Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5

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Abstract
gp91phox is the catalytic subunit of the respiratory burst oxidase, an NADPH-dependent, superoxide generating enzyme present in phagocytes. In phagocytes, the enzyme functions in host defense, but reactive oxygen generation has also been described in a variety of non-phagocytic cells, including cancer cells. We previously reported the cloning of Nox1 (NADPH oxidase1), a homolog of gp91phox, its expression in colon and vascular smooth muscle, and its oncogenic properties when overexpressed [Suh et al. (1999). Nature 401, 79±82]. Herein, we report the cloning and tissue expression of three additional homologs of gp91phox, termed Nox3, Nox4 and Nox5, members of a growing family of gp91phox homologs. All are predicted to encode proteins of around 65 kDa, and like gp91phox, all show 5±6 conserved predicted transmembrane a-helices containing putative heme binding regions as well as a flavoprotein homology domain containing predicted binding sites for both FAD and NADPH. Nox3 is expressed primarily in fetal tissues, and Nox4 is expressed in not only fetal tissues, but also kidney, placenta and glioblastoma cells. Nox5 is expressed in a variety of fetal tissues as well as in adult spleen and uterus. Nox isoforms are aberrantly expressed in several cells derived from human cancers, with Nox4 being the isoform most frequently expressed in the tumor cells investigated. Thus, expression of Nox family members is likely to account for some of the reactive oxygen generation seen in non-phagocytic cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Nox; gp91phox; NADPH oxidase; Reactive oxygen species; Gene expression

1. Introduction

The phagocyte respiratory burst oxidase (a.k.a., NADPH-oxidase) catalyzes the NADPH-dependent reduction of molecular oxygen to generate superoxide, which can dismute to form secondary metabolites including hydrogen peroxide and HOCl. Together, these reactive oxygen species participate in host defense by killing or damaging invading microbes. The respiratory burst oxidase consists of six subunits which include two plasma membrane-associated proteins, gp91phox and p22phox which comprise flavocytochrome b558, and four cytosolic factors, p47phox, p67phox, p40phox and Rac (Babior, 1995). The enzyme is dormant in resting cells, but becomes activated to generate reactive oxygen upon exposure to bacteria or chemical stimuli. Enzyme activation occurs through assembly of the cystosolic factors with the membrane-associated flavocytochrome b558 (Lambeth, 2000). gp91phox is the catalytic subunit of the respiratory burst oxidase. This subunit is anchored to the membrane through a hydrophobic N-terminal half which contains a cluster of five predicted transmembrane alpha helices and which is also thought to contain two bound heme groups (Cross et al., 1995; Nishimoto et al., 1995). The C-terminal half of gp91phox is homologous to known flavoprotein dehydrogenases and contains consensus sequences comprising a putative NAD(P)H-binding site (Rotrosen et al., 1992; Segal et al., 1992).

Abbreviations: ROS, reactive oxygen species; Nox, NADPH oxidase; EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; bp, base pair; kb, kilobase; G3PDh, glyceraldehyde-3-phosphate dehydrogenase; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced

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to be an ‘accidental’ byproduct of mitochondrial respiration. However, in some cases, inhibitor studies have pointed to other sources of reactive oxygen; this prompted us to look for the existence of homologs of gp91phox in non-phagocytic tissues. The first of these to be cloned (Suh et al., 1999), Nox1\textsuperscript{1} (referring to NADPH-oxidase, a.k.a., Mox1), is predicted to encode a \(~65\) kDa protein that is 58\% identical to gp91phox. Nox1 generated superoxide when expressed in NIH 3T3 cells, and surprisingly, produced cell transformation and induced marked tumorigenicity. Suppression of native Nox1 expression in vascular smooth muscle cells induced division, supporting a role in normal cell growth. Large molecular weight homologs of gp91phox were recently reported (Dupuy et al., 1999; De Deken et al., 2000; Lambeth et al., 2000) and are predicted to encode proteins of 175–180 kDa; these homologs, referred to as Duox\textsubscript{1} (also called ThOX1) and Duox\textsubscript{2} (also called p138\textsuperscript{TM} or ThOX2) are expressed in thyroid (Dupuy et al., 1999; De Deken et al., 2000) and a variety of other tissues (G. Cheng, L. Sharling and D. Lambeth, unpublished). Unexpectedly, these homologs also contain an N-terminal domain that is homologous to peroxidases (hence the terminology Duox).

