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Activation of cytosolic Slingshot-1 phosphatase by gelsolin-generated soluble actin filaments

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ABSTRACT

Slingshot-1 (SSH1) is a protein phosphatase that dephosphorylates and activates cofilin, an actin-severing and -disassembling protein. SSH1 is bound to and activated by F-actin, but not G-actin. SSH1 is accumulated in the F-actin-rich lamellipodium but is also diffusely distributed in the cytoplasm. It remains unknown whether SSH1 is activated by soluble (low-level polymerized) actin filaments in the cytoplasm. In this study, we show that SSH1 binds to gelsolin via actin filaments in the cytosolic fraction. Gelsolin promoted solubilization of actin filaments and SSH1 in cell-free assays and in cultured cells. SSH1 was activated by gelsolin-generated soluble actin filaments. Furthermore, gelsolin enhanced cofilin dephosphorylation in neuregulin-stimulated cells. Our results suggest that cytosolic SSH1 forms a complex with gelsolin via soluble actin filaments and is activated by gelsolin-generated soluble actin filaments and that gelsolin promotes stimulus-induced cofilin dephosphorylation through increasing soluble actin filaments, which support SSH1 activation in the cytoplasm.

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1. Introduction

Regulation of actin filament dynamics is essential for cell migration and morphogenesis. Cofilin plays a fundamental role in actin filament dynamics and remodeling by severing and depolymerizing actin filaments [1,2]. Cofilin is inactivated by phosphorylation at Ser-3 by LIMKs and TESKs [3–5] and is reactivated by dephosphorylation by Slingshot (SSH) family protein phosphatases, which comprise SSH1, SSH2, and SSH3 in mammals [6–8]. SSH1 binds to F-actin, and the cofilin-phosphatase activity of SSH1 is markedly enhanced by its binding to F-actin [6–12]. Upon stimulation with growth factors or chemokines, SSH1 accumulates into F-actin-rich lamellipodia at the front of migrating cells [9,13]. These observations suggest that SSH1 plays a critical role in cell migration by activating cofilin and thereby promoting actin filament turnover in lamellipodia [9,13]. On the other hand, a considerable proportion of SSH1 is diffusely distributed in the cytoplasm. However, it remains unknown whether cytosolic SSH1 binds to and is activated by soluble (low-level polymerized) actin filaments.

Gelsolin (GSN) and its closely related protein scinderin (SCIN) are Ca^{2+}-dependent actin-regulatory proteins that stimulate severing of actin filaments, capping of the plus ends of actin filaments, and nucleation of actin assembly [14,15]. The severing and capping activities of GSN produce GSN-capped short actin filaments [14]. Thus, GSN plays important roles in various cell functions, including cell migration and morphogenesis, through the regulation of actin dynamics [14–16].

In this study, we show that SSH1 co-precipitates GSN and SCIN through actin filaments. We provide evidence that SSH1 forms a protein complex with GSN and soluble actin filaments in the cytosolic fraction and is activated by GSN-generated soluble actin filaments. Our results suggest that GSN plays a role in cofilin dephosphorylation through soluble actin filament-induced SSH1 activation.

2. Materials and methods

2.1. Plasmid construction

cDNAs encoding human GSN and SCIN were PCR-amplified from the MegaMan human transcriptome library (Agilent Technologies). The cDNAs were subcloned into FPC1-Myc (GE Healthcare), pGEX (GE Healthcare), and pEYFP-C1 (Clontech) expression vectors.

Abbreviations: GSN, gelsolin; LatA, latrunculin A; NRG, neuregulin; P-cofilin, Ser-3-phosphorylated cofilin; SCIN, scinderin; SSH, Slingshot.

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Plasmids for SSH1 and its mutants were constructed as described previously [12].

2.2. Cell culture, transfection, and staining

293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. MCF-7 cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum. 293T and MCF-7 cells were transfected with plasmids using FuGENE6 (Promega) and Lipofectamine LTX (Invitrogen), respectively. Cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and stained with Alexa Fluor 568-phalloidin (Invitrogen)

2.3. Proteomic analysis

Proteomic analysis of SSH1-binding proteins was performed as described previously [17]. Briefly, lysates of 293T cell lines stably expressing FLAG-SSH1 were immunoprecipitated with an anti-FLAG antibody. The precipitated proteins were eluted with FLAG peptide, separated by SDS–PAGE, and stained with silver. Protein bands were excised, trypsinized, and analyzed by mass spectrometry.

2.4. Immunoprecipitation and immunoblotting

Cell lysates were subjected to immunoprecipitation and immunoblotting, as described previously [17]. Antibodies against FLAG (M2, Sigma), Myc-tag (9E10, Roche), β-actin (AC-15, Sigma), and GFP (Invitrogen) were purchased. Antibodies against SSH1, cofilin, and Ser-3-phosphorylated cofilin (P-cofilin) were prepared as described previously [5,18].

