Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 339 (2006) 985–990
BBRC
www.elsevier.com/locate/ybbrc

Molecular interaction of NADPH oxidase 1 with βPix and Nox Organizer 1

Hye Sun Park [a], Dongeun Park [b], Yun Soo Bae [a,*]

[a] Center for Cell Signaling Research, Division of Molecular Life Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea
[b] School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

Received 27 October 2005
Available online 29 November 2005

Abstract

It is well established that growth-factor-induced reactive oxygen species (ROS) act as second messengers in cell signaling. We have previously reported that βPix, a guanine nucleotide exchange factor for Rac, interacts with NADPH oxidase 1 (Nox1) leading to EGF-induced ROS generation. Here, we report the identification of the domains of Nox1 and βPix responsible for the interaction between the two proteins. GST pull-down assays show that the PH domain of βPix binds to the FAD-binding region of Nox1. We also show that overexpression of the PH domain of βPix results in inhibition of superoxide anion generation in response to EGF. Additionally, NADPH oxidase Organizer 1 (NoxO1) is shown to interact with the NADPH-binding region of Nox1. These results suggest that the formation of the complex consisting of Nox1, βPix, and NoxO1 is likely to be a critical step in EGF-induced ROS generation.

Keywords: NADPH oxidase 1; NADPH oxidase Organizer 1; βPix; Reactive oxygen species; Superoxide anion; EGF

The robust reactive oxidant generation seen in neutrophils and phagocytic cells is mediated by the NADPH oxidase complex. The catalytic component of the complex, gp91phox (Nox2), tightly associates with the membrane-integrated protein p22phox to form cytochrome *b*558 and is subsequently placed under the regulation by the subunits p47phox and p67phox [1]. It is the integral protein complex cytochrome *b*558 that recruits cytosolic proteins including p47phox, p67phox, and Rac protein, and such assembly of cytosolic components is known to be critical for the ROS generation in response to agonist stimulation. Several recent reports demonstrated that catalytic gp91phox homologues, namely Nox1, Nox3, Nox4, and Nox5, are present in various nonphagocytic cells [2,3]. Nox1 is predominantly expressed in colon cells and involved in cell growth and angiogenesis. Nox3 and Nox5 are expressed in fetal tissues and spleen cells, respectively [4,2]. Nox4 expression is detected in several organs including kidney, placenta, pancreas, and endothelial, and smooth muscle cells. Studies on Nox4 in particular revealed a novel link between the insulin receptor and the generation of cellular reactive oxygen species that enhance insulin signal transduction [5].

These Nox proteins are regulated by novel homologues of p47phox (NoxO1) and p67phox (NoxA1) which are capable of supporting the activation of the Nox1 [6–8]. The function and structure of NoxO1 and NoxA1 are very similar to those of p47phox and p67phox. NoxO1 interacts with p22phox via its SH3 domains, identically to the way p47phox does, and NoxA1 interacts with constitutively active form of Rac1, identically as p67phox [9]. Moreover, Nox1 forms a heterodimer with p22phox as like as gp91phox/Nox2, and the formation of heterodimer appears to be essential for EGF-induced ROS generation [10].

Our previous report showed that βPix is involved in ROS generation in response to EGF [11]. The Pix (PAK-interacting exchange factor)/Cool (cloned out of library) proteins have been identified as a new PAK-binding partner and are known to be involved in the regulation of Cdc42/

* Corresponding author. Fax: +82 2 3277 3760.
E-mail address: baeys@ewha.ac.kr (Y.S. Bae).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.108

Rac GTPases and Pak kinase activity [12,13]. The Pix family contains two members, αPix and βPix. Both isoforms share Src-homology 3 (SH3), Db1-homology (DH) domain, and the pleckstrin homology (PH) domain all of which are involved in the formation of signaling complexes [14]. βPix additionally contains a GIT1-binding domain, a proline-rich (PXXP) region, and a leucine zipper (LZ) motif. βPix protein is known to be involved in p38 MAP kinase activation by a Cdc42/Rac/PAK-mediated pathway, resulting in membrane ruffling [15].