The present studies report the cloning of additional 65 kDa homologs of gp91phox and report the tissue and cellular expression of Nox family members, including expression in human cancer cells.

2. Materials and methods

2.1. Cloning of the cDNA for human Nox3

A Blast search (tblastn) using the protein sequence of gp91phox as a query identified a genomic clone, GenBank number HS25719 on chromosome 6q25.1-26. The conceptual protein coding sequence translated from the assembled putative exons showed nearly 60\% identity with about 90\% of gp91phox. To clone this homolog, 5\' and 3\'-RACE were carried out using human fetal kidney marathon-ready cDNA (Clontech, Palo Alto, CA) using the following four primers which were designed based on the genomic sequence: 5\'-RACE: primer 1, 5\'-CTAGAGCTCTCTCTGGTAAATAGA-3\'; Primer 2, 5\'-ATGAACACCTCTGGGGTCA-GCTGA-3\'(for nested PCR); 3\'-RACE: Primer 3, 5\'-CTCGACAAGATGTATTTCACTACCCAG-3\'; Primer 4, 5\'-GGATCCGAGTCACCTCCCTTGCTG-3\'(for nested PCR). The positive PCR bands were sequenced. Primers were designed to subclone by PCR, and the correct full-length cDNA was confirmed by automated sequencing.

2.2. Cloning of the cDNA for human Nox4

A Blast search (tblastn) using the protein sequence of Nox3 as a query identified a 789-base pair sequenced portion of an expressed sequenced tag (EST clone, GenBank No. AI742260) and a 408-base EST clone (GenBank No. AI885681). The first clone exhibited 26\% identity to the cDNA sequence corresponding to amino acid residues 433–560 of Nox3, and the second clone showed 36\% identity to the cDNA sequence corresponding to amino acid residues 5–48 of Nox3. This homolog was cloned using two PCR primers based on the two EST sequences: (Primer 5, 5\'-CAACGAAGGGTTAAA-CACCTCTGC-3\'; Primer 6, 5\'-CACACCTGATTGGTCCGCTGAG-3\'). PCR was carried out using human fetal kidney marathon-ready cDNA (Clontech, Palo Alto, CA), and the 0.85 kb product was sequenced. Based on the sequencing results, we then carried out 5\' and 3\'-RACE using the same library using the following primers: 5\'-RACE: primer 7, 5\'-TACCCGGCTTCCCCGTGTTATAC-3\'; Primer 8, 5\'-TACTTGCTGCCCCCCTTGTTATACA-3\'(for nested PCR); 3\'-RACE: Primer 9, 5\'-TCCATTTACCTCCAATGTG-3\'; Primer 10, 5\'-CTCACCGGAAATCTCAGCGCTGTG-3\'(for nested PCR). The positive PCR bands were sequenced. Primers were designed to subclone the full-length cDNA and the correct sequence was confirmed by automated sequencing.

2.3. Cloning of the cDNA for human Nox5

The Blast search using Nox3 as a query also identified homology with two unfinished genomic clones, GenBank No. AC027088 and AC026512, respectively. These clones exhibit 46 to 50\% identity to Nox3 within three exons. 5\' and 3\'-RACE were carried out using human fetal kidney marathon-ready cDNA (Clontech, Palo Alto, CA), using the following four primers which were designed based on the genomic sequence: Primer 11, 5\'-CTCATTGTCA-CACCTCTCGAGCA-3\'; Primer 12, 5\'-TGCGTCTGA-TGCTGCTGAGAAGCTC-3\'(for nested PCR); 3\'-RACE: Primer 13, 5\'-ATCAAGGGCGCCCTTGGTTAC-3\'; Primer 14, 5\'-CTGCAACATCCACCCATGCTGTG-3\'(for nested PCR). The positive PCR bands were sequenced. Primers were designed to subclone the full-length cDNA and the correct sequence was confirmed by automated sequencing.

