Biochemical and Biophysical Research Communications 443 (2014) 1060–1065

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

N-linked glycosylation of the superoxide-producing NADPH oxidase Nox1

Kei Miyano, Hideki Sumimoto *

Department of Biochemistry, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 10 December 2013
Available online 21 December 2013

Keywords:
Nox1
Glycosylation
p22^{phox}
Superoxide

ABSTRACT

Nox1 is a membrane-integrated protein that belongs to the Nox family of superoxide-producing NADPH oxidases. Here we show that human Nox1 undergoes glycosylation at Asn-162 and Asn-236 in the second and third extracellular loops, respectively. Simultaneous threonine substitution for these residues completely abrogates the glycosylation, but does not prevent Nox1 from forming a heterodimer with p22^{phox}, trafficking to the cell surface, or producing superoxide. In the absence of p22^{phox}, Nox1 is transported to the plasma membrane mainly as a form with high mannose *N*-glycans, although their conversion into complex *N*-glycans is induced by expression of p22^{phox}. These findings indicate that glycosylation and subsequent *N*-glycan maturation of Nox1 are both dispensable for its cell surface recruitment. Superoxide production by unglycosylated Nox1 is largely dependent on p22^{phox}, which is abrogated by glutamine substitution for Pro-156 in p22^{phox}, a mutation leading to a defective interaction with the Nox1-activating protein Noxo1. Thus p22^{phox} directly contributes to Nox1 activation in a glycosylation-independent manner, besides its significant role in Nox1 glycan maturation.

1. Introduction

The Nox family NADPH oxidases are membrane-spanning proteins that deliberately produce reactive oxygen species (ROS), thereby contributing to a variety of functions such as host defense, signal transduction, and synthesis of bioactive compounds [1,2]. Human genome contains seven genes encoding members of the Nox family: Nox1 through Nox5, and dual oxidase 1 (Duox1) and Duox2. Nox1 exists in colon epithelial cells and also in vascular smooth muscle cells, participating in vascular tone control [3]. This oxidase is very close to Nox2 and Nox3; the founding member Nox2, abundant in phagocytes such as neutrophils, produces superoxide, a precursor of microbicidal ROS, during phagocytosis to play a crucial role in host defense; and Nox3 in the inner ear contributes to formation of otoconia, which is required for sensing balance and gravity [3]. Thyroid hormone synthesis requires ROS that is produced by Duox2, an oxidase distantly related to Nox1 [3].

Nox2, as well as Nox1 and Nox3, comprises two regions of a similar size: the N-terminal half comprises six predicted α-helical transmembrane segments, in which two hemes are ligated perpendicularly to the membrane; and the C-terminal moiety is a cytoplasmic domain bearing the NADPH- and FAD-binding sites [1,2] (see Fig. 1A). The structure enables Nox to form a complete electron-transporting apparatus (from cytoplasmic NADPH via FAD and two hemes to molecular oxygen) for production of superoxide on the extracellular side.

The phagocyte oxidase Nox2 exists on the plasma membrane as a heavily glycosylated protein with an apparent molecular mass of about 91 kDa (and thus also known as gp91^{phox}) [4–7]. Nox2 is glycosylated at asparagine residues during translation in the endoplasmic reticulum (ER); after acquisition of hemes, Nox2 with high mannose *N*-glycans (gp65) dimerizes with the nonglycosylated transmembrane protein p22^{phox} in the ER. The nascent heterodimer undergoes conversion of the Nox2 carbohydrate groups to complex *N*-glycans in the Golgi apparatus *en route* to the plasma membrane [8–11]. In the plasma membrane of resting cells, Nox2 is dormant; its activation requires assembly of the Nox2–p22^{phox} dimer with the soluble activating proteins p47^{phox} and p67^{phox}, a process that includes a stimulant-induced association between p22^{phox} and p47^{phox} [1,2]. p22^{phox} also forms a stable complex with Nox1 [12–14] and Nox3 [15], thereby serving as a anchoring site for Noxo1, a p47^{phox}-related protein that is essential for Nox1 activation and is able to enhance Nox3 activity [12,14–17]. Nox3 is co-translationally modified with high mannose *N*-glycans, and subsequently interacts with p22^{phox}; this interaction is a prerequisite for glycan conversion into complex structures and cell surface recruitment of Nox3 [18]. The thyroid oxidase Duox2 is also a

Abbreviations: Nox, NADPH oxidase; ROS, reactive oxygen species; Duox, dual oxidase; ER, endoplasmic reticulum; PNGase F, peptide: *N*-glycosidase F; Endo H, endoglycosidase H.

