Nox protein expression, purification and structure analysis
by Danas Baniulis

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Science
in Microbiology
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Abstract:
Flavocytochrome b558 (Cytb) is a heterodimeric integral membrane protein that serves as the electron transferase of the NADPH oxidase. Six homologues of gp91-phox, the large subunit of Cytb, have been identified (Nox family). Understanding of the structure and function of the Nox proteins is limited. To distinguish solvent-accessible and membrane or conformation sequestered regions on native structure of gp91-phox, a number of proteolytic enzyme cleavage products on the lipid reconstituted protein were identified using mass spectrometry, in this study. Affinity-purified rabbit anti-peptide antibodies binding to intact neutrophils suggested extracellular localization of gp91-phox regions, however, results using control CGD-cells suggested that these antibodies may cross-react with an unusual non-gp91-phox species in the normal and CGD-derived plasma membranes. Further, a monoclonal antibody CL5 epitope was mapped to the region 135-DPYSVALSELGDR on the gp91-phox, the prototype for the Nox family proteins. Epitopes of previously described mAb 54.1 and CL5 in gp91-phox align with Nox family proteins with high degree of identity and the use of these two monoclonal antibodies as immunoprobes for Nox family proteins was evaluated. Ab 54.1 was found to be specifically reactive with homologous Nox protein fragments expressed in E. coli. Nox3 protein expressed in HEK293H cells was also detected by 54.1, but not by CL5. Nox1 expression in stably transfected NIH 3T3 was examined using the antibodies, but no detectable binding to Nox1 was observed in immunoblotting assays and by flow-cytometry analysis. The antibodies were also used to probe for presence of potential truncated forms of gp91-phox expressed in chronic granulomatous disease (CGD) affected neutrophils with premature termination of gp91-phox synthesis. Analysis did not detect any smaller size protein fragments by immunoblotting. In addition, two other proteins were found to be crossreactive with 54.1 and CL5, they were identified as GRP58 and gelsolin, respectively, two universally expressed cytosolic proteins with regulated association with the plasma membrane. Finally, to help in ongoing structural biology efforts, a recombinant human Cytb expressing PLB-985 cell line was used to develop process of large-scale production of the protein for application in structural biology experiments.

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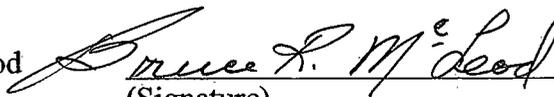Dr. Algirdas J. Jesaitis

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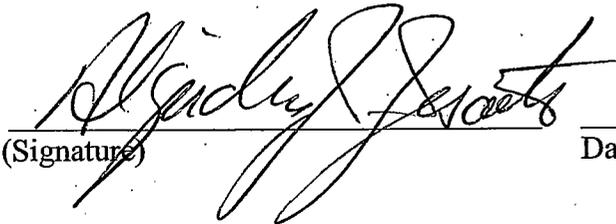
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GLOSSARY

2-DE, two dimensional electrophoresis;

Ac, acetylated;

ASB-14, amidosulfobetaine-14;

ATP, adenosine 5'-triphosphate;

BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium;

BSA, bovine serum albumin;

CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate;

CGD, chronic granulomatous disease;

Cytb, flavocytochrome b558;

CytC, ferrocytochrome c;

DDM, dodecylmaltoside;

DEAE, diethylaminoethyl;

DMEM, Dulbecco's modified Eagle's medium;

DTT, dithiothreitol;

EDTA, ethylenediaminetetraacetic acid;

EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid;

ELISA, enzyme-linked immunosorbant assay;

FBS, fetal bovine serum;

FAD, flavin adenin dinucleotide;

FITC, fluorescein isothiocyanate;

fMLF, *N*-formyl-methionyl-leucyl-phenylalanine;

gp91-PLB-985, gp91-phox transfected X-CGD PLB-985;

HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid];

IEF, isoelectric focusing;

IgG, immunoglobulin G;

IPTG, isopropyl β-D-1-thiogalactopyranoside;

KLH, keyhole limpet hemocyanin;

MAb, monoclonal antibody;

MALDI-TOF, matrix-assisted, laser desorption/ionization - time-of-flight;

Mr, relative molecular mass;

MS, mass spectrometry;

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;

PMA, phorbol myristate acetate;

PMSF, phenylmethylsulfonyl fluoride;

SDS, sodium dodecyl sulfate;

SDS-PAGE, SDS polyacrylamide gel electrophoresis;

SOD, superoxide dismutase;

Tris, tris(hydroxymethyl)aminomethane

YT, yeast extract/tryptone medium.

