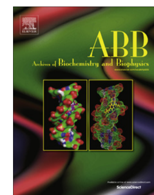
Archives of Biochemistry and Biophysics 568 (2015) 1–7

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



TGFβ1 rapidly activates Src through a non-canonical redox signaling mechanism

Hongqiao Zhang [a], Kelvin J.A. Davies [a,b], Henry Jay Forman [a,c,*]

[a] Ethel Percy Andrus Gerontology Center, Leonard Davis School of Gerontology, The University of Southern California, United States
[b] Division of Molecular & Computational Biology, Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, The University of Southern California, United States
[c] School of Natural Science, The University of California, Merced, United States

ARTICLE INFO

Article history:
Received 24 September 2014
and in revised form 30 December 2014
Available online 10 January 2015

Keywords:
Src
TGF-β
Hydrogen peroxide
Oxidative modification
Redox signaling

ABSTRACT

Transforming growth factor-β1 (TGF-β) is involved in multiple cellular processes through Src activation. In the canonical pathway, Src activation is initiated by pTyr530 dephosphorylation followed by a conformational change allowing Tyr419 auto-phosphorylation. A non-canonical pathway in which oxidation of cysteine allows bypassing of pTyr530 dephosphorylation has been reported. Here, we examined how TGF-β activates Src in H358 cells, a small cell lung carcinoma cell line. TGF-β increased Src Tyr419 phosphorylation, but surprisingly, Tyr530 phosphorylation was increased rather than decreased. Vanadate, a protein tyrosine phosphatase inhibitor, stimulated Src activation itself, but rather than inhibiting Src activation by TGF-β, activation by vanadate was additive with TGF-β showing that pTyr530 dephosphorylation was not required. Thus, the involvement of the non-canonical oxidative activation was suspected. TGF-β increased extracellular H_2O_2 transiently while GSH-ester and catalase abrogated Src activation by TGF-β. Apocynin, a NADPH oxidase inhibitor, inhibited TGF-β-stimulated H_2O_2 production. Furthermore, mutation of cysteines to alanine, 248C/A, 277C/A, or 501C/A abrogated, while 490C/A significantly reduced, TGF-β-mediated Src activation. Taken together, the results indicate that TGF-β-mediated Src activation operates largely through a redox dependent mechanism, resulting from enhanced H_2O_2 production through an NADPH oxidase and that cysteines 248, 277, 490, and 501 are critical for this activation.

© 2015 Elsevier Inc. All rights reserved.

Introduction

Src is a ubiquitously expressed non-receptor tyrosine kinase belonging to the Src family of protein tyrosine kinases (SFKs)[1]. By coupling signals from cell surface receptors and various intracellular signaling pathways [1], Src is involved in various fundamental cellular processes, including proliferation, differentiation, and transformation [2]. Src also plays a critical role in epithelial–mesenchymal transition (EMT) [3–7], a process implicated in both wound healing and cancer metastasis [8–11].

In the canonical Src activation pathway, under basal conditions *in vivo*, a Src molecule remains inactive as a result of the intramolecular interactions between its SH3 domain and linker region, and its SH2 domain and the phosphorylated Tyr530 (pTyr530) in the C-terminal negative regulatory region [12,13]. Upon stimulation, pTyr530 is dephosphorylated thus dissociating the inhibitory intracellular interactions and allowing Src to autophosphorylate at Tyr419 and restoring full activity [14–17]. Thus, in the canonical pathway phosphorylation/dephosphorylation of Tyr530 plays a critical role in Src activation/deactivation. Several kinases and protein tyrosine phosphatases (PTPs) are involved in Src regulation [18], including C-terminal Src kinase (Csk) and Csk homologous kinase (Chk), that phosphorylate Src at Tyr530, and PTP1B, SHP1, PTPα, or PTPγ that reportedly catalyze Tyr530 dephosphorylation [18].

It has been well established that reactive electrophiles, such as H_2O_2, can participate in signal transduction by acting as second messengers through modification of the activity of signaling molecules. This so called redox signaling mechanism underlies the regulation of signaling pathways induced by many stimuli, including various physiological and/or pathological stimuli. Signaling molecules containing redox sensitive moieties such as cysteine, which readily reacts with electrophiles, have been found to be potential targets of regulation through redox mechanisms.

* Corresponding author at: Ethel Percy Andrus Gerontology Center, Leonard Davis School of Gerontology, The University of Southern California, United States.
E-mail address: peroxideman@gmail.com (H.J. Forman).

[1] *Abbreviations used:* TGF-β, transforming growth factor-β1; SFKs, Src family of protein tyrosine kinases; EMT, epithelial–mesenchymal transition; pTyr530, phosphorylated Tyr530; PTPs, protein tyrosine phosphatases; Csk, C-terminal Src kinase; Chk, Csk homologous kinase; FBS, fatal bovine serum; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; KRPG, Krebs–Ringer phosphate glucose; NOX, NADPH oxidases; CBP, CSK binding protein.

http://dx.doi.org/10.1016/j.abb.2015.01.001
0003-9861/© 2015 Elsevier Inc. All rights reserved.

PTPs have been recognized as major switches in redox signaling [19–25]. PTPs all have a catalytic site structure in which a reactive cysteine moiety [26,27] is potentially susceptible to oxidative modification by H_2O_2. The oxidization is however, likely mediated by an enzyme, as the non-enzymatic rate of reaction with H_2O_2 is too slow to account for the inactivation [28]. Redox modification inhibits the activity of PTPs and leads to increased phosphorylation of their corresponding substrate signaling molecules, resulting in altered signaling pathways.