Although we have previously demonstrated that interaction of βPix with Nox1 is essential for EGF-induced ROS generation, the detailed molecular characterization of this interaction has yet to be carried out. In this report, we identify the interaction domains of Nox1, βPix, and the homologue of p47phox, NoxO1, further clarifying the nature and significance of the complex formation and of the regulatory interaction in cellular signaling.

Materials and methods

Cell culture. HEK293T cells were maintained at 37 °C under an atmosphere of 5% CO_2 in culture dishes containing Dulbecco's modified Eagle's medium (JBI) supplemented with 10% fetal bovine serum (JBI) and 1% antibiotic–antimycotic solution (Life Technologies).

GST fusion proteins and GST "pull-down" assay. Glutathione-*S*-transferase (GST) fusion constructs were generated by inserting cDNA fragments of Nox1 and βPix into pGEX4T1. The resulting plasmids were introduced into *Escherichia coli* DH5α (DE3), and the cells were cultured at 25 °C for 5 h. Expression of the fusion proteins was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (ICN), and the cells were subsequently collected by centrifugation at 2000*g* for 15 min and lysed by sonication in 10-fold (weight/volume) PBS containing 1% Triton X-100 and protease inhibitors aprotinin (1 μg/ml), leupeptin (1 μg/ml), and PMSF (0.5 mM). After centrifugation for 30 min at 15,000 rpm, the resulting supernatant was incubated with glutathione–Sepharose 4B (Amersham Pharmacia Biotech) for 4 h at 4 °C. The mixture was centrifuged for 2 min at 5000*g*, and the isolated pellet was washed with 10 bed volumes of PBS containing 1% Triton X-100. For GST pull-down assays, the bead-conjugated GST-Nox1 mutants or GST-βPix mutants were incubated with lysates of HEK293T cells that had been transfected with indicated plasmids for 4 h at 4 °C in PBS containing 1% Triton X-100 and 0.5% NP40. The beads were then separated by centrifugation, washed three times, and subjected to immunoblot analysis.

Immunoprecipitation. pEGFP-C2-Nox1-LC was transiently expressed in HEK293T cells together with pEF-Bos-HA-NoxO1 or NoxA1 as indicated. After 24 h, cell lysates were prepared in lysis buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.5% NP40, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) and then subjected to immunoprecipitation (IP) with antibodies to HA for 4 h at 4 °C. The

