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Src SUMOylation Inhibits Tumor Growth *Via* Decreasing FAK Y925 Phosphorylation

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Abstract

Src, a non-receptor tyrosine kinase protein, plays a critical role in cell proliferation and tumorigenesis. SUMOylation, a reversible ubiquitination-like post-translational modification, is vital for tumor progression. Here, we report that the Src protein can be SUMOylated at lysine 318 both *in vitro* and *in vivo*. Hypoxia can induce a decrease of Src SUMOylation along with an increase of Y419 phosphorylation, a phosphorylation event required for Src activation. On the other hand, treatment with hydrogen peroxide can enhance Src SUMOylation. Significantly, ectopic expression of SUMO-defective mutation, Src K318R, promotes tumor growth more potently than that of wild-type Src, as determined by migration assay, soft agar assay, and tumor xenograft experiments. Consistently, Src SUMOylation leads to a decrease of Y925 phosphorylation of focal adhesion kinase (FAK), an established regulatory event of cell migration. Our results suggest that SUMOylation of Src at lysine 318 negatively modulate its oncogenic function by, at least partially, inhibiting Src-FAK complex activity.

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Introduction

Src is a non-receptor tyrosine kinase protein encoded by the proto-oncogene SRC in normal mammalian cells [1]. Src, also referred to as pp60 c-Src, stands for "cellular Src kinase". It is about 60KD in size [2] and belongs to Src family kinases (SFKs), where other members are Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk [3]. The Src protein is composed of 4 Src homology domains (SH4, SH3, SH2, SH1). The SH4 domain is located on the N-terminal which contains myristoylation sequence for membrane anchoring [4,5]. SH3 domain bounds to proline-rich peptide ligands for protein–protein interactions [6]. SH2 domain recognizes specific phosphopeptide sequences that bind to tyrosine sites [7,8]. In the traditional model, SH1 domain (catalytic domain) has kinase activity, regulated by its tyrosine 419 autophosphorylation site [2],and the C-terminal containing Y530 which negatively regulates Src activity [9]. Mutation at Tyr530 changes Src to be active in kinase and highly oncogenesis [10]. RPTPα and CSK, as critical regulators, dephsphorylates or phosphorylates Tyr530, which cause activation or inactivation of Src [11–13]. Recently, some new evidence shows the activation of Src kinase is regulated by ATP binding site [14] and the phosphorylation of Y419 and Y530 may not be a zero-sum game [15,16]. Activated Src phosphorylates substrates in signal transduction pathways such as Ras/MAPK, PI3K/Akt, the Src/FAK complex and the β-catenin/E-cadherin complex signaling networks. Thus, Src regulates a range of cellular processes, including apoptosis, proliferation, cell adhesion, migration, angiogenesis and metastasis.

Multiple post-translation modifications regulate Src activities and functions. The phosphorylation of Y419 and Y530 regulating mechanism has been described previously. Src is also regulated by ubiquitination, and that the active form is specifically targeted for degradation [17]. Myristoylation at Glycine2, is essential for membrane-binding and for the transforming activity [18]. Besides, certain specific point mutations convert Src activity. Mutation Src E378G in the kinase domain is dramatically more active than Src WT [19]. Src K295 is a critical lysine in

Abbreviations: SFKs, Src family kinases; SH, Src homology; SUMO, small ubiquitin-like modifier; CDS, coding sequence; IP, immunoprecipitation; GST, glutathione S-transferase; FAK, Focal adhesion kinase; SENPs, SUMO-sentrin specific proteases.

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the ATP-binding pocket, and the mutation of this site renders Src completely inactive [20]. Clustered cysteine residues Cys483/Cys487/Cys496/Cys498 in the kinase domain of Src perform critical role for protein stability and cell transformation [21]. Recently, in some of the Src involved signaling pathway, multiple protein, such as PTEN, Grb2, SHP2, FAK, AKT, has been identified to be SUMOylated. SUMOylation is a post-translational modification featured by covalent and reversible attachment of small ubiquitin-like modifier (SUMO) to protein at specific lysine [22]. SUMOylation can affect protein localization, stability, activity or protein–protein interaction [23–27]. It has not been reported whether Src can be SUMOylated. In the present study, we demonstrated that Src could be SUMOylated at K318, which could be inhibited by hypoxia in tumor *via* activating Src Y419. Besides, our results suggested that SUMOylation of Src might be a negative regulator in its oncogenic function by inhibiting Src-FAK complex activity *via* decreasing FAK Y925 phosphorylation.

