A Novel Superoxide-producing NAD(P)H Oxidase in Kidney*

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During phagocytosis, gp91^{phox}, the catalytic subunit of the phagocyte NADPH oxidase, becomes activated to produce superoxide, a precursor of microbicidal oxidants. Currently increasing evidence suggests that nonphagocytic cells contain similar superoxide-producing oxidases, which are proposed to play crucial roles in various events such as cell proliferation and oxygen sensing for erythropoiesis. Here we describe the cloning of human cDNA that encodes a novel NAD(P)H oxidase, designated NOX4. The NOX4 protein of 578 amino acids exhibits 39% identity to gp91^{phox} with special conservation in membrane-spanning regions and binding sites for heme, FAD, and NAD(P)H, indicative of its function as a superoxide-producing NAD(P)H oxidase. The membrane fraction of kidney-derived human embryonic kidney (HEK) 293 cells, expressing NOX4, exhibits NADH- and NADPH-dependent superoxide-producing activities, both of which are inhibited by diphenylene iodonium, an agent known to block oxygen sensing, and decreased in cells expressing antisense NOX4 mRNA. The human *NOX4* gene, comprising 18 exons, is located on chromosome 11q14.2-q21, and its expression is almost exclusively restricted to adult and fetal kidneys. In human renal cortex, high amounts of the NOX4 protein are present in distal tubular cells, which reside near erythropoietin-producing cells. In addition, overexpression of NOX4 in cultured cells leads to increased superoxide production and decreased rate of growth. The present findings thus suggest that the novel NAD(P)H oxidase NOX4 may serve as an oxygen sensor and/or a regulator of cell growth in kidney.

Reactive oxygen species (ROS)[1] are conventionally viewed as

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[1] The abbreviations used are: ROS, reactive oxygen species; NOX, NAD(P)H oxidase; EPO, erythropoietin; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; SOD, superoxide dismutase; FBP, familial benign polycythemia; EST, expressed sequence tag; PAGE,

toxic byproducts of cellular metabolism. On the other hand, organisms possess enzymatic systems that physiologically generate ROS. Among the systems to be well characterized is the superoxide-producing NADPH oxidase in mammalian professional phagocytes, which plays a crucial role in host defense against microbial infection (1–4). The catalytic core of the phagocyte NADPH oxidase is the membrane-integrated flavocytochrome b_{558}, comprising the two subunits p22^{phox} and gp91^{phox}, the latter of which contains a complete electron-transferring apparatus (from NADPH to molecular oxygen) with binding sites for heme, FAD, and NADPH (5–8). The oxidase, dormant in resting cells, becomes activated during phagocytosis to generate superoxide, a precursor of microbicidal ROS. The activation requires the two specialized cytosolic proteins p47^{phox} and p67^{phox}, and the small GTPase Rac, all of which translocate upon cell stimulation to the membrane to interact with and activate the flavocytochrome (9–15). Such a strict regulation of the phagocyte oxidase activity is clearly important, since uncontrolled production of high amounts of ROS is injurious not only to phagocytes themselves but also to their surrounding tissues, and triggers inflammatory reaction (1–4).

A growing body of evidence suggests that similar superoxide-producing NAD(P)H oxidases exist in nonphagocytic cells of vascular smooth muscle (16, 17), endothelium (18), carotid body (19), lung (20, 21), and kidney (22, 23). Although these oxidases are proposed to play a role in a variety of events such as signaling for cell growth or cell death, oxygen sensing, and inflammatory processes (16–25), their *bona fide* functions as well as molecular compositions are largely unknown. Suh *et al.* (26) have recently reported cell transformation by the superoxide-generating oxidase Mox1, which is homologous to gp91^{phox} and highly expressed in colon and to a lesser extent in prostate, uterus, and vascular smooth muscle. Interestingly, alternative splicing of the oxidase NOH-1, equivalent to Mox1, generates a short isoform, which functions as a voltage-gated proton channel (27). Considerable attention has been paid to an NAD(P)H oxidase in kidney; this organ highly expresses p22^{phox}, the small subunit of flavocytochrome b_{558} (28), is susceptible to oxidative damage, suggestive of significance of ROS under pathological conditions (29), and plays a central role in erythropoiesis by synthesizing erythropoietin (EPO), which is regulated by an oxygen sensor (24, 25). Molecular nature of the kidney oxidase, however, has remained elusive.

Here we describe the primary structure and function of a novel superoxide-producing NAD(P)H oxidase whose expres-

polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; RT, reverse transcription; kb, kilobase pair(s); HEK, human embryonic kidney.

sion is almost exclusively restricted to adult and fetal human kidneys. We designate the novel oxidase NOX4 to avoid future confusion about the term, because Drs. Krause, Lambeth, and Lovering have recently established a consensus terminology for the NOX family of NAD(P)H oxidase: NOX1 for Mox1/NOH-1, NOX2 for gp91[phox], and NOX3 for the one, thus far only known from its genomic sequence (accession no. AL031773).[2]

EXPERIMENTAL PROCEDURES

Cloning of Human cDNA Encoding NOX4—-A search of EST data bases with the C-terminal region of human gp91[phox] yielded a cDNA clone (GenBank℗ accession no. AI742260). Using the EST clone as a template, we obtained the PCR product of 500 nucleotides with two unique oligonucleotide primers: 5′-GCTGGATCCCGAATGGTCAAA-GAAAATTT-3′ (forward primer) and 5′-GCTGAATTCATAGTCAG-GTCTGTTCTCTTG-3′ (reverse primer). Here sequences from the EST clone are underlined. The PCR product was used as a probe for screening a human kidney cDNA library (Stratagene). Twelve independent positive clones were obtained from about 6×10^5 plaques, and sequenced in both directions. Among them, six clones encoded the full-length of NOX4.

