SH2-Containing Inositol 5'-Phosphatase Inhibits Transformation of Abelson Murine Leukemia Virus^{▽†}

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v-Abl protein tyrosine kinase encoded by Abelson murine leukemia virus (Ab-MLV) transforms pre-B cells. Transformation requires the phosphatidylinositol 3-kinase (PI3K) pathway. This pathway is antagonized by SH2-containing inositol 5'-phosphatase (SHIP), raising the possibility that v-Abl modulates PI3K signaling through SHIP. Consistent with this, we show that v-Abl expression reduces levels of full-length p145 SHIP in a v-Abl kinase activity-dependent fashion. This event requires signals from the Abl SH2 domain but not the carboxyl terminus. Forced expression of full-length SHIP significantly reduces Ab-MLV pre-B-cell transformation. Therefore, reduction of SHIP protein by v-Abl is a critical component in Ab-MLV transformation.

Abelson murine leukemia virus (Ab-MLV) originated from recombination between Moloney murine leukemia virus (Mo-MuLV) and the cellular proto-oncogene *c-abl* (32). The resulting oncoprotein, v-Abl, is a constitutively active nonreceptor protein tyrosine kinase that includes the Gag region of Mo-MuLV, the SH2 domain, the tyrosine kinase domain, and the carboxyl terminus of *c-abl*, each of which contributes to transformation by activating various signaling pathways (2, 23, 36, 41, 42).

One such pathway critical for Ab-MLV transformation is the phosphatidylinositol 3-kinase (PI3K) cascade (11, 18). PI3K phosphorylates PI-4,5-P₂ phosphatidylinositol lipids, forming PI-3,4,5-P₃ (PIP₃), which creates a docking site and promotes the activation of various pleckstrin homology domain-containing signaling molecules, including Akt (reviewed in reference 3). Levels of PIP₃ are reduced by phosphatase and tensin homolog (PTEN), which converts PIP₃ to PI-4,5-P₂, and by SH2-containing inositol 5'-phosphatase (SHIP) and the related protein SHIP2, which converts PIP₃ to PI-3,4-P₂ (reviewed in references 12, 17, and 30).

The expression of SHIP is restricted to hematopoietic cells, and its loss is associated with myeloproliferative disease and various leukemias, including breakpoint cluster region/Abelson (BCR/ABL)-mediated chronic myelogenous leukemia (CML) (5, 13–15, 21, 22, 25–27). *In vitro* studies have revealed that the kinase activity of BCR/ABL suppresses SHIP protein levels, suggesting that SHIP plays a negative role in transformation by oncogenic Abl proteins (33–35). In the current study, we investigated the regulation of SHIP by v-Abl and directly tested the role of SHIP during Ab-MLV transformation.

To determine if v-Abl regulates SHIP protein levels, Ab-

MLV-transformed pre-B cells (11) expressing P120 v-Abl were treated with the Abl tyrosine kinase inhibitor imatinib mesylate (imatinib) (8) for 2 h and analyzed by Western blotting as described in reference 2 by using an antibody that recognizes the full-length p145 SHIP isoform (see the supplemental material for additional methodology). Increased doses of imatinib resulted in an accumulation of p145 SHIP protein (Fig. 1A, left), which coincided with reduced total cellular phosphotyrosine (Fig. 1A, left). Furthermore, treatment of Ab-MLV-transformed pre-B cells with 1 μ M imatinib for 16 h, followed by removal of the drug, restored SHIP levels to those seen in untreated cells within 4.5 h (Fig. 1A, right). These data demonstrate that the kinase activity of v-Abl reduces total SHIP protein levels in a manner similar to that reported for BCR/ABL (35).