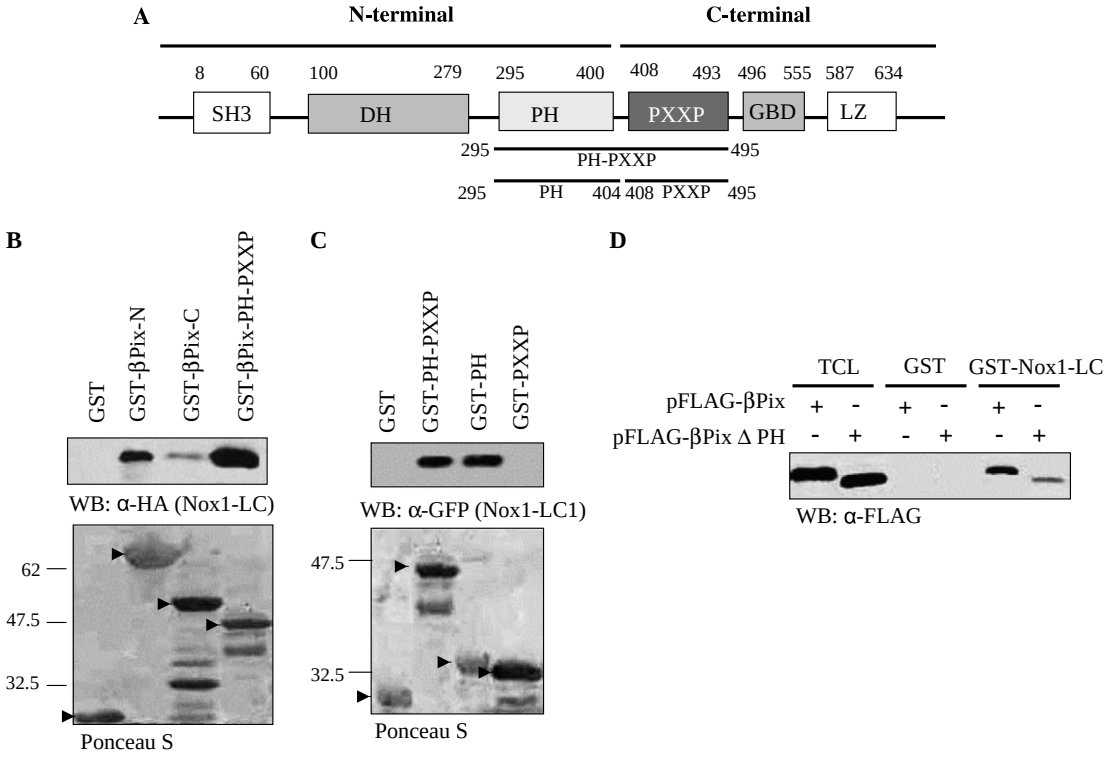

Fig. 1. (A) Schematic representation of βPix and various constructs of βPix mutants. (B,C) The bead-conjugated GST, GST-βPix-NH3 terminal, GST-βPix-COOH-terminal or GST-βPix-PH-PXXP fusion proteins were incubated with lysates (500 μg) of HEK293T cells transfected with pcDNA3.0-HA-Nox1-LC or pEGFP-C2-Nox1-LC for 4 h at 4 °C in 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 1% Triton X-100, and 0.5% NP40. After wash with cold lysis buffer for 3 times, proteins retained specifically by the glutathione–Sepharose 4B beads were subjected to immunoblot analysis with indicated antibodies. Arrowhead indicates GST, GST-βPix-NH3-terminal, GST-βPix-COOH-terminal, GST-βPix-PH-PXXP, GST-PH, or GST-PXXP. (D) GST and GST-Nox1-LC were incubated with lysates of HEK293T cells transfected with the full-length βPix and PH domain deleted mutant form of βPix (βPixΔPH). After wash with lysis buffer, proteins bound specifically by the glutathione–Sepharose 4B beads were subjected to immunoblot analysis with indicated antibodies.

beads were then separated by centrifugation, washed three times, and subjected to immunoblot analysis.

Measurement of superoxide generation. HEK293T cells were transduced with Nox1 virus repeatedly [16], and resulting Nox1 over-expressing HEK293T cells were transfected with pEF-Bos-NoxO1, pEF-Bos-NoxA1, and pcDNA3.1-hEGFR. Superoxide production by HEK293T cells was measured by chemiluminescence using DIOGENES (National Diagnostics), a superoxide-specific chemiluminescence reagent. Adherent cells were trypsinized and washed once with Hanks' balanced salt solution (HBSS). Chemiluminescence was measured for 10 min at 37 °C in 96-well microtiter chemiluminescence plates (5×10^5 cells/well) with a MicroLumat plus LB96V (Berthold) and was expressed in arbitrary units.