* Corresponding author. Fax: +81 92 642 6103.
E-mail address: hsumi@med.kyushu-u.ac.jp (H. Sumimoto).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.086

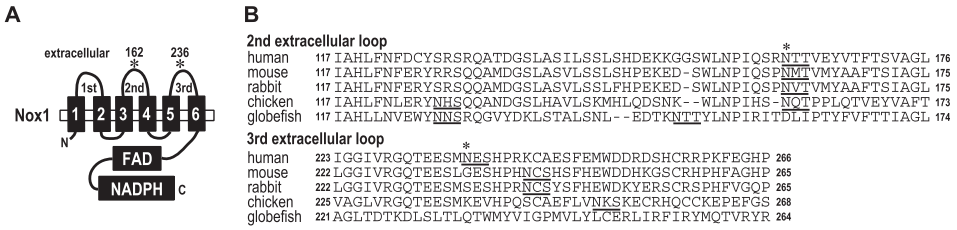

Fig. 1. Membrane topology of human Nox1 and potential *N*-glycosylation sites. (A) Localization of the potential *N*-linked glycosylation sites in the scheme of Nox1 structure. The first to sixth transmembrane fragments in the N-terminal half of Nox1 are numbered. The C-terminal cytoplasmic domain contains the FAD- and NADPH-binding sites. (B) Sequence alignment of the second and third extracellular loops in Nox1 from various species. Human Nox1 comprises 564 amino acid residues; and stars (*) indicate asparagine residues within the consensus sequence for *N*-glycosylation (Asn-X-Ser/Thr). Human, *Homo sapiens*; mouse, *Mus musculus*; rabbit, *Oryctolagus cuniculus*; chicken, *Gallus gallus*; globefish, *Takifugu rubripes*.

glycoprotein, which is synthesized as a form with high mannose *N*-glycans in the ER, and transported to the plasma membrane as a form with complex *N*-glycans [19,20]. On the other hand, little is known about biosynthesis and glycosylation of Nox1.

In the present study, we show that Nox1 is glycosylated at Asn-162 and Asn-236 when expressed in mammalian cells. Nascent Nox1, with high mannose *N*-glycans, can be transported to the plasma membrane without conversion into the mature form with complex *N*-glycans, whereas $p22^{phox}$ induces the conversion and subsequent transport of mature Nox1 to the plasma membrane. Although the blockade of Nox1 glycosylation affects neither its targeting to the cell surface nor its superoxide-producing activity, $p22^{phox}$ likely plays a crucial role even in activation of unglycosylated Nox1 by functioning as an anchoring site for Noxo1.

2. Materials and methods

2.1. Plasmid construction, cell transfection, and immunoblot analysis

The cDNAs encoding human Nox1, Nox2, Nox3, $p22^{phox}$, Noxo1, and Noxa1 were prepared as previously described, and ligated to pEF-BOS for expression in mammalian cells [12,14,21,22]. Mutations leading to the indicated substitution were introduced by PCR-mediated site-directed mutagenesis. All of the constructs were sequenced for confirmation of their identities.

Chinese hamster ovary CHO cells or human lung carcinoma H661 cells were transfected using ExtremeGene HP Transfection Reagent (Roche Applied Science) or Lipofectamine and PLUS™ (Invitrogen), respectively, with the following plasmids: pEF-BOS-FLAG–Nox1, pEF-BOS-FLAG–Nox2, or pEF-BOS-FLAG–Nox3; pEF-BOS-$p22^{phox}$–Myc; and/or pEF-BOS-HA–Noxo1 and pEF-BOS-Myc–Noxa1. HeLa cells or human colon cancer Caco-2 cells, each expressing $p22^{phox}$ endogenously, were transfected with pEF-BOS-FLAG–Nox1 using Lipofectamine (Invitorgen) or using the Nucleofector® apparatus and Cell Line Nucleofector® Kit T (Lonza), respectively. The transfected cells were cultured for 24 h in Ham's F12 medium (CHO cells), RPMI1640 (H661 cells), or DMEM (HeLa or Caco-2 cells), containing 10% fetal bovine serum.

For estimation of protein levels, cell lysates were analyzed by immunoblot [12,14,21] with the anti-FLAG mouse monoclonal antibody M2 (Sigma–Aldrich), the anti-Myc monoclonal antibody 9E10 (Roche Applied Science), the anti-HA monoclonal antibody 16B12 (Covance Research Products), or anti-$p22^{phox}$ polyclonal antibodies (Santa Cruz Biotechnology). The blots were developed using ECL Plus (GE Healthcare Biosciences) for visualization of the antibodies.

2.2. Glycosidase treatment

Cells expressing a FLAG-tagged Nox1, Nox2, or Nox3 were lysed in a solution (150 mM NaCl and 20 mM Tris–HCl, pH 7.4) containing 1% Triton X-100, and the lysate was centrifuged for 10 min at 20,000*g*. The supernatant was subjected to digestion with peptide: *N*-glycosidase F (PNGase F; New England Biolabs) or endoglycosidase H (Endo H; New England Biolabs). Treated proteins were separated by 10% SDS–PAGE, followed by immunoblot analysis with the anti-FLAG antibody.

2.3. Immunoprecipitation assay

Proteins in the lysate of transfected CHO cells were precipitated with the anti-FLAG or anti-Myc mouse monoclonal antibody in the presence of protein G-Sepharose (GE Healthcare Biosciences), as previously described [15]. The precipitants were analyzed by immunoblot with the indicated antibody.

2.4. Cell surface biotinylation

CHO cells in monolayer culture were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4), and treated for 2.5 min at 25 °C with 0.5 mM EZ-link Sulfo-NHS-SS-biotin (Pierce), a membrane-impermeable biotinylation reagent, according to the manufacturer's instruction. After the reaction was quenched with 50 mM glycine, cells were resuspended in PBS containing 1% (v/v) protease inhibitor cocktail (Sigma–Aldrich) and lysed by sonication. The postnuclear fraction of the sonicate was centrifuged for 10 min at 20,000*g*, and the resultant supernatant was ultracentrifuged for 1 h at 150,000*g*. The pellets were solved in 150 mM NaCl and 20 mM Tris–HCl, pH 7.4, containing 1% Triton X-100, and biotinylated membrane proteins were precipitated with streptavidin-Sepharose (GE Healthcare Biosciences). The precipitants were analyzed by immunoblot with the anti-FLAG antibody.

