

Involvement of Fyn tyrosine kinase in actin stress fiber formation in fibroblasts

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Abstract Lysophosphatidic acid (LPA) and sphingosylphosphorylcholine (SPC) activated Fyn tyrosine kinase and induced stress fiber formation, which was blocked by pharmacological inhibition of Fyn, gene silencing of Fyn, or dominant negative Fyn. Overexpressed constitutively active Fyn localized at both ends of F-actin bundles and triggered stress fiber formation, only the latter of which was abolished by Rho-kinase (ROCK) inhibition. SPC, but not LPA, induced filopodia-like protrusion formation, which was not mediated by Fyn and ROCK. Thus, Fyn appears to act downstream of LPA and SPC to specifically stimulate stress fiber formation mediated by ROCK in fibroblasts.

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Keywords: Lysophosphatidic acid; Sphingosylphosphorylcholine; Rho-kinase; Fyn tyrosine kinase; Stress fiber

1. Introduction

Actin stress fibers are one of the major cytoskeleton structures in fibroblasts and play important roles in various cellular functions, including cell motility, contraction, tumorigenicity and morphogenesis [1]. The small GTPase Rho and its effector Rho-kinase (ROCK) are well-known to play a critical role in stress fiber formation and implicated in the formation of stress fibers induced by L- α -lysophosphatidic acid (LPA) and sphingosylphosphorylcholine (SPC) [2,3].

It is known that stress fibers are formed from bundles of actin microfilaments alternating polarity, and myosin II. ROCK is one of the most important Rho effectors and is essential for the formation of stress fibers. ROCK increases the phosphorylation of myosin light chain (MLC), either by directly phosphorylating MLC or negatively regulating myosin light chain phosphatase (MLCP) through the phosphorylation of myosin

phosphatase target subunit 1 (MYPT1), and thus enhances the actin binding and actomyosin-based contractility. The enhanced binding of myosin to actin promotes the bundling of F-actin into stress fibers [4].

Src family protein tyrosine kinases (SrcPTKs), key regulators of signal transduction, are involved in the actin cytoskeleton rearrangement [5]. Among them, c-Src tyrosine kinase was reported to inhibit the formation of stress fibers [6]. Fyn tyrosine kinase, another member of SrcPTKs, is widely expressed in many tissues and involved in a variety of signaling pathways such as integrin-mediated signaling [7] and cell–cell adhesion [8]. However, the effect of Fyn tyrosine kinase on stress fiber formation remains to be elucidated.

We previously found that SrcPTKs, especially Fyn, was involved in Ca²⁺ sensitization of vascular smooth muscle (VSM) contraction mediated by a SPC–ROCK pathway [9]. In this study, we demonstrate the first direct evidence that Fyn tyrosine kinase acts as a novel signaling molecule in ROCK-mediated stress fiber formation in fibroblasts.

2. Materials and methods

2.1. Cell culture and pharmacological treatments

NIH3T3 fibroblasts were grown in DMEM supplemented with 10% bovine calf serum. Cells were serum-starved for 24 h and stimulated with SPC (30 μ M, 3 min) or LPA (1 μ g/ml, 5 min). Inhibitors including Y27632 (10 μ M), PP1 (20 μ M), PP2 (20 μ M), PP3 (20 μ M), and EPA (60 μ M) were applied for 30 min before cell stimulation.

2.2. siRNA transfection

mFyn1 and mFyn2 siRNAs were synthesized by RNAi and Nippon EGT, respectively. Control (non-silencing) siRNA and mitogen-activated protein kinase 1 (MAPK1) siRNA were obtained from Qiagen. Transfection conditions were analyzed with non-silencing Alexa Fluor 488 siRNA (Qiagen). Cells were transfected with 40 nM siRNA using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. At 48 h after the siRNA transfection, cells were serum-starved for 24 h, followed by cell stimulation with SPC or LPA and the determination of knockdown efficiency by Western blot analysis.

2.3. Western blot analysis

Cells were dissolved in lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM Na₃VO₄, 10 mM NaF, 0.5% NP-40, 1% TritonX-100, 1% SDS, and protease inhibitor cocktail (Sigma–Aldrich)]. Cell lysates were separated by SDS–PAGE, and subjected to immunoblotting with proper antibodies against MYPT1 (Santa Cruz), phospho-Thr853 of MYPT1 (Santa Cruz), phospho-Tyr416 of SrcPTKs (pSrcY416, Cell Signaling technology), Fyn (BD transduction laboratories or Santa Cruz), MAPK

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Abbreviations: SPC, sphingosylphosphorylcholine; SrcPTKs, Src family protein tyrosine kinases; ROCK, Rho-kinase; LPA, L- α -lysophosphatidic acid; MYPT1, myosin phosphatase target subunit 1; MLCP, myosin light chain phosphatase; MLC, myosin light chain; FAK, focal adhesion kinase

(Upstate), and GAPDH (Chemicon). Signals were visualized using the SuperSignal West Pico chemiluminescence substrates (Pierce) and evaluated using software named Quantity One with ChemiDoc XRS-J (Bio-Rad).

