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Tyrosine Phosphorylation Controls Nuclear Localization and Transcriptional Activity of Ssdp1 in Mammalian Cells

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Abstract The LIM-HD proteins interact with different cofactors, including Ssdp1 to regulate development in a diverse range of species. The single stranded DNA binding protein (Ssdp1) is a member of an evolutionarily conserved family of proteins that regulate critical transcriptional processes during embryonic development. Ssdp1 is localized predominantly in the cytoplasm of 293T cells but is translocated to the nucleus when co-transfected with Lck, a member of the Src family of non-receptor tyrosine kinases. The Src tyrosine kinase inhibitor PP2 blocked the nuclear translocation of Ssdp1. Western blot analysis showed that co-expression of Ssdp1 and Lck in 293T cells induces Ssdp1 phosphorylation. Mutation of the Ssdp1 N terminal tyrosine residues 23 and 25 markedly reduced both the phosphorylation and the nuclear localization of Ssdp1. Lck enhanced the transcriptional activity of Ssdp1 in the context of known components of a LIM-homeodomain (LIM-HD)/cofactor complex. We propose that phosphorylation involving N-terminal tyrosine residues of Ssdp1 is a means of regulating its nuclear localization and subsequent transcriptional activation of LIM-HD complexes. J. Cell. Biochem. 103: 1856–1865, 2008.

Key words: Ssdp1; intracellular transport; LIM-HD complex; phosphorylation

The single stranded DNA binding protein (Ssdp) family is critical for embryonic development [Nishioka et al., 2005; Enkhmandakh et al., 2006]. Ssdp (also termed Ssbp) family members are components of transcriptional complexes that contain the nuclear co-factor Ldb1 and LIM homeodomain (LIM-HD) proteins. Ldb1, its *Drosophila* homolog Chip, and LIM-HD proteins are well characterized transcriptional regulators of embryonic development in invertebrates and in vertebrates that act in the context of complexes formed in the cell nucleus with other regulators of transcription [Hobert and Westphal, 2000]. More recently, Ldb1 has been shown to interact with members of the Ssdp family. This physical interaction has been observed in immunoprecipitates of mammalian cells [Chen et al., 2002] and in the yeast two-hybrid system [van Meyel et al., 2003]. A genetic interaction of *Ssdp* and *Chip* has been demonstrated in *Drosophila* [Chen et al., 2002; van Meyel et al., 2003]. Mutations of Ssdp1 genes result in severe developmental defects in *Drosophila* [Chen et al., 2002; van Meyel et al., 2003] and in mice [Nishioka et al., 2005; Enkhmandakh et al., 2006].

Ssdp1, Ldb1/Chip and LIM-HD proteins use a modular architecture to assemble transcriptional complexes [Enkhmandakh et al., 2006]. Ldb1/Chip proteins serve as a scaffold for these complexes using an N-terminal self-interaction domain (SID) for dimerization and a C-terminal LIM-interacting domain (LID) for interaction with the LIM domains of LIM-HD and LIM-only (LMO) proteins [Agulnick et al., 1996]. The N-terminal "LUFS" domain of the Ssdp proteins mediates its interaction with a third conserved domain in Ldb1/Chip, the LCCD (Ldb1/Chip Conserved Domain) [van Meyel et al., 2003]. These different binding domains allow for a multifaceted interaction with various proteins,

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a prerequisite for highly versatile transcriptional regulation. Mutations in each of the Chip domains or in interacting domains of binding partners result in *Drosophila* wing phenotypes [Chen et al., 2002; van Meyel et al., 2003].

The Ssdp/Ldb1 interaction is particularly interesting in this context as a previous study in *Drosophila* showed nuclear expression of Ssdp but only if Chip is present [van Meyel et al., 2003]. Ssdp1 does not have a NLS, or nuclear localization sequence, or a NES, or nuclear export sequence to facilitate its nucleocytoplasmic shuttling [van Meyel et al., 2003]. Knowing the mechanism that allows this protein to enter the nucleus and to interact with Chip/Ldb1-containing protein complexes is likely to provide insights into its function as a transcriptional regulator. In this study we show that phosphorylation of Ssdp1 by Src family non-receptor tyrosine kinases, present in the cytoplasm, plays a significant role in its transfer into the nucleus. Our data indicate that phosphorylation of the Tyr^{23} and Tyr^{25} residues of Ssdp1 is a functional prerequisite for its nuclear translocation.

MATERIALS AND METHODS

Plasmid Construction

The Ssdp1 fragment was generated from Ssdp1 cDNA using PCR based methods and cloned into the DsRed2 (C-terminal) (Ssdp1R) (Clontech), pCMV3B (Stratagene) vectors. The Ldb1 fragment was subcloned into the C-terminus of the vector pCMV2B (Stratagene) using the *EcoRI-XhoI* sites. Putative tyrosine phosphorylation sites were predicted by using Scansite 2.0 (scansite.mit.edu) [Obenauer et al., 2003]. Those predicted sites localizing to the LUFS domain were selected for a tyrosine to phenylalanine mutation. The Ssdp1 mutants were generated from the wild-type Ssdp1 cDNA in pCMV3B vector using the Stratagene Quickchange kit as recommended by the manufacturer. The resulting mutations were verified by sequencing.