ABSTRACT

Flavocytochrome b558 (Cytb) is a heterodimeric integral membrane protein that serves as the electron transferase of the NADPH oxidase. Six homologues of gp91-phox, the large subunit of Cytb, have been identified (Nox family). Understanding of the structure and function of the Nox proteins is limited. To distinguish solvent-accessible and membrane or conformation sequestered regions on native structure of gp91-phox, a number of proteolytic enzyme cleavage products on the lipid reconstituted protein were identified using mass spectrometry, in this study. Affinity-purified rabbit anti-peptide antibodies binding to intact neutrophils suggested extracellular localization of gp91-phox regions, however, results using control CGD-cells suggested that these antibodies may cross-react with an unusual non-gp91-phox species in the normal and CGD-derived plasma membranes. Further, a monoclonal antibody CL5 epitope was mapped to the region 135-DPYSVALSELGDR on the gp91-phox, the prototype for the Nox family proteins. Epitopes of previously described mAb 54.1 and CL5 in gp91-phox align with Nox family proteins with high degree of identity and the use of these two monoclonal antibodies as immunoprobes for Nox family proteins was evaluated. Ab 54.1 was found to be specifically reactive with homologous Nox protein fragments expressed in *E. coli*. Nox3 protein expressed in HEK293H cells was also detected by 54.1, but not by CL5. Nox1 expression in stably transfected NIH 3T3 was examined using the antibodies, but no detectable binding to Nox1 was observed in immunoblotting assays and by flow-cytometry analysis. The antibodies were also used to probe for presence of potential truncated forms of gp91-phox expressed in chronic granulomatous disease (CGD) affected neutrophils with premature termination of gp91-phox synthesis. Analysis did not detect any smaller size protein fragments by immunoblotting. In addition, two other proteins were found to be crossreactive with 54.1 and CL5, they were identified as GRP58 and gelsolin, respectively, two universally expressed cytosolic proteins with regulated association with the plasma membrane. Finally, to help in ongoing structural biology efforts, a recombinant human Cytb expressing PLB-985 cell line was used to develop process of large-scale production of the protein for application in structural biology experiments.

INTRODUCTION

Phagocyte Function and NADPH oxidase

Phagocytes are the first line of defense against fungal and bacterial pathogens. Most abundant of this type of immune cells are neutrophils, representing 50-70% of the total number of white blood cells circulating in the blood (1). Neutrophils engulf bacteria by phagocytosis and orchestrate a variety of oxygen dependent and independent systems to kill the ingested pathogens (2). Oxygen-dependent bactericidal species include superoxide anion, hydroxyl radical, hypochlorous acid, nitric oxide, singlet oxygen and ozone (3, 4, 5, 6, 7). Superoxide anion is the common precursor for the production of reactive oxygen species and is generated by the NADPH oxidase in the plasma membrane, phagosomal membrane and at discrete internal membrane sites of these immune cells (8). Recently, the presence of the NADPH oxidase in non-phagocytic cells was also reported and the enzyme activity in such cells may be used for variety of tissue and cell type specific functions, such as host-defense, cell signaling, O_2 sensing or cross-linking reactions (9, 10, 11, 12, 13, 14, 15).

Neutrophils are formed in the bone marrow, where they differentiate to maturity in 7-10 days (1). The cells enter blood stream fully differentiated as polymorphonuclear cells or as nearly mature band cells. Neutrophils have a half life in the blood of approximately 6 hours and ultimately disperse to the tisues in 1-2 days. Mature neutrophils are carried by blood flow but also roll against the blood vessel walls and

sense signals from the underlying endothelium (16). Upregulation of P- and E-selectins and intercellular adhesion molecules, ICAMs, on the endothelial cells is triggered by signals from circulating agents or agents released from the injured cells, e.g. chemokines or bacterial products (17). These interactions enable neutrophil to squeeze between the endothelial cells and to penetrate the basement membrane (an extracellular matrix structure) with the aid of proteolytic enzymes and cell-cell signaling processes. The movement through the vessel wall is known as diapedesis, and enables phagocytes to enter the subepithelial tissues (18). In the tissue they migrate towards infected area along chemical gradients of substances called chemoattractants, such as interleukins, leukotrienes or bacterial products, which are released from bacteria or inflamed cells. This process is called chemotaxis.

Once in an inflammatory site, neutrophils are able to eliminate many pathogens by phagocytosis. One important consequence of the phagocytosis process is the fusion of lysosomes with the phagosome. The process, frequently referred to as phagolysosome fusion, in most cases leads to killing and degradation of the ingested pathogens by peptides, bactericidal enzymes and lipid, protein, and nucleic acid degrading activities (2). Essential to the process is the oxidative degradation of pathogenic components initiated by oxidative metabolites produced via the NADPH oxidase.

Neutrophils contain very few organelles (endoplasmic reticulum, Golgi, mitochondria), apart from the granules. Granule morphogenesis occurs during neutrophil development in the bone marrow as a result of differential expression of granule content proteins during the course of maturation (1, 19, 20). Granules are released selectively

depending on the stimulus (21). Azurophilic granules, also called primary granules, contain proteinases, bactericidal proteins and myeloperoxidase. Gelatinase granules also contain receptors and some proteases (22). The secretory vesicles are the smallest granules and contain plasma proteins, alkaline phosphatase, flavocytochrome b558 (Cytb) and receptors (23). The specific or secondary granules are the most abundant granule type and contain lactoferrin, proteases, components of the NADPH oxidase and receptors for complement and fMLF (24).