2.4. Immunoprecipitation of Fyn

Cells were lysed in immunoprecipitation (IP) buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1 mM Na_3VO_4 , 10 mM NaF, 0.5% NP-40, 1% TritonX-100, and protease inhibitor cocktail] containing 1% SDS. Cell extracts (500 μg) were incubated with 2 μg of the anti-Fyn antibody (BD transduction laboratories) and protein A/G plus agarose (Santa Cruz) for IP. Subsequently, immunoblotting analysis was performed using One-Step Complete IP-Western Kit (GenScript Corporation) for anti-Fyn antibody (Santa Cruz) and pSrcY416 antibody.

2.5. Plasmid construct and transient transfection

The human cDNAs encoding constitutively active Fyn (ca-Fyn, Y530F), dominant negative Fyn (dn-Fyn, K298M), and wild type Fyn (WT-Fyn) were subcloned into the pcDNA6/myc-His A vector (Invitrogen). The constructs were verified by DNA sequencing. NIH3T3 cells were transfected with 2 μg of plasmid DNA using a Nucleofector II under the NIH3T3 conditions recommended by the manufacturer (Amaxa Biosystem). pmaxGFP (Amaxa Biosystem) was used to monitor transfection efficiency (>70%). After transfection, cells were given 8 h to adhere in fresh serum-containing medium, and then serum-starved for 24 h before cell stimulation and Western blot analysis.

2.6. Fluorescence staining and microscopic study

Cells were fixed and stained with anti-myc (clone 9E10, Santa Cruz) antibody or anti-FAK (Upstate) antibody, followed by goat anti-mouse IgG antibody conjugated with AF488 (Invitrogen) or goat anti-rabbit IgG antibody conjugated with AF350 (Invitrogen). F-actin was labeled with AF 488- or rhodamine-conjugated phalloidin (Invitrogen). Stained cells were analyzed on a confocal microscope (LSM-510, Zeiss) or fluorescent microscope (Axiovert 200M, Zeiss) equipped with or without ApoTome (Zeiss). Fluorescence intensity profile analysis was performed using AxioVision Rel. 4.5 fluorescent image analysis algorithms on fluorescent microscope.

2.7. Statistics

Data are expressed as means \pm S.E.M. from three independent experiments. Statistical differences were analyzed with one-way ANOVA. $P < 0.05$ was considered to be significant.

3. Results and discussion

LPA and SPC, well-known regulators of actin cytoskeleton reorganization, induced stress fiber formation (arrows in Fig. 1A, upper panel). In contrast, only SPC, but not LPA, induced filopodia-like protrusion formation (arrowheads in Fig. 1A and Fig. S1 in supplementary data), which was consistent with previous reports [3,10]. Therefore, we investigated the

