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Deletion of the ABL SH3 domain reactivates de-oligomerized BCR-ABL for growth factor independence

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Abstract Biological activities of BCR-ABL, an activated tyrosine kinase oncogene responsible for pathogenesis of human leukemias, can be completely inactivated by a deletion of the BCR aminoterminal sequence with a tetramerizing property (BCR-ABL Δ1–40). We attempted several ways to restore the ability to induce growth factor independence to the de-oligomerized BCR-ABL Δ1–40 and found that an additional deletion of the ABL SH3 domain could. In BCR-ABL Δ1–40 reactivated by the SH3 deletion, transphosphoryation of other cellular proteins like p62 or SHC in vivo and autophosphorylation with recruitment of GRB-2 were also recovered.

Key words: BCR-ABL; Oligomerization; SH3 domain; IL-3 dependence; Tyrosine phosphorylation; GRB-2

1. Introduction

BCR-ABL oncoproteins are found in Philadelphia chromosome-positive chronic myelocytic (CML) or acute lymphocytic (ALL) human leukemias (reviewed in [1]). In vivo CML model utilizing retroviruses and studies on BCR-ABL transgenic mice have demonstrated that BCR-ABL causes leukemias. Transformation of Rat1 fibroblasts and abolishment of growth factor dependence in, for example, interleukin-3 (IL-3)-dependent Ba/F3 cells are convenient ways to test the biological activities of BCR-ABL. Rat1 transformation is inhibited in BCR-ABL with various mutations including (a) 177 Y (tyrosine) to F (phenylalanine) which abolishes GRB-2 binding [2], (b) 1294 Y to F (major autophosphorylation site) [3], (c) SH2 domain mutation [4], and (d) a deletion of aminoterminal 40 amino acids (BCR-ABL Δ1–40) which destroys the BCR tetramer domain [1,5–7] and so forth. However, while the 177F and 1294F mutations still retain their ability to abrogate growth factor dependence, the BCR-ABL Δ1–40 mutation does not. In order to examine how tetramerization can help activate ABL, we attempted to reactivate the ability of BCR-ABL Δ1–40 to induce growth factor independence by the following modifications.

First, we coexpressed BCR-ABL Δ1–40 with MYC, because BCR-ABL not only cooperates with MYC but also dominant negative MYC expressions inhibit biological activities of BCR-ABL [8,9]. Furthermore, it has recently been shown that MYC expression can compliment the SH2 domain mutation of BCR-ABL [4]. Second, we made additional deletion mutations to BCR-ABL Δ1–40. The c-ABL protein can be activated by deletions of either the SH3 domain (ΔSH3) [10–12] or a part of the last exon-encoded sequence (for example, Δ*Pvu*II) [13]. Third, a myristoylation signal was added to BCR-ABL Δ1–40. Both SOS and FES have been shown to be activated by membrane routing by an addition of a myristoylation signal [14,15]. Among these strategies, only the SH3 deletion restored the ability to induce growth factor independence to BCR-ABL Δ1–40.

2. Materials and methods

P185 BCR-ABL ΔSH3 was constructed by excision of *Bgl*II-to-*Hin*cII fragment followed by an in-frame fusion and the *Pvu*II deletion was made as described [13]. The first 41 amino acids from v-ABL (*Pst*I-to-*Pst*I fragment) [10] that contain a myristoylation signal were added to aminoterminus of P185 BCR-ABL Δ1–40 [6]. To simultaneously express both P185 BCR-ABL Δ1–40 and MYC, a modified version of a retrovirus expression vector pSRαMSVtkneo was utilized [9]. Retroviruses were made by the COS cell coexpression method described [6]. Ba/F3 cells were cultured in RPMI/10% fetal calf serum supplemented with 15% conditioned medium from actively growing WEHI 3B cells as a source of interleukin-3 (IL-3). The 96-wells assay to test the ability to induce IL-3 independence was performed as described [3,16,18]. Approximately 10^6 Ba/F3 cells were infected with retroviruses and were cultured in the presence of IL-3. At 3 days postinfection, cells were washed 3 × to remove IL-3 and were plated into 96-wells culture dishes at roughly 10^4 cells/well. After 2 weeks wells that showed an outgrowth of IL-3-independent cells were scored positive. Rat1 transformation assay was performed as described [6,8]. Immunoprecipitation and Western blot analysis of BCR-ABL or GRB-2 were performed as described [4]. Anti-GRB-2 antibody is a gift from Dr. T. Takenawa at University of Tokyo.

3. Results and discussion

BCR-ABL with a deletion of the tetramer domain (BCR-ABL Δ1–40) can no longer induce growth factor independence [7]. Autophosphorylation and transphosphorylation of other cellular proteins like GAP (GTPase activating protein)-associated p62 or SHC are dramatically reduced in BCR-ABL Δ1–40 [7]. In order to obtain a hint to investigate the significance of tetramerization in BCR-ABL, several additional modifications to P185 BCR-ABL Δ1–40 (Fig. 1) were made to reactivate the ability to induce growth factor independence. The modifications include coexpression with MYC [4], deletion of the ABL SH3 domain [10–12], deletion of a part of the ABL last exon-encoded sequence (*Pvu*II/*Pvu*II deletion) [13], aminoterminal tagging with a sequence containing a myristoylation signal from v-ABL, and combinations of those (Fig. 1). Helper-free retroviruses expressing those constructs were made according to the transient COS cell expression system described previously [6].

