Homodimerization of the c-Abl Protein Tyrosine Kinase
c-Abl 蛋白酪氨酸激酶形成同源二聚体

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摘 要 c-Abl 是非受体酪氨酸激酶,它在细胞内被一些基因毒性的、氧化的及其它形式的压力所激活。目前研究证明:应用标记的 c-Abl 发现其在细胞内可以相互形成同源二聚体,并且一分子 c-Abl 的 N 末端区域与相应的另一分子的 C 末端相互作用形成二聚体。实验进一步表明:c-Abl SH3 结构域结合到另一 c-Abl 分子富含脯氨酸的 C-末端约 958-982 氨基酸区域。如果去除 c-Abl 富含脯氨酸的结构域,就会阻止二聚体的形成。这些结果首先证实了 c-Abl 在细胞内可以相互形成同源二聚体,并暗示着二聚体的形成可能影响着 c-Abl 活性的调节。

关键词 c-Abl,相互作用,同源二聚体,SH3
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Abstract The c-Abl nonreceptor tyrosine kinase is activated in the cellular responses to genotoxic, oxidative and other forms of stress. Using tagged forms of c-Abl, the present studies demonstrate that c-Abl forms homodimers in cells. The results show that the c-Abl N-terminal regions interact with the corresponding C-terminal regions of both partners in the dimmer. Specifically, the c-Abl SH3 domain binds to a proline-rich motif at amino acids 958-982 in the c-Abl C-terminal region. Deletion of the proline-rich motif disrupts dimmer formation. These findings provide the first evidence that c-Abl forms homodimers and indicate that homodimerization can contribute to the regulation of c-Abl activity.

Key words c-Abl, interaction, homodimers, SH3

c-Abl is a non-receptor tyrosine kinase, of which the precise functions are not known, but roles for Abl in growth factor and integrin signaling, cell cycle regulation, neurogenesis, and responses to DNA damage and oxidative stress have been suggested[1-5]. c-Abl kinase activity is increased *in vivo* by diverse physiological stimuli including ionizing radiation[6], entry into S phase[7], integrin activation[8], and platelet-derived growth factor stimulation[9]. The mechanism of regulation of Abl tyrosine kinase activity by these processes is not well understood. Abl kinase activity can also be stimulated by the binding of several activator proteins[10]. These observations suggest

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complex regulation of c-Abl at multiple levels through binding or dissociation of activators and inhibitors and via direct phosphorylation[11,12]. The structural organization from the N- to the C-terminus of Abl encoded by a single exon includes a short region containing a myristylation signal, an SH3 domain, an SH2 domain, a catalytic domain and a large C-terminal region. The SH2 domain has high affinity to phosphorylated tyrosine residues, the SH3 domain preferentially binds to proline-rich domains with a PxxP motif and the long C-terminal tail contains nuclear localization (NLS) and nuclear export (NES) signals controlling c-Abl sub-cellular localization[13], a DNA binding domain, an actin binding domain and several proline-rich sequence[14,15]. With all of these structural domains, c-Abl is likely to simultaneously participate in many processes by direct protein-protein interactions[16]. In this report we show that the deletion of C-terminal proline-rich sequences abrogates its interaction with another c-Abl to forms homodimers and indicate that homodimerization can contribute to the regulation of c-Abl activity.

1 MATERIALS AND METHODS

1.1 Cell culture

HEK293 cells (ATCC) were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100μg/mL penicillin and 100μg/mL streptomycin. Transient transfections were performed with Lipofectamine (Life Technologies, Inc.) and cells were harvested 36h after transfection.

1.2 Vectors

Flag-tagged c-Abl and its mutants were expressed by cloning c-Abl and its mutants into the pcDNA3.1-based Flag vector (Invitrogen). His/Exp-tagged constructs were prepared by cloning c-Abl (1-486) into pcDNA4HisMAX which contains N-terminal His and Express tags (Invitrogen). Myc-tagged c-Abl and mutants were prepared by cloning c-Abl and mutants into the pCMV-Myc (Clontech). Glutathione S-transferase (GST) fusion proteins were generated by expression of pGEX4T2-based vectors in *E. coli* BL21 (DE3). These vectors were all constructed by the group of Professor Dr. Cao (Beijing Institute of Biotechnology, Academy of Military Medical Sciences).

