Biochimie 93 (2011) 457–468

Contents lists available at ScienceDirect

Biochimie

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

Research paper

New insight into the Nox4 subcellular localization in HEK293 cells: First monoclonal antibodies against Nox4

Leilei Zhang [a,b], Minh Vu Chuong Nguyen [a], Bernard Lardy [a], Algirdas J. Jesaitis [c], Alexei Grichine [d], Francis Rousset [a], Monique Talbot [e], Marie-Hélène Paclet [a], GuanXiang Qian [b], Françoise Morel [a,*]

[a] GREPI Timc-Imag UMR-CNRS 5525, Enzymologie/CHU Albert Michallon, BP 217, 38043 Grenoble, France
[b] Department of Biochemistry and Molecular Biology, Research Center for Human Gene Therapy, School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, PR China
[c] Department of Microbiology, Montana State University, Bozeman, MT 59717, USA
[d] Platform 'Optical Microscopy – Cell Imaging' – UJF, Inserm U823, Institut Albert Bonniot, 38076 La Tronche, France
[e] UMR-CNRS 8200, Institut de cancérologie Gustave Roussy, 94805 Villejuif, France

ARTICLE INFO

Article history:
Received 7 June 2010
Accepted 3 November 2010
Available online 12 November 2010

Keywords:
NADPH oxidase 4
Monoclonal antibodies
Subcellular localization
O_2^-

ABSTRACT

Nox4, a member of Nox family of NADPH oxidase expressed in nonphagocytic cells, is a major source of reactive oxygen species in many cell types. But understanding of the role of Nox4 in the production of ROS and of regulation mechanism of oxidase activity is largely unknown. This study reports for the first time the generation and characterization of 5 mAbs against a recombinant Nox4 protein (AA: 206–578). Among 5 novel mAbs, 3 mAbs (8E9, 5F9, 6B11) specifically recognized Nox4 protein in HEK293 transfected cells or human kidney cortex by western blot analysis; mAb 8E9 reacted with intact tet-induced T-REx™ Nox4 cells in FACS studies. The other 2 mAbs 10B4 and 7C9 were shown to have a very weak reactivity after purification. Immunofluorescence confocal microscopy showed that Nox4 localized not only in the perinuclear and endoplasmic reticulum regions but also at the plasma membrane of the cells which was further confirmed by TIRF-microscopy. Epitope determination showed that mAb 8E9 recognizes a region on the last extracellular loop of Nox4, while mAbs 6B11 and 5F9 are directed to its cytosolic tail. Contrary to mAb 6B11, mAb 5F9 failed to detect Nox4 at the plasma membrane. Cell-free oxidase assays demonstrated a moderate but significant inhibition of constitutive Nox4 activity by mAbs 5F9 and 6B11. In conclusion, 5 mAbs raised against Nox4 were generated for the first time. 3 of them will provide powerful tools for a structure/function relationship of Nox4 and for physiopathological investigations in humans.

1. Introduction

NADPH oxidase, Nox4, belongs to the Nox family which could generate reactive oxygen species (ROS) by transferring an electron to molecular oxygen. All these members contain six putative transmembrane helices, conserved binding sites for FAD and NADPH, and four heme-binding histidines in the third and fifth transmembrane domain [1]. Nox2 is the prototype of the Nox family [2]: it is essentially present in phagocytes and a well studied characterized source for ROS production. By interacting with the membrane-bound $p22^{phox}$, it becomes the heterodimer flavocytochrome b_{558}. This core unit assembles with various cytosolic regulating and activating factors $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac 1/2 being recruited upon activation, at the membrane level from cytosol [3–5].

Nox4 is a ubiquitous protein with 39% identity to Nox2 [6–8]. Although originally identified and highly expressed in the kidney, Nox4 mRNA was also reported in many human and murine tissues [6,8,9]. Nox4 is a $p22^{phox}$-dependent enzyme which co-immunoprecipates with $p22^{phox}$, and stabilizes the $p22^{phox}$ protein [10]; Contrary to Nox2, the activity of Nox4, is constitutive: it is active without the need for cell stimulation [6,7,11,12] and does not require the assembly of cytosolic factors. $P22^{phox}$ is the only component necessary for its activity. Recently, Poldip2 (polymerase

Abbreviations: IPTG, Isopropyl β-d-1-thiogalactopyranoside; ER, endoplasmic reticulum; ROS, reactive oxygen species; DFP, Diisopropylfluoro-phosphate; eGFP, enhanced Green Fluorescent Protein; SOD, Superoxide dismutases; CAT, catalase; TIRF, Total Internal Reflection Fluorescence; HEK, human embryonic kidney; VSMC, vascular smooth muscle cell; FAD, flavin adenine dinucleotide.

