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Leukemia-associated mutations in SHIP1 inhibit its enzymatic activity, interaction with the GM-CSF receptor and Grb2, and its ability to inactivate PI3K/AKT signaling ☆

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ABSTRACT

The inositol 5-phosphatase SHIP1 is a negative regulator of the PI3K/AKT pathway, which is constitutively activated in 50–70% of acute myeloid leukemias (AML). Ten different missense mutations in SHIP1 have been described in 3% of AML patients suggesting a functional role of SHIP1 in AML. Here, we report the identification of two new SHIP1 mutations T162P and R225W that were detected in 2 and 1 out of 96 AML patients, respectively. The functional analysis of all 12 AML-associated SHIP1 mutations, one ALL-associated SHIP1 mutation (Q1076X) and a missense SNP (H1168Y) revealed that two mutations i.e. Y643H and P1039S abrogated the ability of SHIP1 to reduce constitutive PI3K/AKT signaling in Jurkat cells. The loss of function of SHIP1 mutant Y643H which is localized in the inositol phosphatase domain was due to a reduction of the specific activity by 84%. Because all other SHIP1 mutants had a normal enzymatic activity, we assumed that these SHIP1 mutants may be functionally impaired due to a loss of interaction with plasma membrane receptors or adapter proteins. In agreement with this model, we found that the SHIP1 mutant F28L located in the FLVR motif of the SH2 domain was incapable of binding tyrosine-phosphorylated proteins including the GM-CSF receptor and that the SHIP1 mutant Q1076X lost its ability to bind to the C-terminal SH3 domain of the adapter protein Grb2. In addition, SHIP1 mutant P1039S which does not reduce PI3K/AKT signaling anymore is located in a PXXP SH3 domain consensus binding motif suggesting that mutation of the conserved proline residue interferes with binding of SHIP1 to a so far unidentified SH3 domain containing protein. In summary, our data indicate that SHIP1 mutations detected in human leukemia patients impair the negative regulatory function of SHIP1 on PI3K/AKT signaling in leukemia cells either directly by reduced enzymatic activity or indirectly by disturbed protein interaction with tyrosine-phosphorylated membrane receptors or adapter proteins. These results further support a functional role of SHIP1 as tumor suppressor protein in the pathogenesis of AML.

1. Introduction

SHIP1 (src homology 2 domain-containing inositol phosphatase 1) is a negative regulator of signal transduction in hematopoietic cells [1]. SHIP1 belongs to the class of inositol 5 phosphatases which hydrolyze a phosphate from the D5 position of the inositol ring of phosphoinositides (PI) and inositol phosphates (IP) [2,3]. SHIP1 mediates its regulatory function by converting PI(3,4,5)P_3 to PI(3,4)P_2 and I(1,3,4,5)P_4 to I(1,3,4)P_3, thereby regulating phosphoinositide and inositol phosphate-mediated cellular processes [4–7]. We have recently demonstrated that restoration of SHIP1 activity in Jurkat cells which do not express SHIP1 proteins reduces the level of PI(3,4,5)P_3 and the activity of Akt resulting in a reduced proliferation due to an increased transit time through the G1 phase of the cell cycle [8].

Several lines of evidence show that reduced expression and/or reduced activity of SHIP1 are involved in leukemogenesis. SHIP1-deficient mice as well as mice with a conditional knockout of SHIP1 in the granulocyte/macrophage lineage develop a myeloproliferative disorder due to an increased sensitivity of hematopoietic cells for several cytokines including granulocyte/macrophage colony-stimulating factor (GM-CSF) [9–11]. Expression of SHIP1 is down regulated by BCR–ABL and SHIP1 is barely detectable in BCR/ABL-positive chronic myeloid leukemia [12]. In

Abbreviations: SHIP1, src homology 2 domain-containing inositol phosphatase 1; PI3K, phosphoinositide 3-kinase; AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; SNP, single nucleotide polymorphism; GM-CSF, granulocyte/macrophage colony-stimulating factor; IP, inositol phosphate; rtTA, reverse tet transactivator; dHPLC, denaturing high performance liquid chromatography; GST, glutathione-S-transferase.

