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Characterization of a Myeloid Tyrosine Phosphatase, Lyp, and Its Role in the Bcr-Abl Signal Transduction Pathway*

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The Bcr-Abl protein-tyrosine kinase is implicated in the development of chronic myeloid leukemia. The potential role of protein-tyrosine phosphatase in the regulation of Bcr-Abl signaling was explored. First, expression patterns of tyrosine phosphatases in leukemic cell lines were investigated using degenerate primers for reverse transcription-PCR followed by cloning and sequencing of the cDNA. Distinct patterns of distribution of phosphatase were found in erythroid and myeloid leukemic cell lines. Whereas some phosphatases were ubiquitously expressed, others were limited to specific cell types. Surprisingly, a previously cloned "lymphocyte-specific" phosphatase, Lyp, was frequently detected in a number of myeloid cell lines as well as normal granulocytes and monocytes. Lyp was localized to the cytosol, and overexpression of Lyp caused reduction in the phosphorylation levels of multiple proteins in KCL22 chronic myeloid leukemia blast cells including Cbl, Bcr-Abl, Erk1/2, and CrkL. Co-expression of Lyp and Bcr-Abl in Cos-7 cells resulted in decreased levels of Bcr-Abl, Grb2, and Myc. Overexpression of Lyp markedly suppressed anchorage-independent clonal growth of KCL22 cells. Taken together, the data suggest that Lyp may play an antagonistic role in signaling by the Bcr-Abl fusion protein.

In Philadelphia chromosome-positive human chronic myeloid leukemia (CML),[1] malignant transformation is mediated by a constitutively active tyrosine kinase Bcr-Abl (1, 2). The *abl* gene product is a 145-kDa protein encoding a non-receptor tyrosine kinase (3). The protein contains three src homology domains, SH1 with tyrosine kinase function, and SH2 and SH3 involved in protein-protein interaction. The enzymatic activity of Abl can be regulated through protein-binding domains and stimulated by growth factors and DNA damage (4, 5). The Philadelphia chromosome results from a reciprocal translocation of the *abl* on chromosome 9 transposing to chromosome 22 in the break cluster region (*bcr*) gene (6, 7). Interleukin 3-dependent Ba/F3 cells infected with the fusion gene *bcr-abl* become growth factor-independent and tumorigenic in nude mice (8). The aberrant tyrosine phosphorylation levels of Bcr-Abl activate a series of signaling pathways, and a multitude of proteins exhibit a marked increase in their level of phosphorylation including Bcr-Abl itself, CrkL (an adaptor protein), and phosphatidylinositol 3-kinase (9). Cellular Cbl is a 120-kDa cytoplasmic protein that is ubiquitously expressed with high levels in hematopoietic cells. Cbl can be phosphorylated in response to activation by a variety of growth factors including epidermal growth factor, platelet-derived growth factor, erythropoietin, as well as granulocyte-macrophage colony-stimulating factor (10–12). Tyrosine phosphorylation of Cbl is increased in Bcr-Abl transformed cells (13). Interaction between Cbl and CrkL is tyrosine phosphorylation-dependent, and the complex has been implicated in Bcr-Abl mediated transformation (14).

Protein-tyrosine phosphatases (PTPase) counter the activity of tyrosine kinases by removing phosphate groups from proteins that have been phosphorylated on tyrosyl residues. Whereas protein-tyrosine kinases have been intensely studied over the past decade, the significance of PTPase has just started to be recognized (15). PTPases are involved in the regulation of cellular proliferation and differentiation, as well as cell death (16, 17). CD45 is one of the better studied hematopietic PTPases and is a key regulator of lymphocyte functions (18). Other hematopoietic PTPases, such as SHP-1, are involved in cytokine receptor signaling (19). PTPases display high sequence homology in their catalytic domain (20), which allows the identification of PTPase family members by using degenerate primers in RT-PCR amplification. We have employed this method to investigate the expression pattern of PTPase in leukemic cells. Degenerate PTPase primers were designed and used to amplify different PTPases from RNA isolated from leukemic cell lines. PCR products were cloned, and individual clones were identified by DNA sequencing. Specific PTPase expression patterns were obtained for each cell line.

Lyp, a PTPase that was previously shown to be expressed in lymphoid cells (21), was identified in this study as one of the

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[1] The abbreviations used are: CML, chronic myeloid leukemia; PTPase, protein-tyrosine phosphatase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SHIP, Src homology 2 domain containing inositol phosphatase; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

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TABLE I
Distribution of phosphatase expression in myeloid leukemic cell lines

Phosphatase	K562		HL-60		ML-1	
	n[a]	%	*n*	%	*n*	%
CD45	41	29	71	46	43	57
PEST	41	29	17	11	17	22
PTPs + d	26	19	1	1	ND[b]	ND
Lyp	13	9	48	31	9	12
PTPp	7	5	ND	ND	ND	ND
PTPN4	6	4	6	4	ND	ND
PTPN9	4	3	ND	ND	ND	ND
LCPTP	1	1	ND	ND	ND	ND
PTP-1B	1	1	ND	ND	ND	ND
SHP-1	ND	ND	4	3	2	3
PTYPH	ND	ND	2	1	ND	ND
PTPRF	ND	ND	2	1	ND	ND
HPTPa	ND	ND	1	1	1	1
PTPe	ND	ND	1	1	1	1
PTPRO	ND	ND	1	1	ND	ND
PTPN7	ND	ND	ND	ND	2	3
PTPD1	ND	ND	ND	ND	1	1
Total	140	100	155	100	76	100

[a] *n* represents number of clones. PCR products amplified from degenerate PTPase primers were cloned into pBluescript for sequencing. DNA sequences were compared with the NCBI database using the BLAST program.
[b] ND, not detectable.