SHIP is expressed as multiple isoforms that may be generated by calpain-mediated carboxyl-terminal proteolytic cleavage of full-length p145 SHIP (7). In particular, the p110 isoform expressed in normal hematopoietic cells (7, 9) is not detected by the antibody used for the analyses shown in Fig. 1A, raising the possibility that v-Abl reduces SHIP protein levels by promoting carboxyl-terminal proteolytic cleavage. To test this idea, Ab-MLV-transformed pre-B cells were treated with imatinib for 16 h, and lysates were analyzed by Western blotting with a SHIP antibody that recognizes the p110 isoform. In untreated cells, the 145-kDa band was the most prominent form, while a faint 110-kDa band was also present (Fig. 1B). Imatinib treatment resulted in a similar dose-dependent increase in both p145 and p110 (Fig. 1B), demonstrating that v-Abl kinase activity reduces both forms of SHIP and does not promote carboxyl-terminal proteolytic cleavage of p145 SHIP to reduce SHIP protein levels. Because the normal CD43⁺, B220⁺ target cells that give rise to Ab-MLV transformants (M. Gunthart and N. Rosenberg, unpublished data; see the supplemental material for the methods used) predominantly express the p110 isoform (Fig. 1B), these data suggest that Ab-MLV transformation alters the pattern of SHIP isoforms expressed by the cells. Although the function of the different isoforms has yet to be clarified, differences in association with Shc, a protein prominently involved in v-Abl-mediated signal-

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FIG. 1. SHIP downregulation by P120 v-Abl is inhibited by imatinib. (A) Ab-MLV P120-transformed pre-B cells were treated with the indicated dose of imatinib for 2 h (left) or treated with 1 μ M imatinib for 16 h and resuspended in fresh medium without drug for the indicated time (right) and analyzed by Western blotting using antibodies against the C-terminal portion of SHIP (Santa Cruz Biotechnologies), total phosphotyrosine (p-Tyr) (Millipore), v-Abl (4), and actin (Sigma). (B) Extracts from Ab-MLV P120-transformed pre-B cells treated with the indicated amounts of imatinib for 16 h and untransformed CD43⁺/B220⁺ fluorescence-activated cell sorter (FACS) sorted normal murine bone marrow cells were analyzed by Western blotting using antibodies against the N-terminal portion of SHIP (Santa Cruz Biotechnologies) and actin. (C) Ab-MLV P120-transformed pre-B cells were treated with 10 μ M MG132 for the indicated times and analyzed by Western blotting using antibodies against the C-terminal portion of SHIP and actin. These data are representative of experiments conducted with at least two independent cell lines.

ing (16), and differences in subcellular localization may affect SHIP function (7).

Proteasome-dependent downregulation of proteins has been observed in Abl-expressing cells (6, 10). To determine if v-Abl reduces levels of SHIP protein via proteasome degradation, Ab-MLV-transformed pre-B cells (11) were treated with 10 μ M proteasome inhibitor MG132 and analyzed by Western blotting. Levels of SHIP protein increased by 1 h following MG132 treatment (Fig. 1C), similar to treatment of cells with imatinib, suggesting that SHIP downregulation by v-Abl involves proteasomal degradation in a manner similar to that recently reported for BCR/ABL (33). These findings contrast to the marked stability of SHIP isoforms in normal cells, where half-lives of approximately 10 h have been reported (7). Although one previous report indicated that BCR/ABL does not promote proteasomal degradation of SHIP (34), variations in MG132 treatment length and concentration could explain these differences.

The v-Abl SH2 domain and carboxyl-terminal domains are critical for the formation of signaling complexes that influence v-Abl-mediated transformation, including those involving Shc, a protein known to associate with SHIP (16, 19, 20). To determine if these domains are important for effects on SHIP expression, cells transformed with v-Abl mutants (Fig. 2A) in which the v-Src SH2 domain replaces the v-Abl SH2 domain in the presence of a full-length carboxyl terminus (P120/S2) or in the absence of the carboxyl terminus (P70/S2) were analyzed for SHIP expression (29, 41). Cells expressing P120/S2 or P70/S2 are compromised in Shc/Grb2/Sos complex formation and display reductions in Ras activation (41). To determine if these modifications affect SHIP protein levels, transformed pre-B cells expressing P120 or the mutants were tested for expression of SHIP by Western blotting. Levels of p145 SHIP were elevated in cultures expressing both P120/S2 and P70/S2

FIG. 2. Downregulation of SHIP is dependent upon the v-Abl SH2 domain and not the carboxyl terminus. (A) A schematic of the SH2 variant viruses. IRES, internal ribosome entry site; LTR, long terminal repeat. (B) P120-, P120/S2-, P70-, and P70/S2-transformed pre-B cells were analyzed by Western blotting using antibodies against the C-terminal portion of SHIP and v-Abl. (C) Bone marrow cells were infected with matched titer stocks of P120 ($\diamond$), P120/S2 ($\circ$), P70 ($\square$), or P70/S2 ($\triangle$) viruses and scored as transformed when they contained 2×10^6 cells per ml (1, 24). Fifteen cultures of P120 and P120/S2, 14 cultures of P70, and 8 cultures of P70/S2 were analyzed.

