Two Novel Proteins Activate Superoxide Generation by the NADPH Oxidase NOX1*

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NOX1, an NADPH oxidase expressed predominantly in colon epithelium, shows a high degree of similarity to the phagocyte NADPH oxidase. However, superoxide generation by NOX1 has been difficult to demonstrate. Here we show that NOX1 generates superoxide when co-expressed with the p47phox and p67phox subunits of the phagocyte NADPH oxidase but not when expressed by itself. Since p47phox and p67phox are restricted mainly to myeloid cells, we searched for their homologues and identified two novel cDNAs. The mRNAs of both homologues were found predominantly in colon epithelium. Differences between the homologues and the phagocyte NADPH oxidase subunits included the lack of the autoinhibitory domain and the protein kinase C phosphorylation sites in the p47phox homologue as well as the absence of the first Src homology 3 domain and the presence of a hydrophobic stretch in the p67phox homologue. Co-expression of NOX1 with the two novel proteins led to stimulus-independent high level superoxide generation. Stimulus dependence of NOX1 was restored when p47phox was used to replace its homologue. In conclusion, NOX1 is a superoxide-generating enzyme that is activated by two novel proteins, which we propose to name NOXO1 (NOX organizer 1) and NOXA1 (NOX activator 1).

Superoxide generation by phagocytes plays a crucial role in the elimination of invading microorganisms. It is catalyzed by the phagocyte NADPH oxidase, an enzyme consisting of two transmembrane subunits, p22phox and gp91phox, and at least three cytosolic subunits, p47phox, p67phox, and Rac2 (1). Upon activation, the NADPH oxidase subunits assemble, and electrons are transported from intracellular NADPH to extracellular oxygen by the flavo-heme gp91phox subunit (2).

Recently six gp91phox homologues have been described in mammals: NOX1 1 (3, 4), NOX3 (5, 6), and NOX4 (7, 8) with an overall structure similar to gp91phox (alias NOX2), NOX5 with an N-terminal EF hand-containing extension (9), and DUOX1 and DUOX2 with an additional peroxidase homology domain (10–12). NOX1 is found mainly in colon epithelium (3, 4); NOX3 in embryonic kidney (5, 6), NOX4 in the kidney cortex (7, 8), and DUOX5 in lymphoid organs and testis (9), DUOX1 in thyroid and lung, and DUOX2 in thyroid and colon (10–12).

Based on their primary structure all members of the NOX/DUOX family should be flavo-heme electron transporters. However, it is not established whether all NOX enzymes transfer electrons to oxygen or whether some of them may use other electron acceptors as has been shown for a yeast homologue of gp91phox that functions as a ferric reductase (13). Among NOX enzymes, only gp91phox and NOX5 have appeared capable of generating large amounts of superoxide, both of them in a stimulus-dependent manner (1, 9).

Based on data gained with NOX1-transfected NIH 3T3 cell clones NOX1 has been suggested to be a subunit-independent, low capacity superoxide-generating enzyme involved in the regulation of mitogenesis (4, 14). However, we have not been able to measure any superoxide generation in NOX1-transfected cells, and the question arose whether NOX1 is a subunit-dependent enzyme or possibly not a superoxide-generating enzyme at all.

EXPERIMENTAL PROCEDURES

Cloning of Mouse p47phox and p67phox Homologues—Translated BLAST nucleotide searches were conducted in the mouse genome data base (www.ensembl.org/Mus_musculus/blastview) with the mouse p47phox and p67phox sequences. The exons of the genes identified were predicted with the GENSCAN software (genes.mit.edu/GENSCAN.html), and primers were designed to amplify the coding regions of both predicted transcripts (5′-catggaacggcagccagcgg-3′ and 5′-ctcagccggagcagcctgagg-3′ for the p47phox homologue and 5′-catgagactgatagcag-3′ and 5′-tctgactttctgctgctcct-3′ for the p67phox homologue). Total RNA from mouse colon was purified with the TRIzol® reagent (Invitrogen), and cDNA was synthesized with Superscript® reverse transcriptase (Invitrogen) using random primers followed by PCR with Taq DNA polymerase using “Q buffer” (Qiagen).

Analysis of mRNA—Northern blot analysis and in situ hybridization were performed as described previously (9) using the entire coding region of NOX1 and NOX1A for probe generation in Northern blot experiments and using the region containing base pairs 1–360 of NOX1 and the region containing base pairs 171–490 of NOX1A cDNA for probe generation in the in situ hybridization experiments.

Cell Culture and Transfection—Mouse NOX1 cDNA, mouse NOX1A cDNA, and mouse NOX1A cDNA with inserted Kozak sequences were subcloned into pcDNA3.1 (Invitrogen). HEK293, Chinese hamster ovary, COS-7, and HeLa cells were cultured and transfected as described previously (9).

Superoxide Measurements—Luminol-enhanced chemiluminescence and SOD-inhibitable cytochrome c reduction were measured as described previously (9). For the microscopic NBT test, cells were seeded in 96-well plates and incubated at 37 °C for 15 min in Hanks’ balanced salt solution containing 0.5 mg/ml NBT.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF539799 (mouse NOX1), AF539797 (mouse NOX1), AF539798 (mouse NOX1A), and AF539796 (human NOX1).

