Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1752 (2005) 186 – 196

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Evaluation of two anti-gp91^{phox} antibodies as immunoprobes for Nox family proteins: mAb 54.1 recognizes recombinant full-length Nox2, Nox3 and the C-terminal domains of Nox1-4 and cross-reacts with GRP 58

Danas Baniulis[a], Yoko Nakano[b], William M. Nauseef[b], Botond Banfi[b,c], Guangjie Cheng[d], David J. Lambeth[d], James B. Burritt[a], Ross M. Taylor[a], Algirdas J. Jesaitis[a,*]

[a]*Department of Microbiology, Montana State University, Bozeman, MT 59717, USA*
[b]*Inflammation Program, Department of Medicine, Veterans Affairs Medical Center and University of Iowa, Iowa City, Iowa 52242, USA*
[c]*Department of Anatomy and Cell Biology, University of Iowa, Iowa City, Iowa 52242, USA*
[d]*Department of Pathology, Emory University, Atlanta, GA 30322, USA*

Received 3 June 2005; received in revised form 25 July 2005; accepted 27 July 2005
Available online 10 August 2005

Abstract

Progress in the study of Nox protein expression has been impeded because of the paucity of immunological probes. The large subunit of human phagocyte flavocytochrome b_{558} (Cytb), gp91^{phox}, is also the prototype member of the recently discovered family of NADPH oxidase (Nox) proteins. In this study, we have evaluated the use of two anti-gp91^{phox} monoclonal antibodies, 54.1 and CL5, as immunoprobes for Nox family proteins. Sequence alignment of gp91^{phox} with Nox1, Nox3 and Nox4 identified regions of the Nox proteins that correspond to the gp91^{phox} epitopes recognized by mAb 54.1 and CL5. Antibody 54.1 produced positive immunoblots of recombinant C-terminal fragments of these homologous proteins expressed in *E. coli*. Furthermore, only mAb 54.1 recognized full-length murine and human Nox3 expressed in HEK-293 cells, in immunoblots of alkali-stripped or detergent-solubilized membranes. 54.1 recognized Nox3 expression-specific proteins with Mr 30,000, 50,000, 65,000 and 88,000 for the murine protein and Mr of 38,000–58,000, 90,000, 100,000–130,000 and a broad species of higher than 160,000 for the human protein. We conclude that mAb 54.1 can serve as a probe of Nox3 and possibly other Nox proteins, if precautions are taken to remove GRP 58 and other crossreactive membrane-associated or detergent-insoluble proteins from the sample to be probed.

Keywords: Nox; gp91^{phox}; Monoclonal antibody; Protein expression; Immunoblotting

Abbreviations: 2-DE, two dimensional electrophoresis; ASB-14, amidosulfobetaine-14; ATP, adenosine 5′-triphosphate; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CGD, chronic granulomatous disease; Cytb, flavocytochrome b_{558}; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; IEF, isoelectric focusing; IPTG, isopropyl β-D-1-thiogalactopyranoside; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted, laser desorption/ionization-time-of-flight; Mr, molecular weight; MRB, membrane resuspension buffer (10 mM HEPES, 10 mM NaCl, 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml chymostatin, pH-7.4); NADPH, β-nicotinamide adenine dinucleotidephosphate, reduced; PBS, phosphate buffered saline (10 mM phosphate, 150 mM NaCl, pH-7.4); PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; YT, yeast extract/tryptone medium

* Corresponding author. Tel.: +1 406 994 4811; fax: +1 406 994 4926.
E-mail address: umbaj@montana.edu (A.J. Jesaitis).

1570-9639/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2005.07.018

1. Introduction

Human phagocyte flavocytochrome b_{558} (Cytb) is an integral membrane protein composed of two polypeptides (gp91^{phox} and p22^{phox}) with molecular weights of 91,000 and 22,000 [1,2]. Cytb serves as the electron transferase of the NADPH oxidase complex [3–5]. This membrane-localized enzyme plays an important role in the host defense function of phagocytic cells, including neutrophil granulocytes, monocytes, macrophages and eosinophils [6,7]. During phagocytosis, macrophages and neutrophils produce a variety of toxic products that help to kill the microorganisms engulfed into phagosomes [8,9]. Superoxide anion (O_2^-) is the common precursor for the production of such reactive oxygen species and is generated by the NADPH oxidase in the plasma membrane and at the phagosomal membrane [10].