2.5. Protein purification

SSH1-(Myc-His) was expressed in Sf21 cells using the Bac-to-Bac baculovirus expression system (Invitrogen) and purified using Ni–NTA agarose (Qiagen), as described previously [12]. Cofilin-$(His)_6$ was expressed in 293T cells and purified using Ni–NTA agarose, as described previously [9]. GST-GSN and GST-SCIN were expressed in *Escherichia coli* and purified using glutathione-sepharose (GE Healthcare), as described previously [12].

2.6. In vitro solubility assay

G-actin purified from rabbit skeletal muscle was polymerized to F-actin by adding 10× F-buffer (0.2 M Tris–HCl, pH 7.5, 1 M NaCl, 20 mM $MgCl_2$, and 1 mM DTT) and incubating at 30 °C for 1 h [12]. F-actin was incubated with GST-GSN and/or SSH1-(Myc-His) at 30 °C for 30 min and ultracentrifuged at 100,000×*g* for 30 min at 4 °C. Equal amounts of pellets and supernatants were subjected to SDS–PAGE and analyzed by Coomassie brilliant blue staining.

2.7. Solubility assay of cell lysates

MCF-7 cells were transfected with YFP or YFP-GSN and cultured for 48 h or were treated with 10 μM latrunculin A (LatA) for 30 min. Cells were then lysed in lysis buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM DTT, 0.2 mM ATP, 1% NP-40, 10 μg/ml leupeptin, and 2 mM phalloidin). Cell lysates were ultracentrifuged at 100,000×*g* for 30 min at 4 °C. Equal amounts of pellets and supernatants were subjected to SDS–PAGE and analyzed by immunoblotting with anti-β-actin and anti-SSH1 antibodies.

2.8. In vitro phosphatase assay

F-actin or G-actin was incubated with purified GST-GSN for 30 min at 30 °C. After ultracentrifugation at 100,000×*g* for 30 min, the supernatants were incubated with 152 μg of cofilin-His_6 and 175 ng of SSH1-(Myc-His) at 30 °C for 30 min in 35 μl of F-buffer. Reaction mixtures were analyzed by immunoblotting with anti-cofilin and anti-P-cofilin antibodies.

3. Results

3.1. GSN co-precipitates and co-localizes with SSH1

To examine the mechanisms regulating SSH1 activity, we searched for SSH1-binding proteins using co-precipitation assay. 293T cell lines stably expressing FLAG-tagged SSH1 were established and the cell lysates were immunoprecipitated with an anti-FLAG antibody. The co-precipitated proteins were separated by SDS–PAGE and analyzed by mass spectrometry (Fig. 1A). Actin and several actin-binding proteins, including GSN, SCIN, filamin-A, and the α1 and α2 subunits of capping protein (CapZα1 and CapZα2, respectively), were identified in FLAG-SSH1 co-precipitates. Consistent with a previous result [9], 14-3-3 proteins were also identified in FLAG-SSH1 co-precipitates (Fig. 1A). To confirm the interaction between SSH1 and GSN/SCIN, CFP-tagged SSH1 was co-expressed with Myc-tagged GSN or SCIN in 293T cells and immunoprecipitated with an anti-GFP antibody. Both Myc-GSN and Myc-SCIN were co-precipitated with CFP-SSH1 (Fig. 1B). These results indicate that GSN and SCIN associate with SSH1 directly or indirectly.

SSH1 localizes in F-actin-rich lamellipodia in neuregulin (NRG)-stimulated MCF-7 cells [9]. To examine whether GSN co-localizes with SSH1, CFP-SSH1 and YFP-GSN were co-expressed in MCF-7 cells and their localizations were analyzed before and after NRG stimulation. CFP-SSH1 and YFP-GSN accumulated in F-actin-rich membrane protrusions in non-stimulated cells and in lamellipodia in NRG-stimulated cells (Fig. 1C). They were also diffusely distributed in the cytoplasm before and after NRG stimulation (Fig. 1C).

3.2. SSH1 binds to GSN via actin filaments

GSN and SSH1 are F-actin-binding proteins; therefore, it is possible that GSN co-precipitates and co-localizes with SSH1 via actin filaments. To investigate this possibility, we performed the GSN-SSH1 co-precipitation assay in the absence or presence of LatA, an inhibitor of actin polymerization. 293T cells co-expressing CFP-SSH1 and Myc-GSN were treated with LatA or were untreated and then cell lysates were precipitated with an anti-GFP antibody. Myc-GSN and β-actin co-precipitated with CFP-SSH1 in the absence of LatA, but their co-precipitation was barely detectable after treatment with LatA (Fig. 2A). This suggests that polymerized actin is required for the interaction between GSN and SSH1. A GST pull-down assay using purified proteins revealed that FLAG-SSH1 did not bind to GST-GSN or GST-SCIN (Fig. 2B), further suggesting that SSH1 indirectly binds to GSN and SCIN.