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addition, Friend murine leukemia virus can suppress SHIP1 expression which is associated with accelerated development of erythroleukemia in mice [13]. Downregulation of SHIP1 can also be mediated by oncogenic miR155 which binds to the 3′UTR of SHIP1 [14]. Interestingly, upregulation of miR155 and downregulation of SHIP1 have been observed in diffuse large B cell lymphoma in which reduced levels of SHIP1 correlate with poor survival [15]. In myeloid cells, overexpression of miR155 leads to a myeloproliferative syndrome [16] and upregulation of miR155 has been found in acute myeloid leukemia (AML) [17]. Recently, it has been shown that concomitant deletion of PTEN and SHIP1 in mice leads to the development of lethal B cell lymphomas [18]. In T cell acute lymphoblastic leukemia (T-ALL) expression of full length SHIP1 is frequently down regulated due to alternative splicing and translational inactivation [19]. We have demonstrated that overexpression of SHIP1 in primary leukemia cells from patients with juvenile myelomonocytic leukemia and AML reduces the proliferation of the leukemia cells in vitro [20,21]. These data suggest a tumor suppressor function of SHIP1.

In patients with AML, mutations in the *INPP5D* gene encoding SHIP1 have been detected, thereby implicating mutated SHIP1 in the pathogenesis of AML [22–24]. However, the molecular mechanisms on how SHIP1 mutations may mediate its function as a tumor suppressor have not been analyzed in detail. In this report we have identified 2 new mutations in SHIP1 in 3 AML patients and analyzed the effects of all 13 leukemia-associated SHIP1 missense mutations identified so far in primary AML and ALL cells on the enzymatic activity of SHIP1 and the ability of SHIP1 to reduce PI3K/AKT signaling.

2. Material and methods

2.1. Patient samples and cell lines

Patient samples (n = 96) from 45 AML of childhood and 51 AML of adulthood as well as 45 anonymous healthy donors were obtained from tissue banks at the Clinic of Pediatric Hematology and Oncology Hamburg and the Department of Internal Medicine, Hematology and Oncology at the University Münster. Written informed consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki and with the approval of institutional ethics review boards (W55/08 Ethics Committee Hamburg).

The factor-independent TF-1 mutant I33-5 has been established from the GM-CSF-dependent human erythroleukemia cell line TF-1 after selection for growth factor independence in medium not supplemented with GM-CSF [25–27]. Jurkat is a human T-cell line which had been established from a patient with acute lymphoblastic leukemia [28]. Jurkat Tet-On cells carrying the reverse tet transactivator (rtTA) (BD Clontech, Heidelberg, Germany) were grown in RPMI 1640 Glutamax medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% (vol/vol) fetal calf serum (FCS), 1% (v/v) penicillin G/streptomycin and 1 mM sodium pyruvate. The doxycycline inducible expression of SHIP1 in Jurkat cells (Jurkat-TetOn-SHIP1) by retroviral transduction and selection of cell clones by limiting dilution has been previously described [8]. Generation of inducible Jurkat-TetOn-SHIP1-mutant cells expressing the leukemia-associated SHIP1 mutants was generated in the same way by retroviral transduction and limiting dilution. Expression was induced by the addition of 1 μg/mL doxycycline (MP Biomedicals, Eschwege, Germany) for 48 h. The murine factor-dependent cell line FDC-P1 that had been established from long-term cultures of bone marrow cells from the mouse strain DBA/2 in WEHI-3 conditioned medium [29] was cultivated in DMEM medium supplemented with 10% FCS, 1% (v/v) penicillin G/streptomycin and 10 ng/mL murine IL-3 (PeproTech, Hamburg, Germany). Phoenix Ampho cells (kindly provided by G. Nolan, Stanford University, USA) were used as packaging cells for virion production. Cells were cultivated in DMEM supplemented with 10% FCS, 1% (v/v) penicillin G/streptomycin.

