ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 6, 2009

DOI: 10.1089/ars.2008.2383

Forum Original Research Communication

Identification of Structural Elements in Nox1 and Nox4 Controlling Localization and Activity

Ina Helmcke,[1] Sabine Heumüller,[1] Ritva Tikkanen,[2] Katrin Schröder,[1] and Ralf P. Brandes[1]

Abstract

Nox NADPH oxidases differ in their mode of activation, subcellular localization, and physiological function. Nox1 releases superoxide anions (O_2^-) and depends on cytosolic activator proteins, whereas Nox4 extracellularly releases hydrogen peroxide (H_2O_2), and its activity does not require cotransfection of additional proteins. We constructed chimeric proteins consisting of Nox1 and Nox4 expressed in HEK293 cells. When the cytosolic tail of Nox4 was fused with the transmembrane part of Nox1, Nox1 became constitutively active. The reciprocal construct was inactive, suggesting that cytosolic subunit–dependent activation requires elements in the transmembrane loops. By TIRF-microscopy, Nox1 was observed in the plasma membrane, whereas Nox4 colocalized with proteins of the endoplasmic reticulum. Fusion proteins of Myc and Nox revealed that the N-terminal part of Nox1 but not Nox4 is cleaved. When the potential signal peptide of Nox4 was inserted into Nox1, plasma-membrane localization was lost, and the protein was retained in vesicle-like structures below the plasma membrane. The potential signal peptide of Nox1 failed to translocate Nox4 to the plasma membrane but switched the extracellularly detectable ROS from H_2O_2 to O_2^-. Thus, the very N-terminal part of Nox proteins determines subcellular localization and the ROS type released, whereas the cytosolic tail regulates activity. *Antioxid. Redox Signal.* 11, 1279–1287.

Introduction

The Nox family of NADPH oxidases is a group of proteins that generate reactive oxygen species (ROS) by transferring an electron to molecular oxygen. The best-characterized NADPH oxidase is the phagocyte NADPH oxidase Nox2, which produces large amounts of superoxide anions (O_2^-) to eliminate invaded pathogens. In recent years, a number of homologues of the phagocyte NADPH oxidase were identified (Nox1, Nox3, Nox4, Nox5, Duox1, and Duox2), the functions of which comprise cellular signalling, regulation of gene expression, cell differentiation, and posttranslational modifications of proteins (5).

All Nox family members are transmembrane proteins with six transmembrane domains, conserved binding sites for FAD and NADPH, and four heme-binding histidines in the third and fifth transmembrane domain. The mode of activation, however, differs between the Nox homologues. The phagocyte NADPH oxidase (Nox2) becomes active after assembly with the cytosolic subunits $p47^{phox}$ and $p67^{phox}$, a process that requires phosphorylation and protein–protein interactions (24). In the case of the homologue Nox1, the cytosolic subunits are replaced by the homologues NoxA1 and NoxO1 (4). The activity of Nox4, in contrast, does not require additional cytosolic subunits and is therefore considered constitutive (2, 13).

The structure of the individual Nox proteins is certainly the key to the different molecular functions. The homology between Nox2, Nox1, and Nox4 is 58% and 37%, respectively. The homology between Nox1 and Nox4 is 36%. High similarity exists regarding the localization and sequence of the transmembrane domains, the heme-binding histidine, the FAD and FMN, as well as the NADPH-binding site. Thus, the overall structures of Nox1, Nox2, and Nox4 are similar, and obvious differences are not apparent.

The intracellular localization of Nox proteins has gained considerable attention, and the mechanisms targeting the proteins to the different compartments have not been elucidated in detail.

[1]Institut für Kardiovaskuläre Physiologie, and [2]Institute of Biochemistry II, Goethe-Universität, Frankfurt am Main, Germany.

The Nox2 isoform of phagocytes is in part located at the plasma membranes, but substantial amounts of the proteins are retained in intracellular granules and endosomes (7). Conflicting observations exist regarding the localization of Nox1: In keratinocytes, nuclear staining was shown (8). In vascular smooth muscle cells, localization to the endoplasmic reticulum (ER) (17), the plasma membrane, and vesicle-like structures (15) and to caveolin-enriched fractions (16) was reported. Overexpression experiments also suggested localization in the plasma membrane (10).

Even more uncertainties exist regarding the subcellular localization of Nox4. In endothelial cells (18) and smooth muscle cells (16), Nox4 was reported to be expressed in the nucleus; in vascular smooth muscle cells, localization to focal adhesion points was reported (16). In transfected cells, however, Nox4 was localized in the ER (20). It is uncertain whether these contradicting findings are a consequence of so-far-undefined adaptor proteins changing the localization of Nox4 or of potential problems arising in conjunction with the overexpression of a membrane protein *per se*. It also is possible that the localization of Nox4 changes with the functional state of the cell. In vascular smooth muscle cells, Nox4 relocates from focal adhesions to stress fibers during differentiation (11).

The structural similarities between Nox1, Nox2, and Nox4 would suggest that all three proteins would primarily produce O_2^-, which can further dismutate to H_2O_2. So far, no robust cell-free assay for Nox1 and Nox4 has been developed, and thus, uncertainty still exists regarding the type of ROS particularly produced by Nox4: Almost consistently, overexpression studies revealed only H_2O_2 but not O_2^- production by Nox4 (27). The potentially different subcellular localization of the Nox proteins could serve to explain this somewhat unexpected observation. As Nox4 in such a scenario releases O_2^- to the lumen of the ER, O_2^- would be inaccessible to most assay systems for this radical. Because of its negative charge, O_2^- cannot pass the plasma membrane, but dismutates to H_2O_2, which is freely diffusible and could potentially be measured outside the cell.

In this work, we uncovered some of the molecular differences between Nox1 and Nox4 that might lead to the diverse modes of activation and ROS type released. By exchanging different sequences between Nox enzymes, we generated chimeric proteins and studied them regarding activity and intracellular localization.

Material and Methods

Generation and transfection of fusion constructs

Plasmids encoding human full-length Nox4 and Nox1 were kindly provided by T. Leto (NIH, Bethesda, MD). The plasmids coding for mouse NoxA1 and NoxO1 were generous gifts of B. Banfi (Iowa University, Iowa City, IA).

The plasmids coding for the different chimeric Nox1/Nox4-proteins and the N-terminal Myc-tagged Nox proteins were generated by overlap-extension PCR. All cloned plasmids were confirmed with DNA sequencing.

Transient transfection of HEK293 cells (ATCC, Manassas, VA) was performed by using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions. HEK293 cells stably expressing Nox proteins were generated by using lentiviral infection or transfection with linearized plasmids with Lipofectamine 2000 and subsequent antibiotic selection.