Results and discussion

Our previous data suggested that βPix interacts with Nox1 and that the interaction is important for EGF-induced ROS generation. However, the domains mediating the interaction between them have yet to be identified. To verify which region of βPix is responsible for binding to Nox1, we prepared GST fusion proteins containing either NH3-terminal region (amino acids 1–404) or COOH-terminal region (amino acids 408–647) (Fig. 1A) and conjugated them to glutathione–Sepharose 4B beads. Interaction of βPix mutant containing NH3-terminal region with Nox1 was shown to be substantially stronger than that of βPix mutant containing COOH-terminal region (Fig. 1B). To further define the region of βPix is involved in interaction with Nox1, we prepared GST-PH-PXXP. As shown in Fig. 1B, the PH-PXXP domain of β Pix strongly interacted with long COOH-terminal region of Nox1 (Nox1-LC, amino acid residues 217–550) (Fig. 2A). It is thus that central region of βPix, the PH-PXXP domain, is responsible for the interaction with Nox1. Next we prepared GST-conjugated PH and PXXP domain and then examined their individual interaction properties with Nox1. The PXXP domain failed to interact with Nox1, whereas the PH domain continued to display strong interaction with Nox1 (Fig. 1C), thus revealing that PH is the primary interacting sub-domain of the central region of βPix. To verify the function of PH domain of βPix, we prepared the βPix mutant construct lacking PH domain (βPixΔ PH). While wild type βPix strongly interacted with the COOH-terminal region of Nox1, βPixΔ PH showed a markedly reduced interaction with Nox1 (Fig. 1D). The result indicates that the PH domain in βPix is responsible for the interaction with the COOH-terminal region of Nox1.

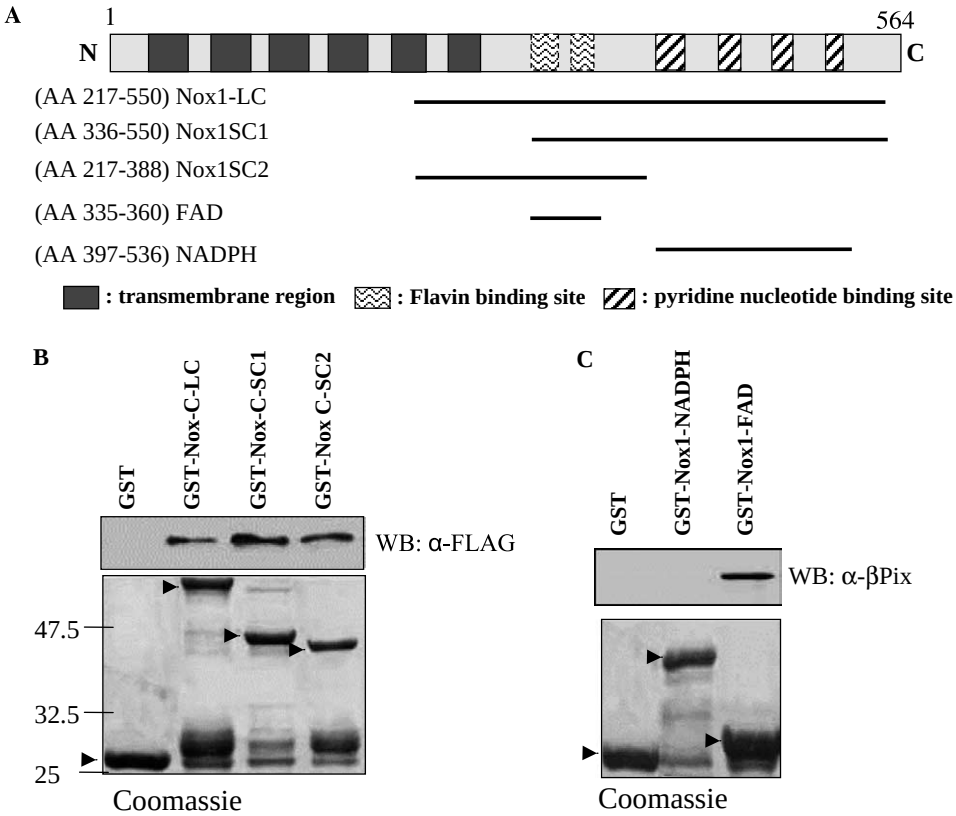

Fig. 2. (A) Schematic presentation of Nox1 and various constructs of Nox1 mutants. (B) The bead-conjugated GST or GST-Nox1 mutants (LC, SC1, and SC2) fusion proteins were incubated with lysates of HEK293T cells transfected with pFLAG-βPix for 4 h at 4 °C in 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 1% Triton X-100, and 0.5% NP40. Arrowhead indicates GST, GST-Nox-C-LC, GST-Nox-C-SC1, or GST-Nox-C-SC2. (C) The bead-conjugated GST or GST-Nox1 mutants (FAD, NADPH) fusion proteins were incubated with lysates of HEK293T cells. After wash with cold lysis buffer for 3 times, proteins retained specifically by the glutathione–Sepharose 4B beads were subjected to immunoblot analysis with antibodies to FLAG or to βPix. Arrowhead indicates GST, GST-Nox1-NADPH, or GST-Nox1-FAD.