The NADPH oxidase plays an essential role in host defensive function of phagocytic cells, i.e., neutrophil granulocytes, monocytes, macrophages and eosinophils (25, 26). Upon phagocytosis, macrophages and neutrophils produce a variety of toxic products that help to kill the microorganisms engulfed into phagosome (3, 5). The NADPH oxidase carries electrons from NADPH across the membrane to oxygen that serves as an electron acceptor and is reduced to superoxide anion. This process is known as the respiratory burst, as it is accompanied by a transient increase in oxygen consumption. Superoxide anion then dismutates to hydrogen peroxide (H_2O_2). Myeloperoxidase, which is released from cytoplasmic granules of neutrophils and monocytes by a degranulation process, reacts with the H_2O_2 to form a complex that can oxidize a large variety of substances (27). Among the latter is chloride, which is oxidized initially to hypochlorous acid, with the subsequent formation of chlorine and chloramines. These products are powerful oxidants that can have profound biological effects, including rapid microbicidal effect or

Table 1. Neutrophil granule content and function (table adapted from (22, 28, 29, 30))

Granule	Factor	Mode of action
Primary (azurophilic)	Microbicidal proteins and peptides (i.e. cathepsin G, defensins, elastase, α_1-antitrypsin, bactenecins)	Destroy bacteria
	Myeloperoxidase	Catalizes the production of hypochlorous acid
	Lysozyme	Hydrolyzes glycosidic linkages in bacterial cell wall
	Neutral and acidic hydrolases	Activated within the phagosome by pH change and readily breakdown microbial products
Secondary (specific)	Membrane constituents of NADPH oxidase, Cytb, Rap 1A, Rac2; also receptors for laminin, fibronectin, fMLF, TNF, vitronectin and thrombospondin	Provides ROS-producing NADPH oxidase complex proteins, cell surface adhesion molecules and variety of receptors
	Lactoferrin	Iron chelator; may also catalyze production of hydroxyl radical
	Collagenase, gelatinase, histaminase, heparinase, plasminogen activator and sialidase	Breakdown microbial products
	Lysozyme	Hydrolyzes glycosidic linkages in bacterial cell wall
Gelatinase	Gelatinase	Collagen cleavage
	Mac-1, FPR	Provides a reserve of adhesion and chemoattractant receptors
Secretory vesicles	Plasma proteins	
	Cytb, Mac-1, FPR, alkaline phosphatase	Provides a reserve of receptors, adhesion molecules and Cytb

induce damage to adjacent tissue and contribute to the pathogenesis of disease when released to the outside of the cell. It has also been shown that nitrogen species can react with superoxide and form substances important for bacterial killing (7).

Generation of O_2^- by NADPH oxidase is not an exclusive attribute of phagocytic cells. It has been demonstrated in B lymphocytes (31), vascular smooth muscles (12, 14), fibroblasts (11), endothelial cells (12, 32, 33), the carotid body (34), kidney (9, 13) and lung (10, 15). In non-phagocytic cells, NADPH oxidase activity may be used for variety of tissue and cell type specific functions, such as host-defense, cell signaling, O_2 sensing, or cross-linking reactions (12, 35).

A function not involved directly in host defense activity was also suggested for phagocyte NADPH oxidase. Activation of NADPH oxidase in phagocytic cells depends on the binding of specific ligands to receptors expressed in the plasma membrane. The NADPH oxidase can be triggered either by stimulation of adhesion molecules or phagocytic receptors or by chemoattractants such as formyl-methionyl-leucyl-phenylalanine (fMLF), interleukin 8 (IL-8), complement fragment 5a (C5a) and platelet activating factor (PAF) (36, 37, 38, 39). There are also ways of triggering the NADPH oxidase, by-passing the receptors, via stimulation of protein kinase C (PKC) with phorbol myristate acetate (PMA) or elevating Ca^{2+} with ionophores (40). Most (80%) of the membrane-bound components of the NADPH oxidase are located in the specific or secondary granules and the rest in the plasma membrane (41, 42). It has been demonstrated that the two different pools of the oxidase can be activated independently. Stimulating neutrophils with fMLF generates a large, rapid generation of O_2^- from the

plasma membrane pool and a smaller intracellular response (43). Phagocytic stimuli such as bacteria and opsonised yeast induce a mainly intracellular response (44). The function of intracellular activation of the NADPH oxidase in the absence of phagocytosis is not yet understood, but reports have suggested that intracellular oxidative metabolites may have signaling capacity (45).