2.4. Analysis of primary structure

Secretion signal sequences were predicted according to web-based SMART program (version 3.1) at EMBL (http://www.smart.embl-heidelberg.de/smart/). Prediction of Open Reading Frames (ORF) was carried out using the EditSeq program (DNAStar), and phylogenetic analysis and multiple sequence alignments were carried out using the clustal method using the Megalign program (DNAStar). Transmembrane alpha helices were predicted using the TMHMM algorithms through the Center for Biological

\textsuperscript{1} As detailed in Lambeth et al. (2000), the terminology Nox (NADPH oxidase) has been approved by the HUGO International Gene Nomenclature Committee and replaces the earlier terminology, ‘Mox’. The terminology Duox (referring to Dual oxidase) is also approved by the HUGO Gene Nomenclature Committee, and replaces former terminologies.
2.5. First-strand cDNA synthesis

Total RNA was extracted from cell lines with Trizol (Life Technologies, Gaithersburg, MD) based on the manufacturer’s protocol or according to (Ishii et al., 1999) for glioma cell lines. RNAs were reverse transcribed into first-strand cDNA with Superscript II (Life Technologies, Gaithersburg, MD) using oligo-dT according to the method provided by the manufacturer.

2.6. PCR detection of mRNA for gp91phox, Nox1, Nox3, Nox4, Nox5, and p22phox

The expression patterns of the above genes were determined using Human Multiple Tissue PCR panels (Clontech, Palo Alto, CA) and reverse transcription products from cell lines (above) using the following primers: (Nox3: primer 2 and primer 4; Nox4: primer 10 and 5'-AGAGGAACACGACATGCCCTTAG-3'; Nox5: primer 13 and 11; gp91phox: 5'-GGAGTTTCAAGATGCGTGGAAACTA-3' and 5'-GCCAGACTCAGATTTGGAATGCT-3'; Nox1: 5'-GTACAAAATTCAGTGACGACCCAC-3' and 5'-CGAGCTGGAAATTCGATGCACGA-3'; p22phox: 5'-ATGGGGCAGATCGAGTGCCATGT-3' and 5'-GATGGAGCCGAACTTCTCAATGGCC-3'). PCR parameters were 95°C for 30 s, 62°C for 20 s, 72°C for 45 s, 25–35 cycles as indicated after denaturing for 1 min 30 s at 95°C.

2.7. Sequencing of gp91, Nox1, Nox3, Nox4, Nox5 and p22phox PCR products

PCR products were purified with a QIAquick PCR purification kit or a gel purification kit (QIAGEN, Valencia, CA) and sequenced by ABI 377 automatic sequencing.

2.8. Real time RT-PCR

The G3PDH PCR product was purified using a QIAquick PCR purification kit or a gel purification kit (QIAGEN, Valencia, CA) and sequenced by ABI 377 automatic sequencing.

2.9. Northern blotting

The Human Fetal and Adult Multiple Tissue Northern Blot (Clontech, Palo Alto, CA) was hybridized with 32P-random primer-labeled Nox3, Nox4, or Nox5 probes according to the manufacturer’s instructions. The probes were prepared by PCR with primers (Nox3: 5'-TTAGTTTCTTATGACCATGCCTCAG-3' and 5'-GATTTTTCATCCCCAGCCGTAAGA-3'; Nox4: Primer 5 and Primer 6; Nox5: 5'-CTGAAACATCCCACTCATTGCTCGC-3' and 5'-GAAGCAGGAATTCCTCAATGGCC-3'). The PCR products represent coding sequences corresponding to amino acids 183–485 (Nox3), 11–294 (Nox4), or 278–557 (Nox5). Because the Nox5 transcript sizes differ between fetal and adult Northern blots, a 420 bp PCR product of the Nox5 3' untranslated region amplified by primers (5'-CTCACTACCTCCTCAAGCTCTGCC-3' and 5'-CTGAACATCCCCACCACCAG-3') was also used to hybridize Northern Blots.

