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SHP-2 inhibits tyrosine phosphorylation of Cas-L and regulates cell migration

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ABSTRACT

The Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-2, plays an important role in cell migration by interacting with various proteins. In this report, we demonstrated that SHP-2 inhibits tyrosine phosphorylation of Crk-associated substrate lymphocyte type (Cas-L), a docking protein which mediates cell migration, and found that SHP-2 negatively regulates migration of A549 lung adenocarcinoma cells induced by fibronectin (FN). We showed that overexpressed SHP-2 co-localizes with Cas-L at focal adhesions and that exogenous expression of SHP-2 abrogates cell migration mediated by Cas-L. SHP-2 inhibits tyrosine phosphorylation of Cas-L, and associates with Cas-L to form a complex in a tyrosine phosphorylation-dependent manner. Finally, immunoprecipitation experiments with deletion mutants revealed that both SH2 domains of SHP-2 are necessary for this association. These results suggest that SHP-2 regulates tyrosine phosphorylation of Cas-L, hence opposing the effect of kinases, and SHP-2 is a negative regulator of cell migration mediated by Cas-L.

Introduction

Cas-L/neural precursor cell expressed, developmentally down-regulated 9 (NEDD9)/human enhancer of filamentation 1 (HEF1) (hereafter designated Cas-L) is a multifunctional scaffolding protein that belongs to the Cas family [1]. It has diverse functions, including having a role in the regulation of cell migration [2–6], invasion [3,7], cell cycle [8–11], and apoptosis [12,13]. With regard to cell migration, we and others have reported that Cas-L is tyrosine phosphorylated on stimulation with integrin and growth factor receptors, and tyrosine phosphorylated Cas-L interacts with Crk, Nck, and C3G [1,14]. This interaction is supposed to result in the activation of small GTPases, which promotes cell migration. Furthermore, recent publications have reported novel findings on the role of Cas-L in tumor progression [3,7]. These reports hence demonstrate the crucial role of Cas-L in the pathogenesis of tumor metastasis *in vivo*.

With regard to the regulation of Cas-L tyrosine phosphorylation, while the role of kinases such as FAK and Src has been well-understood, little is known about phosphatases which regulate tyrosine phosphorylation in an opposite manner. In a previous study, Minegishi et al. reported that SHP-2 associates with Cas-L, and this finding leads us to our present effort to investigate the interaction between SHP-2 and Cas-L.

SHP-2 is a ubiquitously expressed cytoplasmic, protein tyrosine phosphatase (PTP) that has two SH2 domains at the N-terminal region, a PTP domain, and a COOH-terminal tail. There is much evidence that SHP-2 transduces signals from growth factors and cytokines, and regulates the cell migration. For example, Ren et al. reported that SHP-2 associates with Gab1, and is involved in epidermal growth factor (EGF)-induced paxillin dephosphorylation and Src tyrosine kinase activation [15]. Manes et al. reported that SHP-2 dephosphorylates FAK and increases cell migration by promoting turnover of focal adhesions in insulin-like growth factor (IGF)-1-stimulated MCF-7 cells [16]. In these reports, SHP-2 thus functions as a positive regulator of cellular migration. On the other hand, other investigators have reported that SHP-2 can also function as a negative regulator of cell migration [17–19]. Overall, the role of SHP-2 in cell migration appears to be dependent on the assay system.

In this study, we investigate the mechanisms involved in SHP-2 regulation of Cas-L-mediated cell migration and its tyrosine phosphorylation, with the goal of obtaining novel findings relating to SHP-2 function.

Experimental procedures

Reagents and antibodies. Mouse monoclonal antibody (mAb) against Cas-L was from ImmuQuest (Cleveland, UK). Anti-SHP-2

Abbreviations: SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; Cas-L, Crk-associated substrate lymphocyte type.

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mAb was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-myc mAb (9E10) and anti-phosphotyrosine (pTyr) mAb (4G10) were produced from hybridoma obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Anti-Flag mAb was purchased from Sigma–Aldrich (St. Louis, MO, USA). All the chemicals and reagents were obtained from Sigma–Aldrich unless otherwise stated.