To identify the interacting domain of Nox1 with βPix, FLAG-βPix protein was incubated with various C-terminal fragments of Nox1 conjugated with GST protein (Fig. 2A). The Nox1-LC (amino acids 217–550), Nox1-SC1 (amino acids 336–550), and Nox1-SC2 (amino acids 217–388) regions have similar interaction affinities to βPix (Fig. 2B). We thus hypothesized that the FAD region which overlapped between Nox1-SC1 and Nox1-SC2 was responsible for the interaction between Nox1 and βPix. We prepared FAD or NADPH regions of Nox1 conjugated with GST proteins and then incubated with HEK293T cell lysates. Endogenous βPix interacted only with the FAD region of Nox1, suggesting that FAD region of Nox1 is critical for the interaction between these proteins (Fig. 2C).

To investigation whether this interaction between the PH domain of βPix and FAD region of Nox1 is critical for the EGF-induced ROS generation, we measured superoxide generation in cells overexpressing the PH domain as a dominant negative form. As shown in Fig. 3A, the stimulation of HEK293T overexpressing EGFR, Nox1, NoxO1, and NADPH oxidase activator 1 (NoxA1) with EGF resulted in increased superoxide generation by two-fold. However, when we transfected these cells with the PH domain construct, EGF-induced superoxide generation is reduced by 20%. Because protein expression level of the PH domain is relatively lower than those of NoxO1 and NoxA1, the inhibitory effect of the PH domain of βPix on EGF-induced ROS generation is somewhat limited (Fig. 3B). Still, the result is consistent with the fact that the PH domain of βPix blocks the interaction between Nox1 and endogenous βPix leading to the inhibition of EGF-induced ROS generation. It is well known that the PH domain of GEF proteins mediates binding to lipid products in the plasma membrane. Our previous study showed that the PH domain of β Pix interacts with the product of PI3-kinase which is critical for EGF-induced ROS generation [11]. Therefore, we cannot exclude the possibility that the overexpression of PH domain of βPix block the binding of endogenous βPix to PI3-kinase products thereby inhibiting EGF-induced ROS generation. Several lines of evidence suggest that RhoGEF appears to serve as an effector-tethered module in signal transduction. Pix protein binds to Pak and then regulates CDC42 activities. It is likely that PH domain of βPix interacts with FAD-binding domain of Nox1 and Rac1, and then regulates Rac1 activity leading to ROS generation through Nox1 activation.

It is well known that several proteins including the membrane-associated p22phox, the cytosolic subunits p47phox, p67phox, and p40phox, and small GTPase Rac (Rac1/Rac2) regulate gp91phox activity in NADPH oxidase complexes. Recently, several papers have provided evidences that Nox1 is also regulated by the novel homologue of gp47phox (NoxO1) and gp67phox (NoxA1) [6,7]. It has also been reported that Nox1 directly interacts with gp22phox and that Nox1 activity depends on the expression of gp22phox [17]. The PX domain of p47phox and