3. Results and discussion

3.1. cDNA cloning of human Nox3, Nox4 and Nox5

The cDNAs for human Nox3, Nox4 and Nox5 were cloned as described in Section 2. Table 1 shows the basic features of the cDNA and the predicted proteins. Like gp91phox (a.k.a., Nox2) and Nox1, the three new sequences

| Table 1: Molecular Features of Nox3, Nox4 and Nox5 cDNA |
|-----------------|-----------------|-----------------|
| **Nox3** | **Nox4** | **Nox5** |
| cDNA length (bp) | 2044 | 2232 | 2199 |
| Predicted number of aa’s | 568 | 578 | 565 |
| Predicted protein Mw (kDa) | 64.9 | 66.9 | 64.7 |
| pl of protein | 8.0 | 8.7 | 9.7 |
| Kozak sequence | ATGATGA or ATGATGG | GCCATGG | GTGATGG |
| Identity to gp91phox | 58% | 37% | 27% |
| Identity to Nox1 | 55% | 35% | 29% |
| GenBank accession No. | AF190122 | AF254621 | AF317889 |
encode predicted proteins of around 65 kDa, and message sizes are similar in length (2.0–2.2 kb). Nox3, Nox4 and Nox5 show 21–59% identity with gp91phox and with Nox1, with Nox3 being the most similar to gp91phox and Nox1, and Nox5 the most divergent. This is illustrated in the dendrogram shown Fig. 1, which also shows the Dual oxidase (Duox) group of enzymes as well as other homologs that have been identified in Dictyostelium, Drosophila and C. elegans (Lambeth et al., 2000). Nox1, gp91phox, Nox3 and Nox4 cluster within a sub-family that is similar to gp91phox, while the Duox’s form a separate sub-group, which is also characterized by their larger size and the presence of the peroxidase homology domain (Lambeth et al., 2000; De Deken et al., 2000). Nox5 forms a unique group, of which it is the only member identified to date, and which is highly divergent from other members of the family. Based on its position in the family tree, Nox5 may represent the gene which is closest to the primordial Nox.

3.2. Domain organization and sequence comparisons among Nox family members

The alignment of the predicted protein sequences of gp91phox, Nox1, Nox3, Nox4 and Nox5 are shown in Fig. 2. The molecules are roughly divided into two large domains: an N-terminal cluster of hydrophobic membrane-spanning sequences, and a C-terminal flavoprotein domain. The latter shows weak homology with a number of FAD binding proteins including cytochrome P-450 reductase and ferredoxin-NADP oxidoreductase (Rotrosen et al., 1992; Segal et al., 1992). Within the flavoprotein domain are two segments (indicated in Fig. 2) that show homology with known FAD binding sites in other flavoproteins, and four segments nearer the C-terminus that are homologous to documented pyridine nucleotide binding sites in other proteins. The first of these includes the G-X-G-X-X-P canonical sequence that characterizes pyridine nucleotide binding sites. In all Nox forms, this sequence is followed by an F, which is typical of NADPH- rather than NADH-specific enzymes.

While the N-terminal half of Nox1–Nox5 are all hydrophobic, Nox5 differs from the others somewhat in the details of predicted transmembrane alpha helices, as illustrated in Fig. 2. Nox1, gp91phox, Nox3 and Nox4 all contain a predicted transmembrane alpha helix near the extreme N-terminus (light hashed box in Fig. 2). However, this region is also strongly predicted to be a signal peptide sequence in these forms. Predicted proteolytic cleavage sites for each isoform are indicated by the arrows, and cleavage at these positions would lead to a loss of the first putative transmembrane sequence. In contrast, Nox5 does not contain an N-terminal predicted signal peptide, but does contain a predicted transmembrane alpha helix (first hashed box, Nox5 sequence in Fig. 2). According to the prediction algorithm, the extreme N-terminus of Nox5 is located on the inside of the membrane, on the same side as the flavoprotein domain. Five additional transmembrane regions are also predicted in these proteins. The most C-terminal of these is weakly predicted in Nox1, gp91phox, Nox3 and Nox4 and is entirely missed by some prediction algorithms, but is strongly predicted in Nox5 and in some of the Duox isoforms (data not shown). It is necessary to include this
Fig. 2. Alignment of amino acid sequences of human Nox proteins. Predicted amino acid sequences of the human Nox proteins (gp91phox, Nox1, Nox3, Nox4 and Nox5) were aligned as in Section 2.4. Filled circles indicate conserved histidine residues that are candidates for ligating to heme iron. Heavily hashed boxes represent predicted transmembrane alpha helices, while the lightly shaded hashed box is a possible transmembrane helix that lies within a signal peptide motif that is also predicted from the sequences of gp91phox, Nox1, Nox3, Nox4, but not Nox5. Arrows placed within the sequence indicate the predicted proteolytic cleavage site of the signal peptide. Also indicated are putative FAD and NADPH binding sites.
transmembrane region in order to generate a model (Fig. 3) which is consistent with known features of gp91phox, particularly a cytosolic facing location of the flavoprotein domain. In this model, known N-linked glycosylation sites in gp91phox are correctly localized to extracellular loops (although these sites are not conserved in other isoforms). In addition, a polybasic loop of gp91phox that binds to the cytosolic regulatory protein p47phox (Biberstine-Kinkade et al., 1999) is localized on the cytosolic face. In general, extracellular loops tend to be highly variable in length and sequence, whereas the transmembrane helices and intracellular loops tend to be more conserved in sequence and length (Fig. 2).

