Selective Expression and Constitutive Phosphorylation of SHC Proteins in the CD34+ Fraction of Chronic Myelogenous Leukemias

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ABSTRACT

The BCR/ABL fusion protein is a constitutively active tyrosine kinase that is responsible for the pathogenesis of chronic myelogenous leukemia (CML). Clinically, CML is characterized by a chronic phase (CP) that eventually terminates into a blast crisis (BC). BC transformation is associated with accumulation of CD34+ blasts. We investigated the expression and phosphorylation of Src-homology-2 and collagen-homology domains (Shc) proteins in subpopulations of CML primary cells. Shc polypeptides are tyrosine kinase substrates that are constitutively tyrosine-phosphorylated in continuous cell lines of CML. High levels of Shc expression were found in the CD34+ cells from CML-BC, CML-CP and normal bone marrow. In contrast, CD34− fractions from CML-CP and normal bone marrow expressed low levels of p46Shc. Shc proteins were constitutively phosphorylated in the CD34+ fractions from CML cells (both CP and BC), but not in normal CD34+ cells. These data bear implications for the role of Shc in normal hemopoiesis and CML leukemogenesis: (a) dramatic changes of Shc expression during terminal differentiation of hemopoietic cells add a further level of regulation to the signal transduction function of Shc; and (b) constitutive Shc tyrosine-phosphorylation in the rare CD34+ cells of CML-CP might contribute to the selection of this subpopulation during the blast crisis transformation of CML.

INTRODUCTION

The mammalian Shc locus encodes for adaptor proteins, which are involved in the cytoplasmic transduction of mitogenic stimuli from RTKs to Ras (1). The isolated Shc cDNA encoded two proteins of Mr 52,000 and 46,000 (p52/p46), which derive from differential translation initiation (2). They share a COOH-terminal SH2 domain, a central collagen-homology domain (CH1), rich in proline/glycine residues, and an NH2-terminal phosphotyrosine-binding domain. Shc proteins associate with, and are phosphorylated by, a considerable number of RTKs (1). In addition, Shc is rapidly phosphorylated after ligand stimulation of surface receptors that have no intrinsic TK activity and in cells transformed by v-src or v-fps (3, 4), suggesting that it is a common TK target in mitogenic signaling pathways. On phosphorylation, Shc interacts with the SH2 domain of Grb2 and functions as an alternative docking site for the SH2 domain of Grb2/Sos complex (5–7). Several lines of evidence suggest that the Shc/Grb2/Sos complex is involved in Ras activation. Shc overexpression induces transformation of fibroblasts (2) and, in PC12 cells, neurite extension (5), a response that is blocked by expression of a dominant inhibitory Ras mutant.

MATERIALS AND METHODS

Patients and Samples. CML patients in BC (n = 11) or CP (n = 4) and healthy donors (n = 4) were included in this study. The diagnosis of CML and the identification of the phases of the disease were performed according to standard criteria (17). All CML patients were 100% Philadelphia positive by standard cytogenetic analysis. Bone marrow cells were analyzed in all but in two cases (5 and 6), where peripheral blood was used. Normal hematopoietic cells were obtained from healthy donors undergoing bone marrow harvest (n = 3) or peripheral blood progenitor cell mobilization (n = 1) for allogeneic transplantation. Marrow or blood mononuclear cells were separated by centrifugation (400 × g for 30 min at 4°C) on a Ficoll-Hypaque gradient (density 1.077 g/ml). Interface cells were washed in PBS. The samples were directly lysed for protein detection after the separation of CD34+ and CD34− fractions (see below). PMN granulocytes were obtained after Ficoll by resuspending the pellet in dextran. K562 and TOM1 Ph+ cell lines were also analyzed.

Enrichment of CD34+ Cells. BC cells were >90% CD34+. CP CML and normal bone marrow cells were enriched according to CD34 expression by means of a magnetic cell-sorting methodology (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, marrow or blood cells were labeled with a haptenized CD34 antibody (QBEND/10), which is then magnetically labeled in a second-step reaction with an antihapten antibody coupled to super-paramagnetic microbeads. Labeled cells are then separated using a high-gradient magnetic separation column placed in a strong magnetic field. The magnetically stained cells are retained in the column while unstained cells pass through. When the column is removed from the magnetic field, the magnetically retained cells are eluted. The mean (± SD)

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3 The abbreviations used are: Shc, Src-homology-2 and collagen-homology domains; TK, tyrosine kinase; RTK, TK receptor; CML, chronic myeloid leukemia; CP, chronic phase; BC, blast crisis; HRP, horseradish peroxidase; PMN, polymorphonuclear; G-CSF, granulocyte colony-stimulating factor.
percentage of CD34<sup>+</sup> cells within enriched fractions was 86 ± 14% and 85 ± 11% for CML and normal samples, respectively.