We previously showed that the N461 mutant of SSH1 (Fig. 2C) efficiently binds to F-actin, whereas the N456 mutant only binds weakly [10]. When CFP-tagged SSH1(N461) or SSH1(N456) was co-expressed with Myc-GSN and immunoprecipitated with an anti-GFP antibody, Myc-GSN and actin were co-precipitated with SSH1(N461), but not with SSH1(N456) (Fig. 2D). This further suggests that F-actin-binding activity is required for the binding of SSH1 to GSN. Together, these results suggest that SSH1 forms a complex with GSN through actin filaments.

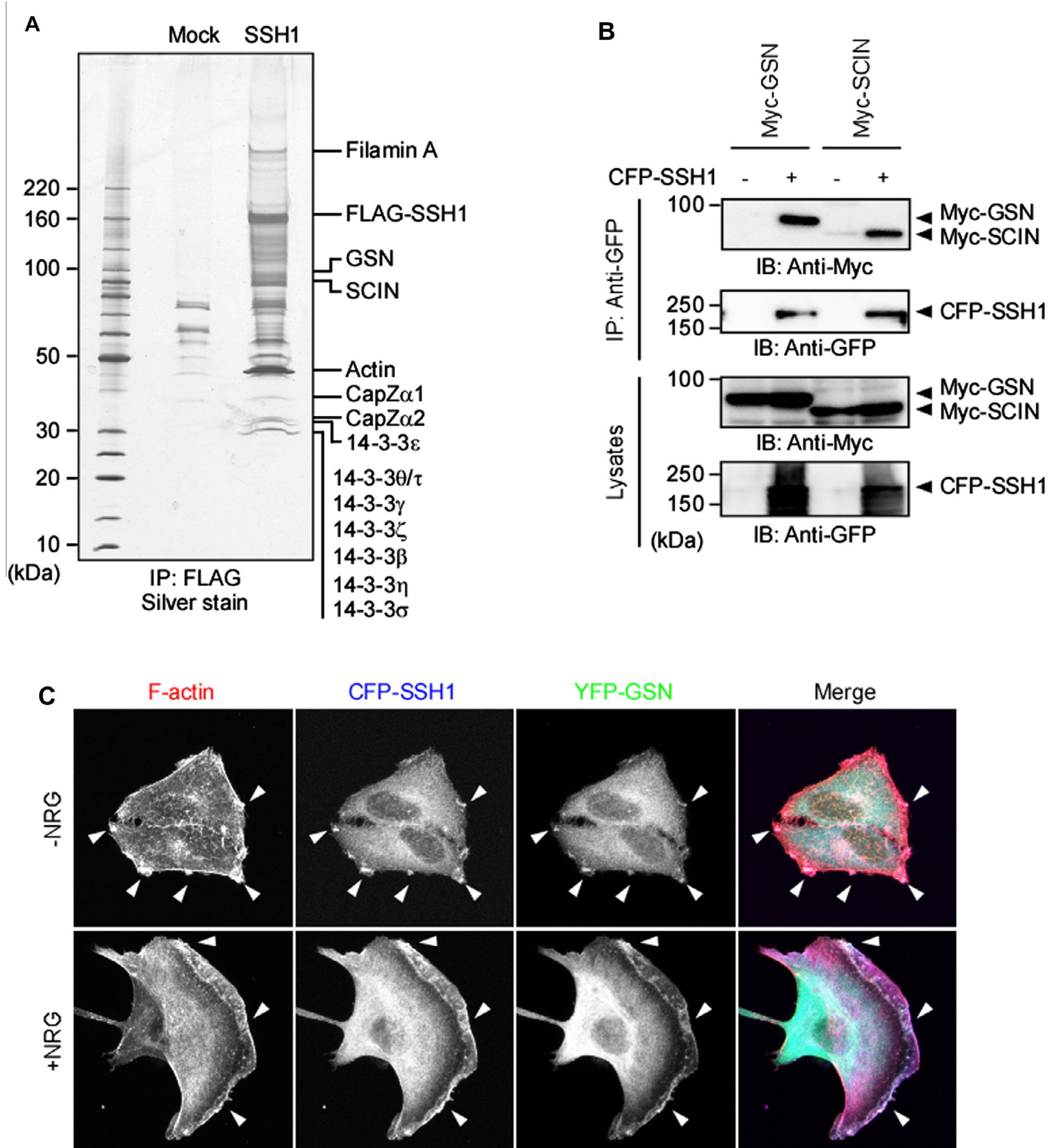

Fig. 1. Gelsolin (GSN) co-precipitates and co-localizes with Slingshot-1 (SSH1). (A) Proteomic analysis of SSH1-binding proteins. Lysates of 293T cells stably expressing FLAG-SSH1 were precipitated with an anti-FLAG antibody. SCIN, scinderin. (B) Co-precipitation assay. CFP-SSH1 and Myc-GSN or Myc-SCIN were co-transfected into 293T cells. Cell lysates were precipitated with an anti-GFP antibody and immunoblotted with the indicated antibodies. (C) SSH1 co-localizes with GSN in F-actin-rich regions. MCF-7 cells were co-transfected with CFP-SSH1 (blue) and YFP-GSN (green) and stimulated for 15 min with 100 ng/ml neuregulin (NRG). Cells were stained with Alexa Fluor 568-phalloidin (red). Arrowheads indicate positions where SSH1 co-localizes with GSN.

3.3. SSH1 forms a complex with GSN and soluble actin filaments in the cytosolic fraction

A proportion of SSH1 and GSN was diffusely distributed in the cytoplasm; therefore, we next examined whether cytosolic SSH1 associates with GSN via soluble (low-level polymerized) actin filaments. CFP-SSH1 and Myc-GSN were co-expressed in 293T cells, the cell lysates were ultracentrifuged to remove insoluble (highly polymerized) actin filaments and their associated proteins, and the supernatants (cytosolic fraction) were immunoprecipitated with an anti-GFP antibody. Myc-GSN and β-actin in the supernatants were co-precipitated with CFP-SSH1 (Fig. 2E), suggesting that cytosolic SSH1, or at least a proportion thereof, forms a complex with GSN and soluble actin filaments. Treatment with LatA

