RAPID COMMUNICATION

Tyrosine Phosphorylation of CRKL in Philadelphia+ Leukemia

By Johanna ten Hoeve, Ralph B. Arlinghaus, Jie Qiang Guo, Nora Heisterkamp, and John Groffen

The chimeric BCR/ABL protein is characteristic of Philadelphia (Ph)" leukemia because it is the direct product of the Ph translocation and it has been shown to play a causal role in the genesis of leukemia. The BCR/ABL protein exhibits a deregulated tyrosine-kinase activity capable of phosphorylating different cellular substrates in vivo and in vitro. CRKL, an adaptor protein consisting of SH2 and SH3 domains in the absence of a catalytic domain, is one potential in vivo substrate for ABL and for BCR/ABL in COS-1 cells. In the current study, we show that in peripheral blood cells a direct correlation exists between the presence of BCR/ABL and the phosphorylation status of CRKL. In Ph" peripheral blood cells, CRKL is present only in the nonphosphorylated form. In contrast, all BCR/ABL+ CML and acute lymphoblastic leukemia patient samples examined showed clear tyrosine-phosphorylation of CRKL. This result strongly suggests that CRKL is a biologically significant substrate for BCR/ABL and is likely to play a major role in the development of Ph" leukemia.

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MATERIALS AND METHODS

Cells and patient samples. Cellular lysates of patients were prepared as described. Blood samples were obtained from patients as part of a program project study sponsored by the National Cancer Institute (Bethesda, MD). All patients signed an appropriate informed consent form. The ALL cell line sup-B15 and the BCWABL cell line K562 were used for immunoprecipitation. The ALL line was provided by J. Groffen (University of Leiden, Leiden, The Netherlands). The BCR/ABL protein was isolated by immunoprecipitation of the ALL cell line sup-B15 and the BCWABL cell line K562. The tyrosine-phosphorylated proteins were precipitated with anti-CRKL, anti-ABL, or anti-Ph+ ALL sera.

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ABL⁺ myeloid cell line KG-1 have been described previously. The CML cell line K562 was from the American Type Culture Collection (ATCC; Rockville, MD).

**Antisera and Western blotting.** The CRKL-specific CH16 antisera was raised against a bacterially expressed CRKL-glutathione-S-transferase (GST) fusion protein containing the CRKL amino acid residues 184-247. This region, which is diverged between CRKL and CRK, was inserted into pGEX-2T as Sau3A-EcoRI fragments. Bacterial cultures expressing the pGEX constructs were induced with 0.25 mmol/L IPTG and lysed by sonication. GST-CRKL fusion proteins were purified on glutathione-Sepharose columns and used to immunize rabbits. Anti-α-p-Tyr (Ab-2) monoclonal antibodies were from Oncogene Science, Inc (OSI; Cambridge, MA). The monoclonal anti-Abl antibody 8E9 has been described elsewhere.

Western blotting and detection of BCR/ABL P210 was as described previously. For detection of CRKL, lysates of 4 × 10⁹ peripheral blood cells or 1 × 10⁹ K562 cells were separated on 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to Hybond-ECL (Amersham, Arlington Heights, IL). Membranes were blocked overnight at 4°C in TBST (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% Tween) plus 5% nonfat milk (BioRad, Hercules, CA) and incubated with CH16 antisera (diluted 1:1,000 in TBST + 2% nonfat milk) for 2 hours at room temperature. Bound antibodies were detected with horseradish peroxidase-labeled secondary antibodies (BioRad: 1:3,000 in TBST + 2% milk). Anti-α-p-Tyr immunoblots were blocked overnight at 4°C in TBST + 2% bovine serum albumin (BSA) + 1% ovalbumin and incubated with the α-p-Tyr monoclonal antibodies at 1 μg/mL in buffer C (TBST + 1% fish gelatin + 0.1% BSA). Blots were developed using the ECL reagents (Amersham) and exposed to Hyperfilm-ECL (Amersham).

**Immunoprecipitations and protein kinase assay.** Extracts of K562 cells and of COS-1 cells transfected with CRKL and BCR/ABL P190 constructs were prepared as described. CRKL and BCR/ABL were immunoprecipitated from 500 μg K562 extract or 100 μg cosCRKL + P190 extract as described, using 2.5 μL CH16 antisera and 1 μg anti-Abl (Ab-1) polyclonal antibodies (OSI), respectively. Immunoprecipitates were washed twice with lysis buffer and four times with ice-cold HNTG (20 mmol/L HEPES, pH 7.0, 150 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, 0.1% ovalbumin and 1% fish gelatin). Kinase reactions were performed on ice for 1 hour in 25 μL 20 mmol/L PIPES, pH 7.0, 20 mmol/L MnCl₂, and 10 μCi [γ-³²P]ATP (6,000 Ci/mmol; Amersham). The immunoprecipitates were boiled in SDS sample buffer and separated by electrophoresis on an 8% SDS-polyacrylamide gel. Phosphorylated protein was subsequently detected by autoradiography.

