博士論文（要約）

Regulation of Slingshot-1 activity by gelsolin-generated soluble actin filaments and the role of a PH-like domain in Slingshot-1 activity

(ゲルゾリンによって産生される可溶性アクチン繊維と PH 様ドメインによるスリングショット‐1 の活性調節)

平成２６年度

東北大学大学院生命科学研究科

分子生命科学専攻

髙橋　克宣

Regulation of Slingshot-1 activity by gelsolin-generated soluble actin filaments and the role of a PH-like domain in Slingshot-1 activity

Preface

Cofilin plays an important role in the regulation of actin filament dynamics during cell migration and morphogenesis by severing and depolymerizing actin filaments [1, 2]. Actin-severing and -depolymerizing activity of cofilin is inhibited by phosphorylation at Ser-3 by LIM-kinases (LIMKs) and testicular protein kinases (TESKs) [3–5] and is reactivated by dephosphorylation by Slingshot (SSH) family protein phosphatases, composed of SSH1, SSH2 and SSH3 in mammals [6–8]. SSH1 binds to filamentous actin (F-actin), and the cofilin-phosphatase activity of SSH1 is dramatically increased by its binding to F-actin [6–12]. During stimulus-induced cell migration, SSH1 accumulates into the F-actin-rich lamellipodia at the front of migrating cells [9, 13]. These results suggest that SSH1 is involved in cell migration by activating cofilin and promoting actin filament turnover in lamellipodia at the leading edge of migrating cells [9, 13]. However, little is known about the molecular mechanisms of regulation of SSH1 activity.

In chapter I of this thesis, I showed the novel mechanism of SSH1 activation in the cytoplasm, in which SSH1 is activated by gelsolin (GSN)-generated cytosolic actin filaments. I identified GSN, scinderin (SCIN), and several actin-binding proteins as SSH1-binding proteins by proteomic analysis. GSN and SCIN belong to a GSN superfamily and play important roles in actin remodeling by severing actin filaments, capping the plus ends of actin filaments, and nucleation of actin assembly [14, 15]. I also showed that SSH1 indirectly binds to GSN via soluble actin filaments in the cytosolic fraction. Previous studies demonstrated that cytoskeletal insoluble actin filaments promote SSH1 activation, but I show here that the GSN-generated soluble actin filaments also activate SSH1 in cell-free assays and in cultured cells. GSN overexpression promoted neuregulin (NRG)-induced cofilin dephosphorylation in MCF-7 cells, and latrunculin A (LatA), an inhibitor of actin polymerization, suppressed the effect of GSN on NRG-induced cofilin dephosphorylation. These results suggest that SSH1 is activated by

GSN-generated soluble actin filaments in the cytoplasm and that cytoplasmic SSH1 is also involved in NRG-induced cofilin dephosphorylation and cell migration. Most of the results in Chapter I were reported [16].

In chapter II of this thesis, I analyzed the role of a pleckstrin homology (PH)-like domain of SSH1 in regulating its cofilin-phosphatase activity. Previous studies demonstrated that F-actin binds to and activates SSH1 and that the N-terminal non-catalytic region of SSH1 (termed SSH-N domain, composed of conserved "A" and "B" domains) is involved in its F-actin-mediated activation. In this study, I found that SSH1 has a PH-like domain in the region crossing over the N-terminal "A" and "B" domains by the secondary structure prediction analysis. Since the PH domains are generally known to bind phospholipids, I performed phospholipid blot assays and found that SSH1 specifically associates with phosphatidylserine (PS) through its N-terminal region (amino acids 1-461). Although the basic residues in the N-terminal β1 and β2 strands of the PH domains were shown to constitute the phospholipid-binding site, replacements of these basic residues (Arg-96, Arg-104 and Arg-106) in the PH-like domain of SSH1 did not affect its PS-binding activity, and the incubation with PS did not affect the cofilin-phosphatase activity of SSH1. By contrast, the replacements of these basic residues in the PH-like domain of SSH1 inhibited its binding to actin filaments and suppressed its cofilin-phosphatase activity in the presence of F-actin. These results suggest that the PH-like domain of SSH1 plays a crucial role in the cofilin-phosphatase activity of SSH1 by binding to F-actin.

Chapter I

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

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 SSH family protein phosphatases, composed of SSH1, SSH2 and SSH3 in mammals [6–8]. 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 scinderin (SCIN) are Ca^{2+}-dependent actin-binding proteins that exhibit multiple actin-regulating functions, such as 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. Thus, GSN plays an important role in cell migration and morphogenesis through the regulation of actin filament dynamics and reorganization [14, 15, 17].