Within the N-terminus are five absolutely conserved histidines (Fig. 2), that are also conserved in all other members of the Nox and Duox enzymes (data not shown). gp91phox contains two heme groups, the irons of which are each ligated by two histidyl nitrogens (Isogai et al., 1993), and these are thought to reside within the N-terminus (Yu et al., 1998). An additional conserved histidine lies within the FAD-binding region and is therefore not a candidate for heme ligation. Thus, four of the five histidines within the N-terminus must participate in heme ligation, providing part of the binding sites for two heme groups, as indicated in Fig. 3.

One additional feature of Nox5 is worth noting. Located at the extreme N-terminus on the cytosolic side of the membrane is a highly cationic proline-rich sequence (the Pro-Arg-rich sequence indicated in Fig. 2 and Fig. 3). Although the function of this region is not known, it is possible that it could serve as a binding sequence for Src-Homology 3 (SH3) domains in another protein. SH3 domains are known to recognize inter- or intra-molecular proline-rich sequences. Precedent for such an interaction in the Nox family comes from p22phox, a membrane-associated subunit that associates with gp91phox. This protein contains a C-terminal, proline-rich sequence (Parkos et al., 1988) that serves as a binding site for a SH3 domain in p47phox, one of the cytosolic subunits that regulates the activity of gp91phox. It is possible that the proline-rich sequence in Nox5 serves as a ‘built-in’ p22phox, allowing interaction with cytosolic regulatory proteins. Alternatively, this polybasic region may interact with negatively charged membrane phospholipids.

3.3. Tissue expression of Nox3, Nox4 and Nox5 mRNA

Northern blot analysis failed to reveal significant expression of the full-length transcript of Nox3, although a smaller (1.2 kb) band was seen in placenta (Fig. 4A). The more sensitive RT-PCR (Fig. 5), revealed expression in fetal kidney, with lesser expression in several other fetal tissues including liver, lung and spleen. The predominant Nox4 2.4 kb message, which corresponds to the size expected for the full-length Nox4 transcript, is highly expressed in adult as
Fig. 4. Northern blotting analysis of tissue expression of human Nox3, Nox4 and Nox5. (A) Membranes were hybridized with probes based on the Nox3 (upper panel), Nox4 (middle panel) and Nox5 (lower panel) coding sequences, as described in Section 2.9. Kilobase molecular size markers are indicated on the left. Bands referred to in the text are indicated on the right side of the figure. (B) The membranes were hybridized with a probe from the 3' untranslated region of
well as fetal kidney (Fig. 4A), confirming recent reports 
(Kikuchi et al., 2000; Geiszt et al., 2000; Shiose et al.,
2000). An additional weak Nox4 band was also detected 
at 4.5 kilobases (kb) in fetal and adult kidney (Fig. 4A).
RT-PCR confirmed kidney expression and also revealed 
expression of Nox4 in all fetal tissues tested as well as in 
several adult tissues including pancreas, placenta, ovary, 
testis and skeletal muscle. Northern blots probed for Nox5 
using a 3′-portion of the coding region (Fig. 4A) revealed 
the presence of a 2.2 kb band corresponding in size to the 
full-length Nox5 transcript in all fetal tissues tested. This 
species was also seen in low amounts in adult spleen and 
testis, along with larger transcripts at 2.6 and 6 kb. A probe 
using a portion of the 3′ untranslated region also revealed 
the presence of the same 2.6 and 6 kb bands (Fig. 4B). Thus, 
these larger bands are larger transcripts derived from the 
same gene. Studies are underway to identify these species, 
which probably represent different splice forms. RT-PCR 
confirmed expression of Nox5 in testis and spleen, and 
also revealed weak expression in ovary, placenta, and 
pancreas (Fig. 5).