Structure of the NADPH oxidase

The NADPH oxidase has been shown to consist of several membrane-bound and cytosolic components. Cytb, the core redox active electron transferase, is an integral membrane protein composed of two polypeptides gp91-phox and p22-phox with molecular weights of 91,000 and 22,000 (46, 47). There are also membrane-bound and cytosolic protein-bound prenylated low molecular weight G proteins Rap 1A and Rac2, and cytoplasmic proteins p47-phox, p67-phox and p40-phox (48, 49). Activation of the NADPH oxidase involves translocation of cytosolic oxidase components to the plasma membrane where they associate with each other and Cytb. This association forms an activation regulated, functional multicomponent electron-transfer system.

The membrane protein heterodimer Cytb, with NADPH and FAD binding domains and transmembrane domain-coordinated hemes, serves as an electron transferase of the NADPH oxidase complex (50). Cytosolic proteins p47-phox, p67-phox and Rac2 are needed in the cell-free system to activate Cytb, and have been shown to be required in recombinant systems (8, 51, 52). Human genetic and engineered deficiencies prove that these proteins are required or necessary *in vivo*. The third cytosolic component, p40-

phox, copurifies with p67-phox and p47-phox, suggesting that it may have a regulatory function, as well (53). p47-phox appears to serve as an adaptor protein, transporting the cytosolic protein complex to membrane during activation (54). Neutrophils lacking p47-phox are unable to transfer p67-phox from the cytosol to the membrane during activation. Serving as a switch to trigger oxidase assembly, phosporylation of p47-phox results in conformational rearrangement, exposing SH3 motifs, proline-rich regions, and a PX domain that together mediate interactions with Cytb and p67-phox (8, 55, 56). Phosphorylation of p47-phox initially occurs in the cytosol, before translocation of p47-phox to the membrane, and continues after membrane association (57). Besides the SH3 domain, which is important for protein-protein interaction in oxidase assembly, p67-phox contains an NADPH binding domain and an activation domain (amino acids ~200-210) those may be involved in electron flow or regulation of the process within Cytb (8). Gorzalczany, et al. (58) proposed that the essential event in activation of NADPH oxidase is the interaction between p67-phox and Cytb, and that Rac2 and p47-phox serve as carriers for p67-phox to the membrane. When prenylated, Rac2 can fulfill the carrier function by itself, supporting oxidase activation by p67-phox in the absence of p47-phox and amphiphile. Likewise, Rac2 was shown to regulate electron transfer from NADPH to FAD by Cytb independently of p67-phox (59). The PX domains of p40-phox, as well as p47-phox, have been demonstrated to bind to specific phosphoinositides and may thus mediate in part the assembly of the oxidase at the plasma or phagosomal membrane (60, 61, 62).

Flavocytochrome b558 Structure and Biosynthesis

The electron transferase function of the glycosylated, heterodimeric, bis-heme, transmembrane Cytb arises from the union of its structural characteristics. The large subunit of Cytb, gp91-phox, is encoded by gene CYBB which is comprised of 13 exons spread over a total of 30 kb on the X chromosome at locus Xp21.1 (63). The gene encoding p22-phox CYBA is localized on chromosome 16 at 16q24 and is divided in 6 exons spaning 8.5 kb. Two subunits of Cytb are tightly associated and required for the active NADPH oxidase complex and integrity of its characteristic heme spectrum (64, 65). In myeloid cells, the absence of p22-phox protein due to genetic defects results in the loss of gp91-phox expression and vice versa, indicating that each of these proteins requires the other for mutual stability (66, 67). Neither gp91-phox alone nor the combination of individual gp91-phox and p22phox subunits, when expressed in COS-7 cell line, are able to replace the intact gp91/p22 heterodimer in supporting superoxide production in cell-free NADPH oxidase reconstitution assays, indicating that assembly of the fully functional enzyme complex requires specific interactions between subunits (65). However, gp91-phox and p22-phox have been stably expressed in the absence of their partner subunit in non-phagocytic cells lines (65, 68).

Heterodimer formation is also important for gp91-phox carbohydrate chain maturation (69). Gp91-phox is a glycoprotein (46, 70) and it has five potential N-linked glycosylation consensus sites. Glycosylation of three asparagine residues (131, 148 and 239) was supported by mutagenesis analysis of these positions in a recombinant expression system (71). The protein core of gp91-phox is 58 kDa and its maturation to

form the highly-glycosylated protein proceeds first in the endoplasmic reticulum and then in the Golgi apparatus. The specific function of the glycosylation, whether it is to protect protein from proteolysis, shield it from immunologic surveillance, or some unknown lectin-like interaction, remains unknown.

Cytb contains at least two non-identical, bis histidinyl coordinated heme groups that are believed to be located in either the transmembrane or extracellular domain of the protein (or possibly in both locations) and mediate the final steps of electron transfer to molecular oxygen (47, 72, 73, 74). The function of the Cytb as an electron transporting component of the NADPH oxidase is completely dependent on the presence of FAD (75, 76, 77, 78) which probably receives electrons directly from NADPH, acting like ferredoxin or nitrate reductase (79). It is uncertain how the enzyme functions as a single electron donor to molecular oxygen from a two electron oxidation of NADPH, although intermolecular electron tunnelling has been proposed (80). The substrate, NADPH, was shown to bind to the large subunit of Cytb (81, 82, 83), although some evidence exists suggesting it binds to p67-phox as well (84, 85).