Transfection, Immunofluorescence, and Fluorescence Microscopy

The Lck and LckY505F constructs obtained from Dr. Lawrence Samelson (NCI/NIH) were used for transfection and immunoprecipitation studies. Transient transfection of 293T and Cos-7 cells was carried out using Fugene6 Transfection Reagent (Roche Applied Science). The 293T and Cos-7 cells were grown in Lab-Tek II chamber slides in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. They were transiently transfected with 1 μg of the appropriate DNA using 3 μl of Fugene6. For inhibition with PP2 (BIOMOL), cells were incubated with 10 μM PP2 for 20 min, and fixed in 4% paraformaldehyde (PFA). The cells were processed by blocking with 2% goat serum and incubation with the primary antibody, anti-Ssdp1, for 3 h. The Ssdp1 antibody was generated by Spring Valley Laboratories against a unique peptide sequence in the C-terminal region of the antigen. This was followed by washing with 1X PBS and incubation in secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular probes). The cover slips were mounted in Fluoromount-G medium (Southern Biotech) and observed under a fluorescence microscope. To visualize the nuclei, the cells were stained with DAPI (Vector Laboratories). The fluorescent images were captured using the Olympus BX60 fluorescence microscope fitted with a Zeiss Axiocam camera.

Statistical Analysis

A two-tailed *t*-test was performed to determine the level of significance in graphical representations.

Immunoprecipitation and Nuclear Extraction

Cell lysates were prepared in 10 mM Tris, 150 mM NaCl, 5 mM NaF, 1 mM Orthovanadate, 2 mM EDTA and 1% Triton X-100, pH 7.4. Complete EDTA Free protease inhibitor cocktail tablets (one tablet in 50 ml lysis buffer, Roche Applied Science) were added to the cell lysate. Sodium orthovanadate was obtained from Sigma and pervanadate was freshly prepared in each experiment. Anti-Myc immunoprecipitation was performed using EZ View Red anti-c-Myc Affinity gel (Sigma–Aldrich). The immunoprecipitates were analyzed on immunoblots with antibodies where indicated. SDS-sample dye was added to the protein extracts along with sample reducing agent, the mixture was boiled, and immunoprecipitates were resolved on a 10% SDS–PAGE followed by immunoblotting with anti-pTyr, anti-Ssdp1 and anti-Myc antibodies. The mouse monoclonal antibody anti-phosphotyrosine, clone 4G10 was obtained from Upstate Cell Signaling

solutions and the polyclonal antibody anti-Myc HRP from Sigma. The secondary antibodies were from Santa Cruz Biotechnology. The blots were detected using chemiluminescence (Pierce technology).

Total cell lysates were collected and centrifuged followed by resuspension in lysis buffer-1 [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA and 1 mM DTT, pH 7.9. Complete EDTA Free protease inhibitor cocktail tablets (one tablet in 50 ml lysis buffer, Roche Applied Science)]. NP40 (Roche Applied Science) was added followed by centrifugation and the supernatant was collected as the cytoplasmic fraction. The pellet was then resuspended in lysis buffer-2 [10 mM HEPES, 400 mM NaCl, 1 mM EDTA and 1 mM DTT, pH 7.9. Complete EDTA Free protease inhibitor cocktail tablets (one tablet in 50 ml lysis buffer, Roche Applied Science)]. This suspension was vortexed and centrifuged and the supernatant was collected as the nuclear fraction. The fractions were then resolved on a 10% SDS–PAGE followed by immunoblotting with anti-Ssdp1, anti-PLCγ1 and anti-Ldb1 antibodies. The polyclonal antibody PLCγ1 used as a cytoplasmic control was obtained from Santa Cruz Biotechnology.

Transfection Assay

293T cells were plated in a 96-well plate and incubated for 16–18 h. Cells were then transiently transfected with different plasmid DNA, the effectors (0.1 μg), the reporter (-492gsc/Luc) and the positive control *Renilla* luciferase (phRL-Tk Vector) (Promega) using Fugene6. At 48 h post-transfection the cells were assayed using the Dual Glo Luciferase Assay System (Promega). The relative luciferase activity was measured by the Victor3 luminometer according to manufacturer instructions. The reporter -492gsc/Luc and pCS2-Xlim1 probes were obtained from Dr. Igor Dawid (LMG/NICHD/NIH).

RESULTS

Ssdp1 Antibody Characterization

To determine the specificity of the antibody we prepared whole cell lysates from 293T as well as Jurkat cells and prepared Western blots with the Ssdp1 antibody (Fig. 1A,B). We immunoprecipitated 293T cells transfected with cMyc-tagged Ssdp1 against the anti-cMyc resin and followed this by Western blotting with Ssdp1 antibody using the untransfected cells as control (Fig. 1C). A band with the expected molecular weight of 50 kDa migrated exclusively in whole cell lysates of both the 293T cells (Fig. 1A) and the Jurkat cells (Fig. 1B). We observed a band of similar size in immunoprecipitates of Ssdp1Myc-transfected cells but not in untransfected cells (Fig. 1C), thus establishing the specificity of the antigen-antibody interaction.