Determination of reactive oxygen species generation

HEK293 cells were seeded on 3.5-cm dishes and transiently transfected with human Nox1, Nox4, or a chimeric Nox1/Nox4-plasmid and mouse NoxA1 and NoxO1, as indicated, and measurements were performed 24 h later. EDTA-detached cells were suspended in 500 μl of HEPES-modified Tyrode solution containing the appropriate chemiluminescence enhancer. As enhancer for hydrogen peroxide (H_2O_2), Luminol (Sigma, St. Louis, MO; 100 μM) and horseradish peroxidase (HRP; Sigma; 1 U/ml) were used. As enhancer for superoxide anions (O_2^-), L-012 (WAKO Chemicals, Richmond, VA; 200 μM) was used.

Western blot analysis

At 24 h after transfection, cells were incubated for 8 h with the proteasome inhibitor MG132 (Calbiochem, Darmstadt, Germany; 10 μM) to stabilize and increase the Nox expression. Then the cells were lysed in SDS sample buffer containing TCEP (Pierce, Rockford, IL) (94 mM Tris/HCl pH 6.8; 12.75% glycerol; 3% SDS; 30 mM DTT; 0.003% bromphenol blue; 50 mM TCEP; 1 mM EDTA; 1 mM EGTA; 500 U/ml DNase I) for detection with the Nox1 antibody or in Triton lysis buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl (sodium chloride); 10 mM $NaPP_i$ (sodium pyrophosphate); 20 mM NaF (sodium fluoride); 1% Triton X-100; 12 μl/ml proteinase inhibitor mix; 0.23 mM phenylmethylsulfonyl fluoride) for detection with the Nox4 antibody.

Proteins were separated with SDS-PAGE and after Western Blot were detected by using appropriate primary antibodies and infrared-fluorescent-labeled secondary antibodies for infrared-based fluorimetric detection with the Odyssey system (Licor, Bad Homburg, Germany). The primary antibody against Nox1 was obtained from Santa Cruz (Mox-1 H-15, Lot G2707; Santa Cruz, CA); the primary antibody against Nox4 was kindly provided by J. D. Lambeth (Emory University, Atlanta, GA). By densitometry, the intensity of the signals of the different Nox proteins was normalized against the signal of β-actin (primary antibody from Sigma).

Confocal and total internal reflection fluorescence microscopy

HEK293 cells stably expressing the different constructs were seeded on μ-Dishes (ibidi, Martinsried, Germany) or on round glass coverslips (24 mm). When the cells reached $\sim$80% confluence, they were incubated for 8 h with the translation inhibitor Anisomycin (Calbiochem; 20 μM) to reduce potential localization of the proteins to the ER in the course of *de novo* synthesis. Then the cells were fixed in phosphate-buffered paraformaldehyde (Sigma) and permeabilized with 0.05% Triton X-100 (Applichem, Darmstadt, Germany). Immunodetection of the Nox proteins was carried out with the same primary antibodies used for Western blot analysis; cell structures were stained by using primary antibody against Pan-Cadherin (Abcam, Cambridge, UK) and GRP78 (Santa Cruz). DNA was stained with DAPI (Sigma). As secondary

Table 1. Nox1-Nox4 Chimeras

No. construct [a]	*Activity*[b]	*Detection in WB*	*Site of switch*
1 **Nox1**(1-206)–**Nox4**(193-578)	−	+	Before fifth TMD
2 **Nox4**(1-192)–**Nox1**(207-564)	−	+++	Before fifth TMD
3 **Nox1**(1-120)–**Nox4**(125-578)	+	+++	After third TMD
4 **Nox4**(1-124)–**Nox1**(121-564)	−	+	After third TMD
5 **Nox1**(1-120)–**Nox4**(125-192)–**Nox1**(207-564)	−	+++	After third TMD and before fifth TMD
6 **Nox4**(1-124)–**Nox1**(121-206)–**Nox4**(193-578)	−	++	After third TMD and before fifth TMD
7 **Nox1**(1-340)–**Nox4**(355-578)	−	++	In FAD binding site
8 **Nox4**(1-354)–**Nox1**(341-564)	−	++	In FAD binding site
9 **Nox1**(1-289)–**Nox4**(304-578)	+	++	After sixth TMD
10 **Nox4**(1-303)–**Nox1**(290-564)	−	+	After sixth TMD
11 **Nox1**(1-289)–**Nox4**(304-354)–**Nox1**(341-564)	−	++	After sixth TMD and before FAD binding site
12 **Nox4**(1-303)–**Nox1**(290-340)–**Nox4**(355-578)	−	++	After sixth TMD and before FAD binding site
13 **Nox1**(1-360)–**Nox4**(377-424)–**Nox1**(397-564)	+	++	After FAD binding site and before NADPH binding site
14 **Nox4**(1-376)–**Nox1**(361-396)–**Nox4**(425-578)	−	+	After FAD binding site and before NADPH binding site
15 **Nox1**(1-29)–**Nox4**(36-578)	+	++	After predicted signal peptide
16 **Nox4**(1-35)–**Nox1**(30-564)	+	++	After predicted signal peptide

[a]Numbers in brackets indicate the amino acids of the individual Nox protein included in the chimera.
[b]Activity and protein expression as detected by Western blot analysis (WB) are provided on a semiquantitative basis (using ±) to allow an easy assessment of the activity of the constructs.

antibodies, appropriate Alexa-labeled antibodies (Molecular Probes, Eugene, OR) were used.

Confocal microscopy was carried out by using the Zeiss LSM 510 Meta confocal microscope. TIRF microscopy was performed on Olympus cell microscope with cell tool TIRFM.

Isolation of membrane proteins

Subcellular fractionation was performed by using the PromoKine Membrane Protein Extraction Kit (Promocell, Heidelberg, Germany) according to manufacturer's instructions. Subcellular fractions were loaded on an SDS polyacrylamide gel and, after separation, blotted onto nitrocellulose membrane. After blocking in 5% milk, fractions were verified by detection of specific proteins: Na^+/K^+-ATPase [monoclonal antibody α6F, Hybridoma facility, University of Iowa, Iowa City, IA (30)] for plasma membrane and GRP78 (Santa Cruz) or Calnexin (Chemicon, Billerica, MA) for the ER in the fraction of the remaining cell membranes.

Statistical analysis

All values expressed as the mean ± SEM. Statistical analysis was carried out by using ANOVA followed by least significant difference (LSD)-*post hoc* testing. Densitometry was performed by using the Odyssey software. A p value of <0.05 was considered statistically significant.

Results

Construction of chimeric Nox proteins

Chimeric proteins consisting of different parts of Nox1 and Nox4 were generated to analyze the putative function of these parts (Table 1). Most of the newly generated proteins were inactive, although all constructs were at least weakly expressed, indicating that the different parts of Nox1 and Nox4 are not freely exchangeable without affecting activity, although the overall structure should remain unaffected by our approach.