Chromosomal Localization—To determine the chromosomal localization of the human *NOX4* gene, fluorescence *in situ* hybridization was performed as described previously (30). In brief, a NOX4 cDNA fragment (corresponding to amino acids 1–578) was labeled with biotin-14-dATP by nick-translation and hybridized to R-banded chromosomes prepared from phytohemaggulutinin-stimulated cultured lymphocytes of normal donors. After overnight hybridization at 37 °C, the slides were washed in 50% formamide, $2\times$ SSC at 37 °C for 10 min, followed by a wash in $1\times$ SSC at room temperature for 15 min. Hybridization signals were amplified using rabbit anti-biotin IgG (Enzo) and fluorescein labeled goat anti-rabbit IgG (Enzo). The chromosomes were counterstained with propidium iodide.

Transfection—Sense and antisense cDNA encoding human NOX4 (amino acids 1–578) were subcloned into pREP10 (Invitrogen) or pEF-BOS (31), a generous gift form Dr. Shigekazu Nagata (Osaka University, Osaka, Japan), for expression of the N-terminal FLAG-tagged proteins. COS-7, HEK293, HeLa, or NIH3T3 cells were transfected with pREP10-NOX4, pREP10-antisense-NOX4, or empty pREP10 vector and/or pREP10-p22[phox], or with pEF-BOS-FLAG-tagged-NOX4 or pEF-BOS-antisense-NOX4, using FuGENE (Roche Molecular Biochemicals).

Western Blot Analysis—The DNA fragments encoding the C-terminal domains of NOX proteins, NOX1 (amino acids 378–564), NOX2 (384–570), and NOX4 (406–578), were prepared by PCR, and the products were ligated to pGEX-2T or -4T (both from Amersham Pharmacia Biotech). Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain BL21 and purified by glutathione-Sepharose-4B (Amersham Pharmacia Biotech), as described previously (9, 11). An anti-human NOX4 rabbit polyclonal antibody was raised against the C-terminal 20 amino acid peptide of human NOX4. Purified proteins or membrane fractions of cells transfected with human NOX4 cDNA were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with the anti-human NOX4 antibody or anti-FLAG (M2) antibody (Sigma-Aldrich). The blots were developed using ECL-plus (Amersham Pharmacia Biotech) to visualize the antibodies.

RT-PCR for NOX1, NOX2, and NOX4—Total RNAs of HEK293, HeLa, KPK13 cells (32) were perpared from with Trizol reagent (Life Technologies, Inc.), and RT-PCR for NOX1, NOX2, and NOX4 was performed with GeneAmp℗ (PerkinElmer Life Sciences) using specific primers: 5′-GATGATCGTGACTCCCAC-3′ (forward primer) and 5′-C-AACAATATTGCTGTCCC-3′ (reverse primer) for NOX1; 5′-GGGAAA-AATAAAGGAATGCC-3′ (forward primer) and 5′-AGCCAGTGAGGTA-GATGTTG-3′ (reverse primer) for NOX2; 5′-GTCATAAGTCATCCCT-CAGA-3′ (forward primer) and 5′-TCAGCTGAAAGACTCTTTAT-3′ (reverse primer) for NOX4.

Superoxide Production—HEK293 cells were lysed by sonication, and the sonicate was centrifuged for 10 min at $10,000 \times g$. The resultant supernatant was further centrifuged for 1 h at $100,000 \times g$. The pellet was used as the membrane fraction. Superoxide production by the membrane was determined as superoxide dismutase (SOD)-inhibitable chemiluminescence detected with an enhancer-containing luminol-based detection system (Diogenes℗; National Diagnostics) as described

previously (11, 14). The membrane (5 μg of protein) was resuspended in 200 μl of 100 mM potassium phosphate, pH 7.0, with 10 μM FAD, 1 mM NaN$_3$, and 1 mM EGTA. After preincubation of the membrane solution with the enhanced luminol-based substrate (200 μl), NADH or NADPH was added at the final concentration of 0.5 mM. The chemiluminescence was assayed using a luminometer (Auto Lumat LB953; EG&G Berthold). The reaction was stopped by the addition of SOD (50 μg/ml).

Cell Growth—COS-7, HEK293, or NIH3T3 cells were seeded with 5.0×10^3 cells/well in 96-well plates, and transfected with pREP10-NOX4 or pREP10-antisense-NOX4 by using FuGENE (Roche Molecular Biochemicals). Transfected cells were maintained in DMEM containing 10% FCS for 56 h, and the cell number was determined photometrically using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma).

Northern Blot Analysis—Human Multiple Tissue Northern℗ blots (CLONTECH) were hybridized with a ^{32}P-labeled NOX4 cDNA fragment, encoding the region that corresponds to amino acids 324–491, under high stringency conditions using ExpressHyb℗ (CLONTECH).

Immunohistological Staining—Localization of the NOX4 protein in human kidney was investigated by immunohistochemical study using renal tissue samples obtained by renal biopsy from patients, who were admitted to our hospital because of chance proteinuria or hematuria. All samples revealed neither glomerular and tubular abnormalities nor significant deposition in immunofluorescent study. Biopsy specimens were fixed in Bouin's solution and embedded in paraffin. The procedures of the use of the samples in the present study was in accordance with the guidelines of the local Human Subject Committee at Kyushu University (Fukuoka, Japan).