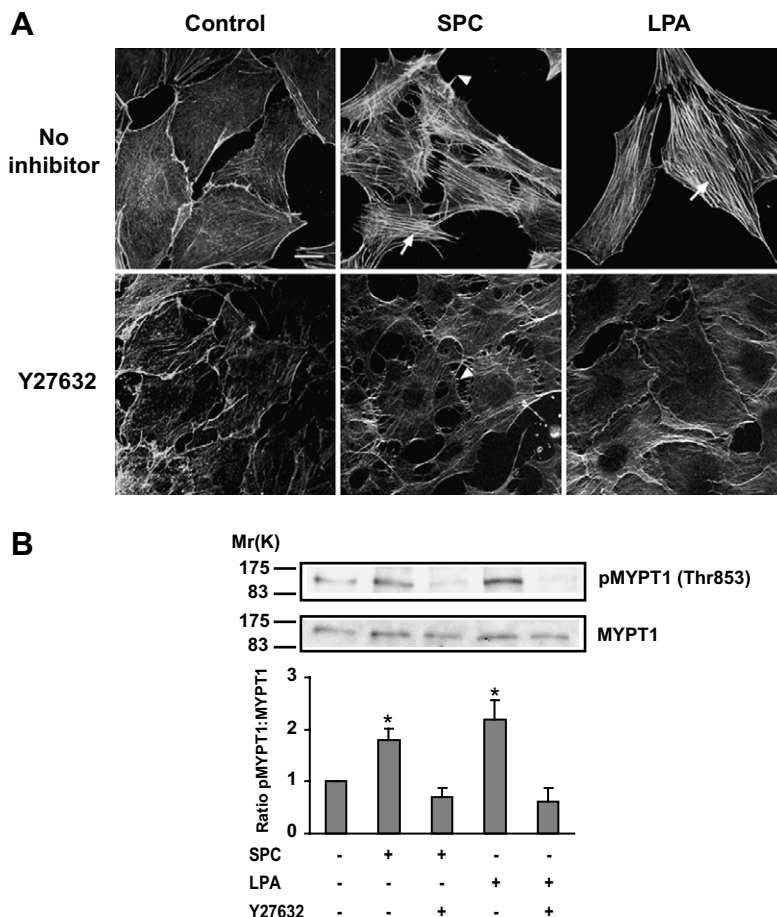


Fig. 1. Effect of Y27632 on stress fiber formation and activation of ROCK. (A) Representative confocal images showing the inhibitory effect of Y27632 on stress fiber formation induced by LPA and SPC. The arrows indicate stress fibers. The arrowheads indicate filopodia-like protrusions. Scale bar = 20 μm . (B) Representative immunoblots using anti-MYPT1 or anti-pMYPT1 (Thr853) antibodies. In bar graph, statistical evaluation of effect of Y27632 on the activation of ROCK was expressed as the ratio of pMYPT1 to MYPT1 (* $P < 0.05$ versus control).

involvement of ROCK in these two types of actin cytoskeleton reorganization. The specificity of the ROCK inhibitor Y27632 is tested *in vitro* by Uehata et al. [11]. Our results showed that specific ROCK inhibitor Y27632 abolished the stress fiber formation induced by either LPA or SPC, but had no effect on the filopodia-like protrusion formation induced by SPC (Fig. 1A, lower panel and Fig. S1 in supplementary data), which supports the specificity of the blocking effect of Y27632 on ROCK-mediated stress fiber formation. Furthermore, Y27632 blocked the activation of ROCK induced by LPA or SPC (Fig. 1B), as assessed by the phosphorylation at Thr853 of MYPT1 [12]. These findings suggest that LPA and SPC indeed activated ROCK, which in turn phosphorylated at Thr853 of MYPT1 and thereby inactivated MLCP, leading to MLC phosphorylation and the resultant formation of stress fibers.

SrcPTKs are involved in a variety of cellular signaling pathways [5]. c-Src and its oncogenic form, v-Src are known to cause disassembly of actin stress fibers [6,13,14], suggesting that SrcPTKs inhibit stress fiber formation. In addition, because redundancy between the effects of each member of SrcPTKs has been well documented in their knockout studies [15], inhibition of SrcPTKs would be expected to enhance stress fiber formation. However, surprisingly, frequently and widely used inhibitor of SrcPTKs, PP1 and PP2, rather inhibited

the stress fiber formation induced by LPA or SPC (Fig. 2), although the observed inhibitions were partial in contrast to the complete inhibition by Y27632 (Fig. 1A). The unexpected inhibitions by PP1 and PP2 are not simply due to non-specific effect because its inactive analogue, PP3, showed no inhibition (Fig. 2). Therefore, it is plausible to hypothesize the presence of a member of SrcPTKs having strong stimulatory effect, which can overcome the inhibitory effect of c-Src, on the formation of stress fibers. To examine this hypothesis, we then used eicosapentaenoic acid (EPA) to inhibit the activities of some members of SrcPTKs without affecting c-Src, because EPA has been reported to inhibit translocation and activation of some of SrcPTKs (especially Fyn), but not of c-Src [9,16]. As shown in Fig. 3A, the EPA treated cells appeared to have much less phalloidin staining in the cytoplasm than Y27632 treated cells. EPA almost completely abolished the stress fiber formation induced by LPA or SPC. The partial inhibition by PP1 or PP2 and the complete inhibition by EPA, of the stress fiber formation, are compatible with the notion that EPA abolished only the stimulatory effect of Fyn, but not the inhibitory effect of c-Src, on the stress fiber formation, whereas PP1 and PP2 inhibited both stimulatory and inhibitory effects of SrcPTKs. These inhibitions are not due to non-specific effects of these inhibitors on the actin cytoskeleton reorganization, because they, like Y27632, selectively suppressed the formation