**RESULTS**

A Western blot analysis procedure has been previously developed to examine the presence of BCR/ABL in peripheral blood lymphocytes of patients. BCR/ABL P210 could easily be detected in the CML cell line K562 and in peripheral blood samples of CML patients (Fig 1). Lower molecular weight proteins are usually also detected by the Ab1 antibodies and are likely to be degradation products of the P210 or may represent some altered form of the BCR/ABL.

**Fig 1.** Western blot analysis of P210 expression levels in CML patient samples. The open arrowhead indicates the nonphosphorylated form of BCWABL. The positions of BCR/ABL P210 and ABL P145 are indicated.

**Fig 2.** CRKL is specifically tyrosine-phosphorylated in CML patient samples expressing P210. (Upper panel) Immunodetection of CRKL protein using CRKL antisera CH16. The locations of the CRKL proteins are indicated to the right. The open arrowhead indicates the tyrosine-phosphorylated form. (Lower panel) Detection of tyrosine-phosphorylated CRKL using α-p-Tyr antibodies (OSI). The position of the 43-kD marker is indicated to the left. The position of the phosphorylated form of CRKL is indicated to the right. Samples include the remission CML samples 2216 and 2225 and the chronic phase CML samples 2222, 2220, 2234, and 2240 (see also Table 1).
protein. BCR/ABL was not detectable in the acute myelogenous leukemia cell line KG-1 or in samples of CML patients who had achieved remission (Fig 1, samples 2216 and 2225).

All the peripheral blood lymphocyte samples contained clearly detectable levels of P38 CRKL. The CRKL proteins migrated as a single band in the remission samples 2216 and 2225 (Fig 2, upper panel) and in KG-1 (Fig 3). However, two distinct but closely migrating CRKL bands were present in the cell line K562 and in the samples of CML patients in chronic phase (Fig 2, upper panel) or blast crisis (Fig 3A, samples C1316 and C1797) expressing the P210 BCR/ABL protein; part of the total amount of CRKL present in these samples had undergone a mobility shift.

Mobility shifts can be caused by secondary modifications and are well known to occur as a consequence of phosphorylation. Probing of a duplicate Western blot with antiphosphotyrosine (a-p-Tyr) antibodies showed that the more slowly migrating band present in the P210-expressing samples contained phosphotyrosine (Fig 2, lower panel). All peripheral blood samples contained an additional tyrosine-phosphorylated protein of a slightly higher molecular weight. However, phosphorylation of this unidentified protein was constant and did not correlate with the presence of BCR/ABL.

A percentage of Ph⁺ ALL patients express P190, a BCR/ABL fusion protein containing only the BCR amino acids encoded by exon 1 but the same ABL moiety as P210. All of the P190-expressing ALL samples examined here showed the P38 doublet characteristic for the presence of phosphorylated and nonphosphorylated forms of CRKL (Fig 3A, samples A0007, A0041, A0055, and B15; Table 1). Indeed, the slower migrating form contained phosphotyrosine (data not shown). In contrast, Ph⁻ ALL patient samples contained only nonphosphorylated CRKL (Fig 3A, samples A0003 and A0018; results not shown). Other Ph⁺ samples examined included peripheral blood lymphocytes of a normal control and of patients with acute myeloid leukemia (AML), diffuse large-cell lymphoma, myeloproliferative syndrome (MPS), Down’s syndrome, chronic lymphocytic leukemia (CLL), and chronic myelomonocytic leukemia (CMML). None of these contained the tyrosine-phosphorylated form of CRKL (Fig 3B; and data not shown).

Some peripheral blood samples expressing BCR/ABL P210 or P190 were analyzed for the presence of tyrosine-phosphorylated proteins in the low as well as high molecular weight range. It appears that CRKL is one of the most prominent phospho-tyrosine-containing proteins in these samples (Fig 4).