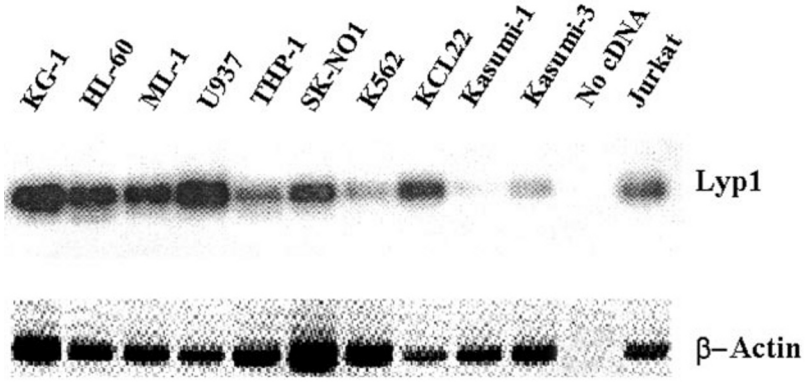

FIG. 1. **Expresssion of Lyp in a panel of leukemic cell lines.** RNA isolated from leukemic cell lines was used for RT-PCR employing β-actin- and Lyp-specific primers. PCR fragments were gel-separated, blotted onto a membrane, and hybridized with either an internal Lyp-specific oligonucleotide or a β-actin cDNA. Detection was performed using the digoxigenin-detection system.

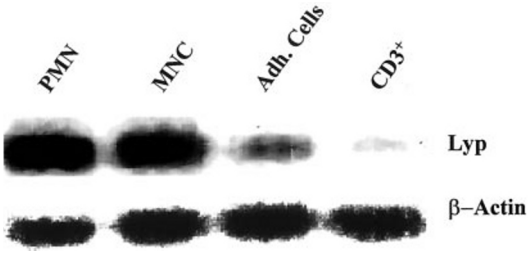

FIG. 2. **Expression of Lyp in peripheral blood white cells.** Subpopulations of human peripheral white blood cells were separated and analyzed by semiquantitative RT-PCR using either β-actin- or Lyp-specific primers. *PMN*, polymorphonuclear neutrophils; *MNC*, peripheral blood mononuclear cells; *Adh. cells*, adherent cells (monocytes); *CD3*$^+$, peripheral blood T-lymphocytes (which express CD3 antigen).

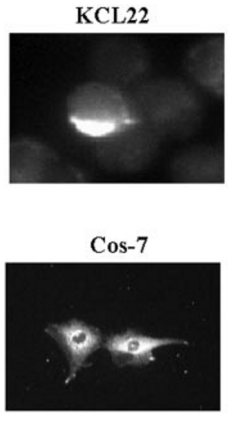

FIG. 3. **Subcellular localization of Lyp in KCL22 and Cos-7 cells.** KCL22 CML and Cos-7 cells were transiently transfected with Lyp-GFP; 48 h later, cells were fixed, and Lyp-GFP protein was detected by green fluorescence microscopy.

major PTPases in myeloid leukemic cells. Lyp was localized to the cytoplasm in the KCL22 CML cell line. Overexpression of Lyp in KCL22 CML cells caused reduction of total cellular phosphorylation levels of proteins. Of particular interest, phosphorylation of Cbl and Bcr-Abl markedly decreased in these cells, and this was associated with markedly decreased levels of Bcr-Abl. Molecules that are substrates of Bcr-Abl, such as CrkL and Erk1/2, also had a decrease in their phosphorylation levels, and amounts of the Grb2 and Myc proteins decreased. Anchorage-independent clonal growth in soft agar markedly decreased in KCL22 cells overexpressing Lyp. Our study suggests a novel mechanism for Bcr-Abl regulation.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and CML Patient Samples—Cell lines were purchased from ATCC (Manassas, VA) except for the following: ML-1 cells were a gift from Dr. M. Kastan (The Johns Hopkins University, Baltimore, MD). Kasumi-1 and Kasumi-3 cells were established by Dr. H. Asou (Hiroshima University, Hiroshima, Japan). Adherent and suspension cells were grown in Dulbecco's modified Eagle's medium and RPMI, respectively, supplemented with 10% fetal bovine serum. Trans-