Ssdp1 Localization in Mammalian Cells

In order to observe Ssdp1 localization in mammalian cells, we generated a full length Ssdp1-DsRed2 fusion protein (Ssdp1R) (Fig. 1D) and transiently transfected this into 293T cells. After 18 h, we fixed the cells with 4% PFA and viewed them under a fluorescence microscope. We found Ssdp1 localized throughout the cell (Fig. 1E). Using the Ssdp1 antibody we determined that Ssdp1 was predominantly present in the cytoplasm (Fig. 1F).

Role of Src Family Kinases in Nucleocytoplasmic Shuttling of Ssdp1

Ssdp1 has a Lck-associated-signal-transduction (LAST) domain (unpublished data, Acc No. AF170906) which may interact with the Src family kinases and potentially contribute to the intracellular signaling of Ssdp1. To determine if Lck plays a role in nucleo-cytoplasmic shuttling of Ssdp1, we transfected Ssdp1R alone or in conjunction with either the wild-type Lck kinase (Lck), a constitutively active form of the Lck kinase (LckY505F) or with an inactive kinase 'dead' Lck (Fig. 2A,B). About 85% of cells co-transfected with LckY505F contained Ssdp1R in the nucleus, whereas nuclear localization was reduced to about 11% in cells co-transfected with the inactive 'dead' Lck kinase. The corresponding values for cells transfected with Ssdp1R alone versus co-transfection with Ssdp1R and Lck were 10% and 44%, respectively (Fig. 2B). These experiments suggest that Lck facilitates the translocation of Ssdp1 into the nucleus.

To confirm that Lck kinase activity plays a role in the nuclear translocation of Ssdp1 we co-transfected the 293T cells with Ssdp1R and Lck followed by treatment with a potent and selective Src tyrosine kinase inhibitor, PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-[3,4-d]pyrimidine) (Fig. 2C). We observed that