compared to cultures expressing P120 or P70 v-Abl (Fig. 2B); imatinib treatment of a P120/S2-transformed pre-B cell line did not further modulate SHIP protein levels (data not shown), indicating that a functional v-Abl SH2 domain is required to regulate SHIP protein levels. Although the specific signals from the v-Abl SH2 domain required for SHIP proteasome degradation have not been elucidated, activation of Ras or PI3K pathways could possibly influence SHIP protein stability in a manner similar to the requirement for PI3K in proteasome-dependent degradation of p27^{Kip} by BCR/ABL (10).

To determine if v-Abl variants, such as P120/S2 and P70/S2, that fail to reduce SHIP protein levels transform pre-B cells poorly compared to cells expressing P120 or P70, the kinetics of bone marrow transformation by these variants (Fig. 2A) were examined using liquid transformation assays that monitor both the time to transformation and transformation frequency (2). Bone marrow cells were infected with matched titer stocks of the different viruses, plated in liquid cultures, and monitored for transformation for 30 days. This assay allows quantitation of transformation by weakly transforming mutants that do not stimulate all of the pathways involved in transformation

FIG. 3. Expression of SHIP inhibits Ab-MLV transformation. (A) A schematic of the v-Abl/SHIP viruses. (B) 293T cells were transfected with 15 μ g of viral DNA encoding either P120 or P120/SHIP or mock transfected. Lysates were prepared 48 h later and analyzed by Western blotting using antibodies against the C-terminal portion of SHIP and v-Abl. (C) Bone marrow cells were infected with matched titer stocks of P120 ($\diamond$) or P120/SHIP ($\blacklozenge$) viruses and scored as transformed when they contained 2×10^6 cells per ml. Twenty-three cultures infected with P120 and P120/SHIP were analyzed, and 7 cultures infected with the kinase-deficient P120/D484N mutant (28) were analyzed.

by wild-type virus (2, 40). Such mutants transform fewer cells, thereby affecting the kinetics with which transformation occurs. No transformants arise in this assay in the absence of virus infection (31). The kinetics of P120/S2 transformation were delayed compared to those of P120 transformation, and similarly, kinetics of P70/S2 transformation were delayed compared to those of P70 transformation (log rank test P120 versus P120/S2, $P < 0.0001$; P70 versus p70/S2, $P = 0.0096$) (Fig. 2C), suggesting a correlation between elevated expression of SHIP and reduced transformation. This pattern contrasts to the observation that P120/S2 transforms NIH 3T3 cells at levels similar to those of P120 (41), a finding likely reflecting the fact that SHIP is not expressed in these cells. The emergence of a reduced frequency of lymphoid transformants with the S2 mutants that continue to express SHIP probably reflects the use of alternative signaling pathways that compensate for SHIP expression. The ability of transformed cells to circumvent tumor-suppressive signals via different pathways has been documented in many types of cancer (39). An example from Ab-MLV transformation involves ways in which cells escape the effects of p53 (28, 37, 38).

To directly test whether expression of SHIP interferes with

Ab-MLV transformation, full-length p145 SHIP was cloned into the pMIA viral vector that allows expression of both v-Abl and a second gene (Fig. 3A). Bone marrow cells were infected with matched titer stocks of the different viruses, plated in liquid cultures, and monitored for pre-B-cell transformation. The kinase-negative P120/D484N mutant that does not transform cells (29) was used as a negative control. Expression of v-Abl was comparable between these viruses, and SHIP was expressed by the virus containing this gene (Fig. 3B). Fewer cultures infected with the v-Abl/SHIP virus (4/23 cultures) underwent transformation than cultures infected with P120 (23/23 cultures), confirming that forced expression of SHIP significantly reduces Ab-MLV transformation (log rank test, $P < 0.0001$) (Fig. 3C). Despite the marked reduction in transformation efficiency, a small number of v-Abl/SHIP-infected cultures became transformed similarly to wild-type (WT) cultures; it is possible that these transformants develop secondary mutations, signal through alternative pathways that bypass the suppressive effects of SHIP, or no longer express exogenous SHIP. The dramatic reduction in transformation by P120/SHIP contrasts to the modest enhancement of Ab-MLV transformation previously observed for bone marrow cells from SHIP^{-/-} mice (27). Because these mice have reduced numbers of pre-B cells (13), the effect of SHIP on Ab-MLV transformation in SHIP^{-/-} mice is difficult to assess.