1.3 Immunoprecipitation and immunoblot analysis

Cell lysates were prepared in lysis buffer (50mmol/L Tris-HCl, pH 7.5, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 10 mmol/L sodium fluoride, and 10μg/mL aprotinin, leupeptin and pepstatin A) containing 1% Nonidet P-40. Soluble protein was subjected to immunoprecipitation with anti-Flag conjugated gel (M2, Sigma, MO), anti-Myc conjugated agarose (Santa Cruz, CA). Immunoblot analysis was performed with HRP conjugated anti-Myc, HRP conjugated anti-Flag (Sigma, MO), HRP conjugated anti-Exp (Invitrogen, CA). The antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech). An aliquot of the total lysate (7% *V*/*V*) was included as a control.

1.4 Binding assays

Cell lysates were incubated with 2μg of GST or GST fusion proteins bound to glutathione beads. After incubation for 2 h at 4℃, the adsorbates were washed with lysis buffer and then subjected to immunoblot analysis. An aliquot of the total lysate (7% *V*/*V*) was included as a control. For *in vitro* binding assays, purified GST fusion proteins were incubated with *in vitro* translated [^{35}S]-labeled Flag-c-Abl (Amersham Biosciences Biotech, Inc). The adsorbates were analyzed by SDS-PAGE and autoradiography.

2 RESULT AND DISCUSSION

2.1 Formation of c-Abl homodimers *in vivo* and *in vitro*

To determine whether c-Abl forms homodimers *in vivo*, lysate of the cells expressing Flag- and Myc-tagged c-Abl were subjected to immunoprecipitation with anti-Flag. Analysis of the immunoprecipitates with anti-Myc demonstrated the coprecipitation of Myc-c-Abl with Flag-c-Abl (Fig. 1a). In the reciprocal experiment, immunoblot analysis of anti-Myc immunoprecipitates with anti-Flag confirmed the detection of complexes containing Myc-c-Abl and Flag-c-Abl (Fig. 1b).

To define the basis for the association, lysates from cells expressing Flag-c-Abl were incubated with GST fusion proteins containing the c-Abl SH3 or SH2 domains. Analysis of the adsorbates with anti-Flag showed no detectable binding of Flag-c-Abl to GST. By contrast, analysis of the adsorbates to the GST fusion proteins demonstrated binding of Flag-c-Abl to c-Abl SH3 (Fig. 2a). In other experiments, GST and the GST-c-Abl fusion proteins were incubated with *in vitro*

translated [^{35}S]-labeled Flag-c-Abl. Autoradiography demonstrated binding of c-Abl to GST-c-Abl SH3 but not to GST or GST-c-Abl SH2 (Fig. 2b). These findings demonstrate that the SH3 domain of c-Abl binds directly to c-Abl.

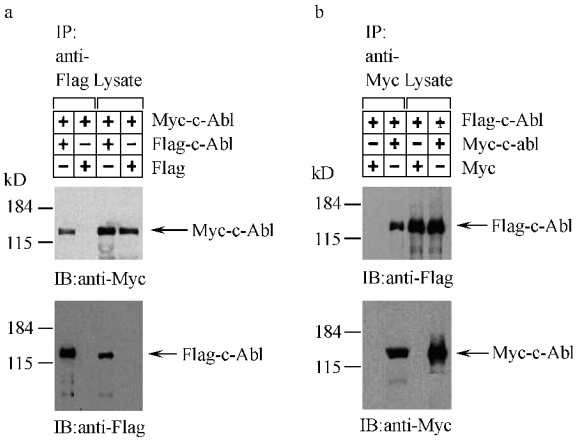


Fig. 1 c-Abl forms homodimer *in vivo*

a 293 cells were cotransfected with Myc-c-Abl and Flag-c-Abl or Flag vector, anti-Flag immunoprecipitates of lysate of the cells were analyzed by immunoblotting with anti-Myc or anti-Flag (left panel); lysate of the cells were analyzed by immunoblotting with anti-Myc or anti-Flag (right panel). b 293 cells were cotransfected with Flag-c-Abl and Myc-c-Abl or Myc vector, anti-Myc immunoprecipitates of lysate of the cells were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Flag or anti-Myc (left panel); lysate of the cells were analyzed by immunoblotting with anti-Flag or anti-Myc (right panel).