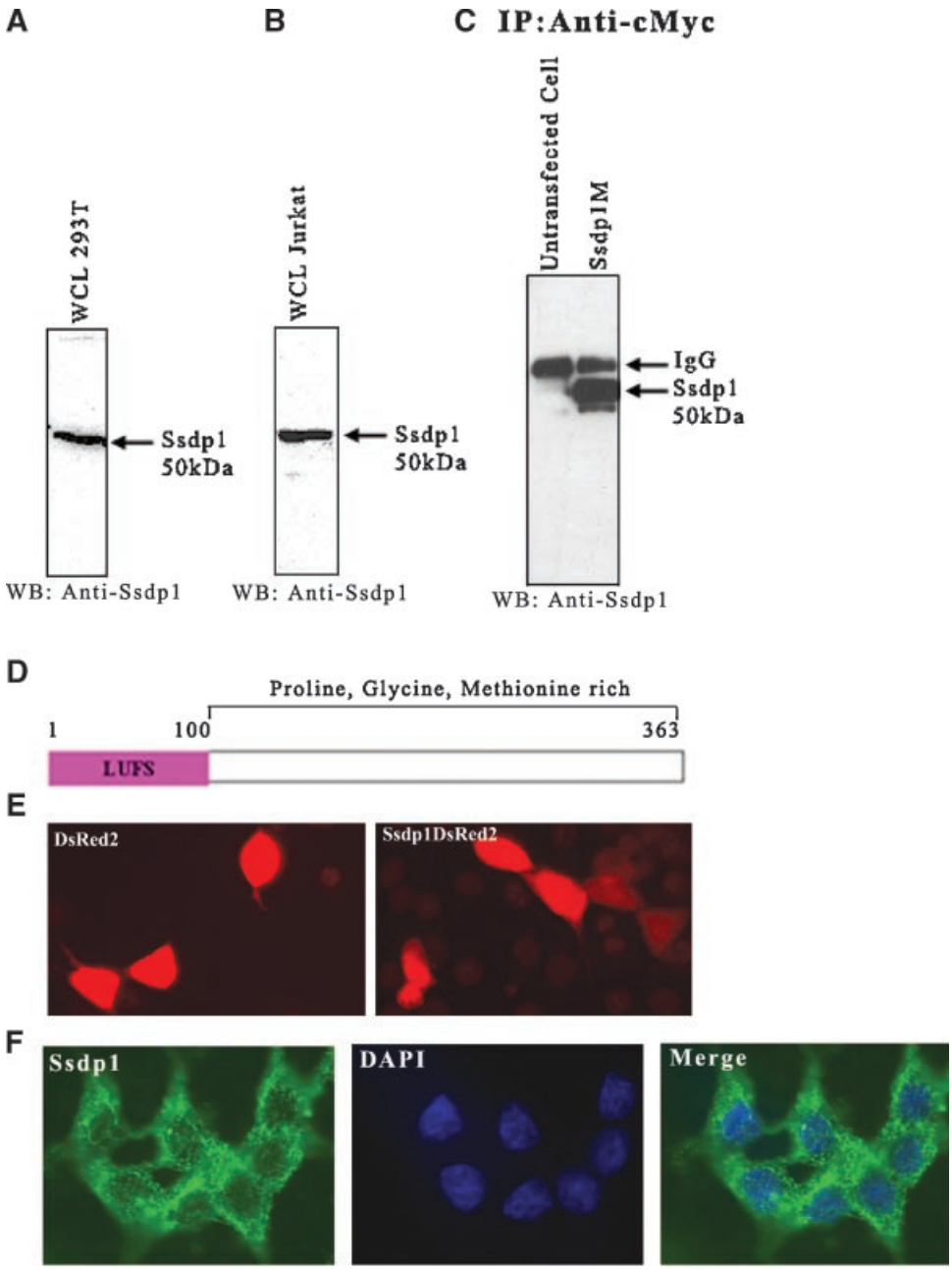

Fig. 1. Characterization and localization of the Ssdp1 antibody in mammalian cells. **A**: Whole cell lysates (WCL) of 293T cells and **B**: Jurkat cells were Western blotted (WB) against Ssdp1 antibody to check for the presence of Ssdp1 in vitro. **C**: Ssdp1 was tagged with Myc (Ssdp1M) and transfected in 293T cells for a period of 18 h followed by immunoprecipitation (IP) with cMyc antibody and Western blotting with Ssdp1 antibody. **D**: A schematic representation of Ssdp1 showing the conserved N-terminal LUFS domain and the proline-methionine-glycine rich C-terminal region. **E**: DsRed2 and Ssdp1DsRed2 vectors were transfected in 293T cells. Ssdp1DsRed2 was observed throughout the cell. **F**: 293T cells were plated on slides. After 18 h the slides were fixed and processed with Ssdp1 antibodies using DAPI as a nuclear marker. Ssdp1 shows localization predominantly in the cytoplasm.

PP2 treatment blocks the LckY505F mediated nuclear transport of Ssdp1 (Fig. 2C,D). These results suggest that the kinase activity of Lck can induce the translocation of Ssdp1 from the cytoplasm to the nucleus. Similar experiments were performed to observe endogenous distribution of Ssdp1 by overexpression of Lck or LckY505F in the cells followed by treatment with PP2. Using the Ssdp1 antibody we observed similar blockade of Lck and LckY505F