Function of the cytosolic tail

Construct 9 Nox1(1-289)–Nox4(304-578) was among the few chimeric proteins that showed activity. This construct consists of the transmembrane part of Nox1 and the cytosolic tail of Nox4 (Fig. 1). This chimeric protein, when transfected into HEK293 cells, produced ROS spontaneously, and co-transfection of the cytosolic subunits NoxA1 and NoxO1 failed to increase ROS formation further (Fig. 2). This shows that the constitutive activity of Nox4 is located in the

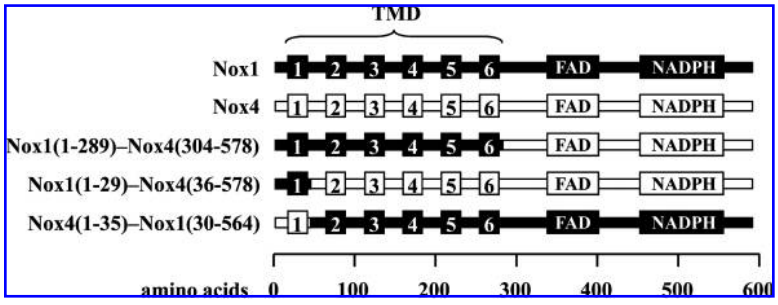

FIG. 1. Schematic illustration of native and chimeric Nox proteins. Boxes represent transmembrane domains (TMD 1-6), FAD-binding site (FAD), and NADPH-binding site (NADPH). *Black boxes*, Nox1; *white boxes*, Nox4. Illustrations are not to scale.

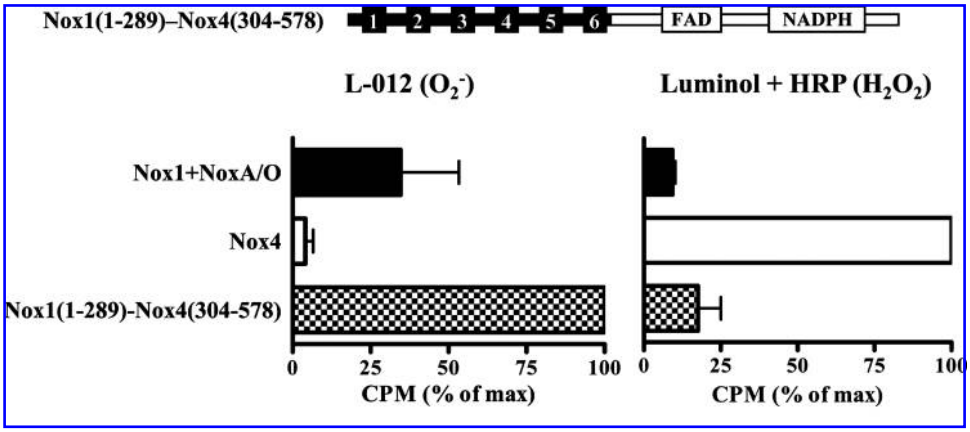

FIG. 2. Function of the cytosolic tail. Determination of the ROS production of HEK293 cells transiently transfected with the plasmids indicated. O_2^- generation was determined with L-012 (*left*); H_2O_2 formation was determined with Luminol+HRP chemiluminescence (*right*). ROS production was normalized against the amount of Nox protein expressed. To allow better comparison of the constructs, the ROS formation of the most active construct is set to 100%. $n \geq 3$, mean ± SEM.

cytosolic tail and that the tail is sufficient to confer constitutive activity to Nox proteins. Obviously the whole tail is required for this property, because chimeric Nox proteins with shorter Nox4-derived C-terminal fragments showed no activity in ROS production (Table 1, No. 7). Construct 3, containing the N-terminal part of Nox1 and the last three transmembrane domains, as well as the cytosolic tail of Nox4, showed activity similar to that of Nox1(1-289)–Nox4(304-578) (No. 9).

The chimeric protein with the opposite combination, Nox4 (1-303)–Nox1(290-564) (Table 1, No. 10), was not active and could not be activated by cytosolic subunits (data not shown), indicating that for the activation of Nox1, more than the cytosolic tail is required, and that either the cytosolic subunits or the cytosolic tail has to interact with specific regions in the membrane part of Nox1, which have not yet been identified.

Chemiluminescence was used to estimate the type of ROS produced: L-012 served as enhancer for O_2^-, whereas Luminol+HRP was used specifically to detect H_2O_2. By using such an approach, we observed that Nox1-transfected cells, when NoxA1 and NoxO1 were cotransfected, produced larger amounts of O_2^- than H_2O_2 (34.7 ± 18.7% *vs.* 9.3 ± 0.9%; $n = 3$), whereas Nox4-transfected cells produced much higher amounts of H_2O_2 than O_2^- (100% *vs.* 4.1 ± 2.4%; $n = 3$) (Fig. 2). The chimeric Nox protein Nox1(1-289)–Nox4(304-578) also produced larger amounts of O_2^- than H_2O_2 (100% *vs.* 17.6 ± 7.4%; $n = 3$). These results indicate that the N-terminal part of the Nox proteins influences whether O_2^- or H_2O_2 is released to the extracellular space.

Intracellular localization of Nox1 and Nox4

The difference in the type of ROS released to the extracellular space might be the consequence of different intracellular localizations of the Nox proteins. With confocal microscopy, Nox1 was detected in the plasma membrane and found to colocalize with pan-cadherin. Nox4, in contrast, colocalized with the heat-shock protein GRP78, suggesting localization in the ER (Fig. 3A, left panels). These observations were confirmed with TIRF microscopy, in which the cell membrane is visualized selectively (Fig. 3A, right panels). A TIRF signal was obtained only in cells expressing Nox1 but not in cells expressing Nox4.

Subcellular fractionation of HEK293 cells stably expressing Nox1, Nox4, or GFP with subsequent Western blot analysis demonstrated the presence of Nox1 in the plasma-membrane fraction but also in the remaining cellular membranes, whereas Nox4 was completely absent from the plasma-membrane fraction and was present only in the fraction of the remaining cell membranes (Fig. 3B).

Intracellular localization of N-terminal Myc-tagged Nox1 and Nox4

To understand the basis of the different localization of the two proteins, we hypothesized that Nox1 may contain a signalling peptide, responsible for membrane translocation. To address this aspect, N-terminal Myc-tagged constructs were cloned. As expected, the Myc tag prevented the translocation of Nox1 to the plasma membrane. Most important, we could not observe any co-staining of Nox1 and the Myc tag, indicating that the Myc tag is processed or cleaved together with the N-terminus of Nox1 (Fig. 4, left panel). Western blot analysis confirmed the absence of the Myc tag on the vast majority of the Nox1 proteins expressed (data not shown). In contrast, colocalization of Myc and Nox4 was found, suggesting that the processing of the N-terminal part is specific for Nox1 (Fig. 4, right panel).

Role of the predicted signal peptide

Further to investigate the role of the predicted signal peptides, we exchanged them between Nox1 and Nox4 (Table 1, No. 15 and 16; Fig. 1). Measurements of the radical production of the chimeric proteins showed that Nox1(1-29)–Nox4(36-578), similar to Nox1(1-289)–Nox4(304-578), was active without cytosolic subunits (Fig. 5, upper panel). Most important, this construct produced larger amounts of O_2^- than H_2O_2 (104 ± 34.7% *vs.* 2.3 ± 1.1%; $n = 3$); thus, although it was almost exclusively derived from Nox4, including the catalytic sites, it produced the radical type generated by Nox1.