2.5. Superoxide production

The superoxide-producing activity of Nox1 expressed in CHO cells was determined by superoxide dismutase (SOD)-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics). Chemiluminescence change was monitored at 37 °C using a luminometer (Auto Lumat LB953; EG&G Berthold), as described previously [21,23].

3. Results

3.1. Nox1 N-glycosylation and $p22^{phox}$-induced glycan maturation

The current model for Nox membrane topology [1,2] indicates that, like Nox2, Nox1 has the N-terminus located to the cytoplasmic side of the plasma membrane, followed by six transmembrane α-helical segments (Fig. 1A). Human Nox1 of 564 amino acid

residues contains four potential *N*-glycosylation sites within the consensus sequon Asn-X-Ser/Thr (X, any amino acid except proline) [24]: amino acid positions at 63, 97, 162, and 236. Among them, Asn-63 localizes in the second transmembrane segment and Asn-97 is present in the first intracellular loop. On the other hand, Asn-162 and Asn-236 exists in the second and third extracellular loops, respectively (Fig. 1B), suggesting that these two asparagine residues possibly undergo *N*-linked glycosylation, which occurs solely on the extracellular side [24].

To test the possibility of Nox1 *N*-glycosylation, we used CHO cells, in which oxidase subunits are not expressed [12,15,25] but Nox2, ectopically expressed together with $p22^{phox}$, undergoes glycosylation and subsequent maturation [25]. In these cells, we first confirmed the presence of Nox2 with high mannose *N*-glycans, which can be digested with PNGase F or Endo H [26], and demonstrated that $p22^{phox}$ induces Nox2 conversion into the form with complex *N*-glycans, susceptible to PNGase F but resistant to Endo H [26] (Fig. 2A). In addition, *N*-glycosylation and $p22^{phox}$-induced maturation of Nox3 were also observed in CHO cells (Fig. 2B), as previously demonstrated in HEK293 cells [18]. Under the same conditions, Nox1 was expressed as a protein with a molecular mass of ~60 kDa, which was shifted to ~55 kDa by treatment with PNGase F or Endo H (Fig. 2C). These findings indicate that Nox1 is expressed mainly as a protein carrying high mannose *N*-glycans in CHO cells. Coexpression of $p22^{phox}$ markedly increased the amount of Nox1 proteins with a higher molecular mass, which were digested with PNGase F to the ~55 kDa protein, but resistant to treatment with Endo H (Fig. 2C). Thus, in the presence of $p22^{phox}$, Nox1 *N*-glycans appear to be efficiently modified to complex oligosaccharides, which modification is known to occur in the Golgi apparatus [26]. Similar Nox1 *N*-glycosylation and its $p22^{phox}$-dependent maturation were observed in H661 cells (Fig. 2D), which do not express endogenous $p22^{phox}$ [27]. As expected, in endogenously $p22^{phox}$-expressing cells such as HeLa and Caco-2 cells [14], Nox1 *N*-glycan maturation occurred without transfection with $p22^{phox}$ cDNA (Fig. 2E and F).

3.2. Nox1 glycosylation at Asn-162 and Asn-236

To determine glycosylated residues in Nox1, we expressed mutant proteins with threonine substitution for the putative *N*-glycosylation sites Asn-162 and Asn-236 (Fig. 1). As shown in Fig. 3A, apparent molecular masses of Nox1 (N162T) and Nox1 (N236T) were slightly lower than that of wild-type Nox1 with a

