Effects of a Leukemia-associated Gain-of-Function Mutation of SHP-2 Phosphatase on Interleukin-3 Signaling*

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Mutations in SHP-2 phosphatase that cause hyperactivation of its catalytic activity have been identified in human leukemias, particularly juvenile myelomonocytic leukemia, which is characterized by hypersensitivity of myeloid progenitor cells to granulocyte macrophage colony-stimulating factor and interleukin (IL)-3. However, the molecular mechanisms by which gain-of-function (GOF) mutations of SHP-2 induce hematopoietic malignancies are not fully understood. Our previous studies have shown that SHP-2 plays an essential role in IL-3 signal transduction in both catalytic-dependent and -independent manners and that overexpression (5–6-fold) of wild type (WT) SHP-2 attenuates IL-3-mediated hematopoietic cell function through accelerated dephosphorylation of STAT5. These results raised the possibility that SHP-2-associated leukemias are not solely attributed to the increased catalytic activity of GOF mutant SHP-2. GOF mutant SHP-2 must have gained additional capacities. To test this possibility, we investigated effects of a GOF mutation of SHP-2 (SHP-2 E76K) on hematopoietic cell function and IL-3 signal transduction by comparing with those of overexpressed WT SHP-2. Our results showed that SHP-2 E76K mutation caused myeloproliferative disease in mice, while overexpression of WT SHP-2 decreased hematopoietic potential of the transduced cells in recipient animals. The E76K mutation in the N-terminal Src homology 2 domain increased interactions of mutant SHP-2 with Grb2, Gab2, and p85, leading to hyperactivation of IL-3-induced Erk and phosphatidylinositol 3-kinase (PI3K) pathways. In addition, despite the substantial increase in the catalytic activity, dephosphorylation of STAT5 by SHP-2 E76K was dampened. Furthermore, catalytically inactive SHP-2 E76K with an additional C459S mutation retained the capability to increase the interaction with Gab2 and to enhance the activation of the PI3K pathway. Taken together, these studies suggest that in addition to the elevated catalytic activity, fundamental changes in physical and functional interactions between GOF mutant SHP-2 and signaling partners also play an important role in SHP-2-related leukemogenesis.

Hematopoietic cell fate is tightly controlled by environmental cues, such as growth factors, cytokines, and extracellular matrix, which exert their functions by activation of intracellular signaling mechanisms. Therefore, intracellular signaling processes play an important role in the determination of hematopoietic cell function. Dysregulation of signal transduction in hematopoietic cells by mutations in cell surface receptors or intracellular signaling components results in malfunction of hematopoietic cells, leading to blood disorders including leukemias. For instance, juvenile myelomonocytic leukemia (JMML), a clonal myeloproliferative disease characterized by overproduction of myeloid lineage cells, is thought to result from increased Ras signaling (1, 2). Activating mutations in the Ras gene or homozygous inactivation of the neurofibromatosis type 1 (NF1) gene, whose product, neurofibromin 1, is a Ras-GTPase-activating protein (Ras-GAP), have been identified in 50% of JMML (3). Due to activating Ras mutations or inactivation of NF1 mutations, hematopoietic progenitor cells in JMML are hypersensitive to granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3 (4, 5).

GM-CSF, IL-3, and IL-5 are a small family of hematopoietic growth factors that induce intracellular signal transduction mainly through the common receptor β chains (6, 7). They regulate hematopoietic cell survival, proliferation, and differentiation via shared intracellular signaling pathways. Although GM-CSF-, IL-3-, and IL-5-triggered signal transduction has been extensively investigated, the precise regulation of their signaling mechanisms is still not well understood. IL-3 exerts its function through binding to two distinct high affinity receptors composed of a ligand-specific α subunit and one of two β subunits: a ligand-specific β chain (Aic2A) or a common β chain (Aic2B). Upon binding by ligands, IL-3 receptors heterodimerize and become quickly tyrosyl phosphorylated. Activated receptors, in particular, the common receptor β chains, then trigger a signal relay to the targets in the nucleus to induce cellular responses through shared Jak-STAT, Ras-Raf-MAP, PI3K, and Src kinase pathways (6, 7). Upon receptor engagement by IL-3, non-receptor tyrosine kinases such as Jak2 and Src family members are activated. Activated Jak2 and/or Src family kinases in turn phosphorylate the receptor β chains on multiple tyrosine residues, which thereafter serve as docking sites for other Src homology (SH) 2 or phosphotyrosine binding domain-containing signaling molecules, such as the Shc adapter protein or SHP-2 phosphatase, to couple downstream signaling pathways (8–10). Jak2 has been demonstrated to play a central role in receptor β chain signaling. Deficiency of Jak2 abrogates physiological and biochemical responses to IL-3 or GM-CSF in hematopoietic cells (11, 12). However, detailed signaling mechanisms downstream of the common receptor β chain and exact components involved in the signaling processes are still not fully characterized.