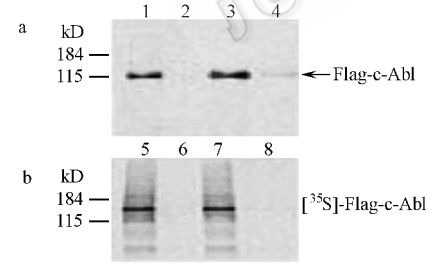


Fig. 2 c-Abl SH3 binds to c-Abl *in vivo* and *in vitro*

a: Lysates of 293 cells expressing Flag-c-Abl were incubated with GST, GST-c-Abl SH3 or GST-c-Abl SH2 conjugated agarose beads, the adsorbates were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Flag. b: *In vitro* translated [^{35}S]-labeled Flag-c-Abl were incubated with GST, GST-c-Abl SH3 or GST-c-Abl SH2 conjugated agarose beads, the adsorbates were subjected to SDS-PAGE and analyzed by autoradiography. 1: Lysate; 2,6: GST; 3,7: GST-c-AblSH_2; 4,8: GST-c-Abl SH2.

2.2 c-Abl SH3 binds to the c-Abl C-terminus

To further define regions of c-Abl that contributes to homodimerization, cells expressing Exp-c-Abl (1-486) and Flag-c-Abl were subjected to immunoprecipitation with anti-Flag or, as a control, with IgG. Analysis of the immunoprecitates with anti-Exp demonstrated coprecipitation of the N-terminal region of c-Abl with full-length c-Abl (Fig. 3a, left panel). Immunoblot analysis of anti-Myc immunoprecipitates from cells expressing Flag-c-Abl (487-1130) and Myc-Abl further demonstrated that the C-terminal region of c-Abl binds to the full-length c-Abl (Fig. 3a, right panel). To extent this analysis, anti-Flag immunoprecipitates from cells expressing Exp-c-Abl (1-486) and Flag-c-Abl (487-1130) were analyzed by immunoblotting with anti-Exp. The results demonstrate that the c-Abl N-terminal region associates with the c-Abl C-terminal (Fig. 3b, left panel). To confirm these results, GST and GST-c-Abl SH3 were incubated with [^{35}S]-labeled Flag-c-Abl (487-1130). Analysis of the adsorbates showed that c-Abl SH3 binds directly to the c-Abl C-terminal region (Fig. 3b, right panel).

2.3 c-Abl SH3 interacts with the c-Abl at amino acids 958-982

The c-Abl C-terminal region contains several proline-rich sites that conform to consensus sequences for binding to the c-Abl SH3 domain. To identify the binding site, anti-Flag immunoprecipitates from cells expressing Exp-c-Abl (1-486) and Flag-c-Abl (487-759) or Flag-c-Abl (616-1130) were analyzed by immunoblotting with anti-Exp. The results indicate that the c-Abl SH3 binding site resides between amino acids 759 and 1130 (Fig. 4a). To more precisely map the site, proline rich sequences in that region of the C-terminus were mutated or deleted (Fig. 4b). Anti-Flag immunoprecipitates from cells expressing Exp-c-Abl (1-486) and mutated or deleted Flag-c-Abl (616-1130) demonstrated that deletion of amino acids 958-982 abrogates binding to the c-Abl N-terminus (Fig. 4c). By contrast, mutation of the other proline-rich sites had no effect on binding. To confirm this finding, GST-c-Abl SH3 was incubated with lysates from cells expressing Flag-c-Abl (616-1130) or Flag-c-Abl (616-1130) (Δ958-982). The results show that deletion of amino acids 958-982 abrogates c-Abl SH3 binding (Fig. 4d, left panel). Similar results were obtained when c-Abl SH3 was incubated with lysates of cell expressing full-length c-Abl deleted at amino acids 958-982 (Fig. 4d, right panel).