In this study, I examined the novel activation mechanism of SSH1 by GSN-generated cytosolic short actin filaments. I identified GSN, SCIN, and several actin-binding proteins as SSH1-binding proteins by proteomic analysis. We also showed that SSH1 binds to GSN via soluble actin filaments in the cytosolic fraction. Previous studies demonstrated that cytoskeletal insoluble F-actin promotes SSH1 activity, but I showed that the GSN-generated soluble actin filaments also activate the cofilin-phosphatase activity of SSH1 in vitro. GSN overexpression promoted neuregulin (NRG)-induced cofilin dephosphorylation in MCF-7 cells, and latrunculin

A, an inhibitor of actin polymerization, suppressed the effect of GSN overexpression on cofilin dephosphorylation. These results suggest that SSH1 is activated by GSN-generated soluble actin filaments in the cytoplasm and is involved in cofilin dephosphorylation during the migration of NRG-stimulated MCF-7 cells.

Chapter II

A pleckstrin homology-like domain of Slingshot-1 is critical for its cofilin phosphatase activity by binding to actin filaments

The pleckstrin homology (PH) domain is a protein module composed of approximately 100 amino acid residues and is found in a wide variety of proteins involved in signal-transduction, cytoskeletal organization, intracellular trafficking and phospholipid processing [18, 19]. The PH domains have an almost identical core structure, consisting of strongly bent seven-stranded anti-parallel β-strands, closed on one end by a C-terminal α-helix. The basic residues located near the β1 and β2 strands of the PH domain form the binding site of phospholipid headgroups. The PH domains play a crucial role in translocation of proteins to the plasma membrane depending on local lipid concentration, resulting in local activation of proteins, such as Akt, 3-phosphoinositide-dependent protein kinase 1 (PDK1) and phospholipase C-γ (PLC-γ) [20–23].

The PH domain plays a role in recognition of phospholipids but also mediates protein-protein interactions. A subset of PH domains, including those of Bruton's tyrosine kinase (Btk), Emt tyrosine kinase, oxysterol-binding protein (OSBP), and pleckstrin (both N- and C-terminal PH domains), binds to actin filaments and the basic residues near the β1 and β2 strands in their PH domains form an F-actin-binding site [24]. The PH domain of Btk is required for its localization to the actin cytoskeleton. The PH domain of PLC-γ2 lacks the corresponding basic residues in the β1 strand and has no actin-binding capacity, but its mutant with substitutions of three amino acids by lysine residues in the β1 strand gains actin-binding ability, which suggests that the basic residues in the β1 strand are essential for actin recognition [24].

Slingshot-1 (SSH1) is a protein phosphatase, which specifically dephosphorylates and activates cofilin and regulates actin cytoskeletal reorganization and directional cell migration by dephosphorylating and activating cofilin [6]. Previous studies revealed that F-actin binds to and dramatically activates SSH1, and the N-terminal non-catalytic region of SSH1 (called SSH-N domain composed of “A” and “B” domains) (Fig. 8A), is required for F-actin-mediated activation of the cofilin-phosphatase activity of SSH1 [6–12].

To understand the molecular mechanism of SSH1 activation, I analyzed the secondary structure of SSH1. The secondary structure prediction analysis suggests that the N-terminal region of SSH1 contains a PH-like domain in the region spanning between the "A" and "B" domains. Since the PH domains are generally known to bind phospholipids, I performed phospholipid blot assays and found that SSH1 specifically associates with phosphatidylserine (PS) through its N-terminal region (amino acids 1-461). Although the basic residues in the β1/β2 loop of the PH domain commonly form phospholipids-binding sites, replacements of the basic residues to acidic residues or alanine in the β1/β2 loop of the PH-like domain of SSH1(1-461) did not affect its PS-binding ability, and the incubation with PS did not affect the cofilin-phosphatase activity of SSH1. By contrast, replacements of the basic residues in the β1/β2 loop of the PH-like domain inhibited the association of SSH1 with actin and suppressed the cofilin-phosphatase activity of SSH1 in the presence of F-actin. Furthermore, I also showed that while the PH-like domain of SSH1 co-localized with actin filaments in HeLa cells, a mutant of the PH-like domain of SSH1 did not co-localize with actin filaments. These results suggest that the PH-like domain of SSH1 is essential for actin-binding and cofilin-phosphatase activities of SSH1.

References

[1] Bernstein BW & Bamburg JR (2010) ADF/cofilin: a functional node in cell biology. *Trends Cell Biol.* **20**, 187–195.