SHP-2, a ubiquitously expressed SH2 domain-containing tyrosine phosphatase, has been implicated in diverse signaling pathways induced by a number of stimuli including growth factors, cytokines, extracellular...
matrix, and even cellular stress (13–15). In many cases, especially in receptor tyrosine kinase-initiated intracellular signaling, SHP-2 enhances signaling at the transcriptional level. SHP-2 is highly expressed in hematopoietic cells. Our previous studies have shown that SHP-2 plays a critical role in hematopoietic cell development and function (16–18) and that SHP-2 is indispensable in IL-3-mediated hematopoietic cell activities (19, 20). SHP-2 appears to act at multiple sites in IL-3 signaling pathways, functioning in both catalytically dependent and independent manners. While it promotes Erk and PI3K pathways and enhances Jak2 activation (19, 20), SHP-2 does have a negative role in hematopoietic cell survival (20). Overexpression (5–6 fold increase) of wild type (WT) SHP-2 enhances growth factor deprivation-induced apoptosis and compromises hematopoietic cell function in vitro, and this is achieved by direct dephosphorylation of STAT5 (20). Recently, genetic lesions in SHP-2 that cause hyperactivation of its catalytic activity have emerged as major genetic events underlying the developmental disorder Noonan syndrome and JMML. Fifty percent of Noonan syndrome patients and 35% of JMML cases carry gain-of-function (GOF) mutations in SHP-2 (21–24). The SHP-2 mutations appear to play a causal role in the development of these diseases. SHP-2 mutations and other JMML-associated Ras or NF1 mutations are mutually exclusive in leukemic patients. Moreover, a single SHP-2 GOF mutation (D61G) does induce myeloproliferative disease and Noonan syndrome in mice (25). These new findings underscore the importance of the role of SHP-2 in cellular processes, particularly hematopoietic cell development. However, the molecular mechanisms of JMML induced by SHP-2 GOF mutations and detailed signaling activities of SHP-2 GOF mutants in hematopoietic cells are not well characterized.

The hallmark of JMML is hypersensitivity of myeloid progenitor cells to GM-CSF and IL-3 (4, 5). Since our previous studies showed that excessive WT SHP-2 in hematopoietic cells attenuated IL-3-induced cellular responses (20), we reasoned that SHP-2-associated JMML was not solely attributed to the increased phosphatase activity of mutant SHP-2. GOF mutant SHP-2 must have gained other capacities. To test this hypothesis and to gain more insight into the pathogenesis of SHP-2-associated leukemias, we investigated the consequences of SHP-2 E76K, the most frequent SHP-2 GOF mutation seen in JMML, on IL-3 signal transduction by comparing signaling activities of SHP-2 E76K to those of overexpressed WT SHP-2. Our results have suggested that profound changes in physical and functional interactions between GOF mutant SHP-2 and its signaling partners also play an important role in the pathogenesis of SHP-2-related JMML.

**Experimental Procedures**

**Mice, Cell Line, and Reagents**—WT C57BL/6 and congenic HW80 mice were purchased from the Jackson Laboratory (Bar Harbor, MN). All animal procedures complied with the National Institutes of Health Guideline for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Ba/F3, an IL-3-dependent murine hematopoietic cell line, was maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 10% conditioned medium containing mouse IL-3 (19). Anti-SHP-2, anti-STAT5a, anti-STAT5b, anti-Grb2, anti-Sos, anti-Erk, anti-phospho-Erk, anti-phospho-Jnk1, and anti-Jnk1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (PY) (4G10), anti-phospho-Jak2, anti-phospho-JNK, and anti-Grb2 Abs were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phospho-p38, anti-phospho-Akt, and anti-Akt Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Ly5.1 Ab was provided by BD Biosciences. AG490, PP2, LFM-A13, and piceatannol were supplied by Calbiochem. BrdUrd- and fluorescein isothiocyanate-conjugated anti-BrdUrd Ab were purchased from Roche Applied Science.