Accumulating evidence suggests that Src can also be activated through a redox dependent mechanism. Purified Src is activated *in vitro* through either oxidation or alkylation of two cysteine (Cys) residues on the protein [29]. *In vivo*, Src is activated by various reactive oxidants such as cigarette smoke [30], acrolein [30], peroxynitrite, and hydrogen peroxide (H_2O_2) [31–34]. In addition, Src activation by many other stimuli including some growth factors seems to involve redox mechanisms [35–38].

There are nine cysteine residues in human Src that are highly conserved among Src family members and species [39], some of which have been implicated in Src activity regulation by oxidative stimuli. Senga first reported that mutation of Cys487 in v-Src (corresponding to Cys 490 in human Src) suppressed its activity while mutation of others changed Src stability [39]. In an *in vitro* assay, Cys277 seemed critical for the homodimerization and activation of Src [40]. Giannoni et al. found that Cys245 and Cys487 in v-Src (corresponding to Cys248 and Cys490 in human respectively) were involved in Src activation by oxidants, and dephosphorylation of Tyr530 was important for the early (10 min) activation of Src, while a redox mechanism was involved in the late phase of Src activation (45 min) in response to extracellular matrix [35].

Transforming growth factor-β1 (TGF-β) is a potent multifunctional growth factor involved in the regulation of cellular proliferation, differentiation and survival, and plays a predominant role in the EMT process [41]. TGF-β initiates signaling through binding to its type II receptor, which recruits and phosphorylates type I TGF-β receptor. The type I receptor, a serine/threonine protein kinase, phosphorylates and activates diverse downstream signaling pathways, including ERK, JNK, p38MAPK, PI3K/AKT, and transcription factors such as SMAD2/3 [42].

Many effects of TGF-β are mediated through Src-mediated signaling pathways [43–45]. How TGF-β activates Src is not completely clear. Some studies demonstrated that TGF-β could induce the production of oxidants that contributed to TGF-β-mediated effects [43,46], however, little is known about the role of oxidants in the activation of Src by TGF-β. Here, we report that a redox dependent mechanism, most likely involving cysteines of both Src and the PTPs that regulate its activity, is involved in Src activation by TGF-β in a non-canonical redox activation of Src.

Materials and methods

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). Antibodies to Src and phosphorylated Src were from Cell Signaling Technology, Inc. (Danvers, MA). M-PER Mammalian Protein Extraction Reagent was from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL). Amplex Red reagent was from Life Technologies (Grand Island, NY). All chemicals used were at least analytical grade.

Cell culture and treatment

A human non-small cell lung carcinoma cell line (H358) was used. H358 cells were cultured in RPMI-1640 medium with 10% fatal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5% CO_2 at 37 °C. Cells were treated when about 85% confluent. In catalase experiment, catalase (final concentration is 30 U/ml) was added to the culture medium immediately before TGF-β exposure.

Western analysis

Briefly, cell lysate was extracted with M-PER and 30 μg protein was electrophoresed on a 4–20% Tris–glycine acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA). Membranes were blocked with 5% fat-free milk and then incubated overnight at 4 °C with primary antibody in 5% BSA dissolved in Tris-buffered saline (TBS). After being washed with 1×TBS containing 0.05% Tween 20 (TTBS), membranes were incubated with secondary antibody at room temperature for 2 h. After TTBS washing, membranes were treated with an enhanced chemiluminescence reagent mixture (Thermal Fisher Scientific, Rockford, IL) for 5 min and then imaged and analyzed using the Biospectrum imaging system (UVP, Upland, CA).

Immunoprecipitation

Cells were washed with cold phosphate-buffered saline (PBS) and collected in 1 ml lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% NP40, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM EGTA, 1 mM PMSF, 10% glycerol, 100 mM sodium fluoride, 10 μM DETAPAC, 10 ng/ml leupeptin, and 10 ng/ml aprotinin. 500 μg of whole cell lysate was used for immunoprecipitation with Anti-FLAG M2 Affinity Gel (Sigma–Aldrich) overnight at 4 °C. The complexes were washed three times with TBS, re-suspended in 100 μl of TBS containing 3 μg of 3×FLAG peptide, and incubated at 4 °C for 30 min with shaking. After centrifuge, the supernatant was collected and used for Western blots.

Construction of FLAG-tagged Src plasmids, site-directed mutagenesis, and transfection

pcDNA3 c-Src [47] (Addgene plasmid 42202) was digested with Hind III and Xba I to get 1.6 kb cDNA of human c-Src. The 1.6 kb c-Src cDNA was then inserted into p3×FLAG-CMV-8 Expression vector (Sigma). The plasmid was selected with ampicillin and confirmed with DNA sequencing.

Site directed mutagenesis was then performed as described before [48] using a kit (GeneArt Site Directed Mutagenesis System, Invitrogen). The primer sequences were as following (only forward primer was shown): Src248C/A, CCTCACCACCGTGTTCCC CACGTCCAAG; Src277C/A, GCTGGGCCAGGGCTTCTTTGGCGAGGTG; Src490C/A, CTGCCCGCCGGAGTTTCCCGAGTCCCTG; Src501C/A, CCTCATGTGCCAGTTCTGGCGGAAGGAG. The mutations were confirmed with DNA sequencing.

Cells were transfected with plasmids with Lipofectamine 2000 when 90% confluence. Briefly DNA and Lipofectamine were mixed at 1:3.5 ratio and incubated at RT for 15 min before being added to cells. The medium was replaced the next day and 48 h after transfection, cells were treated and collected for experiments.

H_2O_2 measurement

H_2O_2 was measured in the extracellular media using Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). Before treated with TGF-β cell medium was replaced with Krebs–Ringer phosphate glucose (KRPG) (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, pH 7.35). 120 μl of KRPG was collected at different time points

Download English Version:

https://daneshyari.com/en/article/1925007

Download Persian Version:

https://daneshyari.com/article/1925007

Daneshyari.com