Cytb biosynthesis studies have demonstrated that the heme incorporation is a critical step in the assembly of Cytb subunits. PLB-985 myeloid leukemia cells in which heme synthesis was prevented by addition of succinyl acetone to cell culture medium had decreased expression levels of both p22-phox and the mature glycosylated form of gp91-phox, but not that of the 65 kDa precursor (86, 87). When the histidine residues 101, 115, 209 and 222, putative ligands for the two heme prosthetic groups in gp91-phox, are

replaced by Leu or Arg, p65 is no longer processed and the p65 does not form a

heterodimer with p22-phox, resulting in cells that lack functional Cytb (88).

Little is known of secondary structure, transmembrane topological organization or

function of the small subunit of Cytb, p22-phox. Primary structure of p22-phox has three

hydrophobic domains in the N-terminal two-thirds of the molecule, and a proline-rich

domain in the C-terminal cytoplasmic tail. Such proline-rich regions can mediate protein-

protein association by binding to SH3 domains that are found in variety of proteins

involved in signal transduction, including cytosolic phox proteins. The proline-rich

domains 156-PPRPP and 177-GGPPGGP of p22-phox binds the N-terminal SH3 domain

of p47-phox, and this interaction is believed to play a dominant role in promoting the

association of cytosolic complex, containing p40-phox, p47-phox, and p-67-phox, with

flavocytochrome b558 (52, 89). Surface features of p22-phox were mapped with

antibodies raised in rabbits against synthetic peptides corresponding to various regions of

the protein or monoclonal antibodies which epitopes were identified using phage display

libraries. In this way, conclusions were drawn that the amino and carboxy termini of the

p22-phox subunit are exposed to the cytosol (90, 91). Moreover, the phage mapping

analysis combined with nuclear magnetic resonance (NMR) spectroscopy provided a

low-resolution view of the tertiary structure of p22-phox around the epitope of

monoclonal antibody 44.1. The results of the study inferred close spatial proximity of the

epitope components 29-TAGRF and 183-PQVNPI from discontinuous regions of p22-

phox separated apart in the sequence by 150 residues (92). Analysis of the cytochrome

using lithium dodecyl sulfate-polyacrylamide gel electrophoresis followed by

tetramethylbenzidine heme staining demonstrated the presence of heme in both the 91- and 22-kDa subunits (93) suggesting that Cytb is bi-heme molecule with at least one heme residing in the large subunit and one shared between both subunits. However, co-expression of gp91-phox with p22-phox harboring leucine, tyrosine, or methionine amino acid substitutions at histidine 94, the only invariant histidine residue within the p22-phox subunit, did not affect heterodimer formation or Cytb function. The heme spectrum in purified preparations of flavocytochrome b558 containing the p22-phox derivative was unaffected. These findings demonstrate conflicting views about the distribution and coordination of the hemes within the Cytb.

X-linked Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is an immunodeficiency syndrome characterized clinically by severe recurrent bacterial and fungal infections (94, 95, 96). The persistence of catalase positive bacteria and fungal conidia and hyphal forms, often within the phagosomal vacuoles of neutrophils or macrophages, is the stimulus to a chronic inflammatory state with granuloma formation. The most common pathogens encountered in CGD patients are *Staphylococcus aureus*, various *Aspergillus* species and variety of Gram-negative enteric bacilli.

CGD is rare disease, with an estimated incidence of 1 in about 250,000 individuals. Biochemically, CGD is characterized by the inability of phagocytic leukocytes (neutrophils, eosinophils, monocytes and macrophages) to activate the

NADPH oxidase and to generate superoxide as the needed precursor for reactive oxygen species involved in the killing of phagocytosed microorganisms (97).

CGD is a very heterogeneous disorder clinically, because of many antimicrobial systems that can partially compensate for the defect in oxygen-dependent killing systems, and biochemically, because of the complicated genetic origin of CGD (94). The disease is caused by mutations in any one of four genes encoding subunits of the NADPH oxidase, resulting in an absence or low levels of enzyme activity. More than two thirds of all cases are X-linked recessive and results from defects in the CYBB gene that encodes the gp91-phox subunit. A broad distribution of defects in patients with X-linked CGD include small and large deletions, insertions, nonsense and missense mutations, splice-site defects and, rarely, mutations in the 5' regulatory region. The remaining CGD mutation cases are autosomal recessive and caused by defects in CYBA, NCF-1 and NCF-2, which encode p22-phox, p47-phox and p67-phox, respectively (98). To date, there are no reports of CGD caused by defects in the gene for a fifth oxidase subunit, p40-phox. A single patient has been identified with a related immunodeficiency resulting from a defect in the gene for Rac2 (99, 100). Of the 410 known defects in the four affected genes only 19 result in normal level of inactive or weakly active protein: 17 out of 358 mutations in CYBB, 1 out of 25 in CYBA, 1 out of 17 in NCF-2 and none out of 10 in NCF-1 (98, 101, 102). Some of these mutations provide evidence for the function of specific domains in affected subunits and their roles in oxidase activation. The remaining 95% of CGD mutations result in a complete absence or greatly diminished level of protein, either

because the affected gene is partially or completely deleted, or because the aberrant

protein product (or mRNA) is unstable.