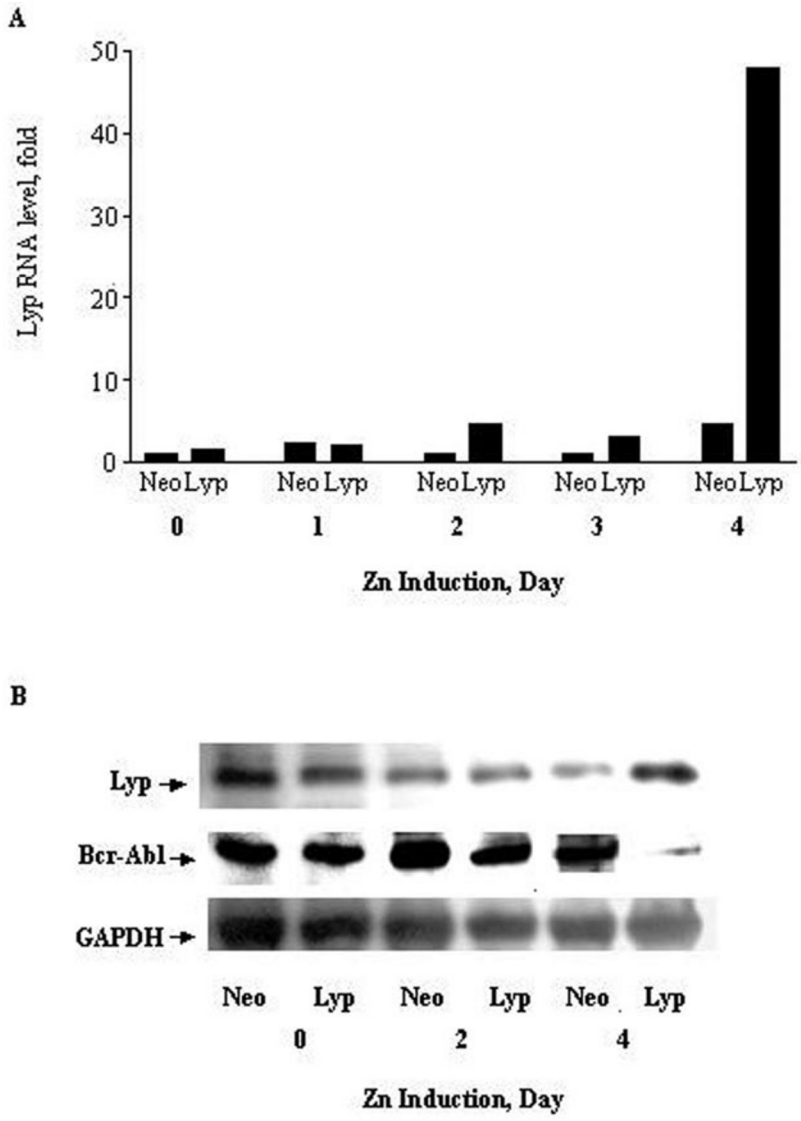

FIG. 4. **Inducible overexpression of Lyp in KCL22 cells.** KCL22 cells were transfected with either pMT-neo or pMT-Lyp expression vector. Single cell clones were selected with G418. As shown in *A*, clones were cultured in the presence of 100 μM $ZnSO_4$ for up to 4 days. Total cellular RNA was prepared after cells were harvested at day 1, 2, 3, and 4 of zinc induction, and Lyp expression was detected by quantitative real-time RT-PCR. The endogenous Lyp expression level in KCL22 is designated as *1*. Other clones were also studied with results similar to those shown on Figs. 6–8. As shown in *B*, protein expression of Lyp and Bcr-Abl was detected by sequential Western blotting with anti-Lyp and anti-Abl antibodies. The anti-Abl antibody recognized the p210 protein of Bcr-Abl. GAPDH antibody was used as loading control. *Neo*, neomycin.

fection of KCL22 cells (5×10^7) was performed by electroporation at 340 V with 20 μg of plasmid in RPMI containing 50% fetal calf serum. Transfection of Cos-7 cells was carried out using LipofectAMINE 2000 (Invitrogen) over 4 h according to the manufacturer's protocol. Proteins and RNA were prepared from the bone marrow of CML patients after their informed consent.

Reverse Transcription and Polymerase Chain Reaction—Two μg of total RNA isolated with TRIzol reagent was reverse-transcribed with Superscript II and random primers according to the manufacturer's protocol (Invitrogen). PCR consisted of 22–30 cycles of denaturation at 95 °C for 1 min, annealing at 62–64 °C for 1 min, and extension at 72 °C for 1 min. With degenerate primers, the annealing cycles were modified by starting the annealing process at 37 °C and heating to 72 °C within 2 min. Primers used are: β-actin-specific, 5′-TACATGGCTGGGGTGTTGAA-3′, 5′-AAGAGAGGCATCCTCACCCT-3′; Lyp-specific, 5′-TGGCCTCCAAGTGGTACCAG-3′, 5′-CATCGGCAAGAAAGAAGGAC-3′. Degenerate primers for PTPase were deduced from amino acid sequences: ACKCCNGCNSWRCARTG (upper strand) and AGYGAYTAYATHAAYGC (lower strand). PCR products amplified from degenerate primers were cloned into pBluescript for sequencing. DNA sequences were compared with the NCBI data base using the BLAST program.

Separation of Peripheral Blood Cells—Polymorphonuclear cells (neutrophils) were isolated from anticoagulated blood using polymorphonuclear neutrophil solution (Robbins Scientific, Sunnyville, CA) according to the one-step density gradient centrifugation method. Briefly, whole blood was layered over polymorphonuclear neutrophil solution and centrifuged for 25 min at 500 × *g*. Mononuclear cells and neutrophils were separated into two distinct bands, whereas erythrocytes pelleted to the bottom of the tube. Neutrophils were obtained from the lower band and were washed twice with serum-free medium. Monocytes were separated from the mononuclear cell fraction by adherence to plastic cell culture dishes. $CD3^+$ cells were isolated from the mononuclear fraction by FACStar flow cytometer (BD Biosciences) using monoclonal murine antibodies against CD3 conjugated to fluorescein isothiocyanate and phosphatidylethanolamine, respectively (Dako, Carpinteria, CA).