Homologs of human gp91^{phox} have been identified in a variety of tissues. Based on their sequence similarity, four different mammalian proteins were identified and assigned to the Nox family, whereas two larger proteins were identified and assigned to the Duox family [11–14]. All of the identified homologs contain a cluster of up to six putative hydrophobic transmembrane domains similar to the gp91^{phox} transmembrane helices in the amino-terminal part of the protein, including conserved histidine residues implicated in heme ligation by gp91^{phox}. Also, there is significant similarity in the carboxy-terminal domain of Nox/Duox family proteins with consensus sequences comprising putative flavin- and NAD(P)H-binding sites found in a variety of FAD-bound redox proteins. Nox5, Duox proteins and homologous plant proteins also contain a larger hydrophilic N-terminal domain not present in other Nox proteins (including gp91^{phox}) [15–19]. This domain contains several Ca^{2+}-binding EF-hand motifs possibly involved in regulation of catalytic activity. In addition, Duox proteins possess a unique, amino-terminal hydrophobic transmembrane α-helix and a putative extracellular domain homologous to peroxidase [15,16]. Recently, these novel proteins have been shown to play unique roles in development and signal transduction [11,12,14,20]. There are few if any specific immunological probes of these proteins [21,22].

Anti-Cytb antibodies have found a variety of applications in research related to NADPH oxidase structure and function. Such antibodies have been widely used for identification and quantitative analysis in Cytb expression experiments and chronic granulomatous disease (CGD) studies [23–29]. Due to the absence of crystallographic data, a variety of experimental approaches have been utilized to explore structural aspects of Cytb. Biochemical analysis combined with epitope mapping of monoclonal antibodies has confirmed aspects of transmembrane topology and has revealed intramolecular interaction features in the tertiary structure of the protein [30–34]. Moreover, such antibodies are finding important application in studies involving biochemical and physiological assays of Cytb and NADPH-oxidase function [32,35].

Few monoclonal antibodies recognizing the heavy chain of Cytb have been reported. Jesaitis and coworkers have characterized several such antibodies, namely 54.1 [36], NL7 [32] produced in their laboratory, antibody 7D5 that was produced by Nakamura et al. [28,31,37] and antibody 48 and 449 produced by Roos et al. [38]. There are no reports describing the use of monoclonal antibodies specific to other Nox family proteins. Polyclonal antibodies have been used to study Nox family protein expression. However an analysis of rabbit anti-gp91^{phox} polyclonal antibody binding to neutrophils showed that the polyclonal antibodies can display low specificity and should be used with caution [39].

The structural similarities among Nox family members suggest that anti-Cytb antibodies may be cross-reactive with some of the other members of this family and might be of use in their detection. Monoclonal antibody cross-reactivity among Nox family proteins has not been previously tested. However, the potential for such cross-reactivity in an unrelated system has been described in the study with several monoclonal antibodies directed against the 65-kDa *Mycobacterium leprae* antigen which cross-react with homologous proteins in human, Chinese hamster, chicken and bacteria [40]. An analysis of the antibody epitopes revealed that corresponding regions in the crossreactive proteins show a high degree of sequence conservation.

An application of anti-gp91^{phox} antibodies as immunoprobes for other Nox family members may have significant impact because few specific immunoprobes of these proteins have been reported [21,22]. In this study, we analyzed the potential of two anti-Cytb monoclonal antibodies (54.1 and CL5) for use as immunoprobes for Nox family proteins and showed that mAb 54.1 may be a probe for Nox3 and possibly other proteins of the family. In this process we partially purified and identified GRP 58, a soluble protein which specifically cross-reacts with mAb 54.1 and must be removed from samples before immunochemical analysis.

2. Materials and methods

2.1. Materials

The pBluescript KS+ vector containing the full-length gp91^{phox} gene insert was kindly provided by M. Dinauer. Mouse anti-ERp57 mAb that binds GRP 58 was obtained from Stressgen Biotechnologies Corporation (Victoria, BC, Canada). Alkaline phosphatase-conjugated goat anti-mouse IgG was from BioRad Laboratories (Hercules, CA). *E. coli* XL1-Blue, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)-pLysS strains were obtained from Invitrogen (Carlsbad, CA), and the expression vector pET-14b from Novagen (Madison, WI). Restriction enzymes *Eco*RI, *Nde*I, *Bam*HI