Taken together, these results directly demonstrate that the v-Abl SH2 domain is required for v-Abl-mediated down-modulation of SHIP and that expression of elevated levels of SHIP reduces Ab-MLV transformation. Together, these data reinforce the regulatory role of SHIP in Abl-mediated oncogenesis.

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REFERENCES

- Afar, D. E. H., A. Goga, J. McLaughlin, O. N. Witte, and C. L. Sawyers. 1994. Differential complementation of Bcr-Abl point mutants with c-Myc. *Science* **264**:424–426.
- Baughn, L. B., and N. Rosenberg. 2005. Disruption of the Shc/Grb2 complex during Abelson virus transformation affects proliferation, but not apoptosis. *J. Virol.* **79**:2325–2334.
- Cantley, L. C. 2002. The phosphoinositide 3-kinase pathway. *Science* **296**:1655–1657.
- Chesebro, B., et al. 1981. Characterization of mouse monoclonal antibodies specific for Friend murine leukemia virus-induced erythroleukemia cells: Friend-specific and FMR-specific antigens. *Virology* **112**:131–144.
- Costinean, S., et al. 2009. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood* **114**:1374–1382.
- Dai, Z., et al. 1998. Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway. *Genes Dev.* **12**:1415–1424.
- Damen, J. E., et al. 1998. Multiple forms of the SH2-containing inositol phosphatase, SHIP, are generated by C-terminal truncation. *Blood* **92**:1199–1205.
- Druker, B. J., et al. 1996. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med.* **2**:561–566.
- Geier, S. J., et al. 1997. The human SHIP gene is differentially expressed in cell lineages of the bone marrow and blood. *Blood* **89**:1876–1885.
- Gesbert, F., W. R. Sellers, S. Signoretto, M. Loda, and J. D. Griffin. 2000. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-kinase/AKT pathway. *J. Biol. Chem.* **275**:39223–39230.
- Gong, L., I. Unnikrishnan, A. Raghavan, K. Parmar, and N. Rosenberg. 2004. Active Akt and functional p53 modulate apoptosis in Abelson virus-transformed pre-B cells. *J. Virol.* **78**:1636–1644.

