} is precipitated by BP-1 from the K562 cells was an extra confirmation of our molecular data, i.e., the absence of a b2-a2 alternative splice in the K562 cell line we used.

In summary, we have shown that breakpoint-specific DNA-encoded sequences are exposed on P210{bcr-abl} molecules and that, under the present experimental conditions, such sequences can be visualized at the protein level by antibodies generated through the use of synthetic peptides encoded by these sequences as immunogens. Obviously, antibodies obtained in this way may further aid in the clinical diagnosis of CML and in the distinction of the various malignant disorders where chromo-
some rearrangements are involved. Studies to detect individual tumor cells using immunohistochemical techniques are now in progress.

Summary

Chronic myeloid leukemia (CML) is characterized by the presence of a 210-kD protein (P210\textsuperscript{bcr-abl}) in the cytoplasm of leukemic cells, generated by the reciprocal translocation between chromosome 9 and chromosome 22. Due to this translocation, the \textit{abl} oncogene is coupled to the \textit{bcr} gene, forming a new determinant in this protein encoded by the \textit{bcr-abl} joining region. In the joining region itself, either the \textit{bcr} exon 2 is coupled to the \textit{abl} exon 2 (b2-a2), or the \textit{bcr} exon 3 is coupled to the \textit{abl} exon 2 (b3-a2). Thus, these joining regions form by definition new tumor-specific determinants in the respective chimeric P210\textsuperscript{bcr-abl} molecules.

This paper addresses the question as to whether these tumor-specific joining regions are exposed on the P210\textsuperscript{bcr-abl} molecule in such a way that antibodies can be generated to detect these sites. To test this possibility a polyclonal antiserum, termed BP-1, was raised against a synthetic peptide representative for the b2-a2 joining region. The reactivity of BP-1 was analyzed in an ELISA system on various synthetic peptides. Peptide inhibition studies showed the presence of antibodies to different parts of the b2-a2 peptide in the polyvalent antiserum.

The reactivity of BP-1 was then tested with native P210\textsuperscript{bcr-abl} molecules in various CML cell lines (K562, LAMA-84, and BV173) using a protein kinase assay. In this context, the \textit{bcr-abl} junctions were first analyzed at the DNA and RNA level. The present study indicates that BP-1 specifically recognizes the b2-a2 junction in native P210\textsuperscript{bcr-abl}. Furthermore, BP-1 clearly discriminates between b2-a2 P210\textsuperscript{bcr-abl} and b3-a2 P210\textsuperscript{bcr-abl}. We conclude that the tumor-specific b2-a2 joining region is antigenically exposed on the native P210\textsuperscript{bcr-abl} molecule.

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