Archives of Biochemistry and Biophysics 638 (2018) 58–65

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Phosphorylation of Ser-525 in βPix impairs Nox1-activating ability in Caco-2 cells

Yuuki Kaito, Ryosuke Kataoka, Tatsuya Mihara, Kento Takechi, Akira Takahira, Shuhei Watanabe, Fei Han, Minoru Tamura*

Department of Applied Chemistry, Graduate School of Science and Engineering, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

ARTICLE INFO

Keywords:
Nox1
Superoxide
βPix
Phosphorylation
Rac
Caco-2 cell

ABSTRACT

βPix activates Nox1, an O_2^--generating NADPH oxidase, through Rac activation. In this study, we found that S525E mutation of βPix eliminated its Nox1-activating ability in transfected Caco-2 cells. Unexpectedly, affinity for Rac was not diminished but rather enhanced by S525E mutation, and guanine nucleotide exchange factor (GEF) activity was not altered. The N-terminal fragment (amino acids 1–400) showed similar Rac-binding and GEF activity to wild-type βPix. In contrast, the C-terminal fragment (amino acids 408–646) had higher Rac-binding activity, particularly for Rac-GTP, than wild-type βPix, and showed no GEF activity. These data suggest that a second Rac-binding site within the C-terminal region is opened by phosphorylation of Ser-525. The site may bind not only Rac-GDP but also Rac-GTP released from the N-terminal catalytic region, which interrupts Rac-GTP translocation to the membrane where Nox1 resides. If one considers that S340E mutation enhances Nox1 activation (Kaito et al., 2014), the present study suggests that βPix can also play an inhibitory role in O_2^- production, depending on the sites of phosphorylation.

1. Introduction

Nox1 is a superoxide (O_2^-)-generating enzyme that belongs to the NADPH oxidase (Nox) family [1]. Nox1 is expressed abundantly in the colon and moderately in vascular smooth muscles and other limited tissues and organs. The enzyme is thought to be involved not only in physiological functions such as host defense, vascular contraction, proliferation, and migration, but also in pathological situations such as hypertension, atherosclerosis, inflammatory bowel disease, and cancers [2,3]. The enzyme is now assumed as a target for drug development as well as other Nox enzymes [4].

Nox1 is an integral membrane protein complexed with $p22^{phox}$ and requires Rac as well as Noxa1 and Noxo1 for its activation [2,5,6]. Specifically, Rac is proposed as a major trigger that can acutely activate Nox1-dependent reactive oxygen species production [7]. Noxo1 is thought to be autoinhibited in the resting state [5,8] and subsequently released by site-specific phosphorylations [9,10]. Noxa1 is also suggested to be masked by an intramolecular interaction, although the exact mechanism is unclear [11–13].

Rac, a member of the Rho family GTPases, is present in the cytosol as a GDP-bound form associated with rhoGDI, and requires rhoGDI release and GDP/GTP exchange for its activation [14]. Consequently, a guanine nucleotide exchange factor (GEF) must be involved in Nox1 activation. βPix is one of the GEFs for Rac, and belongs to the diffuse B-cell lymphoma (DBL) family [15]. Previously, it was demonstrated that βPix is involved in Nox1 activation in epidermal growth factor (EGF)-stimulated cell lines [16,17].

A number of mechanisms have been postulated for βPix regulation, including phosphorylation [15], dimerization [18], and phosphoinositide binding [19]. Regarding phosphorylation, Mayhew et al. [20] demonstrated that mouse βPix exhibits EGF-induced phosphorylation at multiple sites including Ser-340 in transfected HEK cells. Recently, we showed that Ser-340 phosphorylation enhances human βPix activation, resulting in Rac and Nox1 activation in a human cell line [17]. The activation was elucidated by enhanced Rac binding and elevated GEF activity of phosphorylated βPix.