Cells, plasmids, and transfection procedures. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C with 5% CO_2. A549 cells were cultured in RPMI 1640 containing 10% FCS at 37 °C with 5% CO_2. c-myc-tagged Cas-L in pEB6 vector was described previously [20]. SHP-2 cDNA was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). pEB6-Flag-SHP-2 mutants, in which the cysteine 459 was changed to serine and/or aspartic acid 425 to alanine using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), which were designated as SHP-2 CS (catalytically inactive mutant [21]), SHP-2 DA and SHP-2 DM (DA/CS) (substrate-trapping mutant [22]), respectively. CAGGS-Flag SHP-2 CS ΔN-SH2 lacking aa 1–107, ΔN/C-SH2 lacking aa 1–216, ΔPTP lacking aa 214–597 were generated by PCR, using the primers containing the restriction sites for EcoRI and XhoI (ΔN-SH2: forward primer 5′-CGGAATTCACCTCTGAAAGGTGGT-3′, reverse primer 5′-CCCTCG AGTTGCGTCTGTTCTTG-3′; ΔN/C-SH2: forward primer 5′-CGGA ATTCAACACGACTCGTATAAATGCTGCTGAAATAG-3′, reverse primer 5′-CCCTCGAGCATAAACTTTCTTGCGTCTGTTCTTGATCTT-3′; ΔPTP: forward primer 5′-CGGAATTCACATCGCGGAGATGGT-3′, reverse primer 5′-CCCTCGAGTCAGTCAGTCATGTTAAGGGGCTGCTT-3′).

To generate constitutively active Fyn, tyrosine 528 was substituted by phenylalanine by introducing a punctual mutation into the 3′ primer. The resultant mutant Fyn (Y528F) was designated as Fyn (CA). The same method was employed to generate the following mutants: Fyn (K299M) as Fyn (KN) (KN, kinase negative). The plasmids were transfected into cells using Lipofectamine LTX with PLUS reagent (Invitrogen) according to the manufacturer's instructions.

Immunocytochemistry. For fluorescent microscopy experiments using A549 cells, the cells were grown on coverslips, and transfected with c-myc-tagged Cas-L alone, or together with Flag-tagged SHP-2, and were treated and stained as described previously [6].

Migration assay. A549 cell migration was assayed using 6.5 mm-diameter Transwell inserts (Corning, Inc., Life Science, Acton, MA, USA) with FN-coated polycarbonate filters (8.0 μm pore size) as previously described [4].

Immunoprecipitation and immunoblotting. The cells were lysed and subjected to immunoprecipitation and immunoblotting as previously described [6].

In vitro Cas-L tyrosyl dephosphorylation. GST (glutathione *S*-transferase)-SHP-2 fusion proteins used in the Cas-L dephosphorylation assay were kind gifts from Dr. Anton M. Bennett (Yale University School of Medicine), and purified using Microspin GST Purification Module (Amersham Biosciences), according to the manufacturer's instructions. Tyrosine-phosphorylated Cas-L was immunoprecipitated from A549 cellular lysates using anti-Cas-L antibodies, incubated with GST fusion proteins as previously described [23], subjected to immunoblotting.

Results

Exogenous SHP-2 co-localizes with Cas-L at focal adhesions

We first examined subcellular localization of SHP-2 in A549 human lung carcinoma cells. As previously reported, Cas-L co-localized with paxillin at focal adhesions [24,13] (Fig. 1, upper). Co-transfection studies of Flag-tagged SHP-2 with c-myc-tagged Cas-L analyzed by confocal microscopy showed that SHP-2 and Cas-L co-localized at focal adhesions (Fig. 1, lower). These observations suggested that SHP-2 may associate with Cas-L at focal adhesions and have a role in integrin-dependent functions of Cas-L.