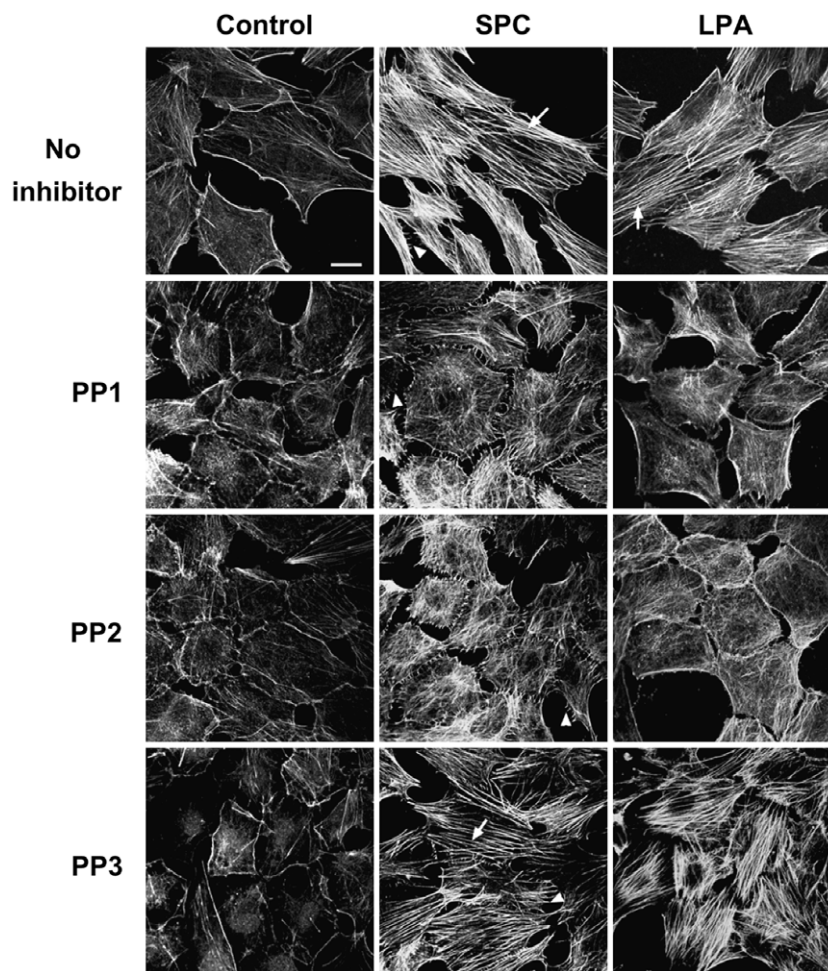


Fig. 2. Representative confocal images showing the inhibitory effect of SrcPTKs inhibitors on stress fiber formation induced by LPA and SPC. The arrows indicate stress fibers. The arrowheads indicate filopodia-like protrusions. Scale bar = 20 μ m.