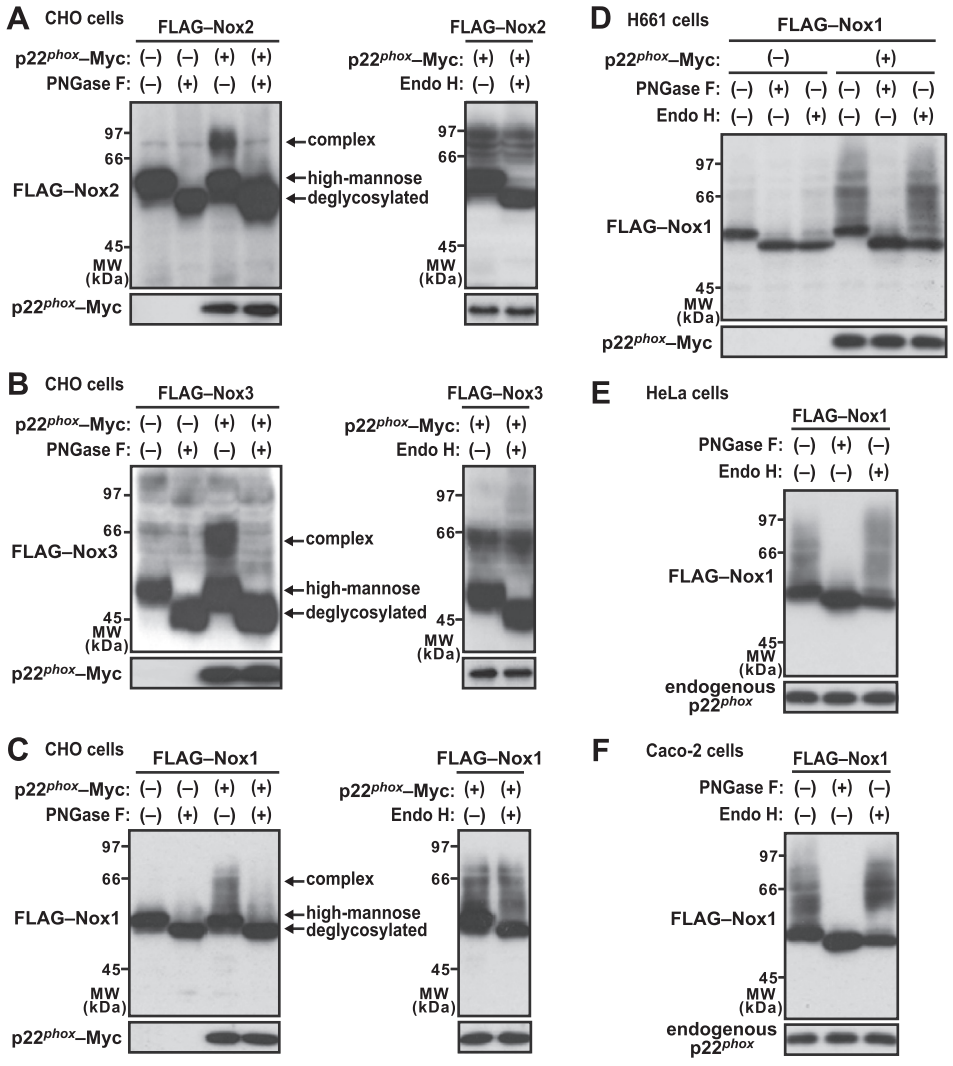

Fig. 2. *N*-glycosylation of human Nox1, Nox2, and Nox3. The lysate from the following cells was extracted with Triton X-100, and digested with PNGase F or Endo H: CHO cells expressing FLAG–Nox2 (A), FLAG–Nox3 (B), or FLAG–Nox1 (C) with or without $p22^{phox}$–Myc; H661 cells expressing FLAG–Nox1 with or without $p22^{phox}$–Myc (D); or HeLa (E) or Caco-2 (F) cells expressing FLAG–Nox1. Proteins were analyzed by immunoblot with the anti-FALG or anti-Myc antibody. Positions for marker proteins are indicated in kDa.