Immunohistochemical staining was performed as described previously (33). Briefly, paraffin sections were cut at 3 μm, deparaffinized, and treated with 10 mM citrate, pH 6.0, in 800-watt microwave oven for 15 min for antigen retrieval before immunostaining. After inactivation of endogenous peroxidase with 3% H_2O_2 in methanol for 30 min at room temperature, the sections were preincubated for 1 h with 5% dry milk and 5% bovine serum albumin in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, and 1.4 mM KH$_2$PO$_4$, pH 7.4). The samples were subsequently incubated with the anti-human NOX4 rabbit polyclonal antibody (1:2000 dilution) for overnight at 4 °C, washed in PBS with 0.1% Triton X-100, and probed with biotinylated anti-rabbit IgG antibody (Nichirei Corp., Tokyo, Japan) for 30 min at room temperature. After washing in PBS with 0.1% Triton X-100, the sections were reacted with HRP-conjugated streptavidin (Nichirei Corp.) for 30 min at room temperature, and the sites of HRP were visualized with diaminobenzidine (Nichirei Corp.) and H_2O_2, and counterstained with hematoxylin for 2 min.

RESULTS

Human cDNA Encoding a Novel Homologue of gp91[phox]—A search of EST data bases with the C-terminal region of human NOX2/gp91[phox] yielded a cDNA clone (GenBank℗ accession no. AI742260). Using a DNA fragment of the EST clone as a probe, we screened a human kidney cDNA library and cloned a cDNA of ~2.4 kb. The cDNA clone contains an open reading frame of 1,734 nucleotides with the first methionine codon surrounded by a consensus Kozak sequence, two in-frame preceding termination codons, and a consensus polyadenylation signal (AATAAA) present in the 3′-region (GenBank℗ accession no. AB041035). The size of the cloned cDNA is identical to that of the major transcript of about 2.4 kb (see below), supporting that the obtained clone indeed contains the full-length cDNA.

The predicted protein consists of 578 amino acids (Fig. 1), with 39% and 35% identity to NOX2/gp91[phox] and NOX1/Mox1, respectively. Regions, that are considered to be binding sites for FAD and pyridine nucleotide (5–8), are nearly identical among the NOX proteins (Fig. 1). Six putative membrane-spanning segments are also well conserved with four histidine residues that are proposed to be ligated to heme in NOX2/gp91[phox] (8); His[105] and His[119] of the third segment, and His[194] and His[207] of the fifth one in NOX4. Since these features in the primary structure indicate the function as a superoxide-producing NAD(P)H oxidase, we designated the protein NOX4, a novel member of the NAD(P)H oxidase (NOX) family.

Genomic Organization and Chromosomal Localization of the

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[2] K.-H. Krause, personal communication.

FIG. 1. **Deduced amino acid sequence of human NOX4 in comparison with human NOX2/gp91**[phox] **and NOX1/Mox1.** Residues identical to NOX4 are *boxed*. *Overlined* residues are presumed membrane-spanning regions, and binding sites for heme, FAD, and NAD(P)H. *Asterisks* indicate conserved histidine residues that are candidates for heme ligation.

FIG. 2. **Genomic organization and chromosomal localization of the human NOX4 gene.** *A*, genomic organization of the human *NOX4* gene in comparison with the human *NOX2/gp91*[phox] gene. Exons and introns are shown as *horizontal* and *vertical bars*, respectively. *NOX4* comprises 18 exons, whereas *NOX2* does 13 exons. Boxed numbers represent those of coding nucleotides in each exon. *B*, chromosomal localization of the human *NOX4* gene. Partial metaphase plate showing doublet signals on human chromosome 11q14.2-q21 (*arrow*). The biotinylated cDNA probe for human *NOX4* gene was hybridized to chromosomes with replication bands prepared from cultured lymphocytes. After hybridization and washing, hybridization signals were amplified using rabbit anti-biotin and fluorescein-labeled goat anti-rabbit IgG. The chromosomes were counterstained with propidium iodide.

Human NOX4 Gene—The human *NOX2* gene consists of 13 exons, all with lengths similar to the 13 exons of the *NOX1* gene (27). To know genomic structure of the *NOX4* gene, we screened a pooled human genomic library by PCR and obtained the BAC clone RP11–745I13 (GenBank accession no. AP002404), that contains exons 2–16 of NOX4. In addition, a data base search revealed that the two BAC clones RP11–345O16 and RP11–97D10 (GenBank accession nos. AC012198 and AP001815, respectively) also contain exons 1–7 and 3–18 of NOX4, respectively. The *NOX4* gene comprises 18 exons, covering a minimum of 160 kb, and its organization is similar to those of *NOX2* (Fig. 2*A*).

To determine the chromosomal localization of the human *NOX4* gene, we performed fluorescence *in situ* hybridization analysis. As shown in Fig. 2*B*, specific hybridization signals were observed on chromosome 11q14.2-q21, whereas no other hybridization sites were detected. Thus, the human *NOX4* gene is located on chromosome 11q14.2-q21, while the human *NOX1* and *NOX2* genes are located on chromosome Xq22 and Xp21.1, respectively (27).