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§ The abbreviations used are: NOX, NADPH oxidase; DPI, diphenylene iodonium; DUOX, dual domain oxidase; NBT, nitro blue tetrazolium; NOX1, 2, NOX activator 1, 2; NOX1A, -2, NOX organizer 1, 2; PMCA, phospholipid 1-myristate 1-acetate; SOD, superoxide dismutase; SH5, Src homology 3.

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RESULTS AND DISCUSSION

Activation of NOX1 by Cytoplasmic Subunits of the Phagocyte NADPH Oxidase—As NOX1 expression by itself did not lead to superoxide generation in transfected cells (Fig. 1), we considered that NOX1, like gp91phox, might be a subunit-dependent enzyme. We therefore co-transfected HEK293 cells with NOX1 and the cytoplasmic subunits of the phagocyte NADPH oxidase, p47phox and p67phox. Cells transfected with NOX1 alone (Fig. 1) or with p47phox and p67phox alone (not shown) did not generate superoxide either with or without stimulation by the phorbol ester PMA. In contrast, HEK293 cells shown) did not generate superoxide either with or without stimulation. These results suggested that NOX1 is a subunit-dependent enzyme. However, p47phox and p67phox generated superoxide but only after addition of PMA. These results suggested that NOX1 is a subunit-dependent enzyme. However, p47phox and p67phox generated superoxide but only after addition of PMA. These results suggested that NOX1 is a subunit-dependent enzyme. However, p47phox and p67phox are expressed mainly in myeloid cells. We therefore searched for homologues that may activate NOX1 in the colon.

Molecular Cloning of Putative NOX1 Cytoplasmic Subunits—A search for p47phox homologues yielded a full-length human sequence deposited as colon cancer antigen NY-CO-31 (GenBank™ accession number AAC18046). Subsequently, we found the open reading frame of the mouse p47phox homologue in chromosome 17 and cloned the cDNA of the coding region by reverse transcription PCR from mouse colon RNA (GenBank™ accession number AF539797). The homologue had an expected molecular mass of 39 kDa and displayed 25% sequence identity with p47phox (Fig. 2A). A search for motifs located a phox homology domain, which targets proteins to the phosphoinositide-tyrosine hydroxylase of membranes (15), two Src homology SH3 domains, and a C-terminal proline-rich region that is crucial in p47phox for interaction with p67phox (16) (Fig. 2A). Interestingly, the homologue was missing the proline-proline-arginine-arginine-containing region of p47phox, which is involved in autoinhibition (17) through binding to the N-terminal SH3 domain, as well as the adjacent serine phosphorylation sites (18), which relieve autoinhibition when phosphorylated (19). This suggested a stimulus-independent activity of the homologue.

A search of the mouse genome for p67phox homologues yielded an open reading frame on chromosome 2. Based on the genomic sequence we cloned the mouse p67phox homologue by reverse transcription PCR (GenBank™ accession number AF539798). The predicted protein had a molecular mass of 49 kDa and displayed 30% sequence identity with p67phox (Fig. 2B). A computer search for motifs revealed tetratricopeptide repeats, which are important for Rac binding in p67phox (Fig. 2B). The so-called activator domain of p67phox, which interacts with gp91phox, was also found in the homologue (20) and it conserved the C-terminal but not the N-terminal SH3 domain of p67phox. The p40phox-binding PB1 domain of p67phox was weakly conserved (22). In addition the homologue had a C-terminal hydrophobic stretch, which may represent a transmembrane tail (Fig. 2B).

After discussions with Dr. Ruth Lovering from the Human Genome Organization Nomenclature Committee and with colleagues working in the field, the novel factors have been named NOX1 (NOX organizer 1) and NOXA1 (NOX activator 1). NOX1 is the p47phox homologue, and NOXA1 is the p67phox homologue. The terms NOX02 and NOXA2 have been intro-
produced as aliases for p47phox and p67phox, respectively.

**NOXO1 and NOXA1 mRNAs Are Expressed Predominantly in Colon Epithelium**—We next investigated the tissue distribution of NOXO1 and NOXA1 by Northern blotting (Fig. 3A). The probe derived from NOXO1 labeled a ∼1.5-kb mRNA, while the NOX1 probe labeled a ∼1.7-kb mRNA. Interestingly, both mRNAs showed a relatively similar tissue distribution with a predominant expression in the colon. Low level expression was seen in uterus, small intestine, and stomach for both transcripts. Testis expressed NOXO1 but not NOXA1 (the increased length of the testis NOXO1 mRNA is due to differences in the non-coding regions, not shown). *In situ* hybridization with NOXO1 and NOXA1 antisense probes labeled colon epithelial cells strongly, while neither the NOXA1 sense probe (Fig. 3B, control) nor the NOXO1 sense probe (not shown) hybridized. Thus, NOXO1 and NOXA1 were expressed in the same tissue and same cell type as NOX1.