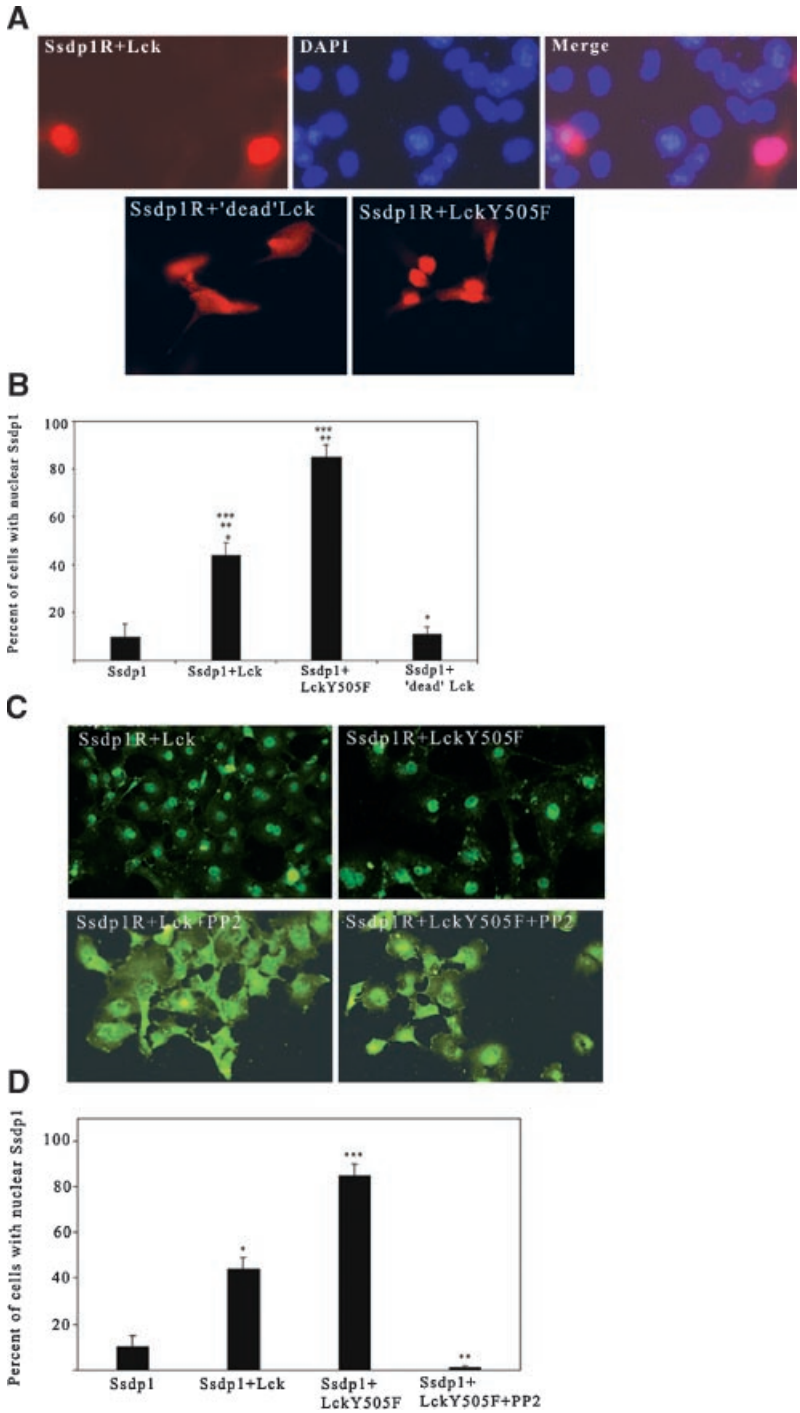

Fig. 2. Phosphorylation of Ssdp1 with Lck tyrosine kinases results in nuclear import of Ssdp1. **A**: 293T cells were transfected with Ssdp1DsRed2 (Ssdp1R) and co-transfected with inactive 'dead' Lck kinase (LckK273E), or wild-type Lck or a constitutively active form of Lck (LckY505F). Co-transfection of Ssdp1 with the wild-type Lck or the constitutively active form of Lck results in translocation of Ssdp1 into the nucleus as shown using DAPI as a nuclear marker. **B**: Graph shows the percentage of cells ± SD with nuclear expression of Ssdp1 after co-transfection with Lck or LckY505F as compared to Ssdp1 alone in 293T cells. $^{*}P < 0.05$ compared to Ssdp1, $^{***}P < 0.001$ compared to Ssdp1, $^{**}P < 0.05$ compared to Ssdp1 + 'dead' Lck, $^{****}P < 0.05$ compared to Ssdp1 + LckY505F. **C**: 293T cells were transfected with Ssdp1R and co-transfected with Lck or constitutively active form of Lck (LckY505F). After 18 h PP2 (1 μm), a Src family tyrosine kinase inhibitor was added to the culture. Subsequently immunocytochemistry with Ssdp1 antibody was performed. PP2 blocks nuclear localization of Ssdp1. **D**: Graphical representation of the effect of PP2 on 293T cells co-transfected with Ssdp1R and Lck/LckY505F. $^{*}P < 0.05$ compared to Ssdp1, $^{***}P < 0.001$ compared to Ssdp1, $^{**}P < 0.01$ compared to Ssdp1 + LckY505F.

mediated Ssdp1 nuclear transport in the presence of PP2.

To further validate our observations that Lck plays an active role in Ssdp1 translocation into the nucleus we did biochemical fractionation of the 293T cells transfected with either LckY505F or the inactive kinase 'dead' Lck. On Western blotting with Ssdp1 antibody in the nuclear versus cytoplasmic fractions we observe increased concentration of Ssdp1 in the nucleus of cells transfected with the active Lck as compared to cells with the inactive 'dead' Lck or untransfected cells (Fig. 3). Ldb1 antibody was used as a nuclear control and PLCγ1 antibody was used as a cytoplasmic control.

Tyrosine Phosphorylation of Ssdp1 by Lck

To determine if Lck can induce Ssdp1 phosphorylation, we transfected 293T cells with cMyc-tagged Ssdp1 (Ssdp1M) with or without Lck/ LckY505F. After 18 h we prepared whole cell lysates and incubated with resin coated with anti-cMyc antibodies to immunoprecipitate Ssdp1M. Precipitates were analyzed by Western blot with anti-phosphotyrosine antibody. We failed to observe any signal for phosphorylation with Ssdp1 alone whereas co-transfection with Lck resulted in a strong band of 50 kDa (Fig. 4A). When co-transfected with the catalytically inactive 'dead' Lck kinase, no Ssdp1 phosphorylation was detected (Fig. 4B). These results indicate that Ssdp1 is phosphorylated upon co-expression with an active Lck kinase. Pervanadate, a potent phosphotyrosine phosphatase inhibitor [Swarup et al., 1982], increased Ssdp1 phosphorylation even in the absence of Lck which suggests that other tyrosine kinases are endogenously expressed in 293T cells (Fig. 4A).

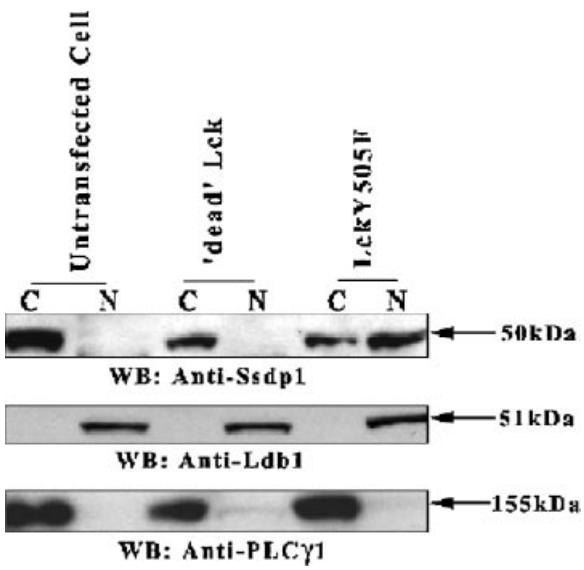

Fig. 3. Nucleo-cytoplasmic transport of Ssdp1 mediated by Lck. 293T cells were transfected with the inactive 'dead' Lck kinase or the constitutively active form of Lck (LckY505F) followed by biochemical fractionation of the total cell lysates into nuclear (N) and cytoplasmic (C) fractions. The membrane was Western blotted (WB) against Ssdp1 antibody followed by stripping and Western blotting against the Ldb1 antibody (nuclear protein) and then stripping and blotting against PLCγ1 antibody (cytoplasmic protein). There is higher concentration of Ssdp1 in the nucleus of cells transfected with LckY505F.