Meanwhile, Koh et al. [21] demonstrated that βPix is phosphorylated by p21-activated protein kinase (PAK) at several sites including Ser-525 by *in vitro* study. Subsequently, it was demonstrated that an S525A/T526A double-mutant of mouse βPix impaired neurite

Abbreviations: GEF, guanine nucleotide exchange factor; Nox, NADPH oxidase; EGF, epidermal growth factor; NGF, nerve growth factor; FGF, fibroblast growth factor; GBD, GIT1-binding domain; G-Sepharose, glutathione-Sepharose; Rac, Rac1(C189S); Rac61L, Rac1(Q61L, C189S); GST, glutathione-S-transferase; S525E, βPix(S525E); S525A, βPix (S525A); S340E, βPix(S340E); S340E/S525E, βPix(S340E, S525E); N-fragment, βPix (1–400); C-fragment, βPix(408–646); SOD, superoxide dismutase

* Corresponding author.
E-mail address: tamura@ehime-u.ac.jp (M. Tamura).

https://doi.org/10.1016/j.abb.2017.12.010
Received 17 September 2017; Received in revised form 1 December 2017; Accepted 9 December 2017
Available online 11 December 2017
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outgrowth of rat cell line PC12 in response to fibroblast growth factor (FGF) or nerve growth factor (NGF) [22,23]. However, the relationships of these phosphorylations with Nox1 regulation have never been investigated.

In the present study, we focused on Ser-525 of human βPix and examined the effects of phosphomimetic and non-phosphomimetic mutation of the residue on O_2^- production in transfected Caco-2 cells. We found that S525E mutation suppressed O_2^- production from the cells, and based on subsequent studies, we propose a mechanism in which βPix phosphorylation at Ser-525 impairs its ability for Nox1 activation.

2. Materials and methods

2.1. Cells

Caco-2 cells (Riken BRC, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biowest, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin at 37 °C under 5% CO_2.

2.2. Reagents

Human EGF, FGF, and superoxide dismutase (SOD) (bovine erythrocyte) were purchased from Sigma-Aldrich (St. Louis, MO). Human NGF was obtained from Alomone Labs (Jerusalem, Israel). PreScission Protease, glutathione-Sepharose (G-Sepharose), HiTrap SP HP column (5 ml), HiTrap CM FF column (5 ml), and Superdex 75 column (1.6 × 60 cm; Hi Load) were purchased from GE Healthcare (Little Chalfont, UK). 2′(3′)-*O*-(*N*-methyl anthraniloyl)-β:γ-imidoguanosine 5′-triphosphate (Mant-GppNHp) was obtained from Biolog Life Science Institute (Bremen, Germany). Diogenes was obtained from National Diagnostics (Atlanta, GA). Lipofectamine LTX, PLUS Reagent, and Opti-MEM were purchased from Thermo Fischer Scientific (Waltham, MA). A chicken anti-Noxo1 antibody and a rabbit anti-Rac antibody were kind gifts from Dr. David Lambeth (Emory University, Atlanta, GA). An anti-Myc monoclonal antibody was purchased from Roche. Peroxidase-conjugated rabbit anti-mouse antibody, rabbit anti-chicken antibody, and goat anti-rabbit antibody were purchased from MP Biomedicals (Santa Ana, CA).

2.3. Plasmid construction

Human βPix cDNA (isoform a; a generous gift from Dr. Takashi Nagase, Kazusa DNA Research Institute, Chiba, Japan) was subcloned into the pEF-BOS vector developed by Mizushima et al. [24] and modified by Noda et al. [25] to have myc as a tag, or into the pGEX-6P vector between the *Bam*HI and *Eco*RI sites. The vectors pEF-BOS-myc-Noxa1 and pEF-BOS-HA-Noxo1 were generous gifts from Dr. Hideki Sumimoto (Kyushu University, Fukuoka, Japan). pGEX-2T-Rac1 was a generous gift from Dr. Dave Lambeth (Emory University, Atlanta, GA). For mutation of βPix, the cDNA in the pEF-BOS-myc or pGEX-6P vector was mutated using a QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by dideoxynucleotide-based sequencing.