Expression of the NOX4 Protein in COS-7 and HeLa Cells Transfected with Its cDNA—We prepared an antibody raised against the C-terminal peptide of human NOX4 as a tool for characterization of the NOX4 protein. As expected, the antibody interacted with the C-terminal cytoplasmic domain of NOX4 expressed as a GST fusion protein, but not with that of NOX1/Mox1 or NOX2/gp91[phox] (Fig. 3*A*). We next expressed NOX4 as a FLAG-tagged protein in COS-7 cells, and detected the protein by Western blot analysis. As shown in Fig. 3*B*, an anti-FLAG antibody bound to a protein with the apparent molecular mass of 66 kDa, the value which is essentially the same as the calculated molecular mass of NOX4. Additional two minor bands with much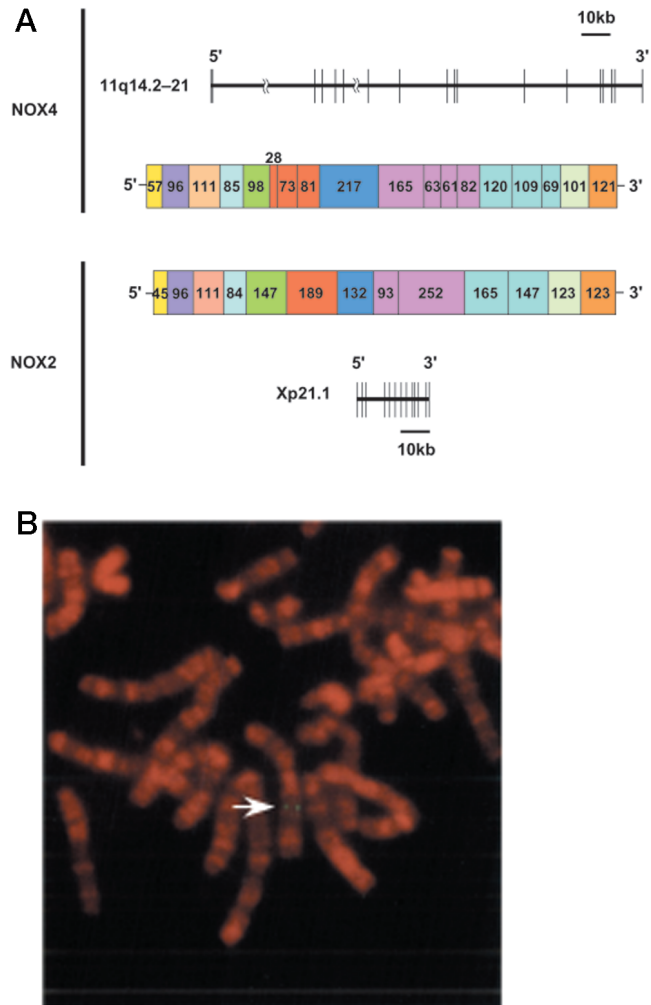 higher molecular masses were also observed on the blot (Fig. 3*B*). The bands likely indicate dimer and/or oligomer(s) of NOX4, since they increased with concomitant decrease of the 66-kDa band when SDS-PAGE was performed under oxidized conditions (data not shown). The antibody to NOX4 only interacted with the same protein as that recognized by the anti-FLAG antibody, among proteins in COS-7 cells (Fig. 3*B*). These observations indicate that the antibody raised to the C terminus of human NOX4 specifically recognizes NOX4.

We also tested effect of expression of p22[phox] on the protein level of NOX4. The protein p22[phox] is the heterodimeric partner of NOX2/gp91[phox] in the cytochrome b_{558} complex of the phagocyte NADPH oxidase, and is required for stabilization of NOX2: NOX2 is absent in phagocytes lacking p22[phox] due to defect in the p22[phox] gene (1–4). Cotransfection of COS-7 cells with both NOX4 and p22[phox] cDNAs did not alter the protein level of NOX4, compared with transfection with the NOX4 cDNA alone (data not shown). The finding raises a possibility that NOX4 may not be complexed with p22[phox], but does not
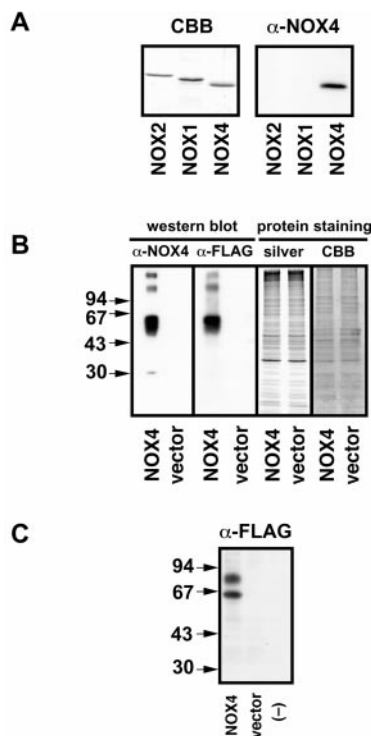
FIG. 3. **Specific recognition of human NOX4 by an antibody raised against its C-terminal peptide.** *A*, Western blot analysis with an anti-NOX4 antibody. The C-terminal cytoplasmic domains of NOX1, NOX2, and NOX4 were expressed as GST fusion proteins. Purified proteins (0.2 μg) were subjected to 10% SDS-PAGE, followed by staining with Coomassie Brilliant Blue (*left panel*) or by immunoblot with an antibody raised against the C-terminal peptide of NOX4 (*right panel*). *B*, Western blot analysis of the membrane fraction of COS-7 cells transfected with the plasmid pEF-BOS encoding FLAG-tagged NOX4 or vector alone. The membrane proteins (0.7 μg) were subjected to 10% SDS-PAGE, followed by immunoblot with the anti-NOX4 or anti-FLAG antibody or by silver or Coomassie Brilliant Blue staining. Positions for marker proteins are indicated in kilodaltons. *C*, Western blot analysis of the membrane fraction of HeLa cells transfected with the plasmid pEF-BOS encoding FLAG-tagged NOX4 or vector alone. The membrane proteins (7.0 μg) were subjected to 10% SDS-PAGE, followed by immunoblot with the anti-FLAG antibody. Positions for marker proteins are indicated in kilodaltons. For details, see "Experimental Procedures." The experiments have been repeated more than three times with similar results.