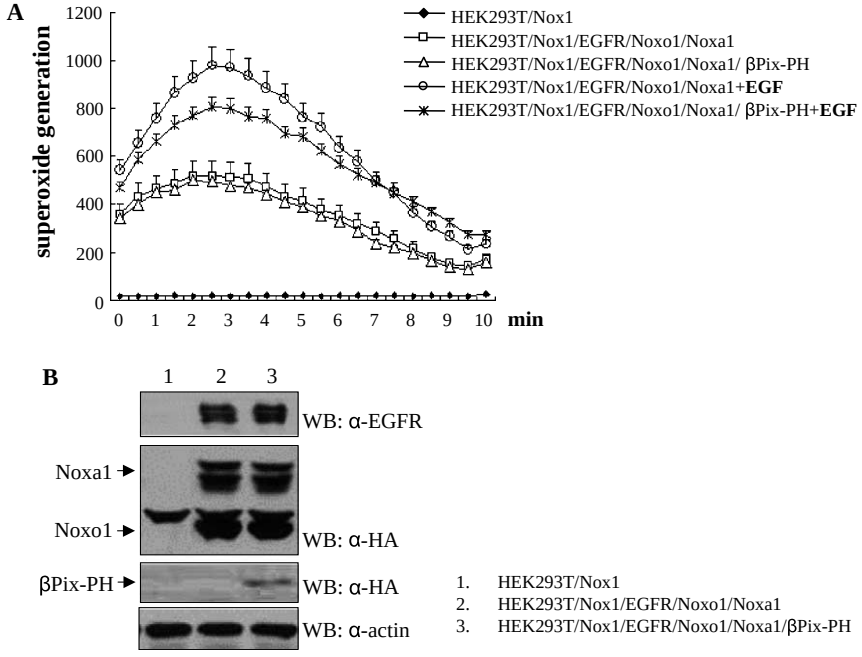

Fig. 3. (A) Nox1 overexpressing HEK293T cells were transfected with pEF-Bos-NoxO1, NoxA1, and pcDNA3.1-hEGFR together with or without pcDNA3.0-HA-βPix-PH. After 24 h, cells were serum starved for 6hrs. Adherent cells were trypsinized and washed once with HBSS. Chemiluminescence was measured using Diogen for 10 min with or without EGF (100 ng/well) at 37 °C in 96-well microtiter chemiluminescence plates (5×10^5 cells/well) with a MicroLumat plus LB96V. Data are means ± SD of values from three independent experiments. (B) Total cell lysates of each sample from panel A were prepared and subjected to immunoblot analysis with indicated antibodies.