Nox4(1-35)–Nox1(30-564), after cotransfection of NoxA1 and NoxO1, also showed activity and produced larger amounts of O_2^- than H_2O_2 (16.8 ± 1.0% *vs.* 0.4 ± 0.2%; $n = 3$) (Fig. 5, lower panel). This observation demonstrates that the very N-terminal part controls whether Nox4 generates O_2^- or H_2O_2, but that the N-terminal part of Nox4 is insufficient to switch Nox1 from an O_2^-- to an H_2O_2-producing enzyme.

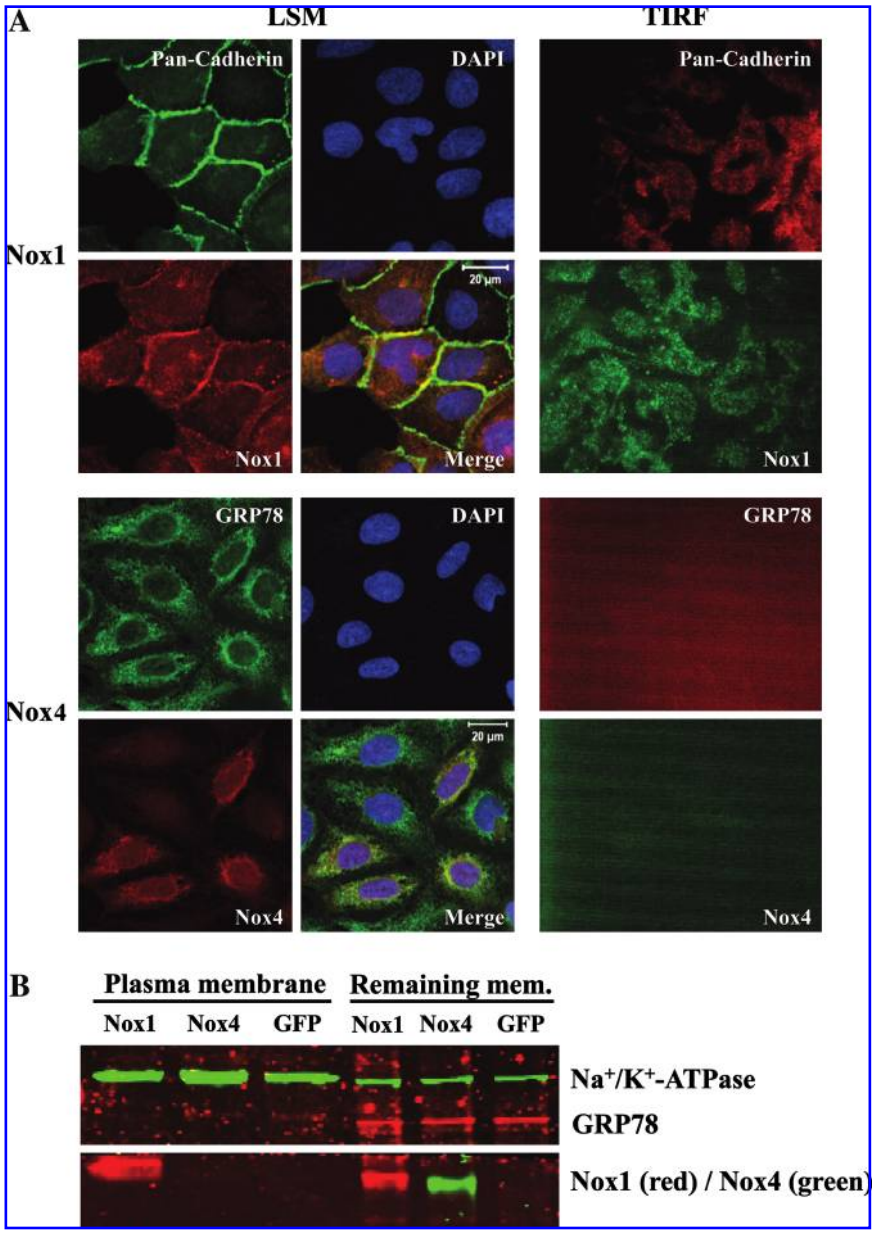

FIG. 3. Intracellular localization of Nox1 and Nox4. HEK293 cells were stably transfected with Nox1, Nox4, or GFP by using a lentiviral system. For microscopy, cells were fixed, and plasma membrane and endoplasmic reticulum were stained by using antibodies directed against pan-cadherin and GRP78, respectively. Nuclei were stained with DAPI. Pictures were taken with confocal microscopy (**A,** *left panel*) or with TIRF microscopy (**A,** *right panel*). Subcellular fractions of the cells were analyzed with Western blot (**B**). Na^+/K^+-ATPase and GRP78 were used as markers for plasma membrane and endoplasmic reticulum, respectively. Images shown are representative of at least four identical experiments. "Remaining mem," the membrane fraction not containing plasma membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Intracellular localization of Nox proteins with switched signal peptides

We speculate that the N-terminal part may change the extracellular ROS release by affecting the subcellular localization. To our surprise, Nox1(1-29)–Nox4(36-578), however, was not detected in the plasma membrane, produced no TIRF signal, and showed co-staining with GRP78 as full-length Nox4, clearly suggesting that ER localization does not preclude O_2^- formation. Nox4(1-35)–Nox1(30-564) was localized in vesicle-like structures in the submembrane space (Fig. 6A). Western blot analysis of the subcellular fractions of cells expressing the chimeric proteins confirmed that Nox1(1-29)–Nox4(36-578) is found predominantly in the fraction of the remaining cell membranes but not in the plasma membrane. Nox4(1-35)–Nox1(30-564) is found only in the fraction of the remaining cell membranes as well, in contrast to full-length Nox1, which is also present in the plasma-membrane fraction (Fig. 6B). These data indicate that the correct intracellular translocation of Nox1 is disturbed when the predicted signal peptide is replaced by the one of Nox4, but it also demonstrates that additional factors determine localization of the Nox proteins.