[2] Ono S (2007) Mechanism of depolymerization and severing of actin filaments and its significance in cytoskeletal dynamics. *Int. Rev. Cytol.* **258**, 1–82.

[3] Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E & Mizuno K (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393**, 809–812.

[4] Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O & Caroni P (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393**, 805–809.

[5] Toshima J, Toshima JY, Amano T, Yang N, Narumiya S & Mizuno K (2001) Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. *Mol. Biol. Cell* **12**, 1131–1145.

[6] Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K & Uemura T (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* **108**, 233–246.

[7] Ohta Y, Kousaka K, Nagata-Ohashi K, Ohashi K, Muramoto A, Shima Y, Niwa R, Uemura T & Mizuno K (2003) Differential activities, subcellular distribution and tissue expression patterns of three members of Slingshot family phosphatases that dephosphorylate cofilin. *Genes to Cells* **8**, 811–824.

[8] Mizuno K (2013) Signaling mechanisms and functional roles of cofilin phosphorylation and dephosphorylation. *Cell. Signal.* **25**, 457–469.

[9] Nagata-Ohashi K, Ohta Y, Goto K, Chiba S, Mori R, Nishita M, Ohashi K, Kousaka K, Iwamatsu A, Niwa R, Uemura T & Mizuno K (2004) A pathway of neuregulin-induced activation of cofilin-phosphatase Slingshot and cofilin in lamellipodia. *J. Cell Biol.* **165**, 465–471.

[10] Yamamoto M, Nagata-Ohashi K, Ohta Y, Ohashi K & Mizuno K (2006) Identification of multiple actin-binding sites in cofilin-phosphatase Slingshot-1L. *FEBS Lett.* **580**, 1789–1794.

[11] Kurita S, Gunji E, Ohashi K & Mizuno K (2007) Actin filaments-stabilizing and -bundling activities of cofilin-phosphatase Slingshot-1. *Genes to Cells* **12**, 663–676.

[12] Kurita S, Watanabe Y, Gunji E, Ohashi K & Mizuno K (2008) Molecular dissection of the mechanisms of substrate recognition and F-actin-mediated activation of cofilin-phosphatase Slingshot-1. *J. Biol. Chem.* **283**, 32542–32552.

[13] Nishita M, Tomizawa C, Yamamoto M, Horita Y, Ohashi K & Mizuno K (2005) Spatial and temporal regulation of cofilin activity by LIM kinase and Slingshot is critical for directional cell migration. *J. Cell Biol.* **171**, 349–359.

[14] Nag S, Larsson M, Robinson RC & Burtnick LD (2013) Gelsolin: the tail of a molecular gymnast. *Cytoskeleton* **70**, 360–384.

[15] Kwiatkowski DJ (1999) Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr. Opin. Cell Biol.* **11**, 103–108.

[16] Takahashi K, Kanno S-I & Mizuno K (2014) Activation of cytosolic Slingshot-1 phosphatase by gelsolin-generated soluble actin filaments. *Biochem. Biophys. Res. Commun.* **454**, 471–477.

[17] Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP & Kwiatkowski DJ (1995) Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* **81**, 41–51.

[18] Scheffzek K & Welti S (2012) Pleckstrin homology (PH) like domains - versatile modules in protein-protein interaction platforms. *FEBS Lett.* **586**, 2662–2673.

[19] Lemmon M & Ferguson K (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem. J.* **18**, 1–18.

[20] Walliser C, Retlich M, Harris R, Everett KL, Josephs MB, Vatter P, Esposito D, Driscoll PC, Katan M, Gierschik P & Bunney TD (2008) Rac regulates its effector phospholipase Cgamma2 through interaction with a split pleckstrin homology domain. *J. Biol. Chem.* **283**, 30351–30362.

[21] Calleja V, Alcor D, Laguerre M, Park J, Vojnovic B, Hemmings BA, Downward J, Parker PJ & Larijani B (2007) Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *PLoS Biol.* **5**, 780–791.

[22] Currie RA, Walker KS, Gray A, Deak M, Casamayor A, Downes CP, Cohen P, Alessi DR & Lucocq J (1999) Role of phosphatidylinositol 3,4,5-triphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem. J.* **337**, 575–583.

[23] Huang BX, Akbar M, Kevala K & Kim H-Y (2011) Phosphatidylserine is a critical modulator for Akt activation. *J. Cell Biol.* **192**, 979–992.

[24] Yao L, Janmey P, Frigeri LG, Han W, Fujita J, Kawakami Y, Apgar JR & Kawakami T (1999) Pleckstrin Homology Domains Interact with Filamentous Actin. *J. Biol. Chem.* **274**, 19752–19761.