Interleukin-3 (IL-3)-dependent mouse Ba/F3 cells were infected with those retroviruses and 2 different types of assay were performed. First, infected cells were deprived of IL-3 72 h after infection and plated into 96-wells to grossly quantitate the

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ability to induce growth factor independence. Second, the whole infected population of cells were cultured in the presence of both IL-3 and G418 at 2 mg/ml for 10 days and then cultures were further continued in the absence of IL-3 for up to 4 weeks. BCR-ABL constructs that showed completely no survival of cells in the second assay were scored negative (−) in Fig. 1. The SH3 deletion turned out to be the only modification that could restore growth factor independence to P185 BCR-ABL Δ1–40. BCR-ABL mutants with the SH3 deletion could cause growth factor independence with a similar efficiency as wild type BCR-ABL when judged by the 96-wells assay (Fig. 1). An antiphosphotyrosine Western blot analysis of the survived cells revealed that autophosphorylation as well as tyrosine phosphorylation of other cellular proteins including SHC and p62, which are good indicators of BCR-ABL-mediated transformations [6,17,18], were also recovered (Fig. 2B). The intensities of autophosphorylation relative to the protein amounts were roughly the same in all forms of BCR-ABL that could induce growth factor independence. The tyrosine 177 (Y177) is one of the autophosphorylation sites in BCR-ABL and is recruited with GRB-2, an SH2/SH3-containing adaptor protein, which links between BCR-ABL and RAS pathway [2,17–19]. An anti-GRB-2 Western blot analysis of anti-ABL immunoprecipitates showed that the GRB-2 proteins bind to the reactivated BCR-ABLs (Fig. 2C).

In Rat1 transformation assay, BCR-ABL with the SH3 domain deletion or *Pvu*II/*Pvu*II deletion was as transforming as wild type BCR-ABL. BCR-ABL Δ1–40 reactivated by the additional deletion of the SH3 domain was poorly expressed in Rat1 cells and its expression level was 10–20% of that of wild type BCR-ABL. The number of Rat1 colonies in soft agar derived from this mutant was roughly 20% of that in wild type BCR-ABL. Therefore, we tentatively conclude that the deletion of the SH3 domain not only reactivates the ability of BCR-ABL Δ1–40 to induce growth factor independence but also the potential to transform Rat1 cells.

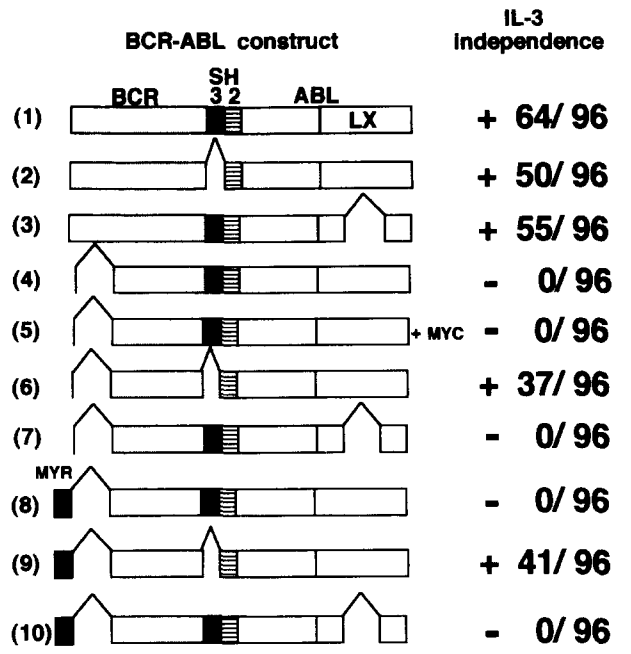

Fig. 1. Deletion of the SH3 domain restores growth factor independence to P185 BCR-ABL Δ1–40. Schematic representation of the mutated P185 BCR-ABLs (1–10). LX, SH3, SH2, MYR (8–10), and MYC (5) show sequences encoded by the ABL last exon, the SH3 and SH2 domains, v-ABL sequences containing a myristoylation signal, coexpressed MYC, respectively. Deletion of amino acids 1–40 in BCR-ABL (4–10), deletion of the *Pvu*II/*Pvu*II sequence (3, 7, 10), and deletion of the SH3 domain (2, 6, 9) are shown. Ability (+) and inability (−) to induce interleukin-3 (IL-3) independence in Ba/F3 cells and results of 96-wells assays are shown on the right (see text).