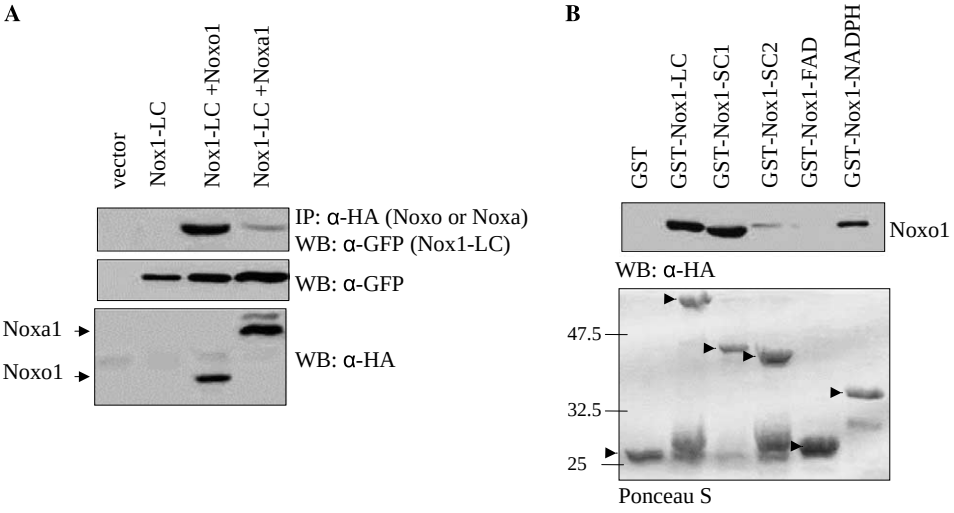

Fig. 4. (A) pEGFP-C2-Nox1-LC was transiently expressed in HEK293T cells together with pEF-BOS-HA-NoxO1 or NoxA1 as indicated. Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to HA, and the resulting precipitates were subjected to immunoblot analysis with antibodies to GFP (upper panel). Lysates were directly subjected to immunoblot analysis with antibodies to GFP (middle panel) or to HA (lower panel). (B) The bead-conjugated GST or GST-Nox1 mutants (LC1, SC1, SC2, FAD, and NADPH) fusion proteins were incubated with lysates of HEK293T cells transfected with HA-NoxO1 for 4 h at 4 °C in 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, 1% Triton X-100, and 0.5% NP40. After wash with cold lysis buffer for 3 times, proteins retained specifically by the beads were subjected to immunoblot analysis with antibodies to HA. Arrowhead indicates GST, GST-Nox1-LC, GST-Nox1-SC1, GST-Nox1-SC2, GST-Nox1-FAD, or GST-Nox1-NADPH.

p40phox binds to the lipid products of phosphatidylinositol 3-kinase, showing a high affinity for phosphatidylinositol 3,4-bisphosphate. Based on its phospholipid binding affinity, NoxO1 is predicted to interact with plasma membranes in cells even without stimulation by an agonist unlike 47phox. Such innate affinity to the membrane lipid induces localization of NoxO1 to the same cellular compartments where Nox1 is located [18].

To investigate whether Nox1 interacts with NoxO1 or NoxA1, we performed immunoprecipitation and GST pull-down assays. Immunoprecipitation shows that Nox1-LC effectively interacts with NoxO1, whereas NoxA1 binds with Nox1 very weakly (Fig. 4A). It is likely that weak interaction of Nox1 with NoxA1 is mediated by the endogenous NoxO1. To identify the domain of Nox1 responsible for interacting with NoxO1, various COOH-terminal fragments of Nox1 conjugated with GST protein were incubated with NoxO1 protein. The NADPH-binding region of Nox1 shows a high affinity for NoxO1 protein in the GST pull-down assay (Fig. 4B). In contrast, the Nox1-SC2 and the FAD regions of Nox1 failed to interact with NoxO1. These results indicate that the NADPH region of Nox1 is responsible for the interaction with NoxO1. Several lines of evidence suggest that COOH-terminal region including NADPH-binding domain of Nox2 is essential for the interaction with p47phox [19]. Biochemical analysis of the COOH-terminal region of Nox2 demonstrates that amino acids 484–500 are responsible for the translocation of p47phox. Aspartic amino acids in the region (amino acids 484–500 in Nox2) are important for the translocation of p47phox in the presence of GTPγ-S [19]. Although we did not determine the identity of amino acids of Nox1 important for binding with NoxO1, it is likely that negatively charged amino acids in NADPH region of Nox1 mediate the interaction. This proposal is based on the high degree of conservation between the sequences of the interaction region of Nox2 with p47phox and that of binding region of Nox1 with NoxO1 [19].

In conclusion, this report demonstrates that the FAD and NADPH domains of Nox1 interact strongly with the PH domain of βPix and NoxO1, respectively, and such an interaction strongly implies that the complex formation is likely to be the key step in EGF-mediated ROS generation.

Acknowledgments

We thank Dr. Sumimoto for the plasmid DNA (pEF-Bos-NoxO1 and pEF-Bos-NoxA1) and Dr. Leto for the retrovirus encoding Nox1. We thank Dr. Jaesang Kim for critical reading. This work is supported by the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling Research at Ewha Womans University, by 21st Century Frontier Functional Proteomics Project (FPR05C2-510), and by Grant M103KV010013-04K2201-01310 to D.P. from Brain Research Center of the 21st Century Frontier Research Program from the Ministry of Science and Technology. H.S.P. is the recipient of BK21 scholarship and of KRF-2005-216-C00126 from Korea Research Foundation.

References

[1] G.M. Bokoch, U.G. Knaus, NADPH oxidases: not just for leukocytes anymore, Trends Biochem. Sci. 9 (2003) 502–508.