**Generation of SHP-2 E76K Retroviral Producer Cell Line and Transduction of Primary Bone Marrow Hematopoietic Stem/Progenitor Cells**—SHP-2 E76K mutation was generated by PCR-based mutagenesis. The mutation was confirmed by cDNA sequencing. SHP-2 E76K cDNA was cloned into the MSCV-IRESP-GFP retroviral vector (26) containing an internal ribosomal entry sequence (IRES) driving expression of a downstream green fluorescence protein (GFP) gene to facilitate tracking of transduced cells. Ecotropic GP+E86-based retroviral producer cell lines were generated by transduction with retroviral supernatant produced by 293T cells that were transiently co-transfected with pQEPAM3 (Minus E) packaging plasmid, pSHoG (vesicular stomatitis virus glycoprotein) envelope plasmid, and the recombinant SHP-2 E76K retroviral plasmid. To transduce primary hematopoietic stem/progenitor cells with SHP-2 E76K, nucleated bone marrow cells harvested from femurs of 4-week-old mice were prestimulated in RPMI 1640 medium containing 10% FBS, SCF (50 ng/ml), IL-3 (20 ng/ml), and IL-6 (50 ng/ml) for 2 days and then co-cultured with irradiated (1500 cGy) retroviral producer cells in the presence of polybrene (6 μg/ml) for 48 h.

**Hematopoietic Progenitor Assay**—Following retroviral-mediated gene transfer, transduction efficiencies of bone marrow cells were examined by fluorescence-activated cell sorting (FACS) based on expression of GFP. Transduced bone marrow cells (5 × 10^6 cells/ml) were then assayed for colony forming units in 0.9% methylcellulose Iscove’s modified Dulbecco’s medium containing 30% FBS, glutamine (10^{-4} μl), β-mercaptoethanol (3.3 × 10^{-5} μl), and GM-CSF or IL-3 at the indicated concentrations. After 7 days of culture at 37 °C in a humidified 5% CO2 incubator, GFP-positive hematopoietic cell colonies were counted under an inverted fluorescence microscope. Colony-forming efficiencies were determined based on numbers of GFP-positive colonies and transduction efficiencies of the starting cells.

**Cell Cycle Analysis**—Cell cycle analysis was performed as previously described (27) with minor modifications. Exponentially growing cells (2 × 10^6) were synchronized by serum starvation for 24 h and then cultured in serum-free IL-3-containing (1 ng/ml) RPMI medium for 24 h. Cells were pulsed with BrdUrd (10 μM) for 45 min, washed twice in cold phosphate-buffered saline (PBS), and fixed in 75% ethanol at −20 °C overnight. After cells were suspended in 2 N HCl containing 0.5% Tween 20 at room temperature for 30 min, cells were washed twice in PBS (pH 7.4) to restore physiological pH and then resuspended with PBS containing 1% bovine serum albumin and 0.5% Tween 20. Fluorescein isothiocyanate-conjugated anti-BrdUrd monoclonal Ab (1 μg/ml) was added and incubated at room temperature for 30 min. Cells were washed twice in PBS, stained with propidium iodide (PI) (50 μg/ml) dissolved in PBS in the presence of 100 μg/ml of RNase A at room temperature for 30 min, and then analyzed by FACS using BD-LSR flow cytometry (BD Biosciences).

**Immunoprecipitation and Immunoblotting**—Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.025% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 2 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 1 mM phenylmethylsulfonyl fluoride). Whole cell lysates (500 μg) were immunoprecipitated with 1–2 μg of purified Abs or 2 μl of antisera Abs. Immunoprecipitates were washed three times with HNTG buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 1% glycerol, 0.1% Triton X-100, and 1 mM Na3VO4) and resolved by SDS-PAGE followed by immunoblotting with the indicated Abs.

**Hematopoietic Cell Transplantation**—Bone marrow cells freshly harvested from C57BL/6 mice were transduced with SHP-2 E76K, WT
SHP-2, or catalytically inactive SHP-2 C459S by using the retroviral producer cell lines as described above. Transduced cells were harvested and examined for transduction efficiencies by FACS based on GFP expression. Transduced cells (1–2 × 10^6 cells) were injected through lateral tail veins into congenic HW80 or isogenic C57BL/6 recipient mice that had been irradiated with 1100 rads. Animals were bled 3 months after transplantation for hematology analyses.