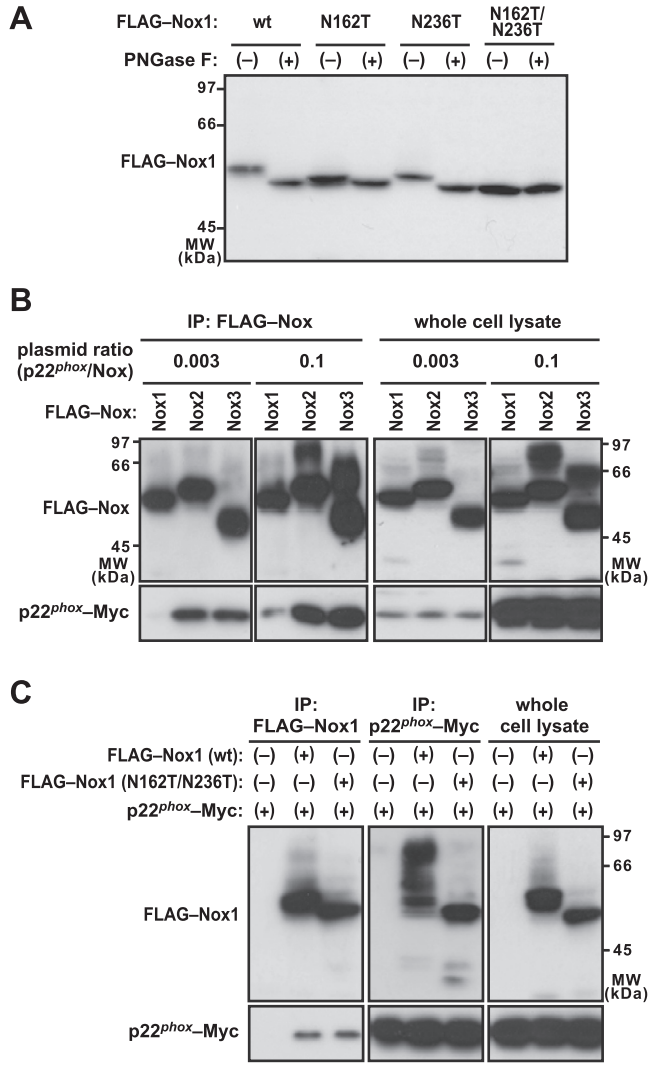

Fig. 3. Nox1 glycosylation and its interaction with p22^{phox}. (A) Glycosylation of Nox1 at Asn-162 and Asn-236. CHO cells were transfected with pEF-BOS-FLAG–Nox1 (wt), pEF-BOS-FLAG–Nox1 (N162T), pEF-BOS-FLAG–Nox1 (N236T), or pEF-BOS-FLAG–Nox1 (N162T/N236T). Proteins in the cell lysate were extracted with Triton X-100, digested with PNGase F, and subjected to SDS–PAGE, followed by immunoblot analysis with the anti-FLAG antibody. (B and C) Nox1 interaction with p22^{phox}. CHO cells were transfected with the following plasmids: pEF-BOS-p22^{phox}–Myc and pEF-BOS encoding a FLAG-tagged Nox protein at the indicated ratio (B); pEF-BOS-p22^{phox}–Myc and pEF-BOS-FLAG–Nox1 (wt) or pEF-BOS-FLAG–Nox1 (N162T/N236T) (C). Proteins in the cell lysate were immunoprecipitated (IP) with the anti-FLAG antibody or anti-Myc antibody, followed by immunoblot analysis with the indicated antibody. Positions for marker proteins are indicated in kDa.

molecular mass of to ~60 kDa. Digestion of these mutant proteins with PNGase F resulted in a shift to ~55 kDa, which was the same as that of PNGase F-treated wild-type Nox1. A mutant protein carrying double substitution for Asn-162 and Asn-236 had an apparent molecular mass of ~55 kDa, which was not changed after treatment with PNGase F (Fig. 3A). These findings indicate that Asn-162 and Asn-236 are the major *N*-glycosylation sites in Nox1.

3.3. N-linked glycosylation of Nox1 and its interaction with p22^{phox}

Coexpression of p22^{phox} induces *N*-glycan maturation of Nox1 but to a much lesser extent than that of Nox2 and Nox3 (Fig. 2), suggesting that Nox1 exhibits a lower affinity for p22^{phox}. To address this issue, we expressed FLAG-tagged Nox proteins and p22^{phox}–Myc in CHO cells, and performed immunoprecipitation experiments. As shown in Fig. 3B, Nox1 precipitated p22^{phox} much less efficiently than Nox2 and Nox3, indicative of a lower affinity of Nox1 for p22^{phox}. We next tested the role for Nox1 glycosylation in its heterodimer formation with p22^{phox}. Because p22^{phox} was co-precipitated with Nox1 (N162T/N236T) as well as wild-type Nox1 (Fig. 3C), Nox1 likely forms a heterodimer with p22^{phox} in a glycosylation-independent manner. When p22^{phox}–Myc was precipitated with the anti-Myc antibody, co-precipitated FLAG–Nox1 proteins were mainly in the mature form with complex *N*-glycans (Fig. 3C), suggesting that p22^{phox}-complexed Nox1 is immediately converted into the mature form. On the other hand, consistent with the finding that only a small portion of Nox1 dimerizes with p22^{phox} (Fig. 3B), the majority of FLAG–Nox1 precipitated with the anti-FLAG antibody was in the immature form with high mannose *N*-glycans (Fig. 3C).

3.4. Cell surface recruitment of Nox1

Nox2 with high mannose *N*-glycans is known to be converted in the Golgi apparatus to the mature form, which is transported to the plasma membrane [1,2]. Consistent with this, in CHO cells, Nox2 was present on the cell surface as an Endo H-resistant form (with complex *N*-glycans) but not as an Endo H-sensitive form (with high mannose glycans), which was indicated by labeling of cell surface proteins with Sulfo-NHS-SS-biotin, a membrane-impermeable biotinylating agent, and subsequent precipitation with streptoavidin-conjugated beads (Fig. 4A). As expected, Nox1 with complex *N*-glycans was targeted to the cell surface (Fig. 4B). Although Nox1 was expressed mainly as a form with high mannose *N*-glycans in the absence of p22^{phox} (Fig. 2C), this form was also efficiently recruited to the cell surface (Fig. 4B), which is in contrast to the inability of high mannose *N*-glycan-harboring Nox2 to reach the plasma membrane. Thus *N*-glycan maturation is likely dispensable for cell surface recruitment of Nox1. Furthermore, even unglycosylated Nox1 (N162T/N236T) was effectively transported to the cell surface (Fig. 4B), indicating that glycosylation by itself is not required for Nox1 transport.

3.5. Role for glycosylation in superoxide-producing activity of Nox1

To investigate the role for glycosylation in superoxide-producing activity of Nox1, we expressed wild-type Nox1 or Nox1 (N162T/N236T) together with the Nox1-activating proteins Noxo1 and Noxa1. As shown in Fig. 4C, unglycosylated Nox1 (N162T/N236T) was capable of fully producing superoxide, indicating that glycosylation is dispensable for catalytic function. It is known that superoxide production by wild-type Nox1 is enhanced by expression of wild-type p22^{phox} but not p22^{phox} (P156Q), defective in binding to Noxo1 [14,16]. p22^{phox} (P156Q) also failed to support superoxide production by Nox1 (N162T/N236T), although this mutant protein was efficiently targeted to the cell surface (Fig. 4C). Thus p22^{phox} likely plays a crucial role in Nox1 activation by interacting with Noxo1 in a glycosylation-independent manner.