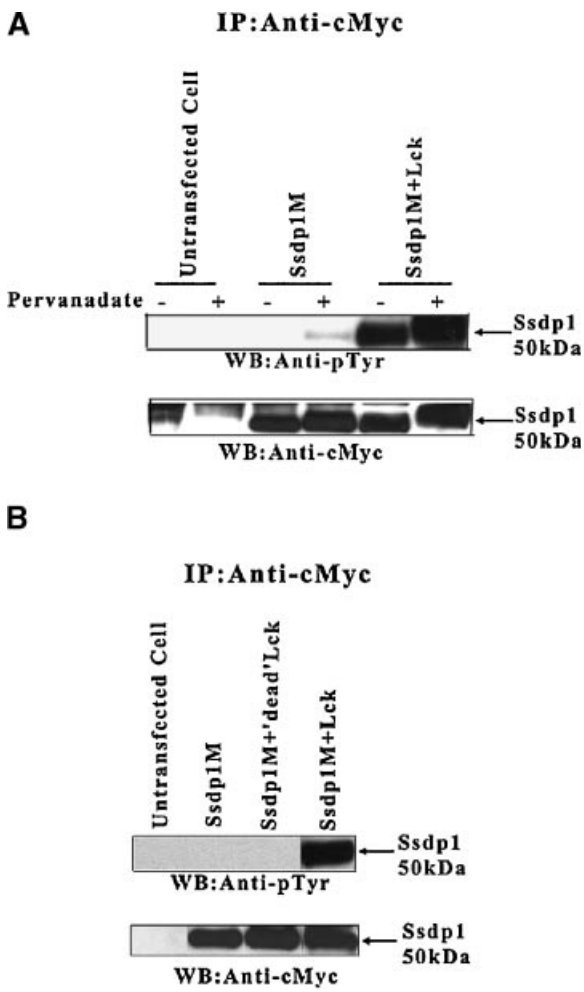

Fig. 4. Tyrosine phosphorylation of Ssdp1 by Lck. **A**,**B**: 293T cells were co-transfected with Ssdp1 tagged with myc (Ssdp1M) and Lck or the inactive kinase 'dead' Lck followed by immunoprecipitation (IP) with the resin coated with anti-cMyc antibodies. The membrane was Western blotted (WB) against 4G10 antibody followed by stripping and Western blotting against the cMyc antibody. Ssdp1 was phosphorylated with Lck. (−) media without pervanadate, (+) media with pervanadate.

Effects of Tyrosine Mutations on Ssdp1 Phosphorylation and Nuclear Translocation

We identified two tyrosines in Ssdp1 that were predicted to be phosphorylated and mutated them to phenylalanine (Fig. 5A). They are located at positions 23 and 25 in the N-terminal LUFS domain that is known to mediate interaction with Ldb1 [van Meyel et al., 2003]. We co-transfected 293T cells with the mutated Ssdp1M and with Lck or LckY505F for a period of 18 h, immunoprecipitated the whole cell lysate using Myc resin, resolved the peptides by 10% SDS–PAGE and carried out a Western blot with 4G10 antibody. There was a very weak signal for Ssdp1Y23F/Y25F with Lck as compared to Ssdp1 with Lck (Fig. 5B). The membrane was subsequently stripped and Western blotted against the cMyc antibody to determine the level of Ssdp1 in the extracts (Fig. 5B). We examined the cellular localization of the tyrosine mutants and observed significantly less import of mutant Ssdp1 to the nucleus ($P < 0.01$) as compared to wild-type Ssdp1 (Fig. 5C–E). These experiments strongly support the notion that phosphorylation of tyrosine residues 23 and/or 25 of Ssdp1 results in enhanced translocation of Ssdp1 into the nucleus.

Enhancement of the Transcriptional Activity of Ssdp1 by Lck

A luciferase assay has been developed to determine the contributions of various components of the Ssdp transcriptional complex to its transcriptional activity [Nishioka et al., 2005]. We used this assay to examine whether Lck has an effect on the transcriptional activity of Ssdp1. In this assay the *Xenopus Gsc* promoter (up to −492 bp) is used to drive expression of a reporter gene since *Gsc* had previously been identified as a target for the Lim1-Ldb1-Ssdp1 complex [Nishioka et al., 2005]. We transfected 293T cells with the luciferase reporter and a variety of plasmid constructs, incubated them for 48 h and assayed for luciferase activity, as shown in Figure 6. Co-transfection of Ssdp1 with LckY505F resulted in high luciferase activity when compared to co-transfection of Ssdp1, Ldb1 and Lim1 together. Ssdp1, Lim1 and LckY505F alone were also able to activate the *Gsc* promoter when compared to the reporter (Fig. 6). On the other hand, Ssdp1 co-transfected with Ldb1 or the inactive 'dead' Lck kinase did not elicit any activity (Fig. 6). However, co-transfection of mutant Ssdp1 (Ssdp1Y23F/Y25F) with LckY505F resulted in a sharp drop in the activity level when compared to co-transfection of Ssdp1 and LckY505F. These results identify Lck as a positive regulator of Ssdp1 transcriptional activity, even without the addition of cDNA encoding Ldb1 and Lim1. Together, these experiments support the notion that tyrosine phosphorylation and subsequent nuclear localization of Ssdp1 are essential for its optimal activity in this setting.