RESULTS

Our previous studies have shown that although SHP-2 phosphatase plays an overall positive role in hematopoietic cell development and function (13–15), overexpression (5–6-fold) of WT SHP-2 enhances growth factor deprivation-induced apoptosis in hematopoietic cells and decreases cellular responses to IL-3-induced proliferation and differentiation in bone marrow progenitor cells (20). To confirm that overexpression of SHP-2 attenuates hematopoietic activity, we transduced bone marrow hematopoietic cells with WT SHP-2 as well as catalytically inactive SHP-2 C459S and then examined hematopoietic potential of the transduced cells in recipient animals. The bone marrow cell transduction efficiencies of WT SHP-2, SHP-2 C459S, and control vector were 28%, 25%, and 30%, respectively. Whole cell populations containing transduced and non-transduced cells were directly used for transplantation. As the catalytic activity of SHP-2 plays an important role in hematopoietic growth factor-induced activation of Erk kinase and Jak2 kinase (19), inhibition of the catalytic activity of endogenous SHP-2 by overexpression of catalytically inactive SHP-2 C459S decreased hematopoietic potential of the transduced progenitor cells. Three months after transplantation, compared with the vector control, contribution of SHP-2 C459S-transduced cells to nucleated cells in peripheral blood of the recipient mice was dramatically decreased (Fig. 1A), even though the hematopoietic system of the recipients was well reconstituted by donor cells (including both SHP-2 C459S-transduced and non-transduced cells), as evidenced by high percentages (>95%) of donor-derived progeny cells (Ly5.1-positive) in peripheral blood (Fig. 1B). Interestingly,
overexpression of WT SHP-2 also decreased hematopoietic activities of the transduced progenitor cells (Fig. 1A). Three months after transplantation, the percentages of nucleated cells derived from WT SHP-2-transduced progenitor cells in peripheral blood of the recipient animals decreased to 1–7% (Fig. 1A). These data suggest that WT SHP-2-overexpressing progenitor cells lost growth advantage when competing with non-transduced donor cells in recipient mice.

GOF mutations in SHP-2 causing hyperactivation of its catalytic activity have recently been identified in human leukemias, in particular JMML (22—24). Since the hallmark of JMML is hypersensitivity of myeloid progenitor cells to GM-CSF and IL-3 (4, 5), these findings prompted us to compare the effects of GOF mutant SHP-2 with those of WT SHP-2 on IL-3 induced cellular responses. We generated SHP-2 E76K, the most common SHP-2 GOF mutant seen in JMML (22), and transduced this mutant into bone marrow hematopoietic progenitor cells via retroviral-mediated gene transfer. Transduced cells were assessed for their sensitivity to GM-CSF and IL-3 by hematopoietic colony assay. As shown in Fig. 2, A and B, SHP-2 E76K transduction increased cellular responses to GM-CSF and IL-3 in hematopoietic progenitor cells. Colony-forming capacities, in particular, at low concentrations of growth factors, were greatly enhanced by SHP-2 E76K transduction, consistent with recent reports (28—30). Furthermore, transduced cells were transplanted into recipient mice. Peripheral blood of these animals was examined three months after transplantation. In direct contrast to recipient mice transplanted with WT SHP-2-overexpressing cells (Fig. 1A), the contribution from SHP-2 E76K-transduced progenitors to the progeny cells in peripheral blood of all recipient mice decreased to 1–7% (Fig. 1A). These data suggest that WT SHP-2-overexpressing progenitor cells lost growth advantage when competing with non-transduced donor cells in recipient mice.

As shown in Fig. 3, A, B, and C, the transduced progenitor cells in peripheral blood of all recipient mice.