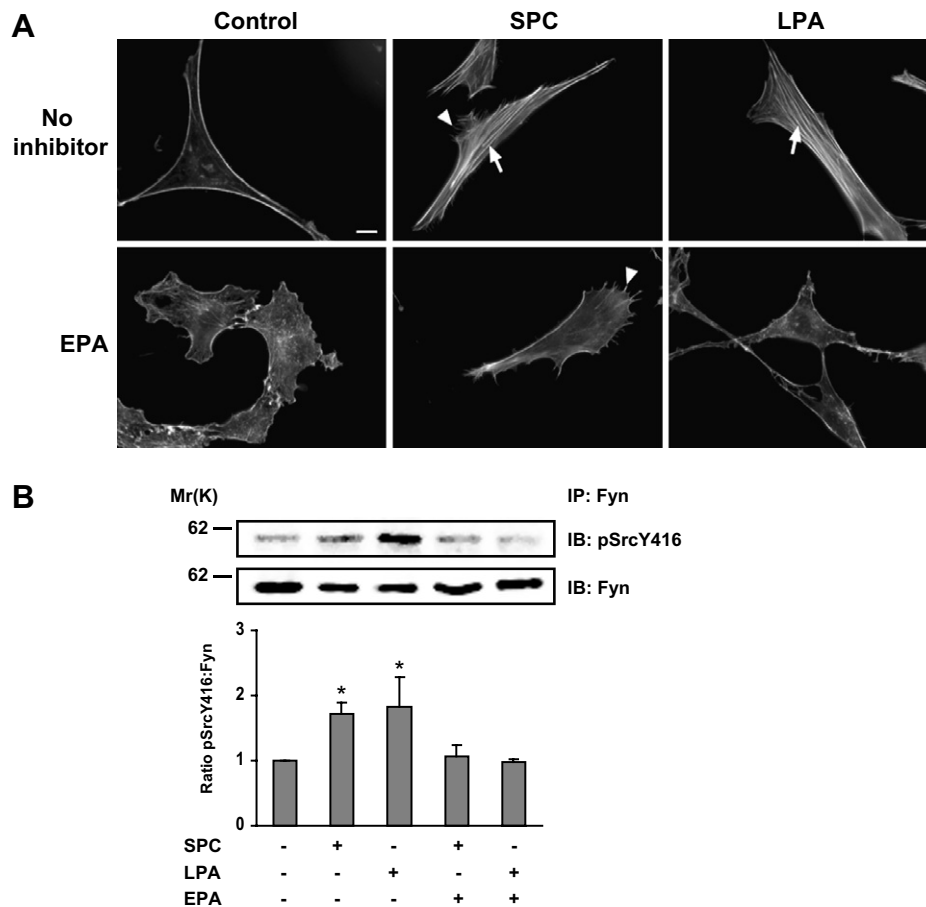


Fig. 3. Effect of EPA on stress fiber formation and activation of Fyn. (A) Representative fluorescence images showing the inhibitory effect of EPA on stress fiber formation induced by LPA and SPC. The arrows indicate stress fibers. The arrowheads indicate filopodia-like protrusions. Scale bar = 50 μ m. (B) Representative immunoblots using anti-Fyn or anti-pSrcY416 antibodies after immunoprecipitation using anti-Fyn antibody. In bar graph, statistical evaluation of effect of EPA on the activation of Fyn was expressed as the ratio of pSrcY416 to Fyn ($*P < 0.05$ versus control).

of actin stress fibers, but not of filopodia-like protrusions (Figs. 2, 3A and Fig. S1 in supplementary data). In addition, LPA or SPC significantly induced the activation of Fyn, which was also inhibited by EPA (Fig. 3B). Taken together, these results also support the specific involvement of some SrcPTKs (probably Fyn) in the stress fiber formation induced by LPA or SPC.

In order to obtain the direct evidence for the stimulatory effect of Fyn on the actin stress fiber formation, we conducted loss-of-function and gain-of-function studies as follows.

Firstly, we attempted to knock down the expression of Fyn by Fyn siRNAs (mFyn1 and mFyn2). The siRNA transfection efficiency was generally >90%, which was monitored by non-silencing AF488 siRNA at either 24 h or 48 h after transfection (Fig. S2 in supplementary data). As a positive control, MAPK1 siRNA induced remarkable knockdown of both p44 and p42 of mouse MAPK, without affecting the expressions of Fyn and GAPDH (Fig. 4A). Transfection with mFyn1 and mFyn2 siRNA specifically knocked down Fyn levels by 82% and 87%, respectively, although less than 20% of Fyn level was left in the cells, without affecting the expressions of MAPK and GAPDH. As a negative control, control siRNA showed no inhibition. These results indicate that Fyn protein was specifically knocked down by its targeting siRNA (Fig. 4A). As shown in Fig. 4B, siRNA-mediated knockdown of Fyn

remarkably blocked the formation of actin stress fibers induced by LPA or SPC. In contrast, neither MAPK1 siRNA nor control siRNA affected the stress fiber formation. Furthermore, we found no inhibition of SPC-induced filopodia-like protrusions formation by any siRNA. Even after transfection with mFyn1 and mFyn2 siRNA, filopodia-like protrusions were still seen clearly (Fig. S3 in supplementary data). These results strongly suggest the involvement of Fyn, but not MAPK, in the stress fiber formation induced by LPA or SPC.

Secondly, Fyn constructs were transfected into NIH3T3 fibroblasts to overexpress the corresponding mutated Fyn and WT-Fyn. We confirmed the overexpression and activation of Fyn constructs in NIH3T3 fibroblasts (data not shown), and then investigated the effects of Fyn constructs on stress fiber formation induced by LPA or SPC. When serum-starved NIH3T3 fibroblasts were stimulated with SPC, stress fiber formation was induced in the cells transfected with mock or WT-Fyn, but not in the dn-Fyn-overexpressed cells (Fig. 5A). Similar results were obtained, when the cells were stimulated with LPA (data not shown). Furthermore, SPC-induced filopodia-like protrusions can be still seen in those cells transfected with dn-Fyn and WT-Fyn (Fig. 5A and Fig. S4 in supplementary data). In contrast, overexpression of ca-Fyn stimulated the formation of stress fibers (arrow in Fig. 5B, upper panel), but not filopodia-like protrusions. Taken to-