Discussion

By using a chimeric-protein approach, the present work aimed to identify mechanisms underlying the different localization, activation, and ROS production of Nox1 and Nox4. We demonstrated that the constitutive activity of Nox4 is mediated by the cytosolic tail. The whole tail is required; chimeric Nox proteins with shorter portions of the cytosolic tail of Nox4 did not produce any ROS. For the activation of Nox1 by assembly with the cytosolic subunits NoxA1 and NoxO1, the cytosolic tail of Nox1 is not the only site of interaction, and probably further interactions with intracellular

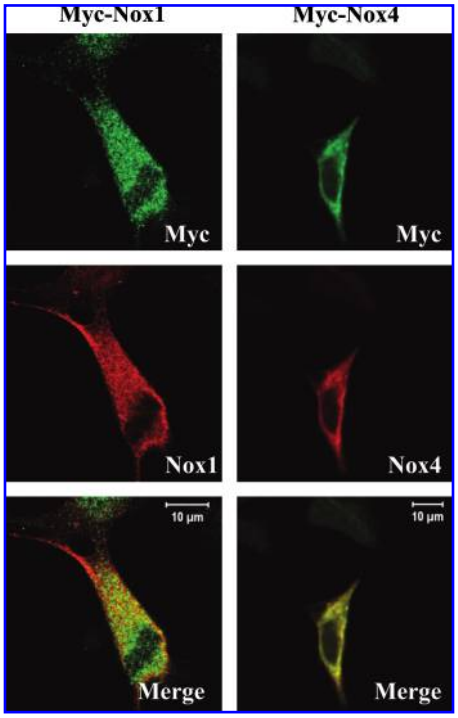

FIG. 4. Intracellular localization of N-terminal Myc-tagged Nox1 and Nox4. HEK293 cells transiently transfected with Myc-Nox1 or Myc-Nox4 were stained with antibodies directed against the Nox proteins and the Myc tag, as indicated. Pictures were taken with confocal microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

loops of the transmembranous parts of Nox1 have to occur. It is likely that the assembly of Nox1 with the cytosolic subunits occurs in a way similar to that in Nox2.

Not only are the cytosolic subunits for Nox1 and Nox2 somewhat homologous (14), but they can even functionally replace each other to some extent (3, 29). For Nox2, it was shown that the first intracellular loop encompassing amino acids 86 to 100 is a potential site of interaction with $p47^{phox}$ (12, 25). Especially the arginines at positions 91 and 92 are essential for Nox2 activity (6). These arginines are conserved between Nox2 and Nox4; in Nox1, the second one is replaced by a lysine, which is also a basic residue. Hence the amino acid sequence is slightly affected in the chimeric construct, and the overall structure may be changed so that correct assembly with cytosolic subunits is not possible.

Furthermore, investigation of the second intracellular loop, also called D-loop (amino acids 191 to 200 in Nox2) by site-directed mutagenesis showed that the residues at position 195 (lysine), 198 (arginine), and 199 (arginine) seem to be essential for Nox2 activity, in particular for the electron transfer from FAD to oxygen (19). The residue 198 is conserved between Nox1, 2, and 4, but residues 195 and 199 are not. However, replacement of the D-loop of Nox2 with the one of Nox1 or Nox4 did not affect functionality. But again, the overall structure may be changed in the chimeric protein. This event might also be an explanation for the large number of chimeric proteins constructed that did not exhibit activity. The second extracellular loop of the Nox proteins differs in length between Nox1 (46 amino acids) and Nox4 (28 amino acids).

In contrast, the third extracellular loop in Nox 4 contains 20 amino acids more than that in Nox1. Because the heme-binding histidines lie in the third and fifth transmem-

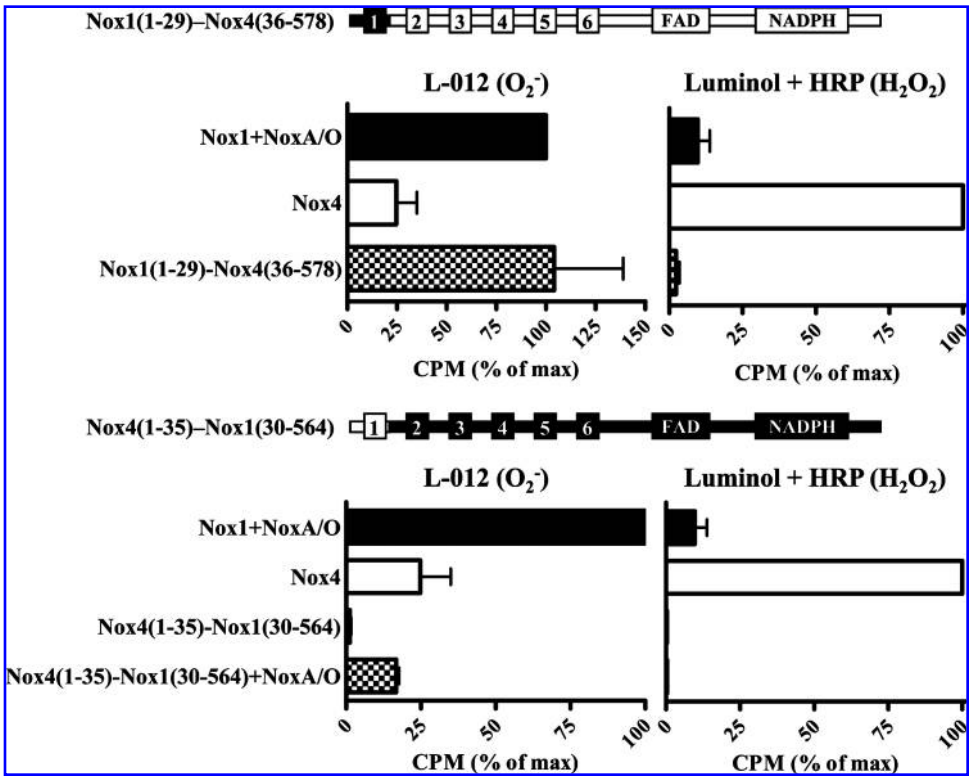

FIG. 5. Role of the predicted signal peptide of Nox proteins. Determination of the ROS production of HEK293 cells transiently transfected with Nox1(1-29)–Nox4(36-578) (*upper panel*) or Nox4 (1-35)–Nox1(30-564) (*lower panel*). O_2^- generation was determined with L-012 chemiluminescence (*left panels*); H_2O_2 formation was determined with Luminol+HRP (*right panels*). ROS production was normalized against the amount of Nox protein expressed. To allow better comparison of the constructs, the ROS formation of the most active construct is set to 100%. $n \geq 3$, mean ± SEM.