Although E76K is an activating mutation and SHP-2 E76K phosphatase activity is about five times higher than that of WT SHP-2 (21, 24), it does not seem that its enhanced catalytic activity is the sole contributing factor to blood disorders, since excessive (5—6-fold increase) WT SHP-2 did not cause myeloproliferative disease in recipient mice but decreased hematopoietic potential of the transduced cells (Fig. 1A). We next decided to dissect signaling activities of SHP-2 E76K by comparing to overexpressed WT SHP-2. As SHP-2 E76K results in hypersensitivity to GM-CSF and IL-3 in myeloid progenitor cells (Fig. 2, A and B), we focused on IL-3 signal transduction. SHP-2 E76K was transduced into an IL-3-dependent hematopoietic cell line, Ba/F3 cells, and transduced cells were sorted based on GFP expression. Sorted cell pools and the WT SHP-2-overexpressing Ba/F3 cell pool that we previously generated (20) were used for subsequent signaling studies. In agreement with the progenitor cell assay data shown in Fig. 2, A and B, transduction of SHP-2 E76K into Ba/F3 cells greatly increased the percentage of S phase cells (Fig. 3A) based on cell cycle analyses. The BrdUrd incorporation assay further showed that compared with WT SHP-2-overexpressing cells, SHP-2 E76K cells in late (upper right panel) rather than early S phases (Fig. 3B, lower right panel) were markedly increased. In addition, the percentage of SHP-2 E76K cells in G2/M phase (Fig. 3B, upper left panel) was also increased.

Analyses of IL-3 signaling showed that transduction of SHP-2 E76K dramatically enhanced and sustained IL-3-induced activation of Erk and Akt kinases (Fig. 4). Although overexpression of WT SHP-2 also resulted in increased Erk and Akt activities, its potential was lower than SHP-2 E76K. IL-3 stimulation also transiently activated Jnk kinase. Jnk1 activation in SHP-2 E76K cells was also increased when compared with that in WT SHP-2-overexpressing cells. Additionally, we found that overnight IL-3 starvation activated p38 kinase, and p38 activity quickly (30 min) returned to the basal level following IL-3 addition. This dynamic change in p38 activity was not altered in SHP-2 E76K-overexpressing cells, suggesting that SHP-2 E76K mutation specifically perturbs IL-3-induced Erk and Akt pathways.

Since IL-3-induced PI3K and Erk kinase pathways are markedly
enhanced by SHP-2 E76K mutation, we next wanted to define the underlying mechanisms. We examined interactions between SHP-2 E76K and other signaling components critical for activation of PI3K and Erk pathways. As shown in Fig. 5, Gab2 detected in the anti-SHP-2 E76K immunocomplex was significantly increased when compared with that in the WT SHP-2 complex from the WT SHP-2-overexpressing cells, even though the expression level of SHP-2 E76K was slightly lower than that of WT SHP-2 in our cell systems. This observation suggests that the interaction between mutant SHP-2 and Gab2 is increased by the E76K mutation in the N-SH2 domain. Reciprocally, SHP-2 detected in the Gab2 immunocomplex was increased in SHP-2 E76K cells (data not shown). Consistent with this result, the interaction between SHP-2 E76K and the p85 subunit of PI3K was also increased when compared with that in the WT SHP-2/p85 interaction in the WT SHP-2-overexpressing cells. Since Gab2 scaffolding protein plays an important role in the IL-3-induced PI3K pathway (10), conceivably, the increased interaction between SHP-2 E76K and Gab2 or p85 leads to enhanced PI3K/Akt activation in the SHP-2 E76K cells.