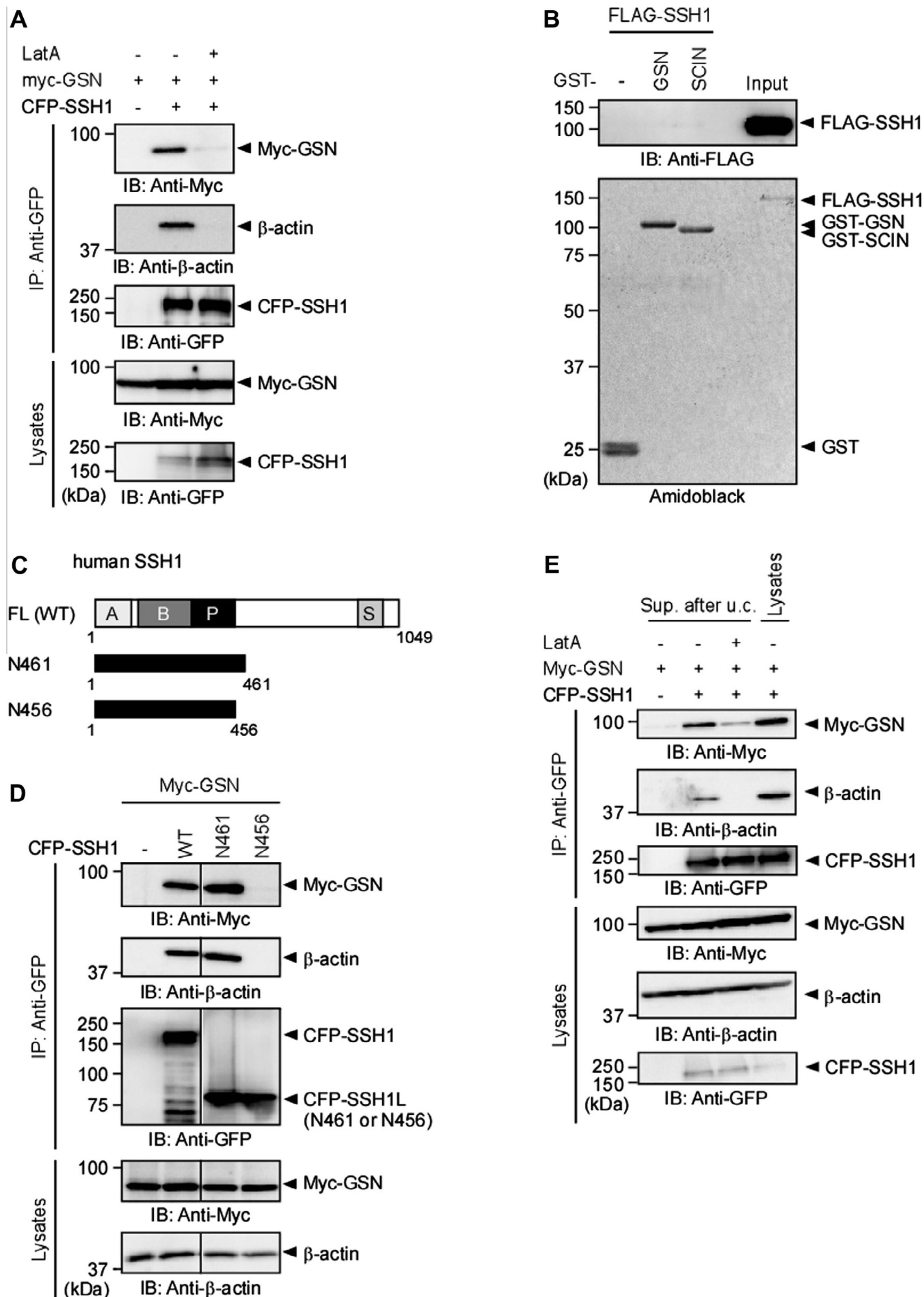

Fig. 2. Slingshot-1 (SSH1) binds to gelsolin (GSN) via actin filaments. (A) Latrunculin A (LatA) inhibits the GSN-SSH1 interaction. Myc-GSN and CFP-SSH1 were co-transfected into 293T cells. Cell lysates were treated with 10 μM LatA for 30 min, precipitated with an anti-GFP antibody, and immunoblotted with the indicated antibodies. (B) SSH1 does not directly bind to GSN or scinderin (SCIN). Purified FLAG-SSH1 was subjected to GST pull-down assays using GST-GSN or GST-SCIN. (C) Structures of SSH1 and its deletion mutants. The conserved regions of SSH family proteins are denoted as A, B, P (phosphatase), and S (Ser-rich) domains. (D) GSN co-precipitates with SSH1(N461), but not with SSH1(N456). Myc-GSN and CFP-SSH1(WT) or its deletion mutants were co-transfected into 293T cells. Lysates were precipitated with an anti-GFP antibody and immunoblotted with the indicated antibodies. (E) SSH1 forms a complex with GSN via soluble actin filaments in the cytosolic fraction. CFP-SSH1 and Myc-GSN were co-transfected into 293T cells. Lysates were treated with or without 10 μM LatA for 30 min and ultracentrifuged (u.c.) to remove highly polymerized actin filaments. The supernatants (Sup) were then immunoprecipitated with an anti-GFP antibody and immunoblotted with the indicated antibodies.