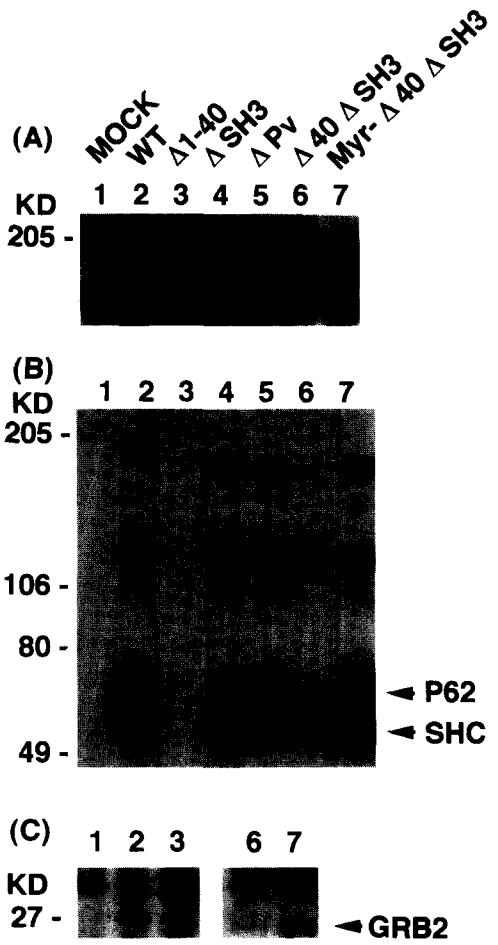

Fig. 2. Deletion of the SH3 domain restores autophosphorylation as well as transphosphorylation to P185 BCR-ABL Δ1–40. (A,B) Anti-ABL (A) and antiphosphotyrosine (B) Western blot analyses of total cell lysates from Ba/F3 cells expressing indicated molecular constructs. Ba/F3 cells were infected with mock (1), P185 BCR-ABL wild type (2: WT), and P185 Δ1–40 (3: Δ1–40) retroviruses, selected by G418 for 2 weeks in the presence of IL-3. Lanes 4 (ΔSH3) (P185 with SH3 domain deletion), 5 (ΔPv) (P185 with deletion of the *Pvu*II/*Pvu*II sequence), 6 (Δ40 ΔSH3) (P185 Δ1–40 with SH3 domain deletion), and 7 (Myr-Δ40 ΔSH3) (P185 Δ1–40 with a myristoylation signal and SH3 domain deletion) show Ba/F3 cells that survived IL-3 deprivation. The tyrosine-phosphorylated P62 and SHC proteins are indicated by arrows. (C) Anti-ABL immunoprecipitates from Ba/F3 cells expressing mock (1), P185 (2), P185 Δ1–40 (3) in the presence of IL-3, and P185 Δ1–40 with SH3 domain deletion (6), P185 Δ1–40 with a myristoylation signal and SH3 domain deletion (7) in the absence of IL-3 were subjected to an anti-GRB-2 Western blot analysis.

Biological function of the SH3 domain is to mediate protein traffic [20,21]. In GRB-2, PLCγ, and type IV ABL, the destruction of the SH3 domain relocalizes the protein from membrane ruffle, actin cytoskeleton, and nucleus, respectively [22,23]. Type IV ABL relocalized from nucleus to cytoplasm by the deletion of the SH3 domain acquires increased tyrosine kinase

activity. BCR-ABL, however, is totally cytoplasmic and a BCR-ABL mutant with a deletion of the first 60 amino acids of BCR with a tetramerizing property has a decreased binding to actin cytoskeleton but is still in the cytoplasm. Since association of BCR-ABL with actin cytoskeleton is governed by carboxyterminal ABL sequences and not by either the SH3 domain or kinase activity of the protein [5,24], the deletion of the SH3 domain in BCR-ABL Δ1–40 may not significantly alter its intracellular localization.

Mutations in the SH3 domain has been reported to activate not only nuclear c-ABL but also cytoplasmic c-SRC [25,26]. Discoveries of molecules such as 3BP-1 [27] and Abi-2 [28] that bind to the ABL SH3 domain strongly suggest that functions of ABL could be regulated by protein/protein interactions through the SH3 domain [20,21]. Although in vivo binding to ABL has not been detected in 3BP-1, it is interesting that this protein functions as a GAP (GTPase activating protein) for RAC family of small GTP-binding proteins [27]. RAC is essential for RAS-mediated transformation of fibroblasts [29] and transforming forms of ABL elicit RAS signals [2,17–19]. Given the fact that GAP accelerates the conversion of GTP-bound active form of GTP-binding proteins to GDP-bound inactive form, the ABL protein might be always accompanied by a negative regulator for transformation. Presence of molecules that inhibit the catalytic function of ABL has been previously suggested [30]. The very recently discovered ABL SH3 domain-binding protein Abi-2 directly modulates the kinase activity as well as the transforming activity of c-ABL through dual SH3 domain/proline-rich sequence interactions [28]. Although we cannot rule out the possibility that deletion of the SH3 domain re-oligomerizes BCR-ABL Δ1–40, our current hypothesis is that BCR sequence-dependent tetramerization might somehow suppress the SH3 domain-mediated negative regulation of ABL.

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