**Cell Lysis.** The cells were lysed on ice in 50 mM Tris-HCl (pH 8), 1.5 mM MgCl<sub>2</sub>, 150 mM NaCl, 5 mM EGTA (pH 7.5), 5% (v/v) glycerol, 1% (v/v) and TritonX-100 containing freshly added protease inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride). Insoluble materials were removed by centrifugation for 10 min at 12,000 × g at 4°C, and protein concentration determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

**Immunoprecipitation.** For immunoprecipitation experiments, cell lysates were incubated with appropriate antibodies and immune complexes isolated with Protein A-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden). Immune complexes were denatured by heating at 95°C in reducing Laemmli buffer and analyzed by SDS-PAGE.

**Immunoblotting.** Either specific immunoprecipitates or total cell lysates were electrotransferred onto polyvinylidene difluoride filters (Millipore Intertech, Bedford, MA) after SDS-PAGE. After blocking nonspecific reactivity, filters were probed 1 h at room temperature with specific antibodies diluted in TBS-T [25 mM Tris-HCl (pH 8), 150 mM NaCl, 0.05% Tween 20] containing 5% nonfat milk. For antiphosphotyrosine experiments, 0.02% Tween 20 and 1% BSA (fraction V; Boehringer Mannheim, Mannheim, Germany) concentrations were used. After extensive washing, immunocomplexes were detected with HRP-conjugated species-specific secondary antibody followed by enhanced chemiluminescence reaction (Amersham International plc, Buckinghamshire, United Kingdom).

**Antibodies.** For immunoprecipitation, the following antibodies were used: rabbit polyclonal antihuman Shc (Upstate Biotechnology, Inc., Lake Placid, NY) and mouse monoclonal antihuman Shc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunoblotting, the following antibodies were used: rabbit polyclonal antihuman Shc (Santa Cruz Biotechnology), mouse monoclonal anti-antiphosphotyrosine (Upstate Biotechnology), goat polyclonal antihuman actin (Santa Cruz Biotechnology), goat antirabbit IgG (H+L)-HRP conjugated (Bio-Rad), goat antimouse IgG (H+L)-HRP conjugated (Bio-Rad), and donkey antigoat IgG (H+L)-HRP conjugated (Santa Cruz Biotechnology).

**RESULTS**

**Higher levels of Shc Protein Expression in CD34<sup>+</sup> versus CD34<sup>-</sup> Ph+ Cells.** To evaluate levels of Shc expression in CML, we performed Western blotting analysis of cellular lysates from 4 cases of CML-CP and 11 cases of CML-BC, using anti-Shc antibodies that recognize p46, p52 and the recently identified p66 Shc isoform (20). Shc expression was markedly higher in the 11 CML-BC cases (see representative Western blots in Fig. 1). In addition, whereas all three Shc isoforms were expressed in the CML-BC samples, only p46 was detected in the cases of CML-CP (Fig. 1). This was not due to the lower levels of Shc protein expression in the CML-CP cases because p46 was the less abundant Shc isoform in the CML-CP cases. Probing of the same Western blots with antiactin polyclonal antibodies revealed that similar amounts of cellular proteins were loaded on each lane (representative results are shown in Fig. 1).

CML-CP samples consist of a heterogeneous population of hematopoietic cells at different stages of differentiation, whereas CML-BC cells mainly consist of immature, CD34<sup>+</sup> myeloid precursors (17, 19). The fraction of CD34<sup>+</sup> cells in CML-CP usually does not exceed 2% of bone marrow mononuclear cells (18). To evaluate whether the different levels of Shc expression in CML-CP versus CML-BC samples reflected differences in cellular composition, we analyzed Shc expression in purified CD34<sup>+</sup> and CD34<sup>-</sup> cells from four different CML-CP samples. Mononuclear cells from CML-CP bone marrow samples were fractionated using an anti-CD34 monoclonal antibody affinity column and the purity of the two fractions (respectively, >95% and <5%; data not shown) assessed by fluorescence-activated cell-sorting analysis. Western blotting revealed low levels of p46 Shc in the CD34<sup>-</sup> CML-CP fraction, whereas high levels of all three Shc isoforms were found in the CD34<sup>+</sup> fraction from the same samples (Fig. 2A). Strikingly, levels of Shc expression in the CD34<sup>+</sup> CML-CP cases were comparable with those found in the CML-BC samples (Fig. 2A). The fact that unfractionated CML-CP samples expressed very low levels of Shc expression is consistent with the low frequency of CD34<sup>+</sup> cells in the same samples (<5%). Taken together, these results suggest that Ph<sup>+</sup> CD34<sup>+</sup> cells, either from BC or CP, express markedly higher levels of Shc expression than Ph<sup>+</sup> CD34<sup>-</sup> cells.