Hybridization with an Internal Oligonucleotide—Gel-separated PCR products were blotted onto nylon membrane (Amersham Biosciences) by capillary transfer in 20× SSC. Prehybridization and hybridization were performed at 42 °C in digoxigenin Easy-Hyb solution (Roche Applied Science). The following internal oligonucleotides were used for hybridization probes: Lyp-specific, 5′-AGTCAGCTGTACTAGCAACTGCTC-3′; β-actin-specific: 5′-ATCGAGCACGGCATCGTCAC-3′. These were end-labeled with digoxigenin using the digoxigenin 3′ end labeling kit (Roche Applied Science). Signal was detected by incubating with chemiluminescent substrate CDP-Star (Tropix, Bedford, MA) followed by autoradiography.

Fluorescent Microscopy—Cells were transfected with Lyp-green fluorescent protein (GFP), fixed at 24 h post-transfection with 2% neutral buffered formaldehyde (2% formaldehyde, 20 mM $NaPO_4$, pH 7.4) in Hanks' balanced salt solution for 15 min at 37 °C, washed with phosphate-buffered saline three times, and examined under a fluorescent microscope. The fluorescence data that are shown (see Fig. 3) are representative of multiple transfection experiments.

Real-time Quantitative RT-PCR—Isolated RNA was reverse-transcribed as described above. Real-time PCR was performed on iCycler thermal cycler (Bio-Rad). PCR reaction contained a total volume of 25 μl and consisted of 5 μl of cDNA, 500 nM of either Lyp or β-actin-specific primer pairs, HotStart *Taq*DNA polymerase (Qiagen, Valencia, CA), 0.25 mM dNTPs, and 1 μl of 1:60,000 SYBRgreen I ® (Molecular Probes,

Eugene, OR). Triplicate samples were analyzed for each cDNA. Expression of Lyp was normalized to β-actin expression.

Western Blot Analysis—Cells were lysed with immunoprecipitation buffer (50 mM Tris, pH 7.6, 5 mM EDTA, 300 mM NaCl, 1 mM dithiothreitol, and 0.1% Nonidet P-40) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, pepstatin, and aprotinin). For immunoprecipitation, 1 mg of lysate was incubated with anti-*abl* antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 2 h and binding with protein A/G-Sepharose (Calbiochem) at 4 °C for 1 h. The beads were washed with lysis buffer and resuspended in the sample buffer before loading onto a 10% SDS-polyacrylamide gel. For blotting, various antibodies were used followed by ECL detection (Amersham Biosciences).

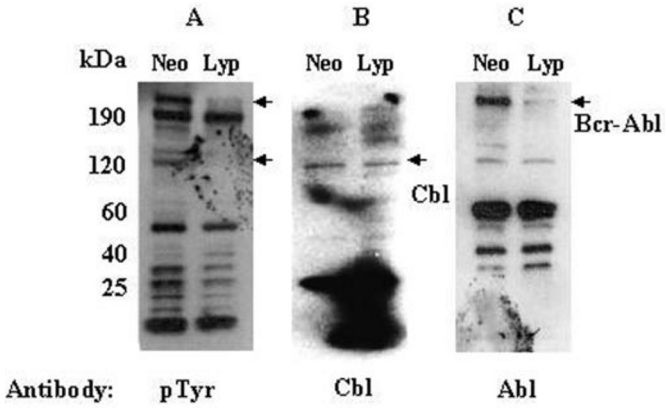

FIG. 5. **Reduction of tyrosine phosphorylation of proteins in KCL22 cells which overexpressed Lyp.** Total proteins were prepared from KCL22 CML cells after 4 days of exposure to 100 μM $ZnSO_4$. Lysates were analyzed by SDS-PAGE followed by sequential immunoblotting with antibody against phosphotyrosine (pTyr), Cbl, and Abl and then visualized by ECL. *Neo*, neomycin.

Soft Agar Colony Assays—Equal numbers of cells (2,000 cells) were plated in 0.3% agar containing 20% fetal bovine serum and RPMI on top of 0.5% agar in 24-well plates. Colony formation was counted and photographed after 2 weeks of culture. Data represent results of triplicate dishes.

RESULTS

Distribution of Protein-tyrosine Phosphatase Expression in Myeloid Leukemic Cell Lines—The distribution of PTPase in myeloid cells was assessed by RT-PCR using degenerate PTPase primers and cloning of individual protein-tyrosine phosphatases. Over 371 individual clones were sequenced from three myeloid cell lines, K562, HL-60, and ML-1. The analysis of a large number of PTPase-containing clones should provide a good reflection of the expression pattern of PTPase in human myeloid leukemic cells and may provide the opportunity to identify a unique PTPase. The K562 cells are erythroleukemic cells, the ML-1 cells are early myeloblasts, and the HL-60 cells are late myeloblasts. CD45 was the most prominently expressed PTPase, representing 29, 46, and 57% of the clones isolated from K562, HL-60, and ML-1, respectively (Table I). In K562 cells, both CD45 and the PTPase PEST were equally abundant with both being expressed in the same number of clones (41 of a total of 140). PEST was the second most prominent PTPase in ML-1 cells (22%). In total, between 8 and 12 different PTPases were identified per cell line with HL-60 myeloblasts showing the greatest diversity in expression (12 different PTPases). Each cell line displayed a distinct set of PTPases. PTPπ, PTPN9, LCPTP, and PTP-1B could only be identified in K562 cells. PTYPH, PTPRF, and PTPRO were only found in HL-60 cells. PTPN7 and PTPD1 were only identified in ML-1 cells, whereas CD45, PEST, and Lyp were present in all three cell lines.