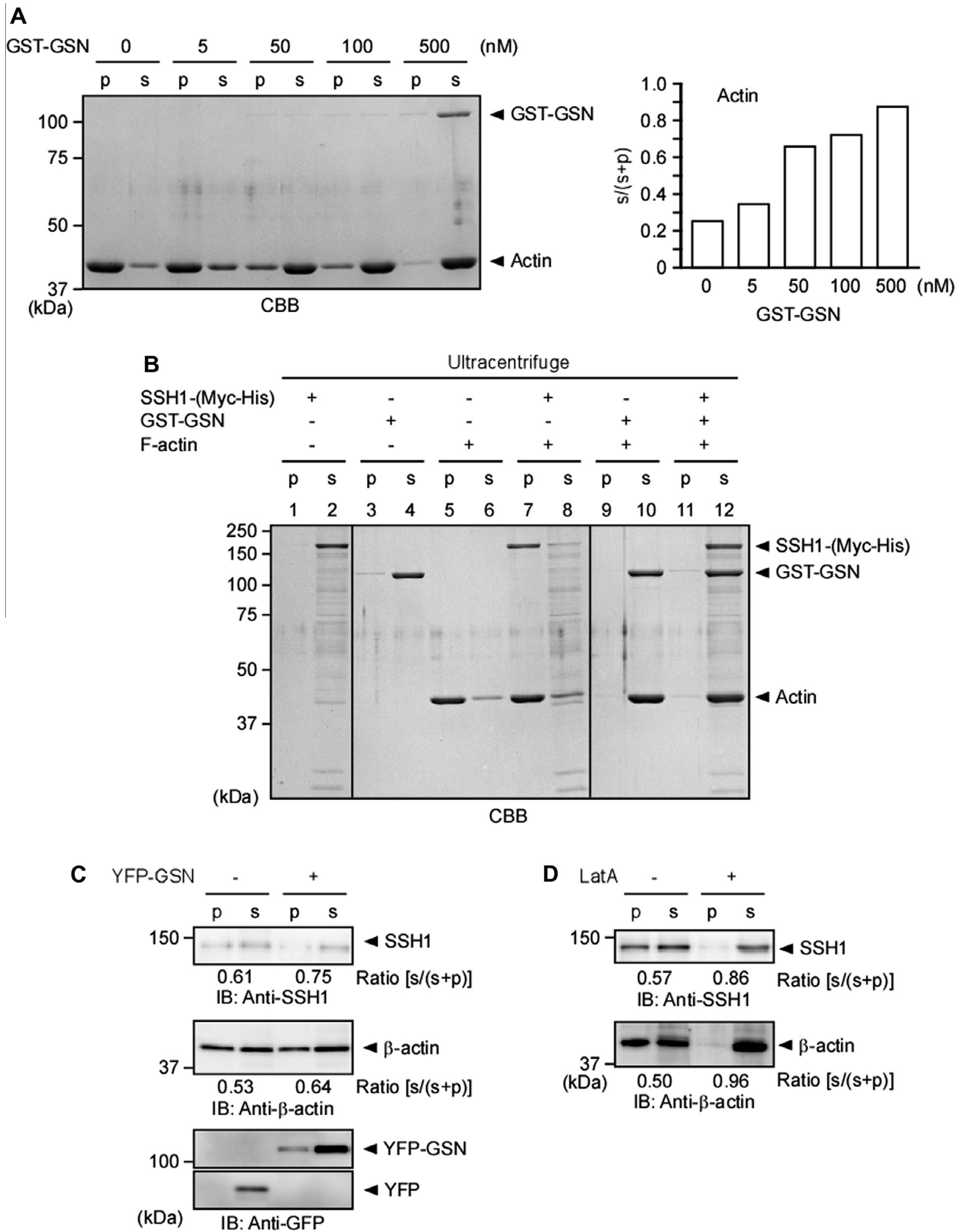

Fig. 3. Gelsolin (GSN) promotes the solubilization of actin filaments and Slingshot-1 (SSH1) in a cell-free assay and in cells. (A) GSN generates soluble actin filaments. F-actin was incubated with GST-GSN and ultracentrifuged. The amounts of actin recovered in pellets (p) and supernatants (s) were analyzed by Coomassie brilliant blue (CBB) staining. The ratios of soluble (s) to total (s + p) actin are shown on the right. (B) GSN promotes the solubilization of actin filaments and SSH1. F-actin was incubated with purified SSH1-(Myc-His) and/or GST-GSN, and ultracentrifuged. The amounts of actin, GSN, and SSH1 in pellets (p) and supernatants (s) were analyzed by CBB staining. (C) GSN promotes the solubilization of actin filaments and SSH1 in cells. MCF-7 cells were transfected with YFP or YFP-GSN and cell lysates were ultracentrifuged. The amounts of actin and SSH1 in pellets (p) and supernatants (s) were analyzed by immunoblotting with the indicated antibodies. The ratios of soluble (s) to total (s + p) actin or SSH1 are indicated below the gel images. (D) Latrunculin A (LatA) promotes the solubilization of actin filaments and SSH1 in cells. MCF-7 cells were treated with LatA and lysates were ultracentrifuged and analyzed as in (C).