4. Discussion

In the present study, we demonstrate that human Nox1 undergoes glycosylation at Asn-162 and Asn-236 in the second and third extracellular loops, respectively (Fig. 3). Nox1 proteins in other species from fishes to mammals also contain putative *N*-glycosylation sites in these extracellular regions (Fig. 1). *N*-linked glycans in Nox1 are not essential for heterodimer formation with p22^{phox} (Fig. 3), which is similar to glycosylation-independent heterodimerization of Nox2 with p22^{phox} [9,11]. The vast majority of Nox2 on the plasma membrane contains complex *N*-glycans

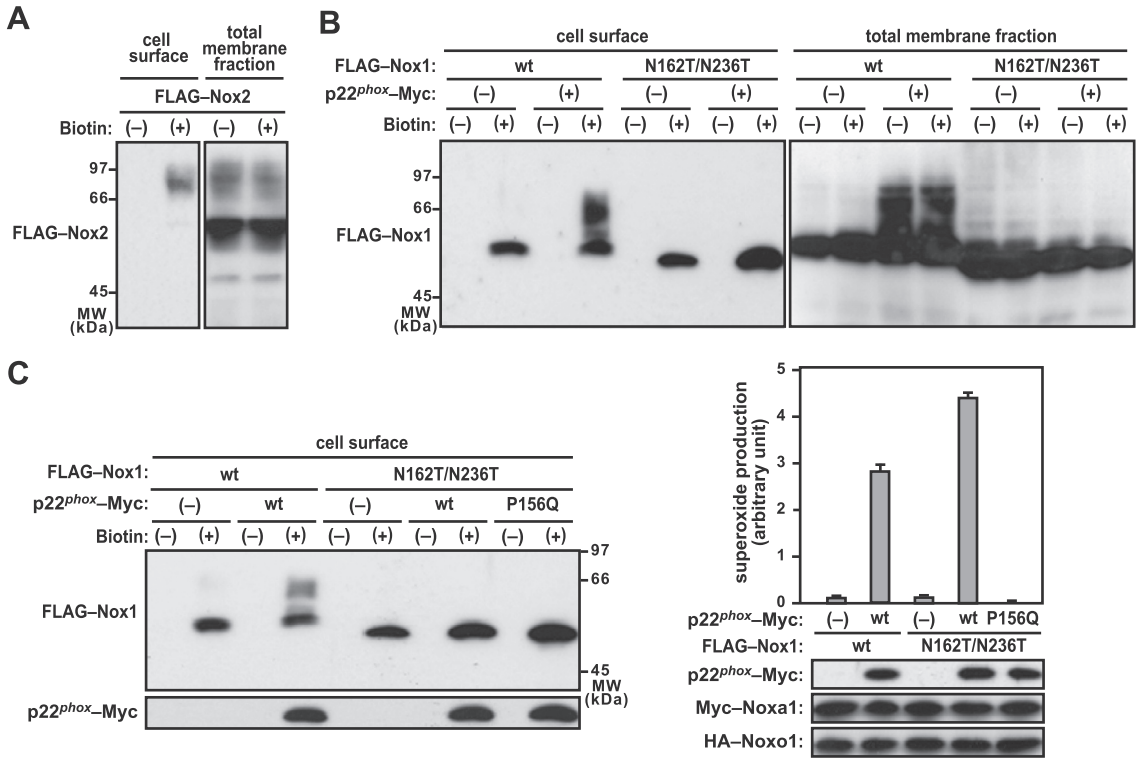

Fig. 4. Role for glycosylation in cell surface recruitment and superoxide-producing activity of Nox1. (A and B) Cell surface recruitment of Nox1 and Nox2. Proteins in the membrane fraction of the following cells were labeled by cell surface biotinylation, followed by precipitation with streptavidin-conjugated beads: CHO cells expressing FLAG–Nox2 (wt) and p22^{phox}–Myc (A); or CHO cells expressing FLAG–Nox1 (wt) or FLAG–Nox1 (N162T/N236T) with or without p22^{phox}–Myc (B). Precipitants were analyzed by immunoblot with the anti-FLAG antibody. (C) Superoxide production by Nox1. CHO cells were transfected with pEF-BOS-p22^{phox}–Myc, pEF-BOS-HA–Noxo1, pEF-BOS-Myc–Noxa1, and pEF-BOS-FLAG–Nox1 (wt) or pEF-BOS-FLAG–Nox1 (N162T/N236T). (*Left panel*) FLAG–Nox1 (wt) and pEF-BOS-FLAG–Nox1 (N162T/N236T), expressed on the cell surface, were analyzed as described in B. (*Right panels*) Transfected cells were incubated for 5 min at 37 °C, and chemiluminescence change was continuously monitored with DIOGENES. Each graph represents the mean ± S.D. of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections. Protein levels of p22^{phox}, Noxa1, and Noxo1 were analyzed by immunoblot with the indicated antibody. Positions for marker proteins are indicated in kDa.

(Fig. 4), suggesting the significance of Nox2 maturation in its cell surface recruitment. In contrast, both the immature form (with high mannose *N*-glycan) and the unglycosylated form of Nox1 are efficiently transported to the cell surface (Fig. 4), indicating that Nox1 *N*-glycosylation is dispensable for its targeting to the plasma membrane. Since the unglycosylated Nox1 is capable of fully producing superoxide (Fig. 4), we conclude that catalytic function of Nox1 does not require glycosylation. It has been reported that Nox2 *N*-glycans are not required for superoxide-producing activity: an *in vitro* activity of the phagocyte oxidase (the Nox2–p22^{phox} complex) is resistant to enzymatic deglycosylation [28].