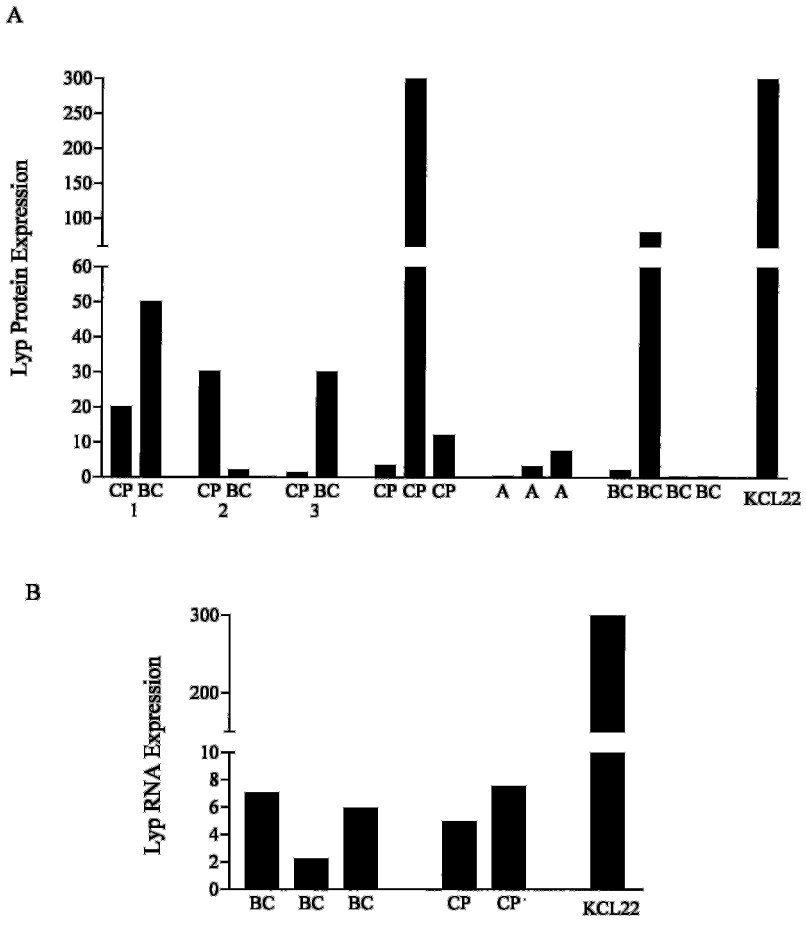

FIG. 6. **Expression of Lyp in CML patient samples.** *A*, Western blot analysis of Lyp in CML samples. Lysates were prepared from bone marrow samples of CML patients, and Western analyses were performed to detect Lyp expression. *CP*, chronic phase; *A*, accelerated phase; *BC*, blast crisis phase of CML. Expression of Lyp protein levels on Western blots were quantitated by densitometry and normalized to GAPDH expression from the same blots. Samples number *1*, *2*, and *3* designate paired specimens from the same patient at different stages of CML. *B*, real-time PCR analysis on RNA prepared from CML samples. Lyp expression was normalized to levels of β-actin.

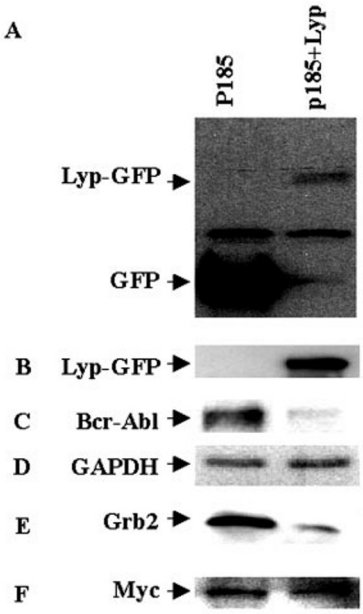

FIG. 7. **Dephosphorylation and degradation of Bcr-Abl caused by expression of Lyp, associated with decreased levels of Grb2 and Myc.** Cos-7 cells were co-transfected with Bcr-Abl (p185) cDNA and either the GFP or Lyp-GFP expression vector. Total lysates were prepared 48 h after transfection. In *A*, antibodies against GFP were used to demonstrate expression of either GFP or Lyp-GFP. In *B*, anti-Lyp antiserum was used to detect Lyp expression. In *D*, total lysates were quantitated with anti-GAPDH to ensure equal amounts of protein in each lane. In *C, E*, and *F*, antibodies against Abl (for Bcr-Abl detection), Grb2, and Myc, respectively, were used separately to detect the expression of the protein.

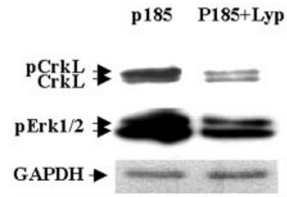

FIG. 8. **Decrease in the phosphorylation levels of CrkL and Erk1/2 after overexpression of Lyp.** Phosphorylation levels of the CrkL and Erk1/2 proteins were examined sequentially using anti-CrkL and anti-phosphorylated Erk1/2 antibodies. The anti-CrkL antibody recognizes both phosphorylated and non-phosphorylated CrkL. The results as shown used Cos7 cells; similar results were obtained using KCL22 cells (data not shown).

