FEBS Letters 581 (2007) 5227–5233

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

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Received 9 July 2007; revised 14 September 2007; accepted 5 October 2007

Available online 12 October 2007

Edited by Michael R. Bubb

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 Ca^{2+} 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 μM, 3 min) or LPA (1 μg/ml, 5 min). Inhibitors including Y27632 (10 μM), PP1 (20 μM), PP2 (20 μM), PP3 (20 μM), and EPA (60 μM) 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 Na_3VO_4, 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

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doi:10.1016/j.febslet.2007.10.010

(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 ± 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

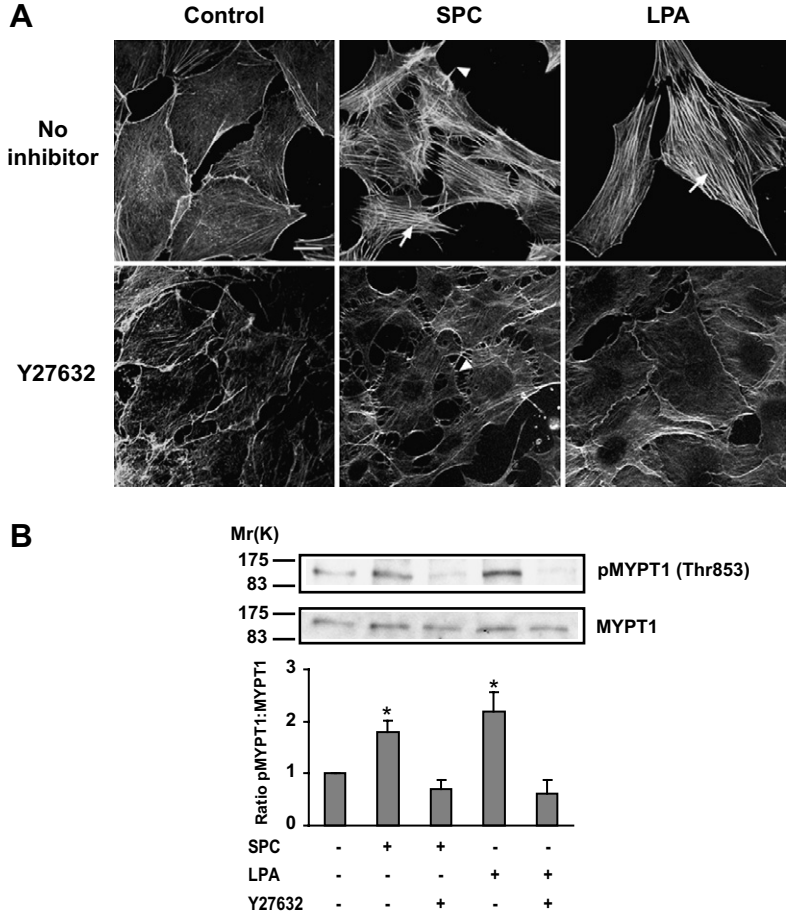

Fig. 1. Effect of Y27632 on stress fiber formation and activation of ROCK. (A) Representative confocal images showing the inhibitory effect of Y27632 on stress fiber formation induced by LPA and SPC. The arrows indicate stress fibers. The arrowheads indicate filopodia-like protrusions. Scale bar = 20 μm. (B) Representative immunblots 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).

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