DISCUSSION

Previous work in *Drosophila* has established that the presence of Chip is required for nuclear localization of Ssdp, as Ssdp is excluded from the nucleus in *Chip* mutants [van Meyel et al., 2003]. In the present study we observed endogenous Ssdp1 predominantly in the cytoplasm of 293T cells (Fig. 2B). When over-expressed from transfected vectors its distribution extended throughout the cell (Fig. 1E). It has been well established that Ssdp1 interacts with the nuclear co-factor Ldb1 [Chen et al., 2002], and so there must be a mechanism for its efficient transport into the nucleus. We found that over-expression of Lck leads to Ssdp1 phosphorylation and its subsequent translocation into the nucleus. Furthermore, inhibitors of Src kinases abrogate this enhanced nuclear localization (Fig. 3). Our mutational analysis of the Ssdp1 protein strongly implies phosphorylation of two specific tyrosine residues, located in the N-terminal LUFS domain, in this mechanism. The LUFS domain of Ssdp1 interacts with Ldb1, and mutating the tyrosine residues in that region affects the translocation of Ssdp1 into the nucleus (Fig. 5). The nuclear membrane serves as a gateway for active and passive transport of molecules into and out of the nucleus [Stoffler et al., 2006]. It is known that macromolecules exceeding 20–40 kDa are carried through the nuclear pore complexes by signal-dependent mechanisms [Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999]. Phosphorylation by members of the Src family of tyrosine kinases is thus a likely means employed by the cell for the purpose of transporting Ssdp1 into the nucleus.

Over-expression of Ssdp1, Ldb1 and Lim1 together is necessary in order to strongly