Gp91-phox Homologues

Homologues 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, two larger proteins were identified and assigned to the Duox

family (35, 103, 104, 105). As shown by a survey of genome data, the Nox and Duox

proteins are widely distributed in nature. Nox orthologues have been identified in

Drosophila and *Dictyostelium discoideum* (104). A protein related to Duox1 has been

found in *Caernorhabditis elegans* (106). Moreover, plants possess homologues of gp91-

phox as well. The *Arabidopsis thaliana* gene called RBOHA (Respiratory Burst Oxidase

Homology A) encodes a protein of 108 kDa, which has carboxy-terminal region highly

similar to the human neutrophil gp91-phox, and a smaller protein (~69 kDa) was

identified in rice (107). A subsequent study revealed the presence in *A. thaliana* of five

other genes encoding homologues of human gp91-phox protein (108). These homologues

are of approximately the same size between 897 and 948 amino acids and their carboxy-

domains are of 60% identity to human gp91-phox. These proteins may be responsible for

NADH-dependent ferricytochrome c reductase activity by plasma membrane cytochrome

b558 in *Z. mais* (109).

The Nox proteins have a relatively specific tissue distribution in human. Nox 1 is

predominantly expressed in colon with lower levels in the prostate, uterus and vascular

smooth muscle cells, where it is induced by platelet derived growth factor (110, 111). Nox2, which designates the gp91-phox, is present mostly in phagocytic and other cell types as described in previous section. Nox3 was cloned from fetal kidney (112), Nox4 also named Renox was found in kidney cortex (113, 114) and osteoclasts (115). Nox5 is expressed in variety of fetal tissues and adult spleen, lymph nodes, uterus, testis (116). The Duox1, also designated as Thox1, is present in the thyroid gland, tracheal, bronchial epithelial cells (117, 118, 119) and Duox2 also designated Thox2 present in thyroid gland and also in the small intestine, colon, epithelial cells in salivary excretory ducts and rectal glands (117, 119, 120).

All of the identified homologues contain a cluster of up to six putative hydrophobic transmembrane domains similar to the gp91-phox transmembrane helicies at amino-terminal part of the protein including conserved histidine residues implicated in heme ligation by gp91-phox. There also 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 larger hydrophilic N-terminal domain not present in other Nox proteins (107, 108, 116, 117, 118). This domain contains two Ca^{2+}-binding EF-hand motifs. In addition, Duox proteins possess a unique, amino-terminal hydrophobic transmembrane α-helix and putative extracellular domain homologous to peroxidase (117, 118). The extended N-terminal domain of plant RbohA also contains EF-hand motifs, and in addition, a region with extended sequence similarity to the human RanGTPase-activating protein (107).

Although neutrophil NADPH oxidase can be activated by Ca^{2+} because of the activation of Ca^{2+}-sensitive second messenger systems as described before (121, 122, 123), it was also shown that another gp91-phox homologue, Nox5, contains an N-terminal extension with three EF hands and when heterologously expressed is able to generate superoxide and conduct H^+ ions in response to cytosolic free Ca^{2+} concentration elevations (116). Activation of Duox enzymes by elevations of the cytosolic free Ca^{2+} concentration has been also suggested based on presence of EF hand motifs and the previously described Ca^{2+}-activated superoxide generation in thyroid cells (124).

The biological function and its mechanism of gp91-phox homologues is scarcely based on experimental data and mostly hypothetical. The Nox, Duox family protein, as well as NADPH oxidase function, may vary depending on the cell type involved. First studies of Nox1 suggested its function in mitogenic regulation and cell transformation (111), and its was shown that production of $O_2^{\cdot-}$ was not enhanced by addition of p47-phox, p67-phox or Rac in cell-free system (125). However, later it was shown that Nox1 expression is induced during differentiation in colon cells and that did not affect the proliferation of colon carcinoma cells (126). Furthermore, coexpression of cytosolic components, p47-phox and p67-phox, augments Nox1 activity in reconstituted K562 or HEK293 cells, and it suggest that Nox1 is likely to be involved in host-defense system of colon epithelium (126, 127). Also it was suggested that the Nox1 activity might be regulated by novel proteins homologous to p47-phox and p67-phox, designated p42-phox and p51-phox, respectively (127, 128, 129).