**NOXO1 and NOXA1 Enable Superoxide Generation by NOX1**—To investigate the function of NOXO1 and NOXA1, we transfected HEK293 cells with NOX1, NOXO1, and NOXA1, or with the empty expression vector (control). The cells transfected with all three constructs generated superoxide, while control-transfected cells did not (Fig. 4, A and B). Addition of a flavoprotein inhibitor, diphenylene iodonium (DPI), inhibited superoxide production (Fig. 4A). The block of NOX1 activity by DPI was slower than that observed for other NOX proteins. The half-time of DPI inhibition of superoxide generation was 239 ± 16 s in NOX1-transfected HEK293 cells, while it was 40 ± 1.5 s in NOX5-transfected HEK293 cells (not shown). This is, to our knowledge, the first clear indication of pharmacological variability among different NOX enzymes.

Individual transfection of NOX1, NOXO1, or NOXA1 did not lead to any detectable superoxide generation (Fig. 4B). Similarly, no superoxide generation was observed upon co-transfection of NOX1 with only one of the novel homologues or cotransfection of NOXO1 and NOXA1 without NOX1 (Fig. 4B).

The co-transfection of NOX1, NOXO1, and NOXA1 led to superoxide generation not only in HEK293 cells but also in Chinese hamster ovary, HeLa, and COS-7 cells (not shown), although we detected the highest activity in HEK293 cells.

Next we verified the NOX1-dependent superoxide generation using the SOD-inhibitable cytochrome *c* reduction assay. As shown in Fig. 4C, cells transfected with NOX1, NOXO1, and NOXA1 reduced cytochrome *c* in an SOD-inhibitable manner. The observed activity was continuous and did not require any stimulus. The rate of superoxide generation was 0.69 ± 0.04 nmol of superoxide/min/10⁷ cells.

Microscopic analysis by the NBT reduction assay showed that ~10% of the transiently NOX1 + NOXO1 + NOXA1-transfected cells but none of the control-transfected cells stained NBT-positive (Fig. 4D). We infer that the superoxide...
generation per cell expressing an active NADPH oxidase was −10 times higher than the mean value given above, or −7 nmol of superoxide/min/10^6 cells. This is in the range reported for phagocyte NADPH oxidase-expressing COS-7 cells (23), and NOX5-expressing HEK293 cells (9).

**Stimulus Independence of NOX1 Activation by Cytoplasmic Factors**—The superoxide generation by NOX1 + NOXO1 + NOXA1-transfected cells shown in Fig. 4 occurred in the absence of external stimuli. In contrast the phagocyte NADPH oxidase requires external stimuli even in reconstituted systems (23, 24). To investigate whether external stimuli are able to enhance the NOX1 respiratory burst, we treated the transfected cells with agents known to activate the phagocyte NADPH oxidase (1). However, none of the following treatments increased significantly the superoxide production compared with non-stimulated cells (100%): 100 nM PMA (84 ± 2%), 100 μM arachidonic acid (108 ± 2%), and 1 μM ionomycin (107 ± 18%).

**Cofactor Requirement for Stimulus-dependent and Stimulus-independent NOX1 Activation**—To understand further the activation of NOX1 by cofactors, we co-transfected NOX1 with the following combinations of cofactors: NOX1 + NOXO1 + p47phox + p67phox + NOXA1, and NOX1 + p67phox (Fig. 4E). Stimulus-independent activation of NOX1 occurred exclusively with the NOXO1 + NOXA1 combination. When NOX1 was replaced by p47phox, stimulus-independent activation disappeared, but the system could be activated by PMA. Under this condition, the superoxide generation was lower than that observed with NOX1 + NOX1 but similar to that observed with p47phox + p67phox. Only minimal NOX1 activity was detected with the combination of NOX1 + p67phox. These results suggested that the differences between p47phox and NOX1 are important for stimulus-dependent versus stimulus-independent NOX1 activity. This is substantiated by the different primary structures of the two proteins, i.e. the lack of the autoinhibitory region and the adjacent phosphorylation sites in NOX1. However, as the combination of NOX1 + p67phox did not display any stimulus-independent activation, some NOX1-specific element might also be involved in the stimulus-independent activity.

In summary, we have described two novel proteins, NOX1 and NOX1A, that support superoxide generation by NOX1. As is the case for NOX1, these proteins are expressed predominantly in colon epithelium and are thus likely to be physiologically relevant partners of NOX1. It is an intriguing question whether other subunits are involved in NOX1 function. In particular p20phox and Rac1 are of interest as both of them have wide tissue distribution and both of them were reported to be expressed in HEK293 cells (25, 26).

The activation of NOX1 by NOX1 and NOX1A is stimulus-independent in the reconstituted system and probably also in colon. However, we cannot exclude that there are some distinct mechanisms of NOX1 inhibition in the colon, which are not reproduced in the HEK293 system. Is the stimulus-dependent activation of NOX1 by p47phox and p67phox of physiological relevance? Superoxide generation by vascular smooth muscle, which contains NOX1, is stimulus-dependent and involves p47phox (27). Thus, an interaction of NOX1 with phagocyte NADPH oxidase subunits may occur in some tissues and would make NOX1 a versatile enzyme that could change activation mechanisms depending on the subunits present in the cell type in which it is expressed.

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