* Corresponding author. Tel.: +33 (0) 4 76 76 57 52; fax: +33 (0) 4 76 76 56 08.
E-mail addresses: zll1981@hotmail.com (L. Zhang), mvchuong@yahoo.fr (M.V.C. Nguyen), blardy@chu-grenoble.fr (B. Lardy), umbaj@montana.edu (A.J. Jesaitis), alexei.grichine@ujf-grenoble.fr (A. Grichine), francisrousset@yahoo.fr (F. Rousset), Talbot@igr.fr (M. Talbot), MHPaclet@chu-grenoble.fr (M.-H. Paclet), qiangx@shsmu.edu.cn (GuanXiang Qian), FrMorel.enzymo@chu-grenoble.fr (F. Morel).

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doi:10.1016/j.biochi.2010.11.001

DNA-directed delta-interacting protein 2) was introduced as a novel Nox4/p22^{phox}-interacting protein: it is a potent positive regulator of Nox4 activity in VSMC [13].

Despite its ubiquitous expression and activity, the primary function of Nox4 derived ROS is not clear. It has been proposed to have a role in oxygen sensing, growth, senescence and differentiation [14]. The abnormal expression and activation of Nox4 may induce tumorigenesis, tumor angiogenesis [15], and be related with the occurrence and development of other diseases, such as hypertension [16,17], atherosclerosis [18], fibrosis [19] and osteoarthritis [20]. Although the reasons for these differences are not quite clear, an important clue to the biological function of Nox4 links to its subcellular localization [21]. Localization studies of Nox4 remain controversial. Nox4 was shown primarily to be located in perinuclear and endoplasmic reticulum regions of COS7, HEK293 and endothelial cells [11], but it was also detected at the plasma membrane [22], focal adhesion and within the nucleus [8]. It may not be surprising that the same protein displays distinct localization in different cell types. However, different Nox4 localization was also reported in human vascular endothelial cells [21,23]. It will be interesting to know whether these different localizations could be due to the specificity between different Nox4 antibodies used. It may also come from the different physiological and pathological state of cells, as different intracellular localization of Nox4 was reported in normal and pathological human thyroid tissues [24].

Although the knowledge of gene expression and Nox4 association with pathologies is rapidly growing, understanding of the role of Nox4 in the production of reactive oxygen species and of regulation mechanism of oxidase activity has been hindered by the lack of specific monoclonal antibodies which are also essential tools to provide direct evidence to topology models and to identify structural features of heterodimer with p22^{phox}.

In this present study, we report for the first time the generation and characterization of 5 novel monoclonal antibodies raised against a truncated recombinant protein (residues 206–578) of Nox4, which were then used to clarify the subcellular localization of Nox4 in human embryonic kidney cell lines. These Nox4-reactive mAbs were epitope mapped by phage-display analysis or by immunodetection of recombinant Nox4 truncated constructions and were examined for effects on Nox4 oxidase activity. Due to their respective properties, these mAbs reported in this study will be valuable in characterizing the regulation of Nox4 activity, the subcellular localization/function relationship of Nox4, therefore to provide new drug targets for the effective prevention of diseases related to ROS.