2.2. Mutation analysis

For mutation analysis of the *INPP5D* gene encoding SHIP1, genomic DNA was amplified by polymerase chain reaction and analyzed by denaturing high performance liquid chromatography (dHPLC) as described previously [30]. All primers used for amplification of the 27 exons of SHIP1 are listed in Supplement Tables S1A–S1B. DNA fragments with an abnormal melting pattern were collected, re-amplified and directly sequenced on an ABI Prism 3730 high-throughput platform. All SHIP1 sequences from normal donors and leukemia patients represented the isoform SHIP1 beta (NP_005532.2) consisting of 1188 amino acids. The SHIP1 beta sequence was used for numbering of SHIP1 in text and figures.

2.3. Site-directed mutagenesis

Mutations were introduced into the retroviral SHIP1 expression vector pLTR-Tet-SHIP1-SV-HPH [8] encoding SHIP1 isoform beta by site-directed mutagenesis using QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. All primers used for mutagenesis are described in Supplement Table S2.

2.4. Western blot analysis

SDS-PAGE and Western blot analysis were performed as described previously [31]. Antibodies used for immunodetection are directed against SHIP1 (P1C1), GAPDH (6C5), Akt1/2/3 (H-136), IL-3/IL-5/GM-CSFRβ (C-20), p-Tyr (PY99) (Santa Cruz Biotechnology, Heidelberg, Germany), Phospho-Akt (Ser473) (193H12) (Cell Signaling, Danvers, MA, USA), and GST (GE Healthcare Lifesciences, Bio-Sciences AB, Uppsala, Sweden). Signals were detected with super signal west pico chemiluminescent substrate (Thermo Fisher Scientific, Schwerte, Germany) and quantified on a luminescent image analyzer with CCD camera LAS-3000 (Fujifilm) using AIDA software.

2.5. Expression of GST fusion proteins and pull down experiments

Pull down experiments with GST or GST-SHIP1-SH2 domains were performed as described previously [32]. For expression as glutathione-S-transferase (GST) fusion protein, the SHIP1 SH2 domain (amino acids 5–103) had been cloned into the BamHI restriction site of pGEX-5X-1 (GE Healthcare Lifesciences, Bio-Sciences AB, Uppsala, Sweden) and was mutated to R31Q and F28L by site-directed mutagenesis. Primers for mutagenesis are described in Table S2. GST and GST SHIP1 SH2 domain fusion proteins (WT, F28L, R31Q) were expressed in BL21 (DE3) pLysS pREP4 by induction with 0.1 mM IPTG for 3.5 h at 32 °C and shaking at 250 rpm. Bacteria were centrifuged 15 min at 4 °C and 6000 ×*g*, pellets were frozen at −20 °C, thawed, lysed in NP40 lysis buffer (5 mM HEPES pH 7.5, 150 mM NaCl, 1% Nonidet P40, 200 KIU/mL aprotinin, 2 mM EDTA, 50 mM NaF, 10 mM $NaPP_i$, 10% glycerine, 1 mM $NaVO_4$, 1 mM PMSF), sonicated 3× 30 s (Max Energy, Cycle 30), centrifuged for 10 min at 4 °C and 12 000 ×*g* and the supernatant was aliquoted and frozen at −80 °C. Fusion proteins were quantified on an SDS polyacrylamide gel by Coomassie Brilliant Blue staining next to a BSA standard.

2.6. Verification of SHIP1 mutations introduced into Jurkat Tet-On cells

Genomic DNA from Jurkat Tet-On clones was isolated with the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. For the amplification and the subsequent sequencing of the SHIP1 cDNA sequence that had been introduced into Jurkat Tet-On cells by retroviral transduction, intron-spanning primers were designed to selectively amplify the transduced sequence but not the endogenous SHIP1 sequence of the Jurkat cells. The 5′ part of the