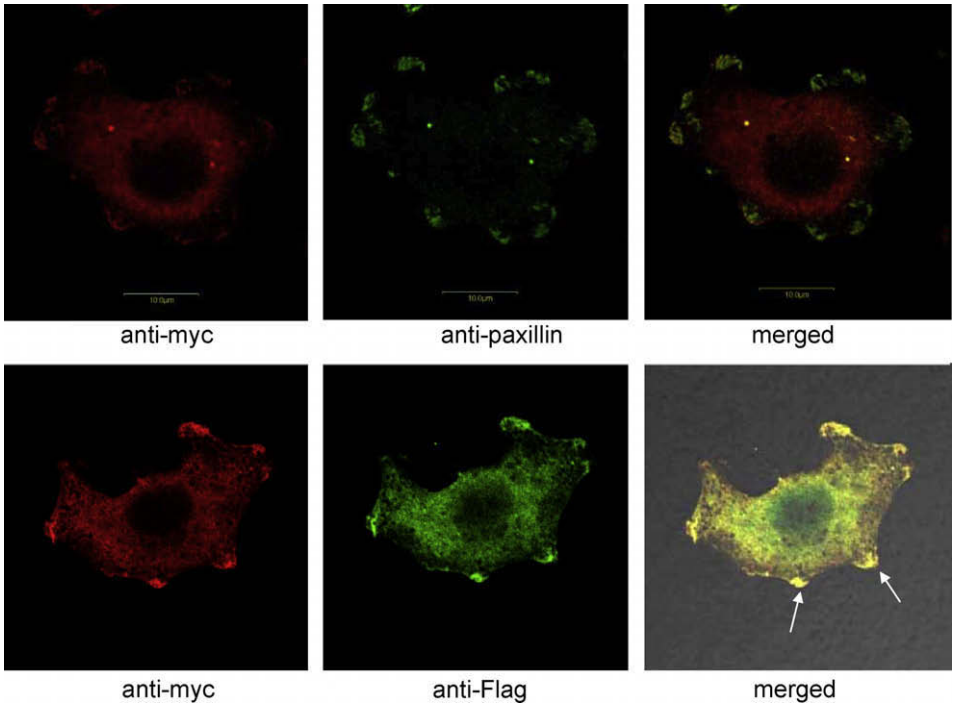

Fig. 1. Overexpressed SHP-2 co-localizes with Cas-L at focal adhesions. (Upper panel) A549 cells were transfected with plasmids expressing c-myc-tagged Cas-L, followed by immunocytochemistry using the indicated antibodies. Paxillin is indicated in green, and Cas-L in red. (Lower panel) A549 cells were transfected with plasmids expressing c-myc-tagged Cas-L and Flag-tagged SHP-2. SHP-2 is indicated in green, and Cas-L in red. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

SHP-2 negatively regulates A549 cell migration mediated by Cas-L

We hypothesized that SHP-2 may participate in integrin-induced cell migration mediated by Cas-L. We examined the motile behavior of A549 cells co-transfected with Cas-L and either pEB6 empty vector, wild-type SHP-2, or the catalytically inactive mutant SHP-2 CS (Fig. 2A and B). Upregulation of Cas-L resulted in a significant promotion of FN-induced migration of A549 cells, however, co-transfection with SHP-2 WT led to a remarkable decrease in cell migration. On the other hand, transfection with SHP-2 CS did not lead to a significant change in cell migration. These results suggest that SHP-2 negatively regulates cell migration mediated by Cas-L and that its phosphatase activity is indispensable for this observed regulatory activity.

SHP-2 dephosphorylates Cas-L

For the promotion of cell migration by Cas-L, its tyrosine phosphorylation is supposed to be critical. To investigate whether SHP-2 is involved in the regulation of Cas-L tyrosine phosphorylation, we performed phosphatase assay *in vitro*. Tyrosyl-phosphorylated Cas-L was immunoprecipitated from A549 cellular lysates and incubated with equal amount of GST, GST-SHP-2 WT (full-length wild-type SHP-2), GST-SHP-2 EA (constitutively active mutant of SHP-2) or GST-PTP WT (PTP domain alone). Our data indicated that wild-type SHP-2 exhibited a low basal level of PTPase activity, resulting in a minimal effect on the tyrosyl dephosphorylation of Cas-L (Fig. 3A). In contrast, GST-SHP-2 EA exhibited a greater capability to dephosphorylate Cas-L, and this activity was inhibited by vanadate. Meanwhile, the PTP domain alone also exhibited a high level of PTPase activity, being able to dephosphorylate Cas-L completely. The PTP domain alone was also prevented from dephospho-