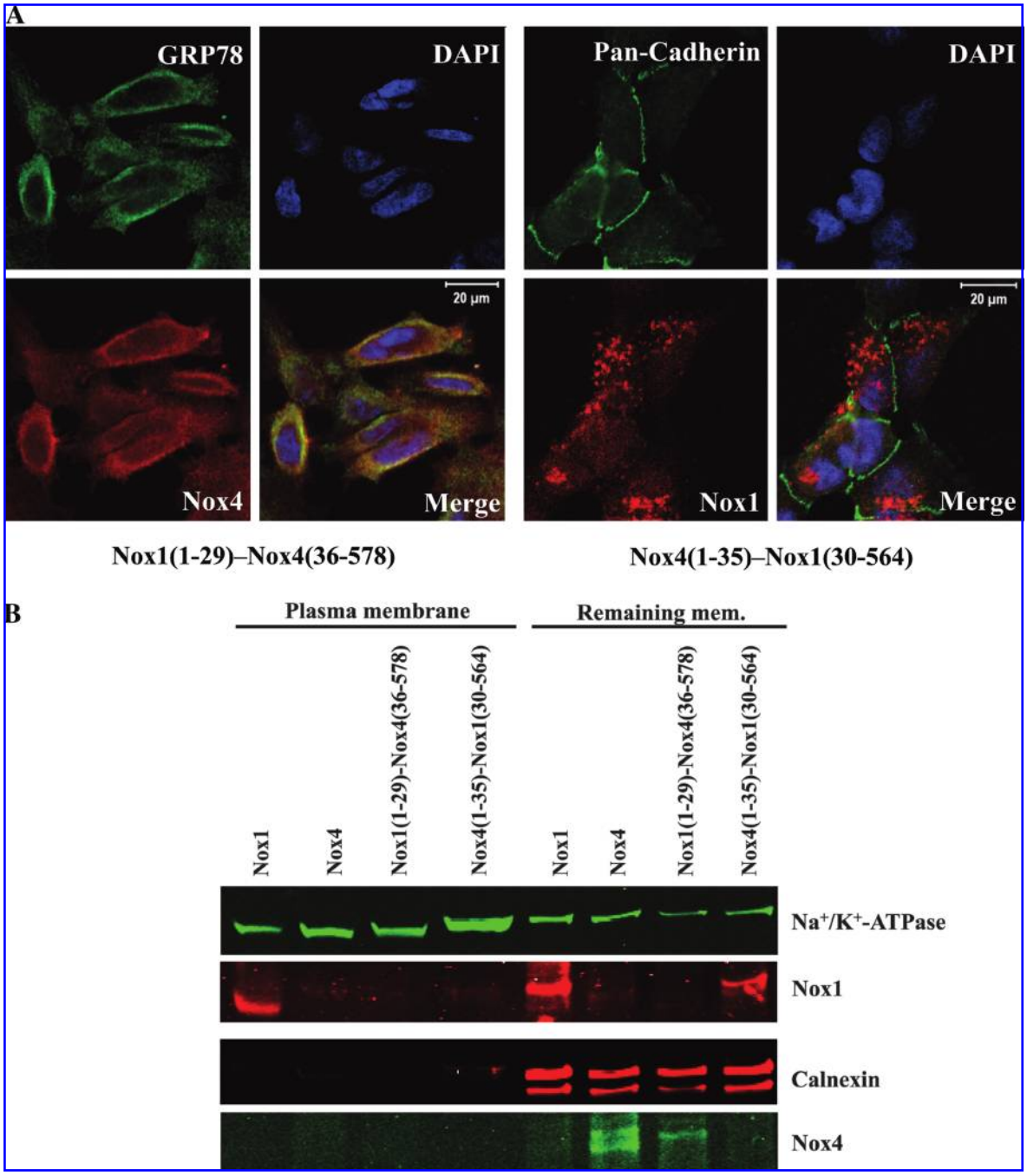

FIG. 6. Intracellular localization of Nox proteins with switched signal peptides. HEK293 cells stably transfected with Nox1(1-29)–Nox4(36-578) or Nox4(1-35)–Nox1(30-564) were stained with the appropriate Nox antibodies and with antibodies against pan-cadherin and GRP78 as markers for plasma membrane and endoplasmic reticulum, respectively. Nuclei were stained with DAPI. Pictures were taken with confocal microscopy (**A**). Subcellular fractions of the cells were analyzed with Western blot (**B**). Na^+/K^+-ATPase and calnexin were used as markers for plasma membrane and endoplasmic reticulum, respectively. Images shown are representative of at least four identical experiments. "Remaining mem," the membrane fraction not containing plasma membranes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

brane domains, the differences in the sequence length may lead to improper distances of the histidines in the chimeric proteins.

We hypothesized that the different types of ROS released by Nox1- and Nox4-transfected cells may be due to the distinct subcellular localization. We demonstrated by immunofluorescence microscopy and by subcellular fractionation of stably transfected cells that Nox1 is located in the plasma membrane and that Nox4 resides in the ER and is totally absent from the plasma membrane.

Proteins that are synthesized *via* the secretory pathway, including membrane proteins, contain a signal peptide at the N-terminus. Usually this signal peptide is cleaved during the translation of the protein, but it can also serve as a transmembrane domain (26). Both Nox1 and Nox4 possess such a predicted signal peptide. Our observations, however, suggest that the N-terminus of Nox1 is processed, in contrast to Nox4. It is important to realize that such transmembrane proteins, which are synthesized in the ER, are usually translocated to the plasma membrane unless they contain an ER-retention signal or other signals such as an endosomal translocation signal. Such an ER-retention signal can be located either at the N-terminus (double arginine motif within the first five amino acids) or at their C-terminus (double lysine motif in −3 and −4/−5 position) (31). Nox4, however, contains none of these signals.

Examples exist of other proteins that are located in the ER without one of the known signals or even after mutational deletion of the signal, indicating that more mechanisms retain a protein in the membrane of the ER. For example, the cytochrome P450 is an integral membrane protein of the ER lacking any classic signal. All members of this family have a characteristic hydrophobic amino-terminal region of 20 to 25 amino acids, which is thought to function as both a signal-recognition particle-dependent ER-membrane insertion and a halt-transfer signal. When the N-terminal 29 amino acids of P450 2C1 are fused to soluble reporter proteins, these proteins are localized in the ER (1). Additionally, P450 2C1 and 2C2 contain a second ER-retention signal in the cytoplasmic domain, which is able to relocate plasma-membrane proteins to the ER. This signal is probably not encoded by a single primary sequence but rather may be the result of a specific three-dimensional structure of the folded protein (28). Another ER-membrane protein, UDP-glucuronosyltransferase, remains located in the membrane when the dilysine motif at the C-terminal end is deleted, suggesting that the signals for ER residency are independent of this motif (21).

Additionally, arginine-based ER-sorting motifs play a role in the assembly of heteromultimeric membrane proteins (22). These motifs with the core-sequence RXR can occur anywhere in the cytosolic domain of the protein and are not restricted to the N- or C-terminal end.

It is likely that the predicted signal peptide of Nox4, including the first transmembrane domain, serves as a localization signal to the ER as fusion of the N-terminal 35 amino acids of Nox4 to catalase leads to localization of the usually peroxisomal protein to the ER (9). However, in our study, fusion of the predicted signal peptide of Nox4 to Nox1 did not lead to localization of the protein to the ER, but normal plasma-membrane localization of Nox1 was hindered. Furthermore, when the signal peptide of Nox4 was replaced by the signal peptide of Nox1, the protein still showed an ER-like staining pattern. These results suggest that several signals together lead to the proper intracellular localization of Nox proteins. The type of ROS released by cells transfected with this chimeric protein, however, was changed for Nox4. This observation is remarkable, as it suggests that Nox4, even when retained in the ER, can produce detectable amounts of O_2^-.

Although we can only speculate about potential explanations, the N-terminal part of Nox4 might potentially dock the protein to a so-far-unidentified O_2^- acceptor like a thioloxidoreductase. Another explanation might be that the first transmembrane domain of Nox4 also has an enzymatic function like a superoxide dismutase, which is, however, unlikely, given the length and structure of this element and the fact that this amino acid stretch failed to convert Nox1 into an H_2O_2-generating enzyme. When the potential signal peptide of Nox1 was replaced by that of Nox4, the protein still produced O_2^-, but to a much lesser extent. Nevertheless, the subcellular localization was altered, as the protein did not show plasma-membrane localization any more. Instead, it was retained in submembrane vesicle-like structures. It was shown in smooth muscle cells that Nox1 generates ROS in early endosomes after extracellular activation by cytokines (23). Potentially, the Nox4 signal peptide leads to a fast turnover of the protein and subsequent submembranal retention.