2. Materials and methods

2.1. Materials

DMEM, fetal bovine serum, neomycin (G418, geneticin) were purchased from GIBCO; Alexa Fluor 488 F(ab′) fragment of goat anti-mouse IgG(H + L) was purchased from Invitrogen; ECL Western Blotting Detection reagents were purchased from Amersham Biosciences; complete mini EDTA-free protease inhibitor EASYpack, Atto-565-concanavalin A, $Na_4P_2O_7$, Na_3VO_4, PMSF, luminol, isoluminol, Triton X-100, Chaps, Isopropyl β-D-1-thio-galactopyranoside(IPTG) and monoclonal antibody anti-poly-Histidine-peroxidase were purchased from SIGMA; okadaic acid, leupeptin, pepstatin, trypsin inhibitor, TLCK, and protein G Agarose were purchased from Roche; Hoechst 33258 was purchased from Molecular Probes; DFP was purchased from Acros Organics; GFP mAb and isotype antibodies were purchased from Santa Cruz; Lab-Tek II chamber slide and Lab-Tek chambered coverglass were purchased from Thermo; Goat anti-Mouse IgG-HRP was purchased from GE healthcare; HiPerFect Transfection Reagent was purchased from QIAGEN; HEK293 T-REx™ Nox4 cells, siRNA a and c were generous gifts from PATIM laboratory, Pr KH. Krause, Geneve university, Switzerland; pET15b ΔT/Stop ZEBRA/MD4-eGFP plasmid was obtained from TheREx laboratory, Pr JL. Lenormand (TIMC-Imag, UMR-CNRS 5525, Grenoble, France).

2.2. Cell culture

All cells were cultured in DMEM containing 4.5 g/L glucose and 0.11 g/L sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin and 2 mM glutamine at 37 °C in air with 5% CO_2. Selecting antibiotics, blasticidin (5 μg/ml) and neomycin (400 μg/ml) were used with HEK293 T-REx™ Nox4 cells, while neomycin (500 μg/ml) was used with HEK293 Nox4GFP cells.

2.3. Generation of plasmids for the expression of recombinant Nox4 proteins

2.3.1. Truncated Nox4 proteins coupled with a His-tag at the N terminus

pET30b plasmid (Novagen) was used to express NHS-Nox4-1TM protein corresponding to the amino acid 206–578 of Nox4. PCR fragments corresponding to the Nox4 (206–578)-truncated form were obtained by using pEF/Nox4 plasmid as a matrix with the forward primer 5′-GGA ATT CTC CAT GGT CTT GCA TGT TTC AGG AGG GCT GC-3′including a *Nco*I site (underlined) and the reverse primer 5′-GCG TTA CTC GAG TCA GCT GAA AGA CTC TTT ATT GTA TTC-3′. The *Xho*I restriction site of the reverse primers is underlined. The purified PCR product was digested with *Nco*I and *Xho*I and ligated into linearized pET30b plasmid to obtain a plasmid encoding for NHS-Nox4-1TM protein. PCR fragments corresponding to the other various Nox4 truncated forms 1–10 (Table S1) were obtained by using pET30b Nox4-1TM as a matrix with each forward primers and reverse primers (Table S1) including a *Nco*I and *Xho*I restriction site (underlined). The purified PCR products were digested with *Nco*I and *Xho*I and ligated into linearized pET30b plasmid to obtain plasmids encoding for various Nox4 truncated forms (Table S1).

2.3.2. Truncated Nox4 proteins coupled with a His-tag at the C-terminus

pIVEX2.3MCS plasmid (Roche) was used to express Nox4-1TM-CH (AA: 206–578) and Nox4qc-CH (AA: 309–578). PCR fragments corresponding to the Nox4 (206–578) or (309–578)-truncated forms were obtained by using pEF/Nox4 plasmid as a matrix with the forward primer 5′-GGA TGA GCG GCC GCG CCT TGC ATG TTT CAG GAG GGC TGC-3′ for Nox4 (206–578) or with the forward primer 5′-GGA TGA GCG GCC GCC AG TCA CCA TCA TTT CGG TC-3′ for Nox4 (309–578) including a *Not*I site (underlined) and the reverse primer 5′-GCG TTA CTC GAG TTG CTG AAA GAC TCT TTA TTG TAT TC-3′. The *Xho*I restriction site of the reverse primers is underlined. The purified PCR product was digested with *Not*I and *Xho*I and ligated into linearized pIVEX2.3MCS plasmid.

2.4. Nox4 truncated proteins produced by in vitro translation (RTS, rapid translation system)

Expression for each protein was performed using the RTS™ HY100 (Roche Applied Science) according to the manufacturer's instructions. 5 μl of the reaction was used for western blot experiments [25,26].