EXPERIMENTAL CELL RESEARCH 314 (2008) 2040–2054

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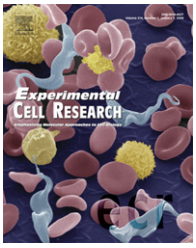

Research Article

Role of Src-family kinases in formation of the cortical actin cap at the dorsal cell surface

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ARTICLE INFORMATION

Article Chronology:
Received 20 October 2007
Revised version received
23 February 2008
Accepted 29 March 2008
Available online 7 April 2008

Keywords:
Src-family tyrosine kinase
Cortical actin cap
Lyn
c-Src
Actin filaments
Protein-tyrosine phosphatase
Tyrosine phosphorylation
Csk
Cdc42

ABSTRACT

Protein-tyrosine phosphorylation is regulated by protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs). Src-family tyrosine kinases (SFKs) participate in the regulation of the actin cytoskeleton. Actin filaments can be accumulated in a cap at the dorsal cell surface, which is called the cortical actin cap. Here, we show that SFKs play an important role in formation of the cortical actin cap. HeLa cells normally exhibit the cortical actin cap, one of the major sites of tyrosine phosphorylation. The cortical actin cap is disrupted by SFK inhibitors or overexpression of the Lyn SH3 domain. Csk-knockout cells form the cortical actin cap when the level of tyrosine phosphorylation is increased by Na_3VO_4, a PTP inhibitor, and the formation of the cortical actin cap is inhibited by SFK inactivation with re-introduction of Csk. SYF cells lacking SFKs minimally exhibit the cortical actin cap even in the presence of Na_3VO_4, and transfection with Lyn restores the cortical actin cap in the presence of Na_3VO_4. Disruption of the cortical actin cap by dominant-negative Cdc42 causes loss of tyrosine phosphorylation at the cell top. These results suggest that SFK(s) is involved in formation of the cortical actin cap, which may serve as a platform of tyrosine phosphorylation signaling.

Introduction

Protein-tyrosine phosphorylation is regulated by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) [1–3]. A wide variety of stimuli, including growth factors, cytokines, hormones, extracellular matrix components, and cell adhesion molecules, transmit signals via pathways involving tyrosine phosphorylation of specific cellular proteins. These signal transduction pathways dictate whether a cell will grow and divide, change shape, move, differentiate, or die [1–3].

Src-family tyrosine kinases (SFKs) belong to a group of non-receptor-type tyrosine kinases. SFKs participate in a wide range of signaling events, including cell proliferation, differentiation, migration, cell shape change, and cell division [4,5]. SFKs consist of proto-oncogene products and structurally related proteins and include at least eight highly homologous proteins: c-Src, c-Yes, Lyn, Fyn, Lck, Hck,

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doi:10.1016/j.yexcr.2008.03.018

Blk, and c-Fgr [4,5]. c-Src, c-Yes, Fyn, and Lyn are widely expressed in a variety of cell types, whereas Blk, c-Fgr, Hck, and Lck are found primarily in hematopoietic cells [4–6]. Multiple combinations in expression of each SFK member in a single cell are implicated to serve specific and overlapping functions [4–15].

SFKs are composed of: (i) a Src homology (SH) 4 domain, which contains lipid modification sites, (ii) a poorly conserved "unique" domain, (iii) an SH3 domain, which can bind to specific proline-rich sequences, (iv) an SH2 domain, which can bind to specific sites of tyrosine phosphorylation, (v) an SH1 tyrosine kinase catalytic domain, and (vi) a negative regulatory tail for autoinhibition of kinase activity [4,5]. The kinase activity of SFKs is repressed through creating a distinctive "closed conformation" due to the intramolecular bindings of the SH2 domain to the tyrosine-phosphorylated tail catalyzed by the Csk-family and of the SH3 domain to the SH2-kinase linker. Activated SFKs are autophosphorylated at a tyrosine residue in the activation loop of the kinase domain, and the autophosphorylation is required for full SFK catalytic activity [4,5]. SFKs can interact with a large number of upstream regulators and downstream substrates through protein-protein interactions [4,5].

Activation of SFKs leads to rapid and profound changes in the cytoskeleton. Indeed, a number of SFK substrates are shown to regulate the actin cytoskeleton [4,5,16,17]. The actin cytoskeleton maintains cellular shape and plays a pivotal role in cell motility, adhesion, endocytosis, and cytokinesis [18–21]. A cell forms a number of discrete types of actin-based structures designed to fulfill specialized roles: for example, actin stress fibers, lamellipodia and ruffles, and filopodia [18,22,23]. Actin filaments can be accumulated in a cap at the dorsal cell surface, which is called the cortical actin cap [24].