In summary, we showed that the cytosolic part of Nox4 is responsible for the constitutive activity. The very N-terminal parts of the proteins might be involved in subcellular localization or, through an unknown mechanism, affect the type of ROS released.

Acknowledgments

This study was supported by grants from the Deutsche Forschungsgemeinschaft to R.P.B. and the excellence cluster cardiopulmonary system (ECCPS), the Schauffler-Stiftung (K.S.), the Fachbereich Medizin of the Goethe-University (K.S.), and by the European Vascular Genomic Network, a Network of Excellence supported by the European Community's sixth Framework Program (Contract LSHM-CT-2003-503254).

Abbreviations

ER, endoplasmic reticulum; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; Nox, NADPH oxidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TIRF, total internal reflection fluorescence.

Disclosure Statement

The authors have no competing financial interests to disclose, despite the support provided by the previously mentioned funding organizations.

References

1. Ahn K, Szczesna-Skorupa E, and Kemper B. The amino-terminal 29 amino acids of cytochrome P450 2C1 are sufficient for retention in the endoplasmic reticulum. *J Biol Chem* 268: 18726–18733, 1993.
2. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, and Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem* 279: 45935–45941, 2004.
3. Ambasta RK, Schreiber JG, Janiszewski M, Busse R, and Brandes RP. Noxa1 is a central component of the smooth muscle NADPH oxidase in mice. *Free Radic Biol Med* 41: 193–201, 2006.
4. Banfi B, Clark RA, Steger K, and Krause KH. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *J Biol Chem* 278: 3510–3513, 2003.

5. Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245–313, 2007.
6. Biberstine-Kinkade KJ, Yu L, and Dinauer MC. Mutagenesis of an arginine- and lysine-rich domain in the gp91(phox) subunit of the phagocyte NADPH-oxidase flavocytochrome b558. *J Biol Chem* 274: 10451–10457, 1999.
7. Borregaard N, Heiple JM, Simons ER, and Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* 97: 52–61, 1983.
8. Chamulitrat W, Schmidt R, Tomakidi P, Stremmel W, Chunglok W, Kawahara T, and Rokutan K. Association of gp91phox homolog Nox1 with anchorage-independent growth and MAP kinase-activation of transformed human keratinocytes. *Oncogene* 22: 6045–6053, 2003.
9. Chen K, Kirber MT, Xiao H, Yang Y, and Keaney JF Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol* 181: 1129–1139, 2008.
10. Cheng G and Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J Biol Chem* 279: 4737–4742, 2004.
11. Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, Schmidt HH, Lassegue B, and Griendling KK. Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol* 27: 42–48, 2007.
12. DeLeo FR, Yu L, Burritt JB, Loetterle LR, Bond CW, Jesaitis AJ, and Quinn MT. Mapping sites of interaction of p47-phox and flavocytochrome b with random-sequence peptide phage display libraries. *Proc Natl Acad Sci U S A* 92: 7110–7114, 1995.
13. Geiszt M, Kopp JB, Varnai P, and Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97: 8010–8014, 2000.
14. Geiszt M, Lekstrom K, Witta J, and Leto TL. Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *J Biol Chem* 278: 20006–20012, 2003.
15. Hanna IR, Hilenski LL, Dikalova A, Taniyama Y, Dikalov S, Lyle A, Quinn MT, Lassegue B, and Griendling KK. Functional association of nox1 with p22phox in vascular smooth muscle cells. *Free Radic Biol Med* 37: 1542–1549, 2004.
16. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, and Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 24: 677–683, 2004.
17. Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CX, and Laurindo FR. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. *J Biol Chem* 280: 40813–40819, 2005.
18. Kuroda J, Nakagawa K, Yamasaki T, Nakamura K, Takeya R, Kuribayashi F, Imajoh-Ohmi S, Igarashi K, Shibata Y, Sueishi K, and Sumimoto H. The superoxide-producing NAD(P)H oxidase Nox4 in the nucleus of human vascular endothelial cells. *Genes Cells* 10: 1139–1151, 2005.
19. Li XJ, Grunwald D, Mathieu J, Morel F, and Stasia MJ. Crucial role of two potential cytosolic regions of Nox2, 191TSSTKTIRRS200 and 484DESQANHFAVHHDEEKD500, on NADPH oxidase activation. *J Biol Chem* 280: 14962–14973, 2005.
20. Martyn KD, Frederick LM, von LK, Dinauer MC, and Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* 18: 69–82, 2006.
21. Meech R and Mackenzie PI. Determinants of UDP glucuronosyltransferase membrane association and residency in the endoplasmic reticulum. *Arch Biochem Biophys* 356: 77–85, 1998.
22. Michelsen K, Yuan H, and Schwappach B. Hide and run: arginine-based endoplasmic-reticulum-sorting motifs in the assembly of heteromultimeric membrane proteins. *EMBO Rep* 6: 717–722, 2005.
23. Miller FJ Jr, Filali M, Huss GJ, Stanic B, Chamseddine A, Barna TJ, and Lamb FS. Cytokine activation of nuclear factor kappa B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and ClC-3. *Circ Res* 101: 663–671, 2007.
24. Nauseef WM. Assembly of the phagocyte NADPH oxidase. *Histochem Cell Biol* 122: 277–291, 2004.
25. Park MY, Imajoh-Ohmi S, Nunoi H, and Kanegasaki S. Synthetic peptides corresponding to various hydrophilic regions of the large subunit of cytochrome b558 inhibit superoxide generation in a cell-free system from neutrophils. *Biochem Biophys Res Commun* 234: 531–536, 1997.
26. Schatz G and Dobberstein B. Common principles of protein translocation across membranes. *Science* 271: 1519–1526, 1996.
27. Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Forro L, Schlegel W, and Krause KH. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochem J* 406: 105–114, 2007.
28. Szczesna-Skorupa E, Ahn K, Chen CD, Doray B, and Kemper B. The cytoplasmic and N-terminal transmembrane domains of cytochrome P450 contain independent signals for retention in the endoplasmic reticulum. *J Biol Chem* 270: 24327–24333, 1995.
29. Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, and Sumimoto H. Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem* 278: 25234–25246, 2003.
30. Takeyasu K, Tamkun MM, Renaud KJ, and Fambrough DM. Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. *J Biol Chem* 263: 4347–4354, 1988.
31. Teasdale RD and Jackson MR. Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annu Rev Cell Dev Biol* 12: 27–54, 1996.

Address reprint requests to:
Ralf P. Brandes
Institut für Kardiovaskuläre Physiologie
Goethe-Universität
Theodor-Stern-Kai 7
60596 Frankfurt am Main, Germany

E-mail: r.brandes@em.uni-frankfurt.de

Date of first submission to ARS Central, November 27, 2008; date of acceptance, December 6, 2008.