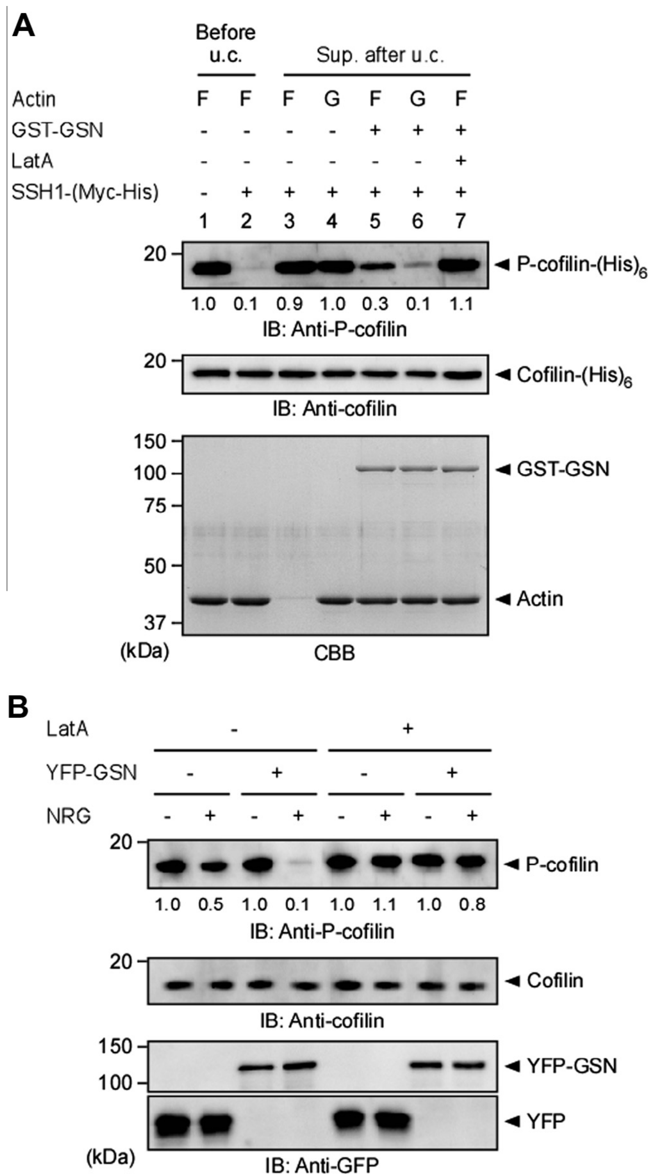

Fig. 4. Slingshot-1 (SSH1) is activated by gelsolin (GSN)-generated soluble actin filaments *in vitro* and GSN enhances neuregulin (NRG)-induced cofilin dephosphorylation in cells. (A) SSH1 activation by GSN-generated soluble actin filaments. F- or G-actin was incubated with or without GST-GSN or latrunculin A (LatA) and ultracentrifuged (u.c.). The supernatants (Sup.) were incubated with purified SSH1-(Myc-His) and subjected to *in vitro* phosphatase assays using cofilin-His$_6$ as a substrate. Reaction mixtures were analyzed by immunoblotting with anti-cofilin and anti-Ser-3-phosphorylated cofilin (P-cofilin) antibodies and Coomassie brilliant blue (CBB) staining. (B) GSN, but not LatA, enhances NRG-induced cofilin dephosphorylation in cells. MCF-7 cells were transfected with YFP or YFP-GSN and treated with or without LatA. Cells were then stimulated with 100 ng/ml NRG for 20 min and P-cofilin levels were analyzed by immunoblotting with anti-cofilin and anti-P-cofilin antibodies. The relative levels of P-cofilin are indicated below the gel image.

markedly decreased the amounts of β-actin and Myc-GSN that co-precipitated with CFP-SSH1 (Fig. 2E), suggesting that cytosolic SSH1 forms a complex with GSN via soluble short actin filaments.

3.4. GSN promotes the solubilization of actin filaments and SSH1

GSN has an F-actin-severing activity [14,15]. When actin filaments were incubated with 0–500 nM purified GST-GSN *in vitro* and then ultracentrifuged, GSN increased the ratio of soluble actin to total actin in a dose-dependent manner (Fig. 3A). More than 80% of total actin was soluble after treatment with 500 nM GSN. Purified SSH1-(Myc-His) was soluble after ultracentrifugation (Fig. 3B, lanes 1 and 2). When purified SSH1-(Myc-His) was incubated with actin filaments in the absence or presence of GSN and ultracentrifuged, SSH1-(Myc-His) mostly co-sedimented with F-actin in the absence of GSN, but was soluble in the presence of 500 nM GSN (Fig. 3B, lanes 7, 8, 11, and 12). These results suggest that GSN has a function to promote the solubilization of actin filaments and SSH1 by severing actin filaments *in vitro*.