For comparison, expression of Nox1, gp91phox and 
p22phox are also shown (Fig. 5). Confirming our earlier 
study (Suh et al., 1999), Nox1 is highly expressed in 
colon. p22phox is widely expressed, as was reported 
previously (Parkos et al., 1988). gp91phox shows a narrower
expression than p22phox, not only in peripheral blood leukocytes, but also particularly in lung, pancreas and placenta. Because each of the tissues is complex and contains multiple cell types, it is possible that the apparent gp91phox expression in some of these tissues is due to contaminating leukocytes, macrophages or monocytes, which also express gp91phox. In any case, these data illustrate that expression patterns of Nox family members are tissue specific, and do not correspond to the expression of gp91phox.

3.4. Expression of Nox3, Nox4 and Nox5 mRNA in cancer cells

Many cancer cells overproduce reactive oxygen species (Szatrowski and Nathan, 1991), and this may be causative in the transformed phenotype (Suh et al., 1999). We therefore investigated the expression of Nox1-5 in a variety of human tumor and other cell lines, to see if these enzymes might account for reactive oxygen generation seen in some tumors. As shown in Fig. 6A,B, Nox isoforms were expressed in 12 out of the 14 tumor or transformed cell lines examined. Nox1 is expressed in two colon cancer lines, Caco-2 and T-84, as well as in the transformed cell line HEK293, and to a lesser extent in Hela cells. Nox4 was seen in 11 of these cell lines, while Nox5 was seen in 7. gp91phox was also expressed in more than half of the cell lines. This is somewhat surprising, since gp91phox is thought to be nearly specific for phagocytes. The identity of the mRNAs was confirmed by sequencing as indicated in Figs. 5,6A, and B.

In five brain tumor cell lines derived from human glioblastomas, Nox 4 was always expressed, along with variable expression of Nox5 and gp91phox (Fig. 6B). Real time PCR revealed that the ratio of expression of Nox to G3PDH varied significantly in the various tumor cell lines compared with primary human astrocytes (Fig. 6C). Although the cellular origin of glioblastomas has not been definitively established, this cancer type is thought by many workers to have arisen from the astrocytic lineage.

The expression of Nox forms in cancer and transformed cell lines did not correlate strictly with the expression in normal tissue, indicating that expression of Nox isoforms is sometimes altered in cancer cells. Thus, aberrant expression or regulation of Nox isoforms could account for the increased reactive oxygen generation seen in some cancer cells.

4. Summary

Herein, we report the molecular cloning and tissue/cell expression of new members of the Nox family. The existence of multiple homologs of gp91phox in non-phagocytic tissues/cells implies that generation of reactive oxygen in these tissues is a deliberate biological strategy that is distinct from host defense, and suggests novel roles for reactive oxygen. Such roles may include oxygen sensing; Nox 4 is expressed at the site of erythropoietin production in the kidney and has been proposed to play a role in inducing this factor in response to hypoxia (Geiszt et al., 2000). In addition, Duox2 (p138Tox) and possibly Duox1 plays a role in the thyroid by providing hydrogen peroxide which serves as a substrate for the thyroid peroxidase-catalyzed iodination of thyroid hormone (Dupuy et al., 1999). In addition,
Nox1 plays a role in growth, transformation and tumorigenecity (Suh et al., 1999). Shown herein, expression of Nox isoforms in tumor cell lines is frequently aberrant compared with normal tissue. Expression data are consistent with Nox family members as sources for reactive oxygen in some cancer cells, and these proteins could play a role in the neoplastic growth of some cancer cells.

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