This article has been cited by:

1. Sebastian Altenhöfer, Pamela W. M. Kleikers, Kim A. Radermacher, Peter Scheurer, J. J. Rob Hermans, Paul Schiffers, Heidi Ho, Kirstin Wingler, Harald H. H. W. Schmidt. 2012. The NOX toolbox: validating the role of NADPH oxidases in physiology and disease. *Cellular and Molecular Life Sciences* **69**:14, 2327-2343. [CrossRef]
2. Timo Kahles, Ralf P. Brandes. 2012. NADPH oxidases as therapeutic targets in ischemic stroke. *Cellular and Molecular Life Sciences* **69**:14, 2345-2363. [CrossRef]
3. Estela Bevilacqua, Sara Zago Gomes, Aline Rodrigues Lorenzon, Mara Sandra Hoshida, Andrea M. Amarante-Paffaro. 2012. NADPH oxidase as an important source of reactive oxygen species at the mouse maternal–fetal interface: putative biological roles. *Reproductive BioMedicine Online* **25**:1, 31-43. [CrossRef]
4. Francisco R.M. Laurindo, Luciana A. Pescatore, Denise de Castro Fernandes. 2012. Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radical Biology and Medicine* **52**:9, 1954-1969. [CrossRef]
5. Jin Qian, Feng Chen, Yevgeniy Kovalenkov, Deepesh Pandey, M. Arthur Moseley, Matthew W. Foster, Stephen M. Black, Richard C. Venema, David W. Stepp, David J.R. Fulton. 2012. Nitric oxide reduces NADPH oxidase 5 (Nox5) activity by reversible S-nitrosylation. *Free Radical Biology and Medicine* **52**:9, 1806-1819. [CrossRef]
6. Jinah Choi. 2012. Oxidative stress, endogenous antioxidants, alcohol, and hepatitis C: Pathogenic interactions and therapeutic considerations. *Free Radical Biology and Medicine* . [CrossRef]
7. Jennifer Streeter, William Thiel, Kate Brieger, Francis J. Miller Jr.. 2012. Opportunity Nox: The Future of NADPH Oxidases as Therapeutic Targets in Cardiovascular Disease. *Cardiovascular Therapeutics* no-no. [CrossRef]
8. Mathieu Da Silva, Grayson K. Jaggers, Sandra V. Verstraeten, Alejandra G. Erlejman, Cesar G. Fraga, Patricia I. Oteiza. 2012. Large procyanidins prevent bile-acid-induced oxidant production and membrane-initiated ERK1/2, p38, and Akt activation in Caco-2 cells. *Free Radical Biology and Medicine* **52**:1, 151-159. [CrossRef]
9. Kim A Radermacher, Kirstin Wingler, Pamela Kleikers, Sebastian Altenhöfer, Johannes JR Hermans, Christoph Kleinschnitz, Harald HHW Schmidt. 2012. The 1027th target candidate in stroke: Will NADPH oxidase hold up?. *Experimental & Translational Stroke Medicine* **4**:1, 11. [CrossRef]
10. Ina Takac, Katrin Schröder, Ralf P. Brandes. 2011. The Nox Family of NADPH Oxidases: Friend or Foe of the Vascular System?. *Current Hypertension Reports* . [CrossRef]
11. Alison C. Brewer, Thomas V.A. Murray, Matthew Arno, Min Zhang, Narayana P. Anilkumar, Giovanni E. Mann, Ajay M. Shah. 2011. Nox4 regulates Nrf2 and glutathione redox in cardiomyocytes in vivo. *Free Radical Biology and Medicine* **51**:1, 205-215. [CrossRef]
12. Feng Chen , Deepesh Pandey , Ahmed Chadli , John D. Catravas , Teng Chen , David J.R. Fulton . 2011. Hsp90 Regulates NADPH Oxidase Activity and Is Necessary for Superoxide but Not Hydrogen Peroxide Production. *Antioxidants & Redox Signaling* **14**:11, 2107-2119. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
13. Imad Al Ghouleh, Nicholas K.H. Khoo, Ulla G. Knaus, Kathy K. Griendling, Rhian M. Touyz, Victor J. Thannickal, Aaron Barchowsky, William M. Nauseef, Eric E. Kelley, Phillip M. Bauer, Victor Darley-Usmar, Sruti Shiva, Eugenia Cifuentes-Pagano, Bruce A. Freeman, Mark T. Gladwin, Patrick J. Pagano. 2011. Oxidases and peroxidases in cardiovascular and lung disease: New concepts in reactive oxygen species signaling. *Free Radical Biology and Medicine* . [CrossRef]
14. Celio X.C. Santos, Narayana Anilkumar, Min Zhang, Alison C. Brewer, Ajay M. Shah. 2011. Redox signaling in cardiac myocytes. *Free Radical Biology and Medicine* **50**:7, 777-793. [CrossRef]
15. Leilei Zhang, Minh Vu Chuong Nguyen, Bernard Lardy, Algirdas J. Jesaitis, Alexei Grichine, Francis Rousset, Monique Talbot, Marie-Hélène Paclet, GuanXiang Qian, Françoise Morel. 2011. New insight into the Nox4 subcellular localization in HEK293 cells: First monoclonal antibodies against Nox4. *Biochimie* **93**:3, 457-468. [CrossRef]
16. M. Zhang, A. C. Brewer, K. Schroder, C. X. C. Santos, D. J. Grieve, M. Wang, N. Anilkumar, B. Yu, X. Dong, S. J. Walker, R. P. Brandes, A. M. Shah. 2010. NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. *Proceedings of the National Academy of Sciences* **107**:42, 18121-18126. [CrossRef]
17. Andreas Petry , Michael Weitnauer , Agnes Görlach . 2010. Receptor Activation of NADPH Oxidases. *Antioxidants & Redox Signaling* **13**:4, 467-487. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
18. R.-M. Liu, K.A. Gaston Pravia. 2010. Oxidative stress and glutathione in TGF-#-mediated fibrogenesis. *Free Radical Biology and Medicine* **48**:1, 1-15. [CrossRef]

19. XiaoYong Tong, Katrin Schröder. 2009. NADPH oxidases are responsible for the failure of nitric oxide to inhibit migration of smooth muscle cells exposed to high glucose. *Free Radical Biology and Medicine* **47**:11, 1578-1583. [CrossRef]
20. David I. Brown, Kathy K. Griendling. 2009. Nox proteins in signal transduction. *Free Radical Biology and Medicine* **47**:9, 1239-1253. [CrossRef]
21. Thomas L. Leto , Stanislas Morand , Darrell Hurt , Takehiko Ueyama . 2009. Targeting and Regulation of Reactive Oxygen Species Generation by Nox Family NADPH Oxidases. *Antioxidants & Redox Signaling* **11**:10, 2607-2619. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
22. Silvia Sorce , Karl-Heinz Krause . 2009. NOX Enzymes in the Central Nervous System: From Signaling to Disease. *Antioxidants & Redox Signaling* **11**:10, 2481-2504. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]