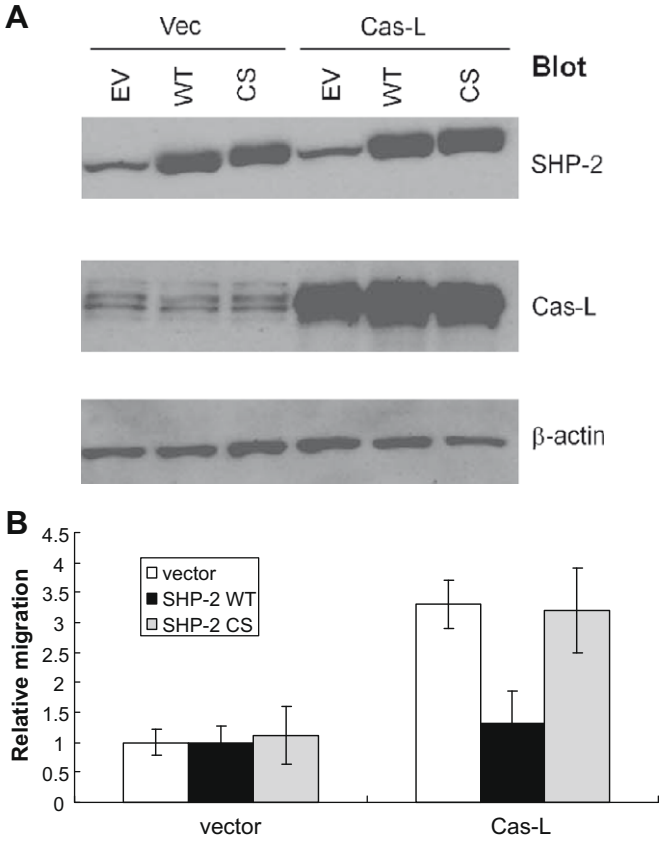

Fig. 2. SHP-2 negatively regulates Cas-L-mediated cell migration. (A) A549 cells were transfected with pEB6 vector, SHP-2 WT (wild-type), or SHP-2 CS (catalytically inactive mutant) in the presence or absence of Cas-L. Cell lysates were subjected to SDS–PAGE and immunoblotted with the indicated antibodies. (B) Transfected A549 cells were incubated for 4 h on FN coated filters with 8.0 μm pore, and fully migrated cells on the lower side were counted. The number of migrating empty vector-transfected cells was designated 1.0. Data shown were from four experiments performed in duplicate. Data were shown as means ± SE. Statistical analyses between the groups were performed by Student's *t* test.