**Higher Levels of Shc Protein Expression in CD34<sup>+</sup> versus CD34<sup>-</sup> Fractions from Normal Bone Marrow.** The finding of higher levels of Shc proteins in CD34<sup>+</sup> Ph<sup>+</sup> cells might reflect a physiological regulation of Shc during differentiation of myeloid cells. Therefore, we analyzed Shc expression in CD34<sup>+</sup> and CD34<sup>-</sup> fractions from three different normal bone marrow samples. Western blotting analysis revealed high levels of Shc expression in the CD34<sup>+</sup> fractions, whereas Shc polypeptides were almost undetectable in the CD34<sup>-</sup> fractions (representative results for two bone marrow samples are shown in Fig. 2B). Notably, low levels of Shc expression, comparable with those found in the CD34<sup>+</sup> fractions, were also found in cellular lysates from purified normal neutrophils (Fig. 2A). Side-to-side comparison of Shc expression levels in the CD34<sup>+</sup> cell fractions from normal bone marrow and CML-CP samples and in cells from CML-BC revealed comparable high levels of Shc expression (Fig. 2B). Taken together, these results reveal that Shc is up-regulated in normal CD34<sup>+</sup> cells, as compared with CD34<sup>-</sup> cells, suggesting that its expression declines in differentiated hematopoietic cells. Therefore, the heterogeneous expression of Shc proteins in the CD34<sup>+</sup> and CD34<sup>-</sup> fractions of CML-CP might reflect the expansion of cell populations with different levels of Shc expression. Accordingly, CML-BC cells, which are mainly CD34<sup>+</sup>, expressed high levels of Shc proteins.

**Higher Levels of Shc Phosphorylation in CD34<sup>+</sup> Ph<sup>+</sup> Leukemic Cells versus CD34<sup>+</sup> Normal Bone Marrow Cells.** We next analyzed levels of Shc phosphorylation in the same samples. Analysis of
Fig. 3. Tyrosine phosphorylation of Shc proteins in CML samples. A, left, antiphosphotyrosine blot of cellular lysates (Lysate) and anti-Shc immunoprecipitates (αShc I.P.) from the K562 and TOM-1 CML cell lines and from one CML-BC sample (BC15). Middle, antiphosphotyrosine blot of anti-Shc immunoprecipitates (αShc I.P.) from the indicated cases. UF, unfractionated; Ig, immunoglobulin cross-reactive polypeptides. Right, antiphosphotyrosine blot of cellular lysates (Lysate) from the indicated cases. B, antiphosphotyrosine blot of cellular lysates from the indicated CML-CP, CML-BC PMN and K562 samples. The same blot was used for antiactin probing (bottom).

Fig. 2. Shc expression in CD34⁺ versus CD34⁻ fractions of CML and normal-derived bone marrow. A, high levels of Shc expression in the CD34⁺ fraction of three CML-CP samples and in two CML-BC samples. p66Shc and p52Shc are not expressed in the CD34⁺ fraction of CML-CP samples, where only low levels of p46Shc are detectable. The same blots were used for anti-Shc and antiactin probing, as indicated. B, side-to-side comparison of Shc expression in the CD34⁺ or CD34⁻ fractions from CML-CP (case 4) and normal bone marrow (N.BM#16) samples, and in the unseparated fraction of one CML-BC sample (BC#14). Comparable high levels of Shc expression are visualized in the different CD34⁺ fractions.
the phosphotyrosine content of Shc proteins requires larger amount of cells, to immunopurify Shc polypeptides from cellular lysates. Sufficient biological material was available from two CML-CP and five CML-BC cases. Antiphosphotyrosine blots of cellular lysates from CD34+ and CD34− CML-CP fractions compared with CML-BC, PMN, and K562 samples. The same blot was used for antiaxin probing (bottom). A, antiphosphotyrosine blots of cellular lysates from CD34+ and CD34− fractions derived from normal human bone marrow (N.BM) and compared with CD34+ and CD34− CML-CP and CML-BC. C, anti-Shc (left) and antiphosphotyrosine (right) blots of cellular lysates from G-CSF mobilized CD34+ cells derived from normal human peripheral blood (P.B.), compared with PMN and K562 lysates. To mobilize CD34+ cells, the donors received rhG-CSF (Neupogen, Roche, Milan, Italy) at a dose of 8 μg/Kg body weight s.c. twice daily for at least 5 days.
We then performed antiphosphotyrosine blotting of CD34+ and CD34− fractions of CML-CP (four cases) and side-to-side comparison with CML-BC samples. In line with Shc expression data, we detected consistently higher levels of tyrosine-phosphorylated Shc proteins in the CD34+ fractions from CML-CP cases, as compared with their corresponding CD34− fractions. In one case (CML-CP #3; Fig. 3A), data were confirmed by antiphosphotyrosine blots of anti-Shc immunoprecipitations. Levels of tyrosine-phosphorylated Shc proteins in the CML-CP CD34+ fractions were comparable with those found in the unfractinated CML-BC lysates (Fig. 4A). Shc phosphorylation was then measured in the CD34+ and CD34− fractions from normal bone marrow (three cases). Low or undetectable tyrosine-phosphorylated Shc polypeptides were observed in either of the two fractions (Fig. 4B). To ascertain that the low levels of phosphorylation of Shc proteins in normal CD34+ cells reflected a functional status, we analyzed CD34+ cells exposed, in vivo, to G-CSF. Lysates from CD34+ cells obtained from one patient treated with G-CSF were analyzed by Western blotting using anti-Shc and anti-tyrosine-phosphorylated Shc antibodies. As shown in Fig. 4C, high levels of Shc polypeptides (left) and tyrosine-phosphorylated Shc proteins (right) were found in the G-CSF-stimulated CD34+ cells.

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