exclude another one that NOX4 alone might be localized in an intracellular membrane compartment, but in the presence of p22^{phox} it may be incorporated into the plasma membrane in a heterodimeric form, a process which has recently been shown to occur in formation of the NOX2/gp91^{phox}–p22^{phox} heterodimer (34). It is also possible that COS-7 cells may express an heretofore unidentified p22^{phox} homologue that could complex with NOX4.

Using the membrane fraction of HeLa cells transfected with the NOX4 cDNA, we detected a protein band of about 75 kDa, in addition to the 66-kDa band, on Western blot (Fig. 3*C*). The larger protein may be a glycosylated form of NOX4, since there exist four putative *N*-glycosylated sites in the second and third extracellular loops of NOX4: Asn^{129}-Phe-Ser, Asn^{133}-Tyr-Ser, Asn^{230}-Arg-Thr, and Asn^{236}-Ile-Ser (Fig. 1). The extent of glycosylation may be dependent on cell types; such a 75-kDa protein was not detected in COS-7 cells transfected with the NOX4 cDNA (Fig. 3*B*). To obtain experimental evidence that NOX4 is glycosylated, we attempted to digest the 75-kDa protein by *N*-glycosidase F, which was unsuccessful. Since the NOX4 protein appears to have a tendency to self-aggregate (Fig. 3*B*), targets for the glycosidase were possibly inaccessible.
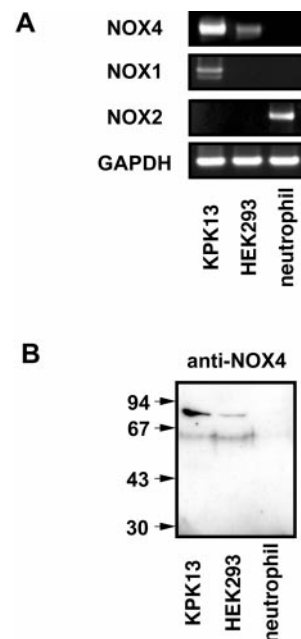

FIG. 4. **Expression of endogenous NOX4 in human cells.** *A*, RT-PCR using RNA from KPK13 cells, HEK293 cells, or human neutrophils, with specific primers for NOX1, NOX2, or NOX4. RT-PCR was performed using RNA (1.0 μg) from KPK13 cells, HEK293 cells, or human neutrophils, with specific primers for NOX1, NOX2, or NOX4. The PCR products were subjected to 1% agarose-gel electrophoresis, and stained with ethidium bromide. *B*, Western blot analysis of the membrane fraction of KPK13 cells, HEK293 cells, or human neutrophils, with the anti-NOX4 antibody. The membrane fraction (5.0 μg) of KPK13 cells, HEK293 cells, or human neutrophils was subjected to 10% SDS-PAGE, followed by immunoblot with the anti-NOX4 antibody. Positions for marker proteins are indicated in kilodaltons. For details, see "Experimental Procedures." The experiments have been repeated more than three times with similar results.

The final conclusion that NOX4 is glycosylated thus waits for further investigation.

Superoxide-producing NAD(P)H Oxidase Activity of NOX4—We searched for cultured cells expressing NOX4, and found that the human renal cell carcinoma KPK13 cells (32) and HEK293 cells express both mRNA and protein of NOX4 by RT-PCR using specific primers (Fig. 4*A*) and by Western blot using the anti-NOX4 antibody (Fig. 4*B*), respectively. Neither NOX1/Mox1 mRNA nor NOX2/gp91^{phox} mRNA was detected in HEK293 cells, while the former mRNA was expressed in KPK13 cells (Fig. 4*A*). In contrast with these cells, human neutrophils exclusively expressed NOX2 (Fig. 4*A*). Thus, HEK293 cells appear to be suitable for study on endogenous NOX4, since they express NOX4 but not other NOXs.

To investigate function of the NOX4 protein, we prepared the membrane fraction of HEK293 cells and assayed its superoxide-producing activity. We initially used the cytochrome *c* reduction assay (9, 11), which is the most reliable one for quantitation of amounts of superoxide produced, but is not highly sensitive to detect the anion. We could detect only small amounts of superoxide produced by the membrane fraction in the presence of NADH or NADPH: the rates were about or less than 0.5 nmol/min/10^6 cell equivalents of membrane, respectively, which are several 10-fold lower than the phagocyte NADPH oxidase activity in human neutrophil membranes (9, 11, 15).