12. **Hamilton, M. J., et al.** 2011. Role of SHIP in cancer. *Exp. Hematol.* **39**:2–13.
13. **Helgason, C. D., et al.** 1998. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev.* **12**:1610–1620.
14. **Horn, S., et al.** 2004. Restoration of SHIP activity in a human leukemia cell line downregulates constitutively activated phosphatidylinositol 3-kinase/Akt/GSK-3 β signaling and leads to an increased transit time through the G₁ phase of the cell cycle. *Leukemia* **18**:1839–1849.
15. **Jiang, X., et al.** 2003. Evidence for a positive role of SHIP in the BCR-ABL-mediated transformation of primitive murine hematopoietic cells and in human chronic myeloid leukemia. *Blood* **102**:2976–2984.
16. **Kavanaugh, W. M., et al.** 1996. Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and Grb2. *Curr. Biol.* **6**:438–445.
17. **Kerr, W. G.** 2011. Inhibitor and activator: dual functions for SHIP in immunity and cancer. *Ann. N. Y. Acad. Sci.* **1217**:1–17.
18. **Kharas, M. G., et al.** 2004. Phosphoinositide 3-kinase signaling is essential for ABL oncogene-mediated transformation of B-lineage cells. *Blood* **103**:4268–4275.
19. **Lioubin, M. N., et al.** 1996. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev.* **10**:1084–1095.
20. **Liu, L., et al.** 1997. The Src homology 2 (SH2) domain of SH2-containing inositol phosphatase (SHIP) is essential for tyrosine phosphorylation of SHIP, its association with Shc, and its induction of apoptosis. *J. Biol. Chem.* **272**:8983–8988.
21. **Liu, Q., et al.** 1999. SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev.* **13**:786–791.
22. **Luo, J. M., et al.** 2003. Possible dominant-negative mutation of the SHIP gene in acute myeloid leukemia. *Leukemia* **17**:1–8.
23. **Mainville, C., et al.** 2001. Alteration of the FLVRES motif in the v-Abl SH2 domain confers a temperature sensitive transformation phenotype. *J. Virol.* **75**:1816–1823.
24. **McLaughlin, J., E. Chianese, and O. N. Witte.** 1989. Alternative forms of BCR-ABL oncogene have quantitatively different potencies for stimulation of immature lymphoid cells. *Mol. Cell. Biol.* **9**:1866–1874.
25. **O'Connell, R. M., A. A. Chaudhuri, D. S. Rao, and D. Baltimore.** 2009. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. U. S. A.* **106**:7113–7118.
26. **O'Connell, R. M., et al.** 2008. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* **205**:585–594.
27. **Oki, S., et al.** 2005. Dok1 and SHIP act as negative regulators of v-Abl induced pre-B cell transformation, proliferation and Ras/Erk activation. *Cell Cycle* **4**:310–314.
28. **Radfar, A., I. Unnikrishnan, H. W. Lee, R. A. DePinho, and N. Rosenberg.** 1998. p19(Arf) induces p53-dependent apoptosis during Abelson virus-mediated pre-B cell transformation. *Proc. Natl. Acad. Sci. U. S. A.* **95**:13194–13199.
29. **Raffel, G. D., K. Parmar, and N. Rosenberg.** 1996. *In vivo* association of v-Abl with Shc mediated by a non-phosphotyrosine-dependent SH2 interaction. *J. Biol. Chem.* **271**:4640–4645.
30. **Rohrschneider, L. R., J. F. Fuller, I. Wolf, Y. Liu, and D. M. Lucas.** 2000. Structure, function, and biology of SHIP proteins. *Genes Dev.* **14**:505–520.
31. **Rosenberg, N., and D. Baltimore.** 1976. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. *J. Exp. Med.* **143**:1453–1463.
32. **Rosenberg, N., and O. N. Witte.** 1988. The viral and cellular forms of the Abelson (*abl*) oncogene, p. 39–81. *In* A. Shatkin (ed.), *Advances in virus research*, vol. 35. Academic Press, New York, NY.
33. **Ruschmann, J., et al.** 2010. Tyrosine phosphorylation of SHIP promotes its proteasomal degradation. *Exp. Hematol.* **38**:392–402.
34. **Sattler, M., et al.** 1997. The phosphatidylinositol polyphosphate 5-phosphatase SHIP and the protein tyrosine phosphatase SHP-2 form a complex in hematopoietic cells which can be regulated by BCR/ABL and growth factors. *Oncogene* **15**:2379–2384.
35. **Sattler, M., et al.** 1999. BCR/ABL directly inhibits expression of SHIP, an SH2-containing polyinositol-5-phosphatase involved in the regulation of hematopoiesis. *Mol. Cell. Biol.* **19**:7473–7480.
36. **Shore, S. K., R. V. Tantravahi, and E. P. Reddy.** 2002. Transforming pathways activated by the v-Abl tyrosine kinase. *Oncogene* **21**:8568–8576.
37. **Unnikrishnan, I., A. Radfar, J. Jenab-Wolcott, and N. Rosenberg.** 1999. p53 mediates apoptotic crisis in primary Ab-MLV-transformed pre-B cells. *Mol. Cell. Biol.* **19**:4825–4831.
38. **Unnikrishnan, I., and N. Rosenberg.** 2003. Absence of p53 complements defects in Abelson murine leukemia virus signaling. *J. Virol.* **77**:6208–6215.
39. **Vogelstein, B., and K. W. Kinzler.** 2004. Cancer genes and the pathways they control. *Nat. Med.* **10**:789–799.
40. **Warren, D., D. Griffin, C. A. Mainville, and N. Rosenberg.** 2003. The extreme carboxyl terminus of v-Abl protein is required for lymphoid transformation by Abelson virus. *J. Virol.* **77**:4617–4625.
41. **Warren, D., A. J. Heilpern, K. Berg, and N. Rosenberg.** 2000. The carboxyl terminus of v-Abl protein can augment SH2 domain function. *J. Virol.* **74**:4495–4504.
42. **Yi, C. R., and N. Rosenberg.** 2007. Gag influences transformation by Abelson murine leukemia virus and suppresses nuclear localization of the v-Abl protein. *J. Virol.* **81**:9461–9468.