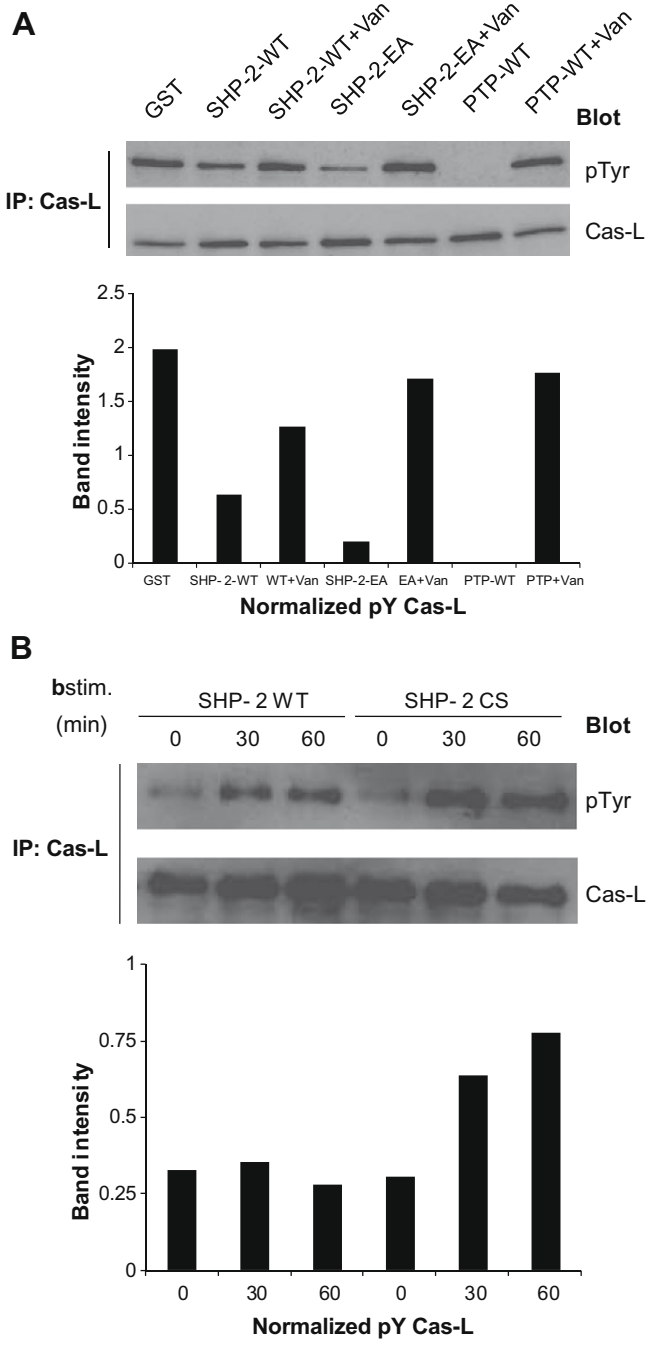

Fig. 3. SHP-2 inhibits tyrosine phosphorylation of Cas-L. (A) Cas-L immune complexes following *in vitro* dephosphorylation with the indicated GST fusion proteins were immunoblotted for phosphotyrosine. Where indicated, GST fusion proteins were incubated with 10 mM vanadate *(+ Van)*. Cas-L proteins were immunoprecipitated and analyzed with anti-phosphotyrosine mAb (anti-pTyr) and anti-Cas-L mAb. The graph below represents the densitometric analysis of tyrosyl-phosphorylated Cas-L normalized to Cas-L from the above experiment. (B) Cas-L was cotransfected with either SHP-2 WT or SHP-2 CS in A549 cells. The cells were then incubated in plates coated with FN for 45 min. Cas-L were immunoprecipitated and analyzed as in (A).

rylating Cas-L by preincubation with vanadate. These data suggest that the catalytic activity of SHP-2 may dephosphorylate Cas-L.

We next examined whether ectopic SHP-2 is capable of altering the tyrosine phosphorylation level of Cas-L in cells. As shown in Fig. 3B, compared with SHP-2 CS, overexpression of SHP-2 WT resulted in a significant decrease in the level of tyrosine-phosphorylated Cas-L in FN-stimulated A549 cells. This observation indicated that SHP-2 phosphatase activity directly and/or indirectly decreases the level of Cas-L tyrosine phosphorylation.

SHP-2 forms a substrate-trapped complex with Cas-L in vivo

To demonstrate that SHP-2 associates with Cas-L *in vivo*, we performed immunoprecipitation assay using substrate-trapping mutants within a cellular context. c-myc-tagged Cas-L was transfected with either the full-length SHP-2 or the three full-length mutants of Flag-tagged SHP-2 (SHP-2 CS, SHP-2 DA and SHP-2-DA/CS) in the presence of constitutively active Fyn (Fyn CA) or kinase negative Fyn (Fyn KN). The previous study showed that SHP-2 DA/CS mutant functions as a trapping mutant while SHP-2 DA does not [22]. As shown in Fig. 4A, ectopically expressed c-myc-tagged Cas-L formed a complex with the substrate-trapping mutants, SHP-2-CS and SHP-2-DA/CS, and the association increased dramatically when Cas-L was tyrosine-phosphorylated, whereas no complex formation was detectable with SHP-2-WT and SHP-2-DA. Collectively, these data suggest that SHP-2 associates with tyrosine phosphorylated Cas-L to form a complex *in vivo*. To determine the domain of SHP-2 necessary for the interaction with Cas-L, truncated SHP-2 mutants were constructed from SHP-2 CS, and coexpressed with Cas-L. As shown in Fig. 4B, SHP-2 ΔN-SH2 precipitated Cas-L, although the interaction was weaker than that observed with full-length SHP-2. ΔN/C-SH2 could interact with Cas-L to a much less extent. While there was almost no interaction observed between ΔPTP and Cas-L, since the Flag blot as control was also weak, the association between them could not be clearly elucidated. These results show that both SH2 domains of SHP-2 are necessary for the interaction with Cas-L.