Primarily, Duox proteins were identified in thyroid gland (118). It was suggested that the proteins possibly complementing function to other thyroid peroxidase and NADPH oxidase enxymes involved in iodination thyroglobulin as they possess amino-terminal peroxidase homologous domain in addition to gp91-phox homologous domains involved in $O_2^{\cdot-}$ production. Later the protein expression was identified in salivary glands, rectum, trachea, and bronchium and Geiszt M et al. postulated that Duox1 and Duox2 might serve as a source of H_2O_2 for lactoperoxidase-mediated antimicrobial defense mechanisms on mucosal surfaces (119).

As a major source of ROS production in kidney, Nox4 has been implicated to have a role under pathological conditions (113, 114). The increase of expression levels and tissue distribution of Nox4 and p22-phox correlated with localization of 8-hydroxy-deoxyguanosine, which is a marker of ROS-induced DNA damage, in the kidney of diabetic rats (130). The other homologue, Nox5, is likely to be involved in Ca2+-actvated, redox-dependent processes of spermatozoa and lymphocytes such as sperm-oocyte fusion, cell proliferation, and cytokine secretion (116).

Rapid generation of oxidants has been shown in many plant-pathogen interactions and is a characteristic feature in the so called hypersensitive response. Plants possess an NADPH oxidase system similar to that of neutrophils (131, 132, 133). Immunological identification of the soybean proteins corresponding to NADPH oxidase components p22-phox, p47-phox and p67-phox has been described. *A. thaliana* protein RbohA amino-terminal Ca^{2+} binding EF hand motifs have been implicated in the rapid stimulation of the oxidative burst in cells challenged to prime the hypersensitive response (132, 134).

Interplay between the Ca^{2+} activation and GTP-ase mediated signal dumping of domain, similar to human GTPase activating protein, at the N-terminus of RbohA may contribute to stringent regulation of the plant NADPH oxidase (107).

Mammalian Membrane Protein Expression

A fundamental requirement of structural biology experiments is to be able to obtain high quantities of pure protein. An analysis of the mammalian membrane protein structures published shows that the majority of the proteins were purified from naturally abundant sources (reviewed by Tate (135)). In contrast, the majority of new soluble protein structural studies utilized recombinant material (136). These statistics reflect the fact that recombinant membrane proteins are difficult to obtain. Currently, four major expression systems can be distinguished: those using bacteria, yeast, insect cells, or mammalian cells. Although overexpression strategies typically have been developed for soluble proteins, these generally perform poorly with integral membrane proteins. However, several cases of successful mammalian integral membrane protein expression in broadly used prokaryotic and lower eukaryotic expression systems, such as *E. coli*, *P. pastoris*, *S. cerevisiae* and baculovirus-mediated expression in insect cells, have been reported during last decade (reviewed in (136, 137)). These include several G-protein coupled receptors, monoamine oxidase, Ca^{2+}-ATPase and the adrenergic receptor.

Use of prokaryotic or lower eukaryotic expression systems for mammalian proteins can lead to misfolding or loss of functionality of the protein expressed. Inclusion bodies are an attractive way of producing large amounts of protein, but this naturally

requires the refolding of the protein into a functional form, which has been achieved for only relatively few membrane proteins (136). Mammalian membrane proteins often require specific lipid requirements, the presence of certain chaperones, and specific post-translational modifications. Although in some cases glycosylation may play a crucial role in correct protein folding and insertion to membrane, reports of successful expression of functional glycoproteins exist. Several G-protein-coupled receptors were expressed in *E. coli* (138, 139), prostaglandin H2 synthase-2 was expressed in insect cells (140), monoamine oxidase-B in yeast (141), and fatty acid amide hydrolase in bacteria (142). However, these three later proteins have relatively small hydrophobic surface area and do not involve more than one subunit.

Obviously, recombinant protein can be best expressed in the expression system that most closely resembles the natural environment of the protein. For mammalian proteins, therefore, mammalian expression systems are likely to give the best results in terms of structure and functionality of the protein. Transient expression in mammalian cells and the creation of stable cell lines are widely used for protein function and physiological studies, though these systems are complex and relatively expensive for production of milligram quantities of protein. Recent progress in this area may make the mammalian expression systems more popular. An efficient system based upon Semliki Forest virus is now available which appears to be capable of producing large quantities of protein from variety of eukaryotic cells (143). In addition, temperature-regulated, inducible Sindbis virus replicon-based expression system has been developed which should prove useful in cases where large doses of the target protein are toxic (144).

Antibody Application for Studies of Cytb Structure and Function

Anti-Cytb antibodies have found variety of applications in research related to NADPH oxidase structure and function. In the absence of crystallographic data, a variety of experimental approaches have been utilized to explore the arrangements of polypeptide chains of the two subunits of Cytb, include analysis of antibody directed against specific sequences access to the epitope. An antibody raised against residues Glu150-Ser172 in the large subunit bound to intact neutrophils, indicating that this region is exposed to the outside of the cells, while the antibodies raised against any of the carboxyl-terminal regions of the large and small subunits or the amino-terminal region of the small subunit, bound to neutrophils only after the cells were made permeable by freezing and thawing (91).