We next assayed superoxide production by the chemiluminescence method, which is much more sensitive to superoxide than the cytochrome *c* assay, but cannot be used for absolute quantitation. As shown in Fig. 5*A*, the addition of NADH to the HEK293 membrane caused enhancement of chemilumines-
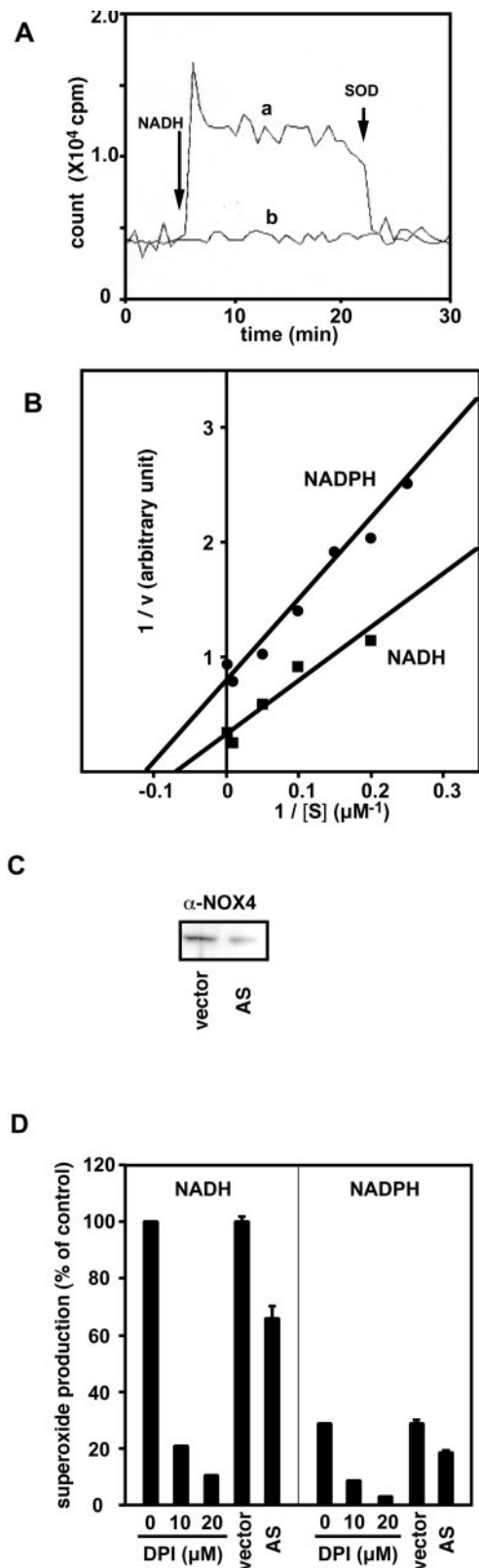
cence. The response was completely inhibited by SOD, indicating the involvement of superoxide. Analysis by this method further revealed that superoxide was also produced by NADPH; the maximal rate with NADH was approximately 2–3-fold higher than that with NADPH, whereas an apparent K_m value for NADH was rather higher than that for NADPH (13 μM for NADH and 9 μM for NADPH) (Fig. 5*B*). The results suggest that a superoxide-producing oxidase in HEK293 cells can use either NADH or NADPH as an electron donor. We also used the chemiluminescence method to assay superoxide production by human neutrophil membranes under cell-free activation conditions (9, 11, 15), and found that the superoxide producing activity is approximately 40- and 100-fold higher than those of HEK293 cell membranes with NADH and NADPH, respectively. This observation is consistent with that obtained by the cytochrome *c* reduction assay as described above.

Both NADH- and NADPH-dependent superoxide-producing activities of HEK293 cell membranes were blocked by the flavoprotein inhibitor diphenylene iodonium (Fig. 5*D*). The oxidase activities were not altered by the presence of the cytosolic fraction of HEK293 cells, by the addition of arachidonic acid, an *in vitro* activator of the phagocyte NADPH oxidase (15, 35), or by added recombinant p47*phox*, p67*phox*, and GTP-loaded Rac2, which are essential for the phagocyte oxidase activation (data not shown).

Transfection of HEK293 cells with a plasmid expressing an antisense mRNA for NOX4 decreased amounts of the NOX4 protein, as determined by Western blot analysis (Fig. 5*C*), and caused a significant decrease in both NADH- and NADPH-supported superoxide producing activities of the membrane (Fig. 5*D*), confirming the identity of NOX4 as a superoxide-producing NAD(P)H oxidase. This observation also indicates that the superoxide-producing NAD(P)H oxidase activity in the HEK293 membrane is at least partly due to NOX4.

Role of NOX4 in Cell Growth—Since it has been reported that overexpression of NOX1/Mox1 increases the growth rate of NIH3T3 cells (26), we estimated a role of NOX4 in cell proliferation. The cells did not express endogenous NOX4 as estimated by RT-PCR (data not shown) and Western blot (Fig. 6*A*). Transfection of NIH3T3 cells with the NOX4 cDNA led to expression of the NOX4 protein (Fig. 6*A*) and a slight but significant increase in NADH-dependent superoxide-producing activity in the membrane (Fig. 6*B*). Unexpectedly, overexpression of NOX4 resulted in a retarded growth of NIH3T3 cells (Fig. 6*B*), with no increase in apoptotic cells (data not shown). We also overexpressed the NOX4 protein in COS-7 and HEK293 cells, in both cases of which the cell proliferation rate was decreased to a similar extent (data not shown). On the other hand, the growth rate was not altered when NIH3T3 cells were transfected with the antisense NOX4 cDNA (Fig. 6*B*). The