To examine whether GSN promotes the solubilization of actin filaments and SSH1 in cultured cells, we analyzed the effect of GSN expression on the amounts of soluble actin and SSH1. When YFP-GSN or control YFP was expressed in MCF-7 cells and the cell lysates were ultracentrifuged, expression of YFP-GSN increased the ratios of actin and SSH1 in the supernatants (Fig. 3C), indicating that GSN increases the amounts of soluble (cytosolic) actin filaments and SSH1 in cultured cells. Treatment with LatA further increased the amounts of soluble actin and SSH1 in MCF-7 cells (Fig. 3D).

3.5. SSH1 is activated by GSN-generated soluble actin filaments

The cofilin-phosphatase activity of SSH1 is remarkably increased by F-actin, but not by G-actin [9,12]. However, it remains unknown whether SSH1 is similarly activated by soluble short actin filaments. To examine whether SSH1 is activated by GSN-generated soluble actin filaments, purified SSH1 was incubated with various F- and G-actin preparations and subjected to *in vitro* phosphatase assays, using cofilin-His$_6$ as a substrate. SSH1 effectively dephosphorylated P-cofilin in the presence of F-actin (Fig. 4A, lanes 1 and 2), but this activity was abolished when F-actin was removed by ultracentrifugation (lane 3), indicating that SSH1 is activated by F-actin. Preincubation of F-actin with GSN caused the solubilization of actin filaments, and soluble actin filaments were detected in the supernatants after ultracentrifugation (lane 5). Under these conditions, SSH1 dephosphorylated P-cofilin, indicating that SSH1 is activated by GSN-generated soluble actin filaments (compare lanes 3 and 5). By contrast, pretreatment of GSN-generated soluble actin filaments with LatA abolished the cofilin-phosphatase activity of SSH1 (lane 7), suggesting that SSH1 is activated by soluble actin filaments but not by G-actin. In fact, G-actin did not activate SSH1 (lane 4). Intriguingly, preincubation of G-actin with GSN promoted SSH1 activity (lane 6). GSN has an actin-nucleating activity; therefore, it is likely that SSH1 is activated by soluble actin filaments that are generated by GSN-mediated actin nucleation. Additionally, addition of purified GSN or SCIN alone had no effect on the cofilin-phosphatase activity of SSH1 (data not shown). These results suggest that SSH1 is not directly activated by GSN or SCIN, but is activated by GSN-generated soluble actin filaments.