Biochemical analysis combined with epitope mapping of monoclonal antibodies has confirmed aspects of transmembrane topology. An epitope bound by monoclonal antibody 7D5 has been mapped using phage display analysis to include residues Arg159-Glu164 and Arg226-Gln231 on gp91-phox and further antibody binding analysis revealed location of the regions on the non-cytosolic aspect of neutrophil membrane (145, 146). Epitopes of monoclonal antibodies NL7 (Glu498-Lys506 on gp91-phox), 44.1 (including regions Ser29-Phe33 and Pro183-Ile188 on p22-phox) and 449 (Gly182-Val185 on p22-phox) were shown to be located in neutrophil cytosol and indicate cytosolic location of the segments on the two Cytb subunits (90, 92, 147, 148). The phage mapping analysis combined with nuclear magnetic resonance (NMR) spectroscopy revealed intramolecular interaction features in the tertiary structure of the Cytb (92). The results of the study

inferred close spatial proximity of the epitope components Ser29-Phe33 and Pro183-Ile188 from discontinuous regions of p22-phox separated apart in the sequence by 150 residues.

Also, it was shown that antibody NL7 epitope Glu498-Lys506 represents a region of gp91-phox important for oxidase function (148). In a cell-free assay, NL7 inhibited in vitro activation of the NADPH oxidase though it bound its epitope on gp91(phox) independently of cytosolic factor or Rac translocation. However, after assembly of the NADPH oxidase complex, mAb NL7 bound the epitope but did not inhibit the generation of superoxide. Three-dimensional modeling of the C-terminal domain of gp91(phox) on a corn nitrate reductase template suggests close proximity of the NL7 epitope to the proposed NADPH binding site, but significant separation from the proposed p47-phox binding sites, suggesting that the Glu498-Lys506 segment resides on the cytosolic surface of gp91-phox and represents a region important for oxidase function, but not substrate or cytosolic component binding.

Except for structural analysis anti Cytb antibodies have a found variety of other important applications, such as Cytb purification (149), identification and quantitative analysis in Cytb expression experiments and chronic granulomatous disease (CGD) studies (13, 33, 65, 66, 146, 150, 151, 152, 153, 154, 155). Moreover, such antibodies are used in studies involving biochemical and physiological assays of Cytb and NADPH oxidase function (148, 156); (R.M. Taylor, A.J. Jesaitis, unpublished data).

Overview of the Dissertation

Nox protein-produced reactive oxygen species participate in variety of biological functions, including host-defense, cell signaling and O_2 sensing. They are also involved in non-specific tissue damage in a variety of inflammatory diseases. Understanding of the structural design of NADPH oxidase core functional element, the prototypical electron transferase flavocytochrome b558 (Cytb) is essential for comprehension and effective control of the ROS mediated processes initiated by Cytb or the recently discovered and widely expressed Nox family proteins. Thus, the fundamental goal of this research was to extend knowledge of Nox protein structure and function by analysis of elementary features of the Cytb structure. In addition, significant effort was extended toward the development of experimental tools required for the Nox protein detection and detailed structure analysis.

To address important structural issues about gp91-phox, transmembrane topology and protein surface features of the protein were characterized in study described in Chapter 2. Amino acid sequence computational analysis was employed to identify hydrophobic segments of the sequence forming transmembrane domains. Further, analysis of published experimental data was used to refine mathematical predictions to Cytb transmembrane topology model. To support topological predictions and to study functional aspects of the Cytb, a classical approach was employed to produce anti-peptide antibodies against synthetic peptides mimicking segments on N-terminal half of the gp91-phox. Proteolytic digestion of purified and lipid-reconstituted Cytb product analysis

by mass spectrometry extended understanding of hydrophilic, solvent exposed segments on gp91-phox.

Anti-Cytb antibodies have found variety of applications in research related to NADPH oxidase structure and function. Similarities of Nox family protein amino acid sequences also suggested the possibility for application of anti-Cytb antibodies in the detection of other Nox proteins. This perspective was explored by the characterization of binding features for two anti-Cytb antibodies and the immunological analysis of Nox protein expression and is described in Chapter 3. In that study, epitope identification of a new monoclonal anti-Cytb antibody to amino-terminal half of the large subunit of the Cytb provided means to investigate the possibility of expression of truncated gp91-phox forms in neutrophils with CGD mutations resulting in premature termination of the large subunit transcription.

In Chapter 4, a new methodology is described for large-scale production of human neutrophil Cytb in a recombinant expression system. Efficient production and purification techniques are essential to obtain quantities of the protein required for detailed structure analysis. Thus, design of high-yield recombinant protein expression systems would be an attractive alternative source for a functional Cytb. Further development of this system is described in Chapter 5. This section includes an unpublished description of attempts to develop a purification procedure for recombinant Cytb.

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