Although Duox2 is expressed in the thyroid as two forms with either high mannose or complex *N*-glycans [19], Duox2, ectopically expressed in non-thyroid cells, exists solely in the ER as the immature form with high mannose *N*-glycans [19,20]. Coexpression of DuoxA2, a membrane-spanning Duox2 partner that is structurally unrelated to p22^{phox}, allows Duox2 maturation and recruitment to the plasma membrane [20,29]. Similar to DuoxA2, p22^{phox} plays an essential role in transport of immature Nox2 (gp65) from the ER to the Golgi apparatus for conversion into gp91^{phox} (the mature form with complex *N*-glycans); the maturation enables cell surface recruitment of the Nox2–p22^{phox} complex [8–11]. p22^{phox} is also necessary for both proper maturation and plasma membrane recruitment of Nox3 [18]. Although p22^{phox} has an ability to mature *N*-glycans of Nox1 as well as Nox2 and Nox3 (Fig. 2), Nox1 is efficiently targeted to the cell surface even in the absence of p22^{phox} (Fig. 4). Thus p22^{phox}-mediated *N*-glycan maturation is likely required for cell surface recruitment of Nox2 and Nox3 [8–11,18] but not for that of Nox1 (Fig. 4). The molecular basis for Nox1 difference from Nox2 and Nox3 remains unknown at present. This may be related to the current finding that Nox1 interacts with p22^{phox} to a much lower extent than Nox2 and Nox3 (Fig. 3). It is tempting to postulate that Nox1 affinity for p22^{phox} has decreased during evolution after Nox1 obtained an ability to translocate to the plasma membrane without p22^{phox}-dependent glycan maturation. Some specific residues might be involved in Nox1 independence from p22^{phox}: a lysine in the first intracellular loop (Lys-91 in human Nox1) is evolutionarily well conserved in Nox1, but replaced with arginine in Nox2 and Nox3; the invariant glycine in the second extracellular loop of Nox2 and Nox3 (Gly-142 in human Nox2 and Gly-143 in human Nox3) is substituted with a residue other than glycine in Nox1.

The present study shows that, although p22^{phox}-associated Nox1 preferentially undergoes *N*-glycan maturation (Fig. 3), the maturation is not necessary for catalytic function of Nox1 (Fig. 4). Nevertheless, p22^{phox} appears to play a crucial role in Nox1-catalyzed superoxide production by functioning as an anchoring site for Noxo1, a protein required for Nox1 activation [12,14]. Indeed, whereas wild-type p22^{phox} facilitates superoxide production by unglycosylated Nox1 (N162T/N236T) as well as by the wild-type protein, a mutant p22^{phox} (P156Q), defective in binding to Noxo1, fails to support activation of Nox1 (N162T/N236T) (Fig. 4). Thus p22^{phox} likely regulates Nox1 activity in a glycosylation-independent manner.

Because high mannose *N*-glycans on proteins are modified to complex structures in the Golgi apparatus [26], the modification of *N*-glycans on p22^{phox}-complexed Nox1 is expected to occur in

the Golgi apparatus, followed by transport to the plasma membrane. On the other hand, Nox1 can be efficiently recruited to the cell surface as the form with high mannose *N*-glycans and even as the unglycosylated form, irrespective of the dimerization with $p22^{phox}$ (Fig. 4). It is presently unknown whether these forms of Nox1 are transported via the Golgi apparatus. Interestingly, several membrane-integrated glycoproteins are delivered from the ER to the plasma membrane without passing through the Golgi apparatus [30,31]. Future studies should address the question how high mannose *N*-glycan-harboring or unglycosylated Nox1 traffics to the cell surface.

Acknowledgments

We thank Yohko Kage (Kyushu University) and Namiko Kubo (Kyushu University) for technical assistance, and Minako Nishino (Kyushu University) for secretarial assistance. This work was supported in part by Grants-in-Aid for Scientific Research and Targeted Proteins Research Program (TPRP) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