Discussion

In this study we demonstrated that SHP-2 inhibits tyrosine phosphorylation of Cas-L, and negatively regulates the cell migration induced by Cas-L. Furthermore, our data raise the possibility that Cas-L is a direct substrate for SHP-2, although SHP-2 may inhibit Cas-L phosphorylation indirectly by regulating kinase activity. On integrin/growth factor receptor stimulation, SHP-2 dephosphorylates focal adhesion-associated proteins such as paxillin and FAK [15,16,25,26,18]. This dephosphorylation may result in turnover of focal adhesion sites and occasionally, activation of ERK pathway. In those reports, SHP-2 functions as a positive regulator of cell migration, however, SHP-2 may also act as a negative regulator. Mathew et al. reported that SHP-2 inhibits tyrosine phosphorylation of the adaptor protein villin by inactivating c-src and negatively regulates cell migration [19]. Taken together with our results, we propose a scheme in which SHP-2 has a diverse and intricate regulatory role in the presence of downstream adaptor molecules such as villin and Cas-L. SHP-2 has been known to be a promoter of cancer progression; however, it may function in a different manner in Cas-L overexpressed tissues.

There are some reports with regard to the association between Cas family protein and other non-receptor type PTPs, which may be clue for further investigation of SHP-2-Cas-L interaction. For example, PTP-proline, glutamate, serine, and threonine sequence protein (PTP-PEST) dephosphorylates p130Cas as a substrate [27]. Ceacareanu et al. reported that nitric oxide (NO) decreases the phosphor-