It was proposed that similar to other Src kinases, the c-Abl kinase domain is under repression by an intra-molecular mechanism[17]. Indeed, Pluk and coworkers showed that the inhibition of c-Abl activity is an intrinsic property and does

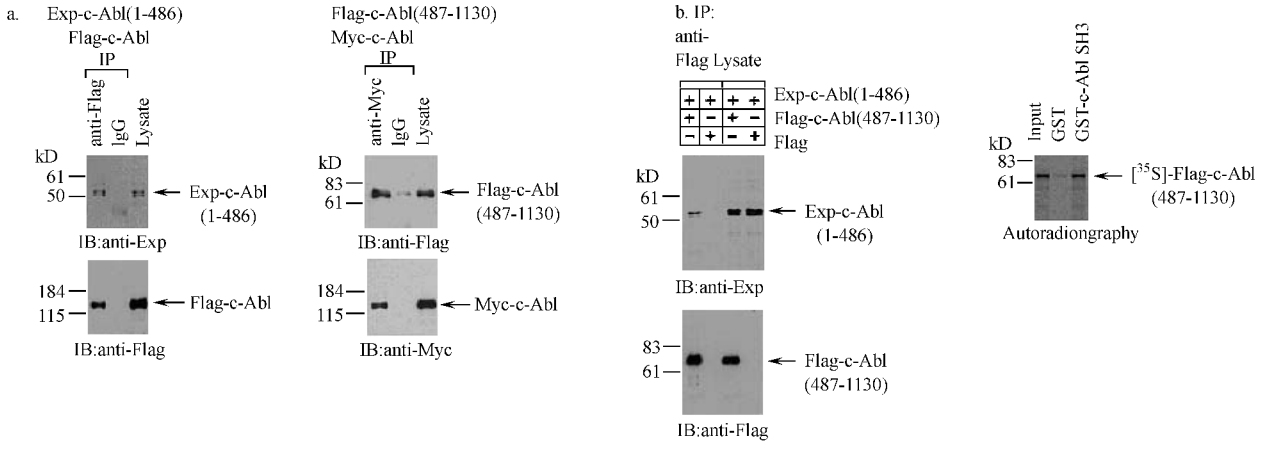
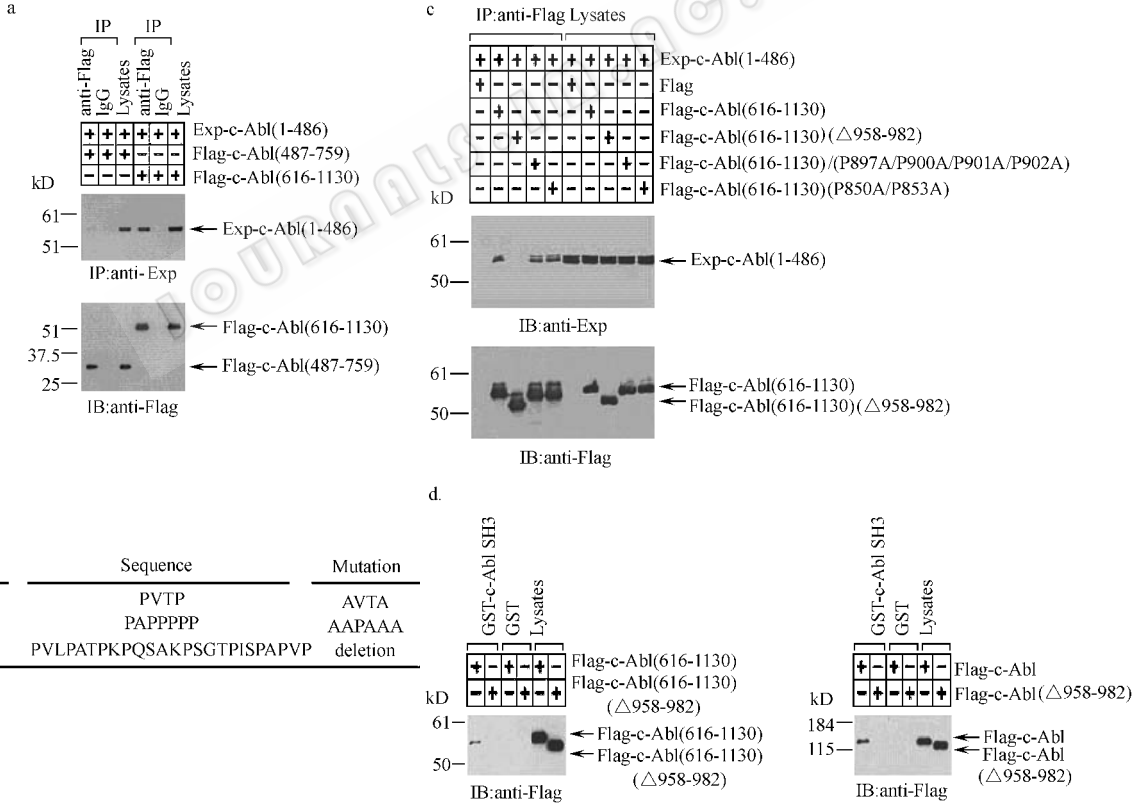