We next examined the interaction between SHP-2 E76K and Grb2, a critical adaptor protein linking the downstream Erk pathway to the IL-3 receptor, and found that the SHP-2 E76K/Grb2 interaction was also increased when compared with WT SHP-2/Grb2 interaction in WT SHP-2-overexpressing cells (Fig. 5). Consistent with this observation, Sos, the guanine nucleotide exchange factor that constitutively associates with Grb2 and activates Ras, was also increased in the anti-SHP-2 E76K immunocomplex (Fig. 5). The increased SHP-2 E76K/Grb2 interaction may be attributed to enhanced tyrosine phosphorylation of SHP-2 E76K (Fig. 5), since the SHP-2/Grb2 binding is mainly mediated by the SH2 domain of Grb2 and two PY residues in the C terminus of SHP-2. Additionally, we observed that the interaction between Sos and Grb2, a critical process for activation of the Erk pathway, was dramatically prolonged in SHP-2 E76K-transduced cells (Fig. 6). These data suggest that increased and sustained interaction between SHP-2 E76K and Grb2 contributes to the enhanced Erk pathway.
Effects of SHP-2 E76K mutation on the IL-3-induced Jak/STAT pathway was next investigated. Overexpression of WT SHP-2 mildly increased IL-3 activation of Jak2 kinase, as defined by its tyrosine phosphorylation response (Fig. 7A) but disproportionately attenuated STAT5 activation (Fig. 7B), consistent with our previous observations (20). IL-3-induced Jak2 activation in SHP-2 E76K was slightly increased when compared with that in WT SHP-2-overexpressing cells (Fig. 7A). In agreement with this result, tyrosine phosphorylation of the IL-3 receptor β chain, one of the putative substrates of Jak2, was also enhanced in SHP-2 E76K cells (Fig. 7A). Intriguingly, compared with WT SHP-2-overexpressing cells, tyrosine phosphorylation of STAT5 was increased and sustained rather than decreased in SHP-2 E76K cells (Fig. 7B). These results pointed to the possibility that, in contrast to WT SHP-2, dephosphorylation of STAT5 by SHP-2 E76K is dampened, despite the increase in its enzymatic activity. To test this hypothesis, we examined in vivo dephosphorylation of STAT5 following IL-3 starvation. When compared with vector-transduced cells, overexpression of WT SHP-2 accelerated dephosphorylation of STAT5. Tyrosine phosphorylation of STAT5 in WT SHP-2-overexpressing cells decreased to the basal level after 2 h of starvation. However, STAT5 dephosphorylation rate in SHP-2 E76K cells is not significantly changed when compared with the vector control cells (Fig. 8). These results suggest that the efficiency of mutant SHP-2 in dephosphorylation of STAT5 is decreased, as a result of the E76K mutation.

Since IL-3-induced Jak2 activation is slightly enhanced in SHP-2 E76K cells (Fig. 7A), we next wanted to determine whether enhanced activation of Jak2 or other cytosolic tyrosine kinases may be responsible for the increased SHP-2 E76K/Gab2 interaction (Fig. 5). We treated the SHP-2 E76K cells with Jak2 kinase inhibitor AG490, Src kinase inhibitor PP2, Btk kinase inhibitor LFM-A13, or Syk kinase inhibitor piceatannol; SHP-2 E76K/Gab2 interaction was then examined. As shown in Fig. 9, IL-3 stimulation dramatically induced tyrosine phosphorylation of Gab2 and SHP-2 E76K as well as their interaction. Inhibition of Jak2, but not Src or Btk kinases, completely blocked Gab2 phosphorylation and thereby SHP-2 E76K/Gab2 interaction. Inhibition of Syk kinase activity also showed mild effects. These results suggest that enhanced Jak2 acti-
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As shown in Fig. 12, the interaction between SHP-2 E76K/C459S and Gab2 is also increased and sustained, similar to the SHP-2 E76K/Gab2 interaction. This result indicates that the catalytic activity of SHP-2 E76K may not be essential for its increased interaction with Gab2. Consistent with this result, IL-3-induced Akt activation in SHP-2 E76K C459S-overexpressing cells is also enhanced when compared with WT SHP-2-overexpressing cells (Fig. 12). These data suggest that the E76K mutation in the N-terminal SH2 domain directly contributes to the increased interaction with Gab2 and thereby the enhanced activation of the PI3K pathway.

**DISCUSSION**

In this report, we investigated effects of the E76K mutation, the most common JMML-associated SHP-2 mutation, on hematopoietic cell...
function and IL-3 signal transduction by comparing with those of over-expressed WT SHP-2. We confirmed that SHP-2 E76K mutation enhanced cellular responses to GM-CSF and IL-3 and that overexpression (5–6-fold increase) of WT SHP-2 in hematopoietic stem/progenitor cells compromised their hematopoietic potential. When compared with WT SHP-2, the E76K mutation in the N-SH2 domain increased interactions of the mutant SHP-2 with Grb2, Gab2, and p85, leading to enhanced activation of Erk and PI3K pathways. Furthermore, despite the elevated phosphatase activity of SHP-2 E76K, the efficiency of mutant SHP-2 in dephosphorylating STAT5 in vivo was alleviated. These results together have suggested that multiple mechanisms contribute to enhanced IL-3 signaling and cellular responses by SHP-2 E76K mutation. In addition to increased catalytic activity, altered physical and functional interactions between mutant SHP-2 and its signaling partners also play an important role in the pathogenesis of SHP-2-related hematopoietic malignancies.