[1] J.D. Lambeth, NOX enzymes and the biology of reactive oxygen, Nat. Rev. Immunol. 4 (2004) 181–189.
[2] H. Sumimoto, Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species, FEBS J. 275 (2008) 3249–3277.
[3] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, Physiol. Rev. 87 (2007) 245–313.
[4] M.C. Dinauer, S.H. Orkin, R. Brown, et al., The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome *b* complex, Nature 327 (1987) 717–720.
[5] C.A. Parkos, R.A. Allen, C.G. Cochrane, A.J. Jesaitis, Purified cytochrome *b* from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000, J. Clin. Invest. 80 (1987) 732–742.
[6] T.M. Wallach, A.W. Segal, Analysis of glycosylation sites on gp91*phox*, the flavocytochrome of the NADPH oxidase, by site-directed mutagenesis and translation *in vitro*, Biochem. J. 321 (1997) 583–585.
[7] R.M. Taylor, D. Baniulis, J.B. Burritt, et al., Analysis of human phagocyte flavocytochrome b_{558} by mass spectrometry, J. Biol. Chem. 281 (2006) 37045–37056.
[8] C.D. Porter, M.H. Parkar, A.J. Verhoeven, p22-*phox*-deficient chronic granulomatous disease: reconstitution by retrovirus-mediated expression and identification of a biosynthetic intermediate of gp91-*phox*, Blood 84 (1994) 2767–2775.
[9] L. Yu, F.R. DeLeo, K.J. Biberstine-Kinkade, et al., Biosynthesis of flavocytochrome b_{558}: $gp91^{phox}$ is synthesized as a 65-kDa precursor (p65) in the endoplasmic reticulum, J. Biol. Chem. 274 (1999) 4364–4369.
[10] L. Yu, L. Zhen, M.C. Dinauer, Biosynthesis of the phagocyte NADPH oxidase cytochrome b_{558}: role of heme incorporation and heterodimer formation in maturation and stability of $gp91^{phox}$ and $p22^{phox}$ subunits, J. Biol. Chem. 272 (1997) 27288–27294.
[11] F.R. DeLeo, J.B. Burritt, L. Yu, et al., Processing and maturation of flavocytochrome b_{558} include incorporation of heme as a prerequisite for heterodimer assembly, J. Biol. Chem. 275 (2000) 13986–13993.
[12] R. Takeya, N. Ueno, K. Kami, et al., Novel human homologues of $p47^{phox}$ and $p67^{phox}$ participate in activation of superoxide-producing NADPH oxidases, J. Biol. Chem. 278 (2003) 25234–25246.
[13] R.K. Ambasta, P. Kumar, K.K. Griendling, et al., 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.
[14] K. Miyano, N. Ueno, R. Takeya, et al., Direct involvement of the small GTPase Rac in activation of the superoxide-producing NADPH oxidase Nox1, J. Biol. Chem. 281 (2006) 21857–21868.
[15] N. Ueno, R. Takeya, K. Miyano, et al., The NADPH oxidase Nox3 constitutively produces superoxide in a $p22^{phox}$-dependent manner: its regulation by oxidase organizers and activators, J. Biol. Chem. 280 (2005) 23328–23339.
[16] T. Kawahara, D. Ritsick, G. Cheng, J.D. Lambeth, Point mutations in the proline-rich region of $p22^{phox}$ are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation, J. Biol. Chem. 280 (2005) 31859–31869.
[17] T. Ueyama, M. Geiszt, T.L. Leto, Involvement of Rac1 in activation of multicomponent Nox1- and Nox3-based NADPH oxidases, Mol. Cell. Biol. 26 (2006) 2160–2174.
[18] Y. Nakano, B. Bánfi, A.J. Jesaitis, et al., Critical roles for p22phox in the structural maturation and subcellular targeting of Nox3, Biochem. J. 403 (2007) 97–108.
[19] X. De Deken, D. Wang, J.E. Dumont, F. Miot, Characterization of ThOX protein as components of the thyroid H_2O_2-generating system, Exp. Cell Res. 273 (2002) 187–196.
[20] H. Grasberger, S. Refetoff, Identification of the maturation factor for dual oxidase: evolution of an eukaryotic operon equivalent, J. Biol. Chem. 281 (2006) 18269–18272.
[21] K. Miyano, H. Sumimoto, Assessment of the role for Rho family GTPases in NADPH oxidase activation, Methods Mol. Biol. 827 (2012) 195–212.
[22] Y. Maehara, K. Miyano, S. Yuzawa, et al., A conserved region between the TPR and activation domains of $p67^{phox}$ participates in activation of the phagocyte NADPH oxidase, J. Biol. Chem. 285 (2010) 31435–31445.
[23] A. Yamamoto, R. Takeya, M. Matsumoto, et al., Phosphorylation of Noxo1 at threonine 341 regulates its interaction with Noxa1 and the superoxide-producing activity of Nox1, FEBS J. 280 (2013) 5145–5159.
[24] M. Aebi, N-linked protein glycosylation in the ER, Biochim. Biophys. Acta 2013 (1833) 2430–2437.
[25] Y. Zhu, C.C. Marchal, A.-J. Casbon, et al., Deletion mutagenesis of $p22^{phox}$ subunit of flavocytochrome b_{558}: identification of regions critical for $gp91^{phox}$ maturation and NADPH oxidase activity, J. Biol. Chem. 281 (2006) 30336–30346.
[26] P. Stanley, Golgi glycosylation, Cold Spring Harb. Perspect. Biol. 3 (2011) a005199.
[27] K. von Löhneysen, D. Noack, A.J. Jesaitis, et al., Mutational analysis reveals distinct features of the Nox4-$p22^{phox}$ complex, J. Biol. Chem. 283 (2008) 35273–35282.
[28] M.-H. Paclet, A.W. Coleman, S. Vergnaud, F. Morel, P67-phox-mediated NADPH oxidase assembly: imaging of cytochrome b_{558} liposomes by atomic force microscopy, Biochemistry 39 (2000) 9302–9310.
[29] S. Morand, T. Ueyama, S. Tsujibe, et al., Duox maturation factors form cell surface complexes with Duox affecting the specificity of reactive oxygen species generation, FASEB J. 23 (2009) 1205–1218.
[30] A.G. Grieve, C. Rabouille, Golgi bypass: skirting around the heart of classical secretion, Cold Spring Harb. Perspect. Biol. 3 (2011) a005298.
[31] C. Rabouille, V. Malhotra, W. Nickel, Diversity in unconventional protein secretion, J. Cell Sci. 125 (2012). 5251–5225.