Fig. 5. **Superoxide production by the membrane fraction of HEK293 cells.** *A*, NADH-dependent chemiluminescence by the membrane fraction of HEK293 cells. After preincubation of the membrane fraction of HEK293 cells (12.5 μg/ml) in the presence (*trace b*) or absence (*trace a*) of SOD (50 μg/ml) for 5 min, 500 μM NADH was added to the reaction mixture and chemiluminescence change was continuously monitored with an enhanced luminol-based substrate, Diogenes®. Where indicated, SOD was added at the final concentration of 50 μg/ml (*trace a*). *B*, Lineweaver-Burk plot for superoxide production. The membrane fraction was incubated with various concentrations of NADH (*closed squares*) or NADPH (*closed circles*), and the rate of

superoxide production was determined by SOD-inhibitable chemiluminescence. *C*, effect of expression of antisense NOX4 mRNA on its protein level. Membrane fractions were prepared from HEK293 cells transfected with pREP10-antisense-NOX4 (*AS*) or empty pREP10 vector (*vector*). The membrane proteins (10 μg) were subjected to 10% SDS-PAGE, followed by immunoblot with the anti-NOX4 antibody. *D*, superoxide-producing activity of the membrane fraction of HEK293 cells in the presence of 0.5 mM NADH (*left panel*) or NADPH (*right panel*). NADH- and NADPH-dependent superoxide-producing activities of the membrane fraction from HEK293 cells transfected with pREP10-antisense-NOX4 (*AS*) or empty pREP10 vector (*vector*) were determined as SOD-inhibitable chemiluminescence. For details, see "Experimental Procedures." When indicated, the membrane was preincubated with 10 or 20 μM diphenylene iodonium. Superoxide production is expressed as the percentage of activity relative to untransfected cells. Each *graph* represents the mean of data from six independent transfections, with *bars* representing the S.D. for percentage of activity.
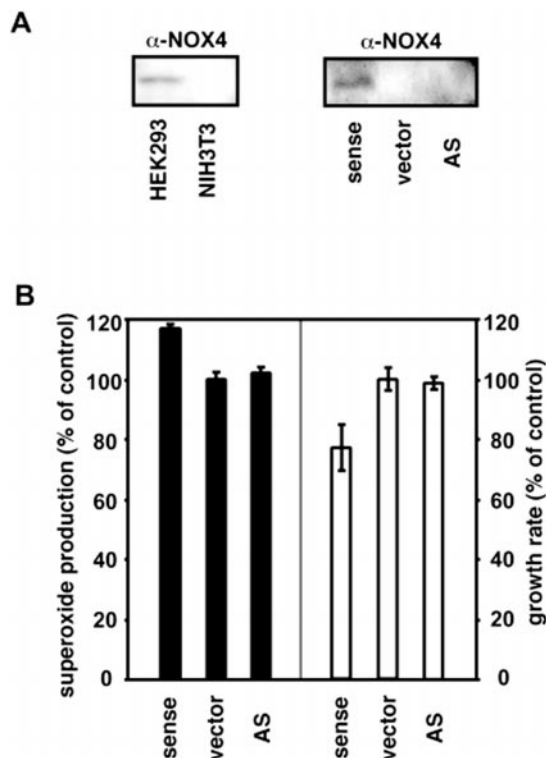
A

B

FIG. 7. **Northern blot analysis for expression of NOX4 mRNA in human tissues.** Human Multiple Tissue Northern® blots (CLON-TECH) were hybridized with ^{32}P-labeled human NOX4 cDNA fragments (corresponding to amino acids 324–491) under high stringency conditions using ExpressHyb® (CLONTECH). The results are representative of two independent blotting experiments. Positions of RNA molecular size markers are shown in kilobases.

FIG. 8. **Expression of the NOX4 protein in human renal cortex.** Immunohistochemical localization of anti-NOX4 antibody in human renal cortex. The sections were incubated with antisera to NOX4 (*B* and *C*) or preimmune sera (*A*), probed with biotinylated anti-rabbit IgG antibody, and reacted with HRP-conjugated streptavidin. The sites of HRP were visualized with diaminobenzidine and H_2O_2, and counterstained with hematoxylin for 2 min. For details, see "Experimental Procedures." The results are representative of three independent experiments.

FIG. 6. **Effect of NOX4 expression on cell growth.** *A*, expression of human NOX4 in NIH3T3 cells. NIH3T3 cells were transfected with pREP10-NOX4, pREP10-antisense NOX4, or empty pREP10 vector. The membrane fraction (10 μg) from untransfected NIH3T3 or HEK293 cells (*left panel*) or that from the transfected NIH3T3 cells (*right panel*) was subjected to 10% SDS-PAGE, followed by immunoblot with the anti-NOX4 antibody. *B*, effect for expression of NOX4 in NIH3T3 cells on NADH-dependent superoxide producing activity (*left panel*) and cell growth (*right panel*). For details, see "Experimental Procedures." Both superoxide-producing activity and cell growth rate of untransfected cells are set as 100%. Each *graph* represents the mean of data from four independent transfections, with *bars* representing the S.D. for percentage of activity.

oxidases NOX4 and NOX1 thus appear to have the opposite effect on regulation of cell growth.

Expression of Human NOX4—We finally studied expression of NOX4 in various human tissues. Northern blot analysis revealed that human NOX4 mRNA of about 2.4 kb is expressed almost exclusively in kidney among adult tissues tested, with faint signals in heart, skeletal muscle, and brain (Fig. 7). Human fetal kidney also abundantly expressed the message of NOX4 (Fig. 7). Thus, the tissue distribution of the NOX4 mRNA was totally different from those of NOX1 and NOX2; the former is most abundantly expressed in colon (26, 27), and the latter in phagocytic leukocytes (1–4).