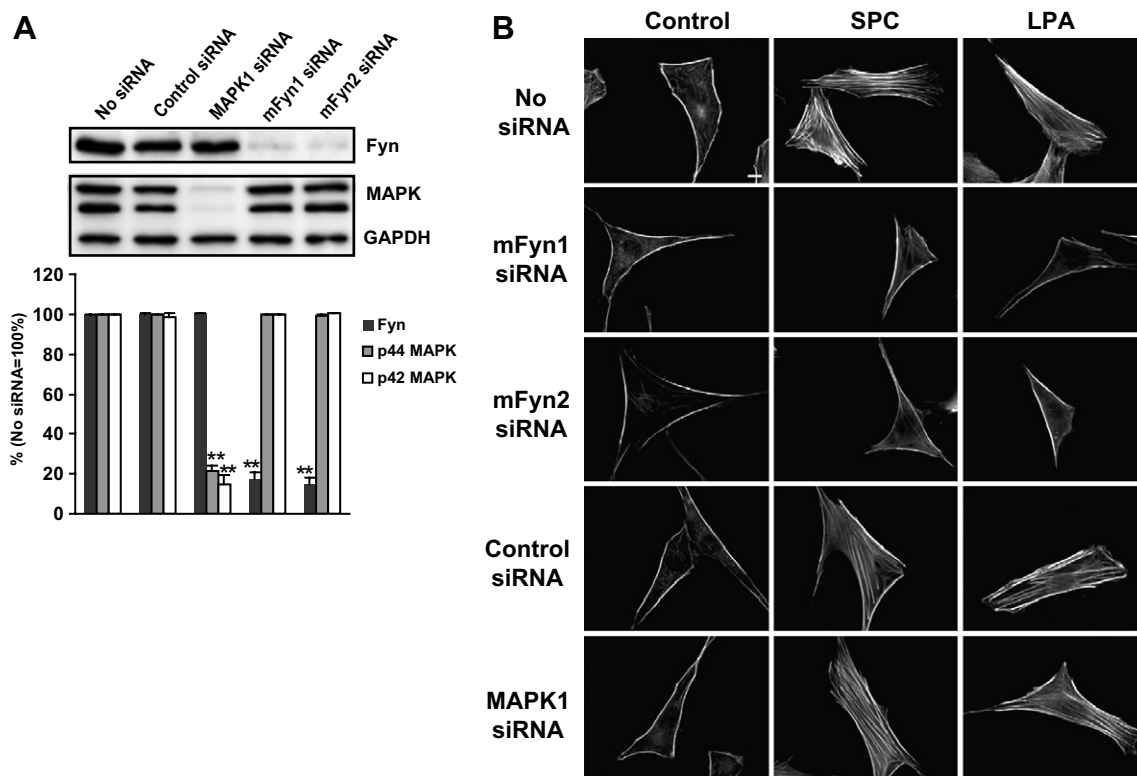


Fig. 4. Effects of Fyn siRNAs on stress fiber formation induced by SPC and LPA. (A) Representative immunoblots using anti-Fyn, anti-MAPK or anti-GAPDH antibodies at 48 h after siRNA transfection. In bar graph, statistical evaluation of effects of siRNAs on Fyn and MAPK levels was expressed as the ratio of Fyn or MAPK to GAPDH (** $P < 0.001$ versus siRNA-untransfected cells). (B) Representative fluorescence images showing the effect of siRNAs on the formation of stress fibers induced by LPA and SPC. Scale bar = 50 μ m.

gether, these data provide the first direct evidence that Fyn activation is necessary for either LPA or SPC to trigger the formation of stress fibers.

Varying observations have been reported with respect to the steady-state intracellular distribution of Fyn. Fyn protein is synthesized and *N*-myristoylated on cytosolic polysomes and then rapidly targeted to the plasma membrane, where it is palmitoylated. In human T-lymphocytes, Fyn was mainly associated with centrosomal structures or exclusively in association with the T-cell receptor complex at the plasma membrane. Immunofluorescence studies in NIH3T3 cells revealed that Fyn is localized to both the plasma membrane and intracellular, perinuclear membrane [17]. We have seen that endogenous Fyn is mainly present in the cytosol and little on the membrane (data not shown). Similarly, in the present study we found that WT-Fyn was mainly present in cytosol without stimulation, whereas it translocated to cell membrane and enriched at the ends of F-actin when activated by LPA or SPC (Fig. S4 in supplementary data and Fig. 5A).

Interestingly, similar with activated WT-Fyn by SPC (Fig. 5A, bottom panel), ca-Fyn was exclusively localized at the ends of F-actin (Fig. 5B, arrowheads). This was evidenced by a profile analysis as shown in histograms (Fig. 5C), where the fluorescence intensity in ca-Fyn (green) channel arrived at peaks on merged location on line a but not other positions on line b (Fig. 5C enlarged photo), along with increased fluorescence intensity in F-actin (red) channel. ROCK inhibitor Y27632 abolished stress fiber formation induced by ca-Fyn (Fig. 5B lower panel), without affecting the localization of

ca-Fyn. These findings indicated that Fyn may be upstream signal molecule of ROCK to induce stress fiber formation. In addition, it was also suggested that ROCK may play an important role in not only the formation but also the maintenance of stress fibers.