In the peripheral blood samples examined there seems to be a direct correlation between the level of CRKL phosphorylation (as determined by the percentage of the CRKL proteins that had undergone a mobility shift) and the amount of BCR/ABL P210 or P190 detected (Table 1). As shown by us previously, CRKL and BCR/ABL P190 can be coimmunoprecipitated from COS-1 cells overexpressing both proteins using either CRKL or ABL antibodies (Fig 5). To confirm that CRKL and BCR/ABL are also in complex with each other in hematopoietic cells, CRKL was immunoprecipitated from extracts of the human cell line K562 and subjected to a kinase reaction. As shown in Fig 5, CRKL is also in complex with and a substrate for BCR/ABL P210 in K562 cells.

**DISCUSSION**

The deregulated tyrosine kinase activity displayed by BCR/ABL has prompted a number of investigators to exam-
Table 1. Peripheral Blood Samples Examined for CRKL Phosphorylation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Disease</th>
<th>Phase</th>
<th>P210</th>
<th>P190</th>
<th>% P-Y-CRKL*</th>
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<tr>
<td>K5621</td>
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<tr>
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* Determined by visual comparison of the intensities of the two CRKL bands; approximately equal levels of total CRKL protein were present in the peripheral blood lymphocyte samples. The cell line K562 contained higher levels of total CRKL protein. Samples S15 through S336 do not contain BCR/ABL P210 or P190.

† Cell lines.

We have found that P38 CRKL is expressed at clearly detectable levels in human peripheral blood cells and that CRKL is present in a non-tyrosine-phosphorylated form in these cells. However, CRKL becomes phosphorylated on tyrosine in peripheral blood cells expressing BCR/ABL P210 or P190, and the level of CRKL phosphorylation was found to correlate well with the amount of BCR/ABL detected by Western blotting. This suggests that a single BCR/ABL molecule is capable of phosphorylating only a limited number of CRKL molecules or, alternatively, that CRKL and BCR/ABL form a relatively permanent or stable complex.

CRKL is capable of forming complexes with both ABL and BCR-ABL when coexpressed in COS-1 cells. Also bacterially expressed CRKL can bind both overexpressed ABL and BCR/ABL in a Western blot binding assay. Binding of CRKL to BCR/ABL is therefore likely to involve the ABL part of the molecule. CRKL can be phosphorylated by both ABL and BCR/ABL when coexpressed in COS-1 cells. However, in peripheral blood cells, CRKL is only phosphorylated to detectable levels by BCR/ABL and not by the relatively abundant ABL protein (Fig 1). We therefore conclude that the high degree of CRKL phosphorylation in Ph+ cells can be considered abnormal.

CRKL is homologous to the adaptor protein CRK, which also consists exclusively out of SH2 and SH3 domains. In HeLa cells, CRK appears to be present solely in the tyrosine-phosphorylated form, reportedly caused by rapid posttranslational modification. CRK binds to p145 ABL via its first SH3 domain and is subsequently phosphorylated on tyrosine residue Y251. In vitro, this phosphorylated Y251 residue can provide a binding site for the CRK SH2 domain, and it has been proposed that the tyrosine-phosphorylation of CRK would result in intramolecular folding, thereby blocking the interaction of the CRK SH3 domain with other proteins such
as ABL. In analogy to SRC, tyrosine-phosphorylation of CRK would therefore result in the generation of an "inactive" CRK molecule that is unable to participate in signaling.

From our results it is clear that in hematopoietic cells CRKL is only present in a nonphosphorylated form. Whether this form is unable to engage in other molecular interactions or whether the tyrosine-phosphorylated form is "inactive" remains to be determined. If phosphorylation of CRKL would induce the interaction with other cellular proteins, it is easy to envision that this could activate certain signal transduction pathways and potentially impair the further terminal differentiation of myeloid precursor cells. Alternatively, if tyrosine-phosphorylation would inactivate CRKL, expression of BCR/ABL will result in loss-of-function of CRKL, the degree of which is correlated with the amount of BCR/ABL present. This postulated loss-of-function is not incompatible with the current views on the pathobiology of CML, which is characterized by an expansion of the most mature proliferating myeloid cell compartment. CML progenitor cells most likely undergo one or more transit times in the maturation compartments than do normal precursors. If the signal transduction pathway in which CRKL is involved conveys signals that will eventually lead to terminal differentiation, partial abrogation of CRKL function could slowly result in an expanding pool of myeloid precursors. Support for a possible role for CRKL in terminal differentiation comes from the finding that microinjection of the related CRK protein into the rat pheochromocytoma cell line PC12 induces neuronal differentiation. Although we currently are unable to distinguish between these alternative hypotheses of CRKL activation, both are amenable to experimental investigation. Regardless of the consequences of CRKL phosphorylation, the phosphorylation of CRKL is a clear hallmark of Ph+ leukemia, which might be used for diagnostic purposes.

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