Expression Pattern of Lyp—Lyp has recently been described as a lymphoid-specific PTPase (21); thus, we were surprised to find it to be one of the more frequently expressed PTPases in the three myeloid cell lines used in this study (Table I, Fig. 1). Lyp represented 30% of the PTPase-containing clones in HL-60 cells and 10–12% of clones in K562 and ML-1 cells. Therefore, we studied Lyp expression in a panel of human leukemic cell lines as well as in normal peripheral blood lymphocytes using semiquantitative RT-PCR with Lyp-specific primers. In the myeloid cell lines, we observed high levels of Lyp mRNA in KG-1 (very early myeloblasts), HL-60 and ML-1 (myeloblasts), U937 (myelomonoblasts), as well as SK-NO1 and KCL22 (CML blasts) (Fig. 1). The expression was comparable with that seen in the Jurkat T-cell line (Fig. 1). In contrast, Lyp was less robustly expressed in THP-1 (monoblasts), K562 (erythroblasts), Kasumi-1 (mature myeloblasts expressing the AML1-ETO fusion protein), and Kasumi-3 (very early myeloblasts) cells (Fig. 1). We also analyzed a series of colon cancer cell lines and found very low or undetectectable expression of Lyp (data not shown). Next, we isolated different types of normal human peripheral white blood cells to analyze Lyp expression. We found that Lyp was expressed at a higher level in neutrophils and monocytes as compared with $CD3^+$ T lymphocytes (Fig. 2).

The subcellular localization of the Lyp phosphatase was determined. The Cos-7 and KCL22 CML myeloid blast cells were transiently transfected with an expression vector of Lyp fused to GFP and examined by fluorescence microscopy 48 h after transfection. We found that Lyp was predominantly cytoplasmic (Fig. 3).

Role of Lyp in Myeloid Cells—The Lyp phosphatase has previously been shown to interact with and regulate the tyrosine phosphorylation of Cbl in T lymphocytes (21). Cbl itself has been demonstrated to be a target for tyrosine phosphorylation by the Bcr-Abl tyrosine kinase (22). Thus, we were interested to determine whether Lyp might have a role in Bcr-Abl-expressing CML cells, such as KCL22. Human Lyp cDNA was inserted into a Zn^{2+}-inducible vector, and the resulting Lyp expression vector (pMT-Lyp) and the empty vector (pMT-neo) were transfected separately into KCL22 cells. Stable cell lines were isolated by G418 selection. The induction of Lyp was measured by quantitative real-time PCR for up to 4 days of exposure to $ZnSO_4$. We found a 3-fold induction of Lyp mRNA when comparing KCL22 stably transfected with pMT-Lyp to the empty pMT vector alone after 2 and 3 days of zinc induction (Fig. 4*A*). Only after 4 days in the presence of zinc did we observe more than 8–10-fold of Lyp mRNA induction. Total cellular proteins were isolated from 2 and 4 days of zinc induction, and the total cell lysates were analyzed for Lyp protein expression by Western immunoblotting (Fig. 4*B*). Because Bcr-Abl is a major protein involved in the transforming phenotype of KCL22, we looked at the Bcr-Abl protein levels in these Lyp-overexpressing cells. We found that after 4 days of zinc exposure, Bcr-Abl protein level is decreased by more than 10-fold (Fig. 4*B*).

Since Lyp dephosphorylates tyrosine residues, we first probed a Western blot from the protein lysates with an anti-phosphotyrosine antibody to determine whether overexpression of Lyp affected the overall level of tyrosine phosphorylation as compared with the control cells. We observed a decrease in the level of protein tyrosine phosphorylation upon Lyp overexpression (Fig. 5*A*). Of particular interest was the marked reduction of tyrosine phosphorylation for proteins of 120 and 210 kDa (Fig. 5*A*). We proposed that these proteins were most likely Cbl and Bcr-Abl, respectively, based on their molecular sizes. Therefore, the Western immunoblot was reprobed with antibodies against these two proteins, which confirmed that the proteins at 120 and 210 kDa were indeed Cbl and Bcr-Abl (Fig. 5, *B* and *C*). A comparison of the Lyp-overexpressing KCL22 *versus* control KCL22 cells showed that the overall amount of Cbl protein was unaffected by the induction of Lyp (Fig. 5*B*). However, when the immunoblot was reprobed with an antibody against Abl, we observed that overexpression of Lyp resulted in a decrease in Bcr-Abl protein expression (Fig. 5*C*), similar to what was observed above (Fig. 4*B*).

We also examined the expression of Lyp protein in the leukemic cells from patients with CML. CML has a chronic phase, a short accelerated phase, evolving into the short-lived blast crisis. Lyp was expressed in these samples at various levels, and no correlation was noted between the amount of expression of Lyp protein and stage of the CML (Fig. 6*A*). Similar observations were made when looking at Lyp RNA levels in CML patients by real-time PCR, although the number of cases was small (Fig. 6*B*).

To investigate further the role of Lyp in the regulation of Bcr-Abl, we transiently co-transfected Cos-7 cells with an expression vector for Bcr-Abl and either Lyp-GFP or GFP alone. Total cellular proteins were isolated and analyzed by Western