Fig. 3 c-Abl N terminal(1-486) interacts with c-Abl C terminal(487-1130)

a :293 cells were co-transfected with Flag-c-Abl and Exp-c-Abl(1-486) (left panel), or with Myc-c-Abl and Flag-c-Abl(487-1130)(right panel), cell lysates were subjected to immunoprecipitation with anti-Flag(left panel) or anti-Myc(right panel), and the immunoprecipitates were analyzed by immunoblotting with anti-Exp and anti-Flag(left panel), or anti-Flag and anti-Myc(right panel). b :293 cells were co-transfected with Exp-c-Abl(1-486) and Flag-c-Abl(487-1130) or Flag vector, anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-Exp or anti-Flag(left panel). In vitro translated [35S]-labeled Flag-c-Abl(487-1130) were incubated with GST-c-Abl SH3 or GST conjugated agarose beads, adsorbates were subjected to SDS-PAGE followed by autoradiography.



b

Abl Amino Acids	Sequence	Mutation
850-853	PVTP	AVTA
897-903	PAPPPPP	AAPAAA
958-982	PVLPATPKPQSAKPSGTPISPAPVP	deletion

Fig.4 c-Abl N terminal interacted with c-Abl C terminal at Proline rich domain

a :293 cells were transfected with Exp-c-Abl(1-486) and Flag-c-Abl(487-759) or Flag-c-Abl(616-1130), anti-Flag immunoprecipitates were subjected by immunoblotting with anti-Exp or anti-Flag. b :Mutations in Proline rich region. c :293 cells were co-transfected with Exp-c-Abl(1-486) and Flag-c-Abl(616-1130) or its mutants, anti-Flag immunoprecipitates were subjected to immunoblotting with anti-Exp and anti-Flag. d :293 cells were transfected with Flag-c-Abl(616-1130) or Flag-c-Abl(616-1130)(Δ958-982), cell lysates were incubated with GST-c-Abl SH3 or GST conjugated agarose beads, and the adsorbent were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Flag(left panel). As in left panel except 293 cells were transfected with full length Flag-c-Abl or Flag-c-Abl(Δ958-982)(right panel).

not require a particular cellular inhibitor. The kinase activity of c-Abl is tightly regulated *in vivo*. It has been postulated that the kinase activity is negatively regulated by a factor binding to the SH3 domain of c-Abl[18,19]. The group of Dr. Goff[20] provided the evidence for the intermolecular interactions between c-Abl proteins. They found that deletion of the NH_2-terminal 509 amino acids from one binding partner appears to abolish the interaction. These findings only indicated that NH_2-terminus were required for this interaction. In this study, our results not only demonstrate that the c-Abl N-terminal regions interact with the corresponding C-terminal regions of both partners in the dimmer, but show that the c-Abl SH3 domain binds to a proline-rich motif at amino acids 958-982 in the c-Abl C-terminal region. These results suggest the possibility that interaction of c-Abl may contribute to regulation of its kinase activity and may also play an important role in cytoskeletal reorganization and cell motility.

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