2.4. Transfection

Cells (1.0×10^6) were washed with buffer T (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.3), suspended in the same buffer (0.4 ml), mixed with 5 μg of pEF-BOS-myc-βPix (or a mutant), pEF-BOS-myc-Noxa1, and pEF-BOS-HA-Noxo1 in a pulse cuvette with a 2-mm gap (NEPA Gene, Chiba, Japan), and kept on ice for 10 min. The mixture was then pulsed with an ECM600 electroporator (BTX, San Diego, CA) at 950 V/cm from a 300-μF capacitor. After the electroporation, the cells were incubated at room temperature for 10 min and cultured for 24 h in the presence of fetal bovine serum.

For estimation of protein levels of myc-βPix, myc-Noxa1, and HA-Noxo1, proteins in cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane Hybond-P (GE Healthcare). The membrane was then cut into two pieces and each was treated with an anti-Myc antibody or an anti-Noxo1 antibody. After treated with corresponding second antibodies the blots were developed with ECL plus reagent (GE Healthcare) and detected with LAS-1000 or LAS-4000 (GE Healthcare).

2.5. Stimulation and measurement of O_2^-

The above-treated cells were suspended in buffer C (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, 17 mM HEPES pH 7.4) at 1×10^5 cells/ml. Subsequently, bovine serum albumin (0.03% final concentration) and 10 μl of modified Diogenes solution (diluted twice with phosphate-buffered saline containing 50 mM luminol [26]) was added to the suspension. After incubation for 5 min at 37 °C, the cells were incubated for 5 min at 37 °C in the presence of EGF (in 5% trehalose), NGF (in 20 mM Tris-HCl pH 7.5), FGF (in 20 m Tris-HCl pH 7.5, 1 M NaCl), or vehicle (5% trehalose). The luminescence was measured for 1 min at 37 °C using an AB-2270 luminometer (Atto, Tokyo, Japan).

2.6. Measurement of O_2^- with cytochrome *c*

In case of cytochrome *c* reduction assay, transfection was performed by a lipofection using Lipofectamine LTX according to the manufacturer's protocol. Caco-2 cells (2×10^5) were seeded on a 6-well plate containing the medium (2 ml) and cultured for 24–36 h. The plasmid mixture (2.5 μg each) was mixed with PLUS Reagent (7.5 μl) in Opti-MEM (500 μl), incubated for 15 min at 24 °C, and supplemented with Lipofectamine LTX (4 μl) and incubated for 25 min at 24 °C. The DNA-reagent complex was added to the cells and cultured for 24 h. After separation and counting, 5×10^5 cells were suspended in buffer C (100 μl). The cells were then incubated for 30 min at 37 °C in the presence of EGF (16 μM) and cytochrome *c* (80 μM) with or without SOD (30 units). The suspensions were centrifuged and the supernatants were subjected to measurement of SOD-inhibitable cytochrome *c* reduction with a Shimadzu 120 A spectrophotometer. Data were normalized to the isosbestic point at 542 nm and O_2^- production was calculated with an extinction coefficient of 21.1 mM^{-1} cm^{-1} at 550 nm.

2.7. Preparation of βPix and its mutants

βPix and its mutants were expressed as glutathione-S-transferase (GST)-fused proteins in *Escherichia coli* BL21 cells and purified as previously described [17], except that the purification was performed with a Superdex 75 column instead of a HiTrap Q column. Briefly, the eluate from G-Sepharose was treated with 2 mM DIFP, supplemented with leupeptin (40 μg/ml), aprotinin (10 μg/ml), and 2 mM DIFP, and applied to the Superdex 75 column on an AKTA prime system (GE Healthcare). βPix or a mutant was eluted with 50 mM Tris-HCl buffer (pH 9.0) containing 500 mM NaCl. βPix(1–400) (N-fragment) was also purified with the Superdex 75 column as described above, except that the protein was eluted with 150 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl. βPix(408–646) (C-fragment) was purified using a HiTrap SP column. The eluate from G-Sepharose was applied to the column and the C-fragment was eluted with an NaCl gradient (100–500 mM) in 50 mM Tris-HCl buffer (pH 8.0). All samples from the columns were dialyzed against 20 mM potassium phosphate buffer (pH 7.5) containing 500 mM and concentrated with an Amicon Ultra (30-kD cut-off; Merck Millipore, Darmstadt, Germany). Purity of each purified protein was verified by SDS-polyacrylamide gel electrophoresis (Fig. S1).

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