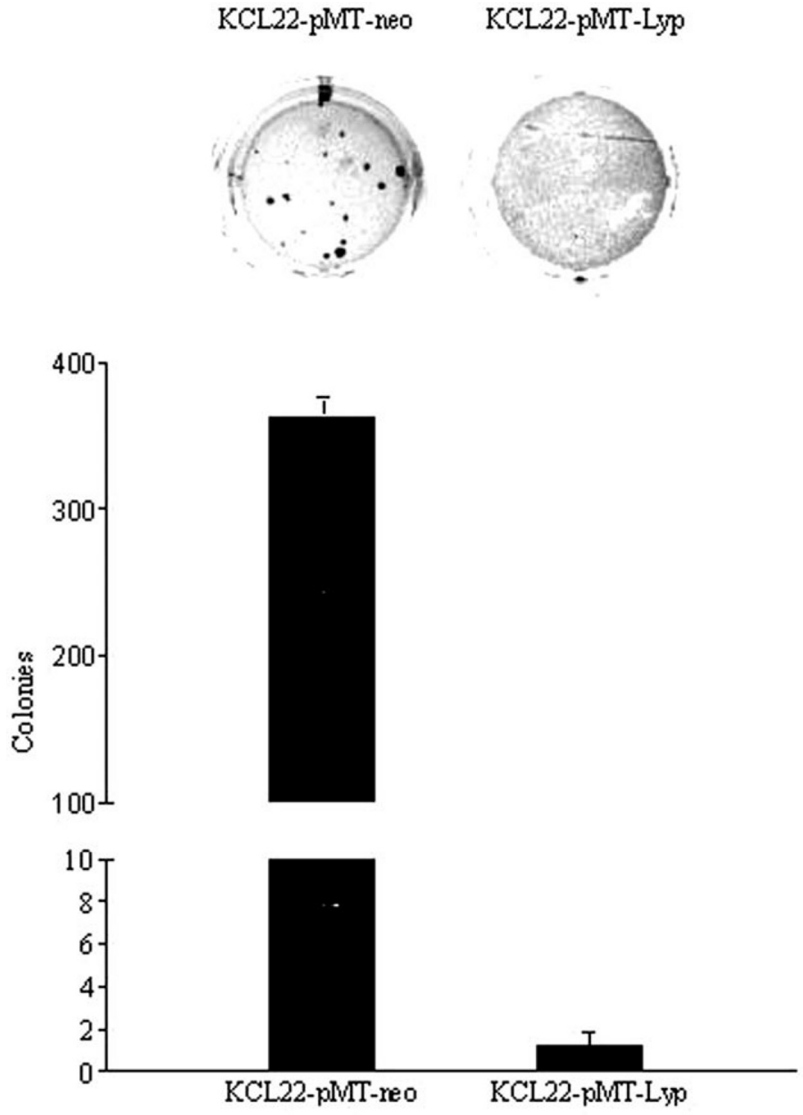

FIG. 9. **Overexpression of Lyp inhibits transforming potential of KCL22 CML cells in soft agar.** An equal number of either KCL22-pMT-Lyp or KCL22-pMT-neo were seeded in 1% agar containing 10% fetal bovine serum, RPMI culture media, and 100 μM $ZnSO_4$ on top of 1.5% agar. Colony formation was counted, and dishes were photographed 2 weeks after plating the cells. Colonies containing ≥100 cells were enumerated. Data represent the mean ± S.D. of triplicate plates.

immunoblots. The use of an anti-GFP antibody and Lyp antiserum demonstrated the expression of the Lyp phosphatase in these cells co-transfected with Lyp-GFP and Bcr-Abl (Fig. 7, *A* and *B*). Negligible Bcr-Abl could be detected from the Cos-7 cells co-transfected with Bcr-Abl and the Lyp-GFP (Fig. 7*C*). GAPDH immunoblotting demonstrated that equal amounts of protein lysates were used (Fig. 7*D*). These results suggest that the expression of Lyp causes a decrease in Bcr-Abl protein expression, presumably via regulating its degradation.

Activation of the Ras signaling pathway by Bcr-Abl involves a series of molecular events including direct interaction between the adaptor protein Grb2 and the phosphorylated Bcr-Abl (23), induction of Myc protein (24), and phosphorylation of CrkL and Erk1/2. We found that when Lyp was expressed, levels of Bcr-Abl and Grb2 markedly decreased, and a slight reduction of Myc protein levels was observed (Fig. 7*E*). Also, a prominent decrease of CrkL and Erk1/2 phosphorylation occurred in these Lyp-expressing cells (Fig. 8).

The effect of Lyp overexpression on anchorage-independent clonal growth in soft agar of KCL22 CML cells was investigated. KCL22 pMT-neo formed robust colonies in soft agar (Fig. 9). In comparison, KCL22 pMT-Lyp cells had markedly decreased clonal growth, both in number and size of the colonies, suggesting that overexpression of Lyp inhibited the transforming potential of KCL22 CML cells (Fig. 9).

DISCUSSION

In this study, we identified a number of PTPases in three human myeloid leukemic cell lines. Of these, CD45, PEST, and Lyp were the most prevalent. CD45, also known as the leukocyte common antigen, is an integral membrane protein expressed on all nucleated hematopoietic cells (25, 26). It regulates the activity of the Src family of kinases (27). Since CD45 has been shown to be expressed on all hematopoietic lineages and stages of development, we were not surprised that it was the predominant PTPase detected in our study. PEST, a major PTPase identified in K562 cells, has been described as an important regulator of cell migration (28) and has been suggested to have a role in leukemia through an interaction with paxillin (29). The third major PTPase detected in the myeloid cells was Lyp. Previously, Lyp was identified as a lymphocyte-specific PTPase (21). We found that Lyp represented 31% of the 155 clones obtained from the late myeloblast HL-60 cells, and a substantial amount of Lyp expression was found in several myeloid cell lines including myelomonoblastic U937 cells and the very early myeloblastic KG-1 cells. Expression of Lyp can also be found in peripheral blood granulocytes and monocytes, suggesting a potential function of Lyp in myeloid cell development.