Materials and Methods

Antibodies and Reagents

Anti-Src rabbit monoclonal(#2109),anti-phospho-Src (Tyr 416) rabbit polyclonal (#2101; used for detecting human Src pY419), anti-phospho-Src (Tyr 527) rabbit polyclonal (#2105; used for detecting human Src pY530), anti-FAK rabbit polyclonal(#3285), anti- phospho-FAK (Tyr 925)rabbit polyclonal(#3284), Anti-Csk rabbit monoclonal(#4980) antibody were purchased from Cell Signaling Technology. Anti-GAPDH polyclonal (#ab37168), SUMO1 (#ab32058), Senp1 (#ab108981) antibody were from Abcam. Protein A/G PLUS Agarose beads (#K0115) were obtained from Santa Cruz Biotechnology. Ni^{2+}-NTA agarose beads were from Qiagen. Glutathione Sepharose 4B beads (#17–0756-01) were from GE Healthcare Life Sciences. Puromycin (#P8833) was from Sigma. Anti-RPTPα (#7–091) is kept in our lab [28].

Plasmids

The human Src CDS was cloned from pCMV-Tag2B-Src plasmid [15], digested with EcoR I and Not I and then subcloned into vector pEF5HA, CD513B, pGEX-4 T-1 and mutant Src K318R was generated using PCR-directed mutagenesis and sequenced. The shRNA sequence targeting Src 3'UTR (shSrc) was from Sigma-Aldrich "Mission shRNA" online: 5′-CATCCTCAGGAACCAACAATT-3′. The shRNA was cloned into pLKO.1 vector. The pE1E2S1 plasmid was a kind gift from Dr. Jiemin Wong in East China Normal University.

Cell Culture

HEK293T, HEK293FT, NIH/3 T3 and DU145 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium(DMEM) containing 10% fetal calf serum (Hyclone) at 37 °C in 5% CO_2 humidified incubator. Cell transfection was performed using Lipofectamine 2000 (Invitrogen).

SUMOylation Assays

Src SUMOylation was analyzed in HEK293T by the method of *in vivo* SUMOylation assay using Ni^{2+}-NTA agarose beads as previously described [29]. Src SUMOylation analysis was also performed by the method of *in vitro E. coli* BL21-based SUMOylation assay with the plasmid pE1E2S1 as described [30].

Soft Agar Colony Assay

The method was performed in six-well plates with a base of 2 ml of DMEM medium containing 5% FBS with 0.6% Bacto agar (Amresco). Stable NIH/3 T3 or DU145 cells were seeded in 2 ml of medium containing 5% FBS with 0.35% agar at 2 or 4 X 10^3 cells per well and layered onto the base. The photos of the colonies developed in soft agar were taken at day 21. Three independent experiments were performed in triplicate.

Migration Assay by RTCA-DP

The method was carried out as described previously [23]. Briefly, stable NIH/3 T3 cells were starvation pre-treated with serum-free medium for 12 hours and then 4×10^4 cells resuspended in 100 μl of serum-free medium were added into the pre-equilibrated upper chambers of the CIM-plate. The lower chamber was filled with 160 μl of normal growth medium containing 10% FBS. The kinetic cell indexes of their migration were recorded every 15 min for 2 days.

Mouse Xenograft Models

Murine xenograft models were established as described previously [31]. Briefly, 5-weeks-old nude mice were subcutaneously injected in the back with 100 μl of medium containing 2.5×10^6 DU145 cells stably re-expressing Src WT and Src K318R. Forty-two days after injection, at the experimental endpoint, mice were sacrificed and the tumors were weighted and photographed. Statistical differences between groups were analyzed by the two-tailed Student's t test. $P < .05$ was considered statistically significant. Animal procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine.

Hematoxylin and Eosin Staining (H&E)

Paraffin-embedded sample preparation, hematoxylin and eosin staining (H&E) were performed as previously described [29].

Immunoprecipitation (IP)

HEK293T cells transfected with the indicated plasmids were lysed in the RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 10 mM NaF, 40 mM N-ethylmaleimide with protease inhibitor cocktail tablet) on ice. Lysates were immunoprecipitated with appropriate antibody overnight at 4 °C and subjected to 8–12% SDS-polyacrylamide gels for Western blotting analysis.

Western Blotting

Cells were lysed in SDS-lysis buffer (25.6 mM Tris, 2% SDS, pH 6.8), and total protein concentrations were determined by Nanodrop 2000. About 100 μg of each total protein was resolved on 8–12% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was subsequently probed with the indicated primary antibodies and second antibodies, and then exposured in ImageQuant LAS 4000 (GE) after incubating with ECL substrate, then analyzed band intensities of the images with the Photoshop CS5. All primary antibodies were used at a 1:1000 dilution. All secondary antibodies were used at a 1:5000 dilution.

Results

Src can be SUMOylated Both In Vivo and In Vitro

To test whether Src protein could be SUMOylated, We transiently transfected HA-tagged Src together with His-SUMO1 and Flag-UBC9 (E2 ligase in SUMOylation) in HEK293T cells. His-SUMO-conjugated Src was pulled down by the method of