Fyn is known to interact with multiple intracellular substrates related to cytoskeleton, including focal adhesion kinase (FAK), catenin, and p130Cas, linked to cellular events such as focal adhesion, cell–cell adhesion, cell motility [8,18,19]. Therefore, these previous findings, taken together with the observation in the present study that ca-Fyn and F-actin are colocalized, strongly suggest that Fyn and both the ends of the actin stress fibers may bind to common structures, presumably the focal adhesion.

It is reported that FAK, a protein tyrosine kinase that is localized at focal adhesion sites, was concentrated at the ends of actin stress fibers in both high- and low-density cells [20]. Therefore, NIH3T3 cells were transfected with ca-Fyn and then co-stained with anti-myc antibody and anti-FAK antibody. Actin filaments were also stained with rhodamine-conjugated phalloidin. As shown in Fig. 5D, Fyn colocalized with FAK to the focal adhesion, which was evidenced by a profile analysis as shown in histograms (Fig. 5E), where the fluorescence intensity in ca-Fyn (green) channel arrived at peaks along with increased fluorescence intensity in FAK (blue) and F-actin (red) channels on merged location on line c indicated in enlarged photo.

Immunofluorescence studies (Fig. 5B–E) in ca-Fyn-transfected cells indicate an association between Fyn, actin cytoskele-