Lyp has cytoplasmic expression and some perinuclear stain-

ing in Jurkat cells (21). We showed in KCL22 CML cells that Lyp is localized to the cytoplasm, where it may interact with Bcr-Abl and play a role in regulating the signaling of this transforming fusion protein. We found that overexpression of Lyp resulted in a decrease in the expression of Bcr-Abl as well as a reduction in the phosphorylation levels of Cbl. The decrease in Cbl tyrosine phosphorylation is probably the result of Lyp phosphatase activity independent of Bcr-Abl function since Cbl has been shown to be a target of Lyp activity in T cells not containing Bcr-Abl (21). In T cells, Lyp regulates T cell receptor signaling by associating with and dephosphorylating Cbl. Alternatively, Cbl is a substrate of Bcr-Abl tyrosine kinase, and down-regulation of Bcr-Abl would cause a reduction in the level of Cbl phosphorylation.

CML is caused in part by activation of various signaling pathways by the aberrant tyrosine kinase activity of the Bcr-Abl fusion protein. This active tyrosine kinase phosphorylates a number of signaling proteins, including STAT5, SHC, Cbl, Grb2, paxillin, and CrkL. The interaction of Bcr-Abl with Cbl probably causes activation of the phosphatidylinositol 3-kinase pathway (14), and interaction between Bcr-Abl with Grb2 can lead to the activation of Ras signaling. Stimulation of both of these pathways is important for Bcr-Abl-induced transformation. Either a dominant-negative form of Grb2 (30) or Bcr-Abl with mutation in the Grb2-binding SH2 domain can suppress Bcr-Abl transformation (31) and leukemogenesis (32). More recently, Lyp has also been shown to interact with the adaptor molecule Grb2 (33). In this instance, wild-type Lyp, but not a catalytically inactive Lyp, had a negative regulatory role in T cell signaling via regulation of interactions of Grb2 with the T cell receptor.

In KCL22 CML cells, lower Grb2 expression levels were found when Lyp was overexpressed. The SH3 domain of Grb2 interacts with Sos (the guanine nucleotide releasing factor son of sevenless) and stimulates Ras and the MAPK kinase pathways. Inhibition of CML blast cell proliferation can be induced by disruption of Grb2-Sos complexes (34). Potentially, the degradation of Grb2 can be a direct effect of overexpression of Lyp, and disruption of the Grb2-Sos complex leads to inactivation of Erk1/2 and consequently contributes to the loss of the transforming potential of KCL22 cells in the soft agar assay. Decreased phosphorylation of CrkL may partially contribute to the loss of transforming potential of Lyp-overexpressing cells. Multiple tyrosine residues are phosphorylated in CrkL when activated by Bcr-Abl, and mutations or deletions in CrkL diminish cell transformation and adhesion in both fibroblasts and hematopoietic cells (35).

To date, a major focus of basic CML research has focused on the aberrant tyrosine kinase activity of Bcr-Abl and the consequences of tyrosine phosphorylation. However, intracellular levels of tyrosine phosphorylation are controlled by the opposing actions of kinases and phosphatases. Bcr-Abl can interact with and regulate phosphatases. In some instances, Bcr-Abl phosphorylates these phosphatases, and subsequently, these phosphatases can dephosphorylate Bcr-Abl, resulting in a decrease in Bcr-Abl kinase activity (36). For example, Bcr-Abl up-regulates the expression of PTP-1B PTPase, which in turn dephosphorylates Bcr-Abl, resulting in the inhibition of Grb2 binding and suppression of Ras activity (37). Bcr-Abl has been shown to interact with and to regulate the expression of SHIP, an SH2-containing inositol phosphatase that regulates the phosphatidylinositol 3-kinase signaling pathway (38). Bcr-Abl can directly inhibit the expression of SHIP phosphatase by down-regulating the amount of mRNA as well as decreasing the half-life of the protein (39). Since SHIP down-regulates the activity of the phosphatidylinositol 3-kinase signaling pathway, direct control of this negative regulator by Bcr-Abl can result in increased myeloid proliferation, as was observed in SHIP−/− mice (40). In previous studies that showed an interaction of a phosphatase such as PTP1B with Bcr-Abl, the Bcr-Abl kinase activity decreased, but no change occurred in the overall expression of the fusion protein (36, 37). Our study is, to our knowledge, the first description of a phosphatase regulating Bcr-Abl signaling via alteration of the levels of Bcr-Abl protein. The mechanism by which this occurs has yet to be determined. We attempted experiments to determine whether Bcr-Abl was ubiquitinated by Lyp. The results of these experiments showed that Bcr-Abl was not ubiquitinated by co-expression of Lyp (data not shown). In K562 cells, the molecular chaperone Hsp90 forms a complex with Bcr-Abl, extending the half-life of Bcr-Abl (41). By interfering with the function of Hsp90, geldanamycin or its analog 17-allyamnogeldanamycin can induce Bcr-Abl protein degradation (41). Perhaps overexpression of Lyp disrupts the association between the Hsp90 and Bcr-Abl complex and promotes Bcr-Abl degradation.

In conclusion, we showed that Lyp is expressed in the myeloid cell lineage, and Lyp overexpression in KCL22 cells induces Bcr-Abl and Grb2 degradation, as well as dephosphorylation of Cbl, CrkL, and Erk1/2. Overexpression of Lyp inhibits transforming potential of KCL22 cells. Our data suggest that Lyp may behave as a tumor suppressor.

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