In this study, we examined the involvement of SFKs in formation of the cortical actin cap, which is one of the major subcellular sites of tyrosine phosphorylation. We show that SFKs play an important role in formation of the cortical actin cap in HeLa cells using SFK inhibitors and dominant-negative Lyn constructs. The requirement of SFKs for formation of the cortical actin cap is further substantiated by an inducible expression system for Csk in Csk-knockout cells and transfection experiments with Lyn into SYF cells lacking expression of ubiquitous SFK members. In addition, disruption of the cortical actin cap is induced by a dominant-negative form of the Rho-family GTPase Cdc42, resulting in loss of tyrosine phosphorylation at the cell top. Our results indicate that an increase in SFK-mediated tyrosine phosphorylation induces formation of the cortical actin cap, which may serve as a platform of tyrosine phosphorylation signaling at the cell top.

Materials and methods

Plasmids

cDNAs encoding human c-Src (provided by D.J. Fujita [25]) and human Lyn (1–512; with 1 designating the initiator methionine) (provided by T. Yamamoto [26]) were subcloned into the pcDNA4/TO vector (Invitrogen) as described [14,27]. LynΔK-HA (1–298), subcloned into the pMKITneo vector (provided by K. Maruyama), was constructed as described [27]. LynΔKΔSH2-HA (1–164 and 245–298) was generated from LynΔK-HA by HindIII and SmaI digestion and blunting. LynΔKΔSH3-HA (1–61 and 118–298) was generated as follows. The DNA fragment corresponding to the amino acid sequence 1–61 of Lyn was amplified by PCR using the sense primer 5′-AAACACGCGTCGAGCGGGAAATATGGGATGT-3′ and the antisense primer 5′-AGAATGGCCACCTCTGGATCTTTAGTTTGAAACC-3′, and the amplified fragment was ligated with the fragment (118–298) of LynΔK-HA. LynΔKΔSH2-HA and LynΔKΔSH3-HA were subcloned into pMKITneo. Lyn118-GFP (1–118) was generated by fusion with EGFP obtained from the pEGFP-C1 vector (Clontech Laboratories, Inc.) and subcloned into pcDNA4/TO. The Trp→Ala mutation at position 99 [Lyn118 (W99A)-GFP] was generated by site-directed mutagenesis using the sense primer 5′-GGGACTTTGCTTTCCAAGCTTCTCCATGCTCCTCCAGG-3′ and the antisense primer 5′-CCTGGAGGAGCATGGAGAAGCTTGGAAAGCAAAGTCCC-3′. Rat Csk cDNA was provided by M. Okada and S. Nada [28]. Csk-HA (1–440) generated previously [27] was subcloned into pcDNA4/TO. The BOSH2BGFP-N1 vector encoding GFP-tagged histone H2B was provided by H. Saya [29,30]. mCherry-β-actin was constructed in the pEGFP-β-actin vector (provided by H. Bito [31]) by substitution of EGFP for mCherry obtained from the pRSET-B-mCherry vector (provided by R.Y. Tsien [32]) using NheI and Bsp1407I. pEF-BOS vectors encoding HA-tagged dominant-negative Cdc42 (HA-Cdc42-T17N), Rac1 (HA-Rac1-T17N), and RhoA (HA-RhoA-T19N) were provided by M. Negishi [33,34].

Cells and cell culture

HeLa (Japanese Collection of Research Bioresources), COS-1, MCF-7 (provided by H. Saya), A431 (provided by M.N. Fukuda), HEK293 (provided by M. Tagawa), NIH3T3 (provided by K. Igarashi), and Csk-knockout D5 and c-Src/c-Yes/Fyn-knockout SYF cells (provided by M. Okada and S. Nada) [11,35] were cultured in Iscove's modified Dulbecco's medium containing 5% fetal bovine serum (FBS). Transient transfection was performed using TransIT transfection reagent (Mirus) [14], LipofectAMINE™ 2000 reagent (Invitrogen) [14], or linear polyethylenimine (25 kDa) (Polysciences) [36]. The kinase activity of SFKs was specifically inhibited by PP2 (Calbiochem) [37] or SU6656 (Calbiochem) [38]. Na_3VO_4 was used to inhibit PTPs [39].

Csk-inducible D5/Csk32-5 cell line

The pCAG-TR (the tetracycline repressor) vector was constructed by subcloning TR obtained from the pcDNA6/TR vector (Invitrogen) into the pCAG vector (provided by J. Miyazaki [40]), as described for generation of the HeLa #3-2 clone [12]. A D5 cell clone co-transfected with pCAG-TR and a plasmid containing the hygromycin-resistance gene was selected in medium containing 5% FBS and 300 μg/ml hygromycin B (Wako Pure Chemicals, Osaka). TR expression in clone #51-13 was detected by Western blotting with anti-TR antibody (MoBiTec) (data not shown). The Csk-inducible D5/Csk32-5 cell line was generated from clone #51-13 by transfection with pcDNA4/TO expressing Csk-HA and selection in medium containing 5% FBS and 400 μg/ml Zeocin (Invitrogen). Doxycycline (Dox) was used at 1 μg/ml for 2 days to induce Csk-HA.