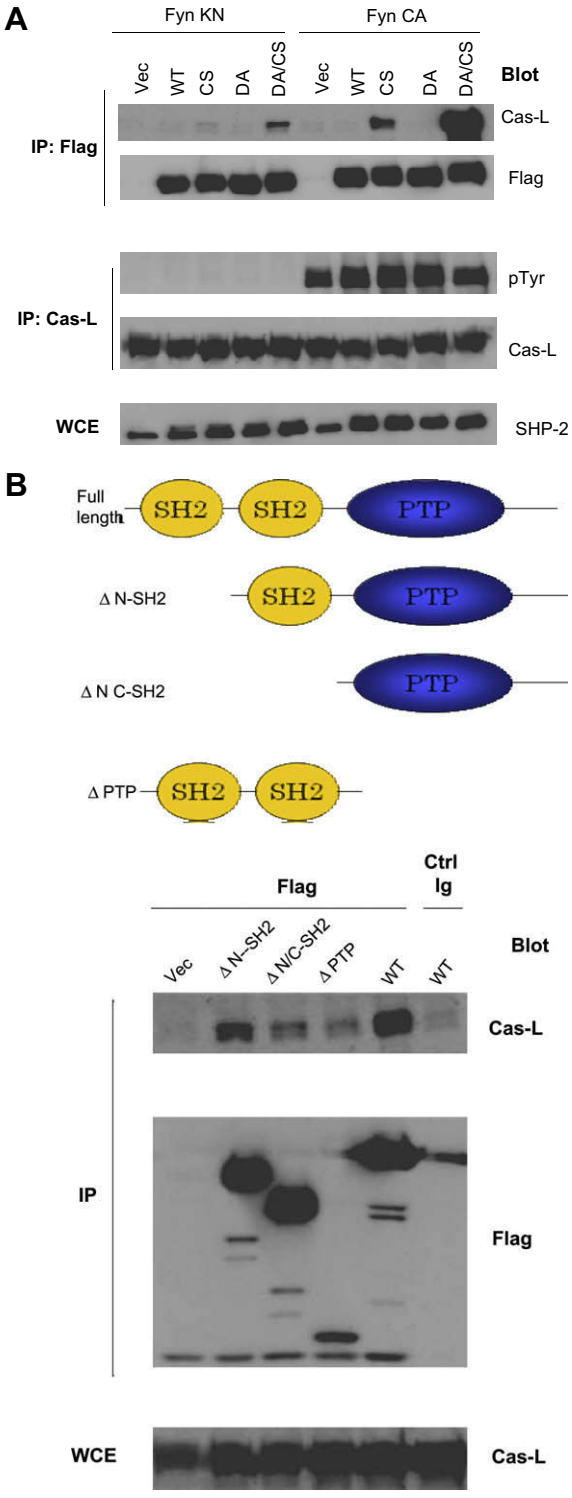

Fig. 4. SHP-2 forms a complex with Cas-L *in vivo*. (A) 293T cells were co-transfected with c-myc-Cas-L along with either pEB6 control vector, Flag-SHP-2 WT, SHP-2 CS, SHP-2 DA, or SHP-2 DM (DA/CS), in the presence of kinase negative Fyn (Fyn KN) or constitutively active Fyn (Fyn CA). Flag immunoprecipitates from these transfectants were resolved and immunoblotted for Cas-L. The immunoblots were re-probed with anti-Flag antibodies. The quantities and tyrosine phosphorylation levels of Cas-L were assessed by immunoprecipitation using anti-c-myc antibody following by immunoblotting with the indicated antibodies. The quantity of SHP-2 was shown by immunoblotting. (B) Flag-tagged full-length SHP-2 CS in a pEB6 vector (FL), pEB6-Flag-SHP-2 CS ΔN-SH2 domain (ΔN-SH2), pEB6-Flag-SHP-2 CS ΔN/C-SH2 domain (ΔN/C-SH2), pEB6-Flag-SHP-2 ΔPTP domain (ΔPTP) were made and co-transfected with Cas-L and constitutively active Fyn in 293 T cells. Flag immunoprecipitates from these transfectants were analyzed as in (A). The quantity of c-myc-Cas-L was shown by immunoblotting.

ylation level of p130Cas and Rac1 activity by activating PTP-PEST [28]. Furthermore, Davidson et al. showed that PTP-PEST interacts with p130Cas, paxillin, Csk and negatively regulates lymphocyte activation by dephosphorylating p130Cas, FAK, Shc, and inhibiting Ras signaling pathway. These results suggest that PTP-PEST, together with SHP-2, may play an important role in the biological processes mediated by Cas-L. We performed the loss-of-function assay using shSHP-2; however, we were not able to obtain any significant result. One possibility for this observation may be that other PTPs such as PTP-PEST compensate for the function of SHP-2 in our assay system.

Another possibility which remains to be investigated is the potential role of Cas-L as a scaffolding protein for SHP-2. As a potentially related example, the SHP-2 binding protein, Grb-2 associated binder (Gab) 2, interacts with and activates SHP-2 when it is tyrosine phosphorylated by Bcr-Abl or overexpressed, and this aberrant interaction results in activation of signaling pathways and cell transformation [29–31]. Bentires-Alj et al. demonstrated that Gab2 is overexpressed in human breast cancer and the activation of SHP-2-Erk pathway promotes the proliferation of cancer cells [32]. It is reported that Cas-L interacts with overexpressed v-Abl and is tyrosine phosphorylated [24], and, considering the example of Gab2-SHP-2 interaction, the role of Cas-L-SHP-2 interaction in leukemia caused by BCR-Abl is an intriguing subject. In addition, since the association of Ras and Cas-L is reported in cancer metastasis [3], Cas-L interaction with SHP-2 to promote and regulate the Ras/ERK pathway may be of importance.

In conclusion, our present data show that SHP-2 forms a complex with Cas-L, inhibits its tyrosine phosphorylation and negatively regulates Cas-L-mediated cell migration. Our work therefore provides novel insights into understanding the diverse functions of SHP-2 in physiological and pathological states.

Acknowledgments

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