Signaling activities of SHP-2 are tightly regulated by its protein confirmation. SHP-2 consists of two SH2 domains, a tyrosine phosphatase domain, and a C-terminal tail containing several important functional motifs and tyrosine residues. The 2.0-Å x-ray crystal structure of SHP-2 reveals that SHP-2 is self-inhibited (31, 32). The backside of the N-SH2 domain loop binds to the deep pocket of the phosphatase domain via hydrogen bonds, leading to occlusion of the phosphatase catalytic site, as well as distortion of the N-SH2 PY binding groove. Ligands with PY residues act to activate SHP-2 by binding the tandem SH2 domains, thereby disrupting N-SH2 domain-phosphatase domain binding and exposing the phosphatase catalytic site (31–33). Mutations in SHP-2 identified in leukemias, such as SHP-2 E76K, lead to changes in amino acids at the interface formed by the N-SH2 and phosphatase domains in the self-inhibited SHP-2 conformation. These mutations thus cause a decrease in N-SH2 domain-phosphatase domain binding affinity, which leads to hyperactivation of its phosphatase activity (21, 22, 24) by allowing access to the phosphatase catalytic site on the enzyme.

Our studies in this report suggest that in addition to enhancing the catalytic activity, leukemia-associated SHP-2 mutations may also have an impact on the function of the N-SH2 domain per se. Compared with WT SHP-2, the E76K point mutation in the N-SH2 domain significantly increases interactions between SHP-2 and Gab2 and p85 (Fig. 5). The SH2 domains, in particular, the N-SH2 domain, play an important role in SHP-2 function. They mediate the binding of SHP-2 to other signaling proteins via their PY residues in a sequence-specific fashion, thereby directing SHP-2 to the appropriate subcellular location and helping determine the specificity of substrate-enzyme interactions for SHP-2.
As the leukemia-associated SHP-2 mutations are located primarily in the SH2 domains, in particular, the N-SH2 domain, and these mutations lie either within or near the PY binding pocket, it is thus possible that these mutations perturb PY-dependent SHP-2 interactions with other signaling proteins.

Additionally, our results suggest that the in vivo substrate specificity of SHP-2 E76K also has been changed. SHP-2 physically interacts and dephosphorylates STAT5 in the IL-3 signaling pathway (20). Dephosphorylation of STAT5 is accelerated by excessive amount (5–6-fold increase) of WT SHP-2 in WT SHP-2-overexpressing cells. Thus, overexpression of WT SHP-2 compromised hematopoietic cell function and enhanced growth factor deprivation-induced apoptosis (20). Intriguingly, in SHP-2 E76K cells, tyrosine phosphorylation of STAT5 in response to IL-3 stimulation was increased and prolonged (Fig. 7B), even though the phosphatase activity of SHP-2 E76K is substantially increased (21, 24). This appears to be due to the decreased efficiency of SHP-2 E76K in dephosphorylating STAT5 in vivo. Compared with WT SHP-2-overexpressing cells, delay of the phosphorylation level of STAT5 in SHP-2 E76K cells following IL-3 withdrawal is delayed (Fig. 8).

SHP-2 phosphatase is a multifunctional protein, playing complicated roles in hematopoietic cell signaling and function. Our previous studies (16–18) showed that SHP-2 was required for the onset of hematopoietic development. Erythroid, myeloid, and lymphoid development in primitive hematopoiesis was blocked by the Exon 3 deletion mutation in SHP-2 (16–18). We also showed that IL-3-induced signaling and cellular responses were decreased by inhibition of the catalytic activity of endogenous SHP-2 through overexpression of catalytically inactive mutant SHP-2 C459S (19, 20). Consistent with these results, overexpression of SHP-2 C459S in hematopoietic stem/progenitor cells greatly suppressed their hematopoietic potential in vivo (Fig. 1A). These observations suggest that the catalytic activity of SHP-2 is important for normal hematopoiesis. Nevertheless, although activating mutations in SHP-2 that cause hyperactivation of its catalytic activity are associated with leukemias (22–24), it does not seem that the enhanced catalytic capability and the altered substrate specificity of SHP-2 E76K. Conceivably, the conversion of negatively charged glutamic acid (Glu) to positively charged lysine (Lys) at amino acid residue 76, which is located within the PY binding pocket of the N-SH2 domain, is likely to increase the binding affinity for the negatively charged PY residues. Alternatively, the E76K mutation may change SHP-2 ability to bind the PY residues through distorted confirmation of the N-SH2 domain.

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