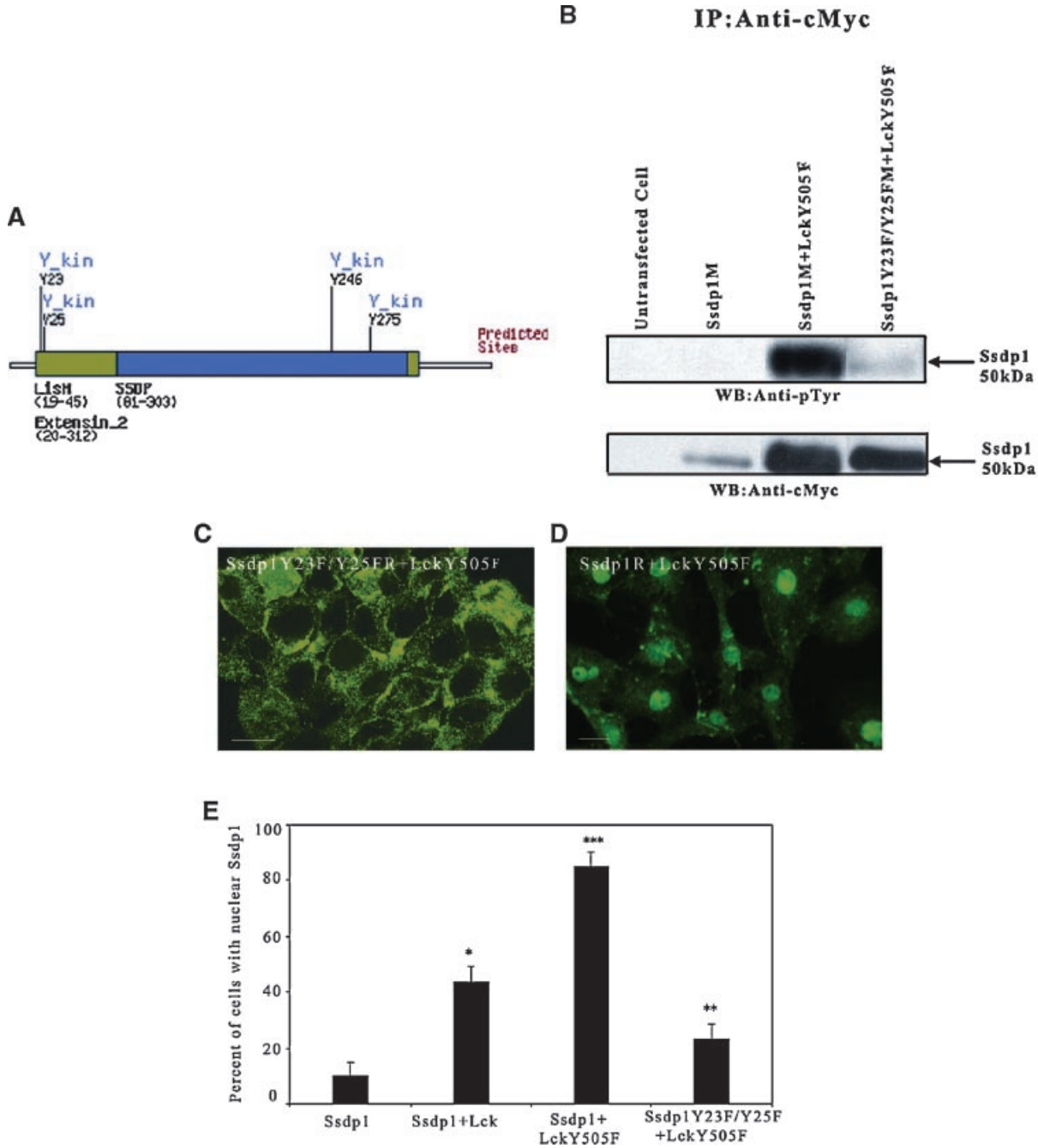

Fig. 5. Mutation of tyrosine residues alters Ssdp1 phosphorylation and localization. **A**: Schematic representation of Ssdp1 phosphorylation. The four tyrosine residues of Ssdp1 are marked with their positions. **B**: 293T cells were co-transfected with the mutant form of Ssdp1 (Ssdp1Y23F/Y25FM) and the constitutively active form of Lck (LckY505F). The whole cell lysates were immunoprecipitated (IP) and Western blotted (WB) against 4G10 antibody. The membrane was stripped and blotted against the cMyc antibody. Ssdp1Y23F/Y25F shows very weak phosphorylation with LckY505F as compared to Ssdp1 with LckY505F. **C**: Immunocytochemistry of 293T cells co-transfected with Ssdp1Y23F/Y25FR and LckY505F and **D**: with Ssdp1R and LckY505F, with Ssdp1 antibody. Mutant Ssdp1 shows localization predominantly in the cytoplasm. **E**: Graph shows percentage of 293T cells ± SD with nuclear localization after co-transfection. Ssdp1Y23F/Y25F shows significantly lower number of cells with nuclear localization as compared to Ssdp1, after co-transfection with LckY505F. $^{*}P < 0.05$ compared to Ssdp1, $^{***}P < 0.001$ compared to Ssdp1, $^{**}P < 0.01$ compared to Ssdp1 + LckY505F.

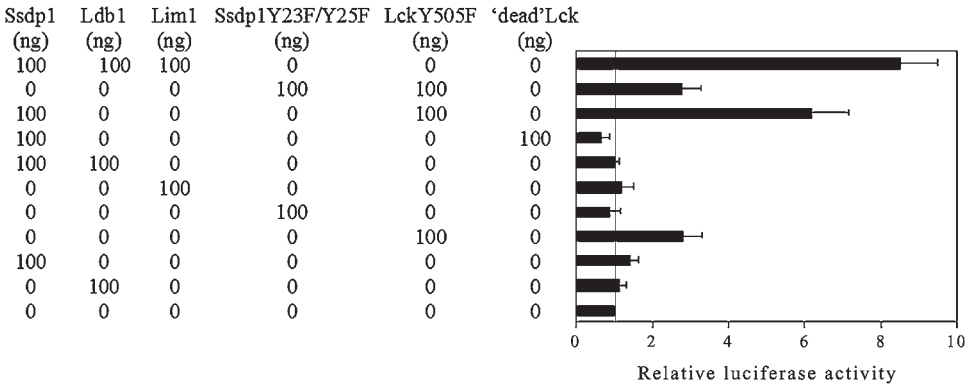

Fig. 6. Activation of Ssdp1 by Src Family kinase, Lck. 293T cells were co-transfected with the dose of plasmids shown above using *Gsc/Luc* as reporter and *Renilla Luc* as control. Graph shows relative luciferase activity ± SD of the proteins as compared to the reporter. The line indicates the reference point for the reporter.

activate a *Gsc/Luciferase* reporter [Nishioka et al., 2005]. We have shown that Ssdp1 and Lck alone can activate this reporter and this transcriptional activation is strongly reduced if the wild-type Ssdp1 is replaced by our Ssdp1 tyrosine mutants (Fig. 6). This provides evidence that tyrosine phosphorylation is critical for the contribution of Ssdp1 to the expression of the reporter construct. Ssdp1 also shows high transcriptional activity with Lck in the absence of transfected Ldb1. It has been shown by yeast two-hybrid interactions that Ssdp1 can exert transcriptional activity independent of Ldb1 [Wu, 2006]. Alternatively, or in addition, endogenous products of the *Ldb1* or the closely related *Ldb2* gene [Matthews and Visvader, 2003] may sustain the observed transcriptional activity of the phosphorylated Ssdp1.

Lck, when co-transfected with Ssdp1, results in marked phosphorylation and efficient translocation of Ssdp1. This suggests that Lck plays an important role in the regulation of intracellular Ssdp1 transport. Lck is a member of the Src family of non-receptor tyrosine kinases that is expressed predominantly in T cells where it plays a pivotal role in T cell antigen receptor-mediated (TCR) signal transduction [Straus and Weiss, 1992]. We have observed that Ssdp1 protein is expressed in the human T-cell line Jurkat (Fig. 1B). This is in keeping with its possible functional significance in T-cells.

In the context of an untransfected 293T cell we failed to notice any Ssdp1 phosphorylation. However, there is weak phosphorylation in the presence of pervanadate (Fig. 4A). Several Src family kinase members are ubiquitously expressed [Parsons and Parsons, 2004], and one or the other of these may well be utilized to phosphorylate basal levels of Ssdp1. We do not know which kinases specifically act on Ssdp1 in various in vivo contexts. However, it stands to reason that its phosphorylation results in conformational changes that allow Ssdp1 proteins to enter the nucleus as a prerequisite for their functional interaction with Ldb1 and other regulators of transcription. As has been proposed before, phosphorylation may be a very efficient way to regulate the activity of preformed transcription complexes [Jurata et al., 2000]. It follows that signaling cascades affecting phosphorylation of Ssdp1 may play a pivotal role in the regulation of transcriptional events mediated by this protein during embryonic development and tissue specification.

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