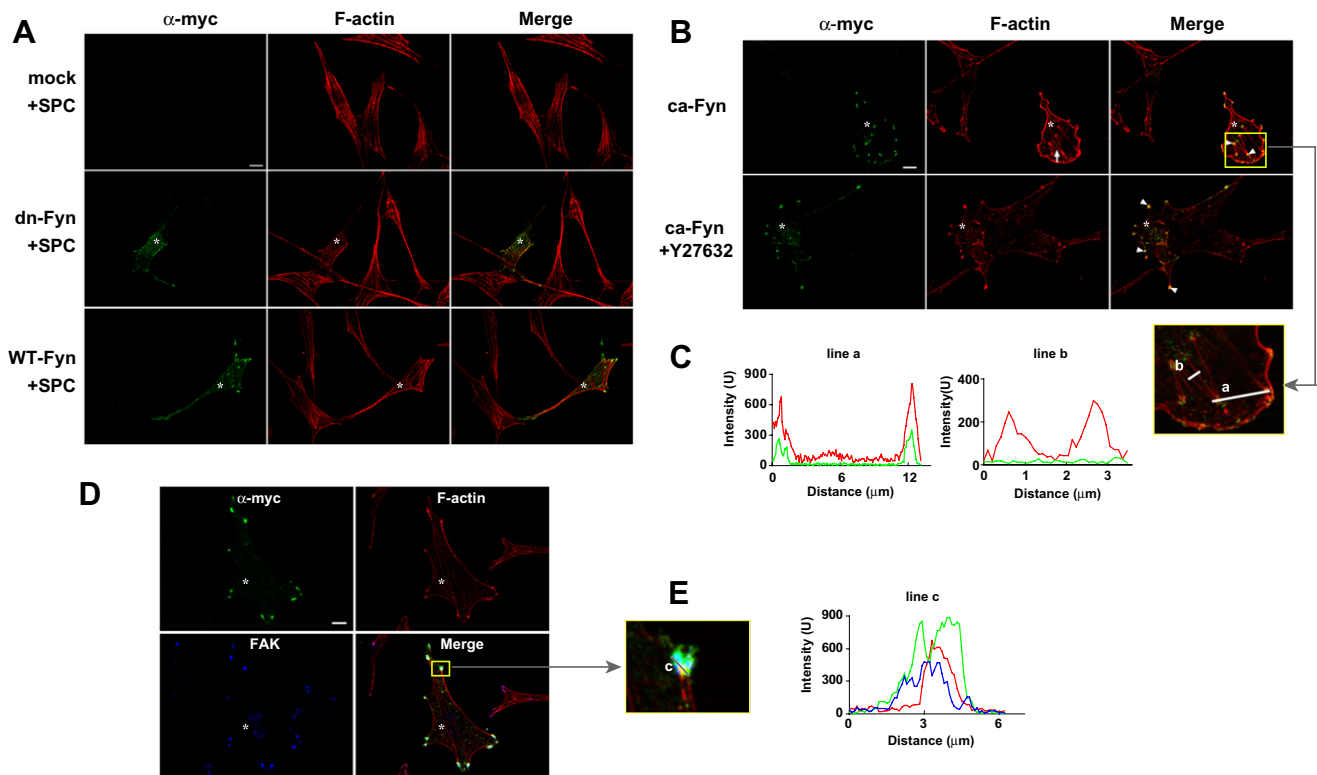


Fig. 5. Effects of myc-tagged Fyn constructs on stress fiber formation induced by SPC and LPA. (A, B and D) Representative fluorescence images with ApoTome showing the immunostaining of myc-tagged Fyn constructs (green), F-actin (red) or FAK (blue in D). Asterisks indicate the cells transfected with Fyn constructs. The arrow indicates stress fibers. The arrowheads indicate the localization of ca-Fyn at both ends of F-actin. Scale bar = 10 μ m. (C and E) Histograms show the fluorescence profiles for the enlarged images of the boxed area from B and D, illustrating the fluorescence intensity (arbitrary units, U) in each channel for c-myc-tagged Fyn (green lines), F-actin (red lines) or FAK (blue line in E) against distance along the lines (a–c) indicated on the micrograph.

ton and FAK on focal adhesion, in which mediators initiate chains of cellular events such as morphological changes. It has been reported that EPA reduced tumor adhesion to a range of matrix components [21], therefore, we suppose that EPA may affect the focal adhesion complex including Fyn and FAK, resulting in the morphological change in the EPA-treated cells (Fig. 3A).

Cell density is reported to affect the localization of FAK associated with the rearrangement of actin stress fibers [20]. However, the distribution of actin stress fiber in our study, either at low or high densities, was much similar to that over the ventral surface of cells at high cell densities in Xu's report. Such difference might be due to the difference of cell types or the matrix component, which could cause the difference in integrin-mediated signaling.

Both LPA and SPC are recognized as important signaling molecules functioning in a variety of intracellular pathways that targeting cytoskeleton rearrangement, cell migration, cell growth and cell survival. In respect to stress fiber formation, LPA and SPC induced the activation of Rho (data not shown), and thereby ROCK (Fig. 1B), which can promote the bundling of F-actin. Therefore, we suppose that ROCK inhibitor Y27632 inhibits stress fiber formation induced by LPA or SPC through the inhibition of F-actin bundling. On the other hand, SrcPTKs inhibitors PP1 and PP2 inhibited both actin polymerization through the inhibition of Rho/mDia and F-actin bundling through the inhibition of Rho/ROCK. However, the inhibition of PP1 and PP2 to stress fiber formation was

partial because those SrcPTKs inhibitors also inhibited c-Src, which is reported to inhibit stress fiber formation.

Distinct from LPA, it is reported that SPC can induce a unique pattern of filopodia-like protrusions (also called as actin microspikes and magunapodia) [3,10], except for actin stress fiber formation. However, a precise mechanism is yet to be sought for the action of SPC on the formation of filopodia-like protrusions. Filopodia have been proposed to function in sensing environmental cues to guide cell migration, epithelial adhesion and wound healing [22,23], which may be helpful for the role of SPC in those fields.

Fyn tyrosine kinase and c-Src belong to the same family of SrcPTKs, and functional redundancy has been observed in the knockout studies of SrcPTKs [15]. Therefore, they are thought to be involved in some common cellular events such as FAK-mediated signaling events [18]. However, Fyn also plays distinct roles in specific pathways, such as Ca^{2+} sensitization of VSM contraction [9] and hyperosmotic-stress-induced caveolin phosphorylation [24]. Moreover, recent studies demonstrated that Fyn and c-Src have opposing effects on the formation of cell-cell junction and focal contacts [7,8]. Fyn and Src have been described to oppositely react to the same stimuli such as hyperosmolarity, which stimulated Fyn and inhibited Src [25]. In the present study using pharmacological inhibitor (Figs. 2 and 3), RNA interference (Fig. 4), and overexpression of mutated Fyn (Fig. 5), we demonstrated for the first time that Fyn surprisingly stimu-

lates stress fiber formation, reversing the inhibitory effect of c-Src.

Fyn is known to phosphorylate Vav [26], an important GEF for Rho and the existence of a positive feedback loop between Fyn and Rho has recently been suggested [8]. Fyn can interact with a brain-enriched Rho GTPase-activating protein TCGAP in the regulation of axon and dendrite outgrowth [27]. We recently found that SrcPTKs inhibitor PP2 can partially inhibit SPC-induced Rho activation (data not shown). Therefore, it seems that Rho activation by these stimuli is downstream of Fyn, and further study is ongoing in our laboratory for the confirmation of this notion.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2007.10.010](https://doi.org/10.1016/j.febslet.2007.10.010).

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