3.6. GSN enhances cofilin dephosphorylation in NRG-stimulated cells

We previously showed that NRG induces cofilin dephosphorylation in MCF-7 cells [9]. To examine the role of GSN in cofilin dephosphorylation in cells, we analyzed the effect of YFP-GSN expression on the level of cofilin phosphorylation in NRG-stimulated and non-stimulated MCF-7 cells (Fig. 4B). As reported previously [8], stimulation with NRG induced cofilin dephosphorylation in control YFP-expressing cells. Expression of YFP-GSN further enhanced NRG-induced cofilin dephosphorylation. By contrast, expression of YFP-GSN had no apparent effect on the P-cofilin level in non-stimulated cells. These results suggest that GSN promotes cofilin dephosphorylation but this effect is limited to NRG-stimulated cells. By contrast, treatment with LatA inhibited NRG-induced and GSN-enhanced cofilin dephosphorylation

(Fig. 4B, lanes 5–8). LatA is an inhibitor of actin polymerization; therefore, these results suggest that actin polymers are required for both NRG-induced and GSN-enhanced cofilin dephosphorylation.

4. Discussion

The cofilin-phosphatase activity of SSH1 is dramatically increased by its binding to F-actin [9,12]. However, it is unknown whether cytosolic SSH1 is activated by soluble (low-level polymerized) actin filaments in the cytoplasm, similar to SSH1 bound to insoluble (highly polymerized) cytoskeletal actin filaments. In this study, we showed that SSH1 forms a protein complex with GSN and soluble actin filaments in the cytosolic fraction of cultured cells. GSN promoted the solubilization of actin filaments and SSH1 in a cell-free assay system and in cells, probably by severing insoluble actin filaments into soluble fragments. SSH1 was activated by GSN-generated soluble actin filaments. These results suggest that cytoplasmic SSH1 can be activated by cytosolic soluble F-actin fragments, similar to SSH1 bound to cytoskeletal insoluble actin filaments. LatA also promoted the solubilization of actin filaments and SSH1 in cells but it abolished SSH1 activation induced by GSN-generated F-actin fragments, which suggests that SSH1 is activated by soluble and insoluble actin filaments, but not by G-actin.

We previously showed that NRG stimulates SSH1-mediated cofilin dephosphorylation in MCF-7 cells [9]. SSH1 activity is regulated by phosphorylation of Ser-937 and Ser-978 in its C-terminal non-catalytic region and the subsequent binding of 14-3-3 proteins [9]. 14-3-3 proteins bind to SSH1 and inhibit its F-actin-binding activity and F-actin-mediated activation, dependent on the phosphorylation of Ser-937 and Ser-978 [9]. NRG induces SSH1 activation probably through Ser-937/Ser-978 dephosphorylation of SSH1 and its dissociation from 14-3-3 proteins and association with actin filaments [8,9,19]. In this study, we showed that expression of GSN further enhanced NRG-induced cofilin dephosphorylation in MCF-7 cells, indicating that GSN has a promotive role in NRG-induced cofilin dephosphorylation. Considering that GSN promotes the solubilization of actin filaments, that SSH1 is activated by GSN-generated soluble actin filaments, and that most P-cofilin is diffusely distributed in the cytoplasm, it is conceivable that GSN promotes cofilin dephosphorylation by increasing the amount of active SSH1 bound to soluble actin filaments in the cytoplasm. On the other hand, expression of GSN did not affect the basal P-cofilin level in non-stimulated cells, which suggests that SSH1 is not activated by GSN without NRG stimulation. It is likely that SSH1 requires NRG-induced Ser-937/Ser-978 dephosphorylation and the subsequent release of 14-3-3 proteins for its activation by GSN-generated short actin filaments.

In the present study, we showed that SSH1 is bound to and activated by soluble actin filaments in the cytoplasm. The functional roles of cytoplasmic short actin filaments and actin oligomers are not well understood. Further studies on the functions and dynamics of these relatively short actin filaments in the cytoplasm will uncover the novel roles of GSN family proteins and short actin filaments in various cell activities and functions.

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