[2] G. Cheng, Z. Cao, X. Xu, E.G. van Meir, J.D. Lambeth, Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5, Gene 269 (2001) 131–140.
[3] K.H. Krause, Tissue distribution and putative physiological function of NOX family NADPH oxidases, Jpn. J. Infect. Dis. 57 (2004) S28–S29.
[4] J.D. Lambeth, Nox/Duox family of nicotinamide adenine dinucleotide (phosphate) oxidase, Curr. Opin. Hematol. 9 (2000) 11–17.
[5] K. Mahadev, H. Motoshima, X. Wu, J.M. Ruddy, R.S. Arnold, G. Cheng, J.D. Lambeth, B.J. Goldstein, The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H_2O_2 and plays an integral role in insulin signal transduction, Mol. Cell. Biol. 24 (2004) 1844–1854.
[6] R. Takeya, N. Ueno, K. Kami, M. Taura, M. Kohjima, T. Izaki, H. Nunoi, H. Sumimoto, Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases, J. Biol. Chem. 278 (2003) 25234–25246.
[7] B. Banfi, R.A. Clark, K. Steger, K.H. Krause, Two novel proteins activate superoxide generation by the NADPH oxidase NOX1, J. Biol. Chem. 278 (2003) 3510–3513.
[8] M. Geiszt, K. Lekstrom, J. Witta, T.L. Leto, Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells, J. Biol. Chem. 278 (2003) 20006–20012.
[9] J.D. Lambeth, NOX enzymes and the biology of reactive oxygen, Nat. Rev. Immunol. 4 (2004) 181–189.
[10] T. Kawahara, D. Ritsick, G. Cheng, J.D. Lambeth, Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation, J. Biol. Chem. (2005).
[11] H.S. Park, S.H. Lee, D. Park, J.S. Lee, S.H. Ryu, W.J. Lee, S.G. Rhee, Y.S. Bae, Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H2O2, Mol. Cell. Biol. 24 (2004) 4384–4394.
[12] E. Manser, T.H. Loo, C.G. Koh, Z.S. Zhao, X.Q. Chen, L. Tan, I. Tan, T. Leung, L. Lim, PAK kinases are directly coupled to the PIX family of nucleotide exchange factors, Mol. Cell 1 (1998) 183–192.
[13] Q. Feng, J.G. Albeck, R.A. Cerione, W. Yang, Regulation of the Cool/Pix proteins: key binding partners of the Cdc42/Rac targets, the p21-activated kinases, J. Biol. Chem. 277 (2002) 5644–5650.
[14] W.K. Oh, J.C. Yoo, D. Jo, Y.H. Song, M.G. Kim, D. Park, Cloning of a SH3 domain-containing proline-rich protein, p85SPR, and its localization in focal adhesion, Biochem. Biophys. Res. Commun. 235 (1997) 794–798.
[15] S.H. Lee, M. Eom, S.J. Lee, S. Kim, H.J. Park, D. Park, beta-PIX-enhanced p38 activation by Cdc42/Rac/PAK/MKK3/6-mediated pathway. Implication in the regulation of membrane ruffling, J. Biol. Chem. 276 (2001) 25066–25072.
[16] M. Geiszt, K. Lekstrom, S. Brenner, S.M. Hewitt, R. Dana, H.L. Malech, T.L. Leto, NAD(P)H oxidase 1, a product of differentiated colon epithelial cells, can partially replace glycoprotein 91phox in the regulated production of superoxide by phagocytes, J. Immunol. 171 (2003) 299–306.
[17] R.K. Ambasta, P. Kumar, K.K. Griendling, H.H. Schmidt, R. Busse, R.P. Brandes, Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase, J. Biol. Chem. 279 (2004) 45935–45941.
[18] G. Cheng, J.D. Lambeth, NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain, J. Biol. Chem. 279 (2004) 4737–4742.
[19] X.J. Li, D. Grunwald, J. Mathieu, F. Morel, M.J. Stasia, Crucial role of two potential cytosolic regions of Nox2—r191TSSTTIRRSr200 and r484DESQANHFAVHHDEEKDr500—on NADPH oxidase activation, J. Biol. Chem. (2005).