To know which types of cells express the NOX4 protein in human kidney, we performed immunohistochemical analysis of samples of the renal cortex using antibodies against NOX4. Strong signals were observed in epithelial cells of distal tubules, whereas only faint signals can be detected in proximal tubules and glomeruli (Fig. 8). Thus, the NOX4 protein is most abundantly expressed in distal tubular cells in human kidney cortex.

DISCUSSION

In the present study, we describe a novel gp91[*phox*] homologue, designated NOX4, that is highly expressed in adult and fetal kidneys. As expected from its primary structure, NOX4 exhibits NADH- and NADPH-dependent superoxide producing activities. NOX4-expressing HEK293 cells show such oxidase activities, which are decreased when transfected with anti-
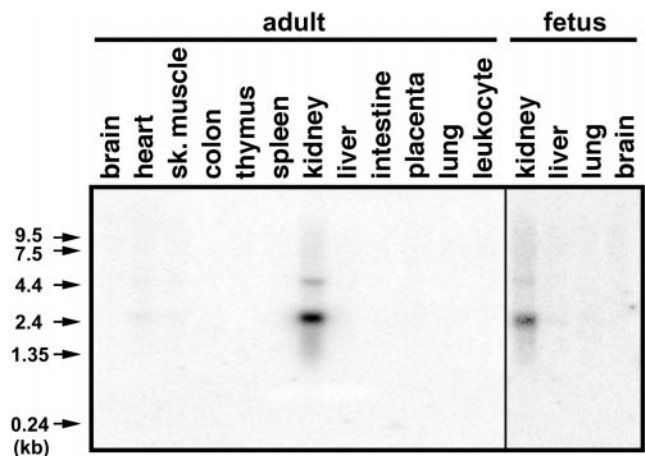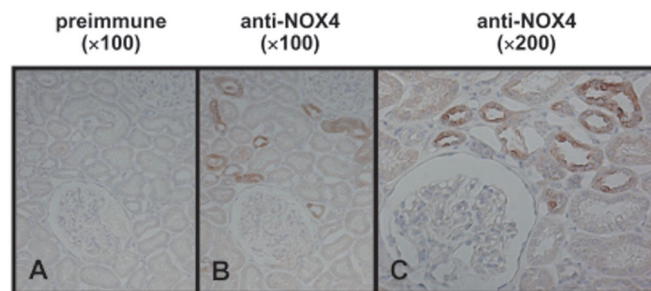
sense NOX4 cDNA; and overexpression of NOX4 in NIH3T3 cells leads to increased superoxide production.

Abundant expression of the NOX4 protein in distal tubular cells may suggest its function as an oxygen sensor for EPO synthesis. A current model for the oxygen sensing proposes that, dependent on oxygen tension, an NAD(P)H oxidase generates superoxide and its derivative ROS, which oxidatively destabilize HIF-1α, a dominant transcriptional activator of the gene for EPO, thereby decreasing expression of EPO (24, 36). EPO is generally considered to be produced by the peritubular interstitial cells in the renal cortex (24), that are adjacent to NOX4-expressing tubular cells and thus easily exposed to ROS produced by NOX4. Diphenylene iodonium, a flavoprotein inhibitor that blocks the oxygen sensing for EPO synthesis (24, 25), blocks superoxide production by NOX4, which is consistent with the idea of NOX4 as an oxygen sensor. In this context, it should be noted that the gene responsible for familial benign polycythemia (FBP), a rare autosomal recessive condition characterized by erythrocytosis usually with increased EPO production, is assigned by linkage analysis of affected families to the region of chromosome 11q23 (37), close to the locus of NOX4 (chromosome 11q14.2–21). It may be possible that the gene for FBP corresponds to the *NOX4* gene. If this is the case, increased levels of EPO in individuals with FBP can be explicable

by defective function of NOX4, a plausible oxygen sensor that negatively regulates production of the cytokine in kidney. This possibility is currently under investigation. On the other hand, the major organ of EPO synthesis in the fetus, *i.e.* liver, expresses a negligible amount of the NOX4 mRNA (Fig. 7). Another oxidase or a different oxygen-sensing system in the regulation of EPO expression might occur in fetal liver.

During the completion of this study, Geiszt *et al.* (38) reported the cloning of essentially the same cDNA as NOX4, designated Renox in their report, and presented that transfection of NIH3T3 cells with the cDNA leads to an increased production of superoxide at the cell level and a decreased rate of proliferation. They have also shown, by *in situ* hybridization, that Renox is expressed in proximal tubular cells of mouse kidney, whereas the present study demonstrates, using an antibody specific to NOX4, that the protein is predominantly present in distal tubular cells, not in proximal tubular cells, of human kidney (Fig. 8). The discrepancy may be due to difference in species or methods used in the two studies. In addition, Geiszt *et al.* (38) showed that mRNA of mouse Renox is exclusively detected in kidney as human NOX4 is (Fig. 8), whereas mouse NOX4 mRNA is expressed not only in kidney but also in liver, but to a lesser extent, as estimated by Northern blot analysis.[3] The reason for this discrepancy is presently unknown.

Also in the fetus, the NOX4 mRNA is abundantly expressed in kidney (Fig. 7), an organ that is not considered to highly produce EPO at this stage. In addition to a suggested function as an oxygen sensor, NOX4 possibly plays a role in other cellular events. One possibility is that NOX4 may participate in regulation of renal cell growth and/or death. ROS are known to be important mediators of many pathophysiological processes in renal diseases, probably by causing cell death via necrosis or apoptosis (29). The present study shows that overexpression of NOX4 results in decreased cell proliferation, although we did not detect an increase in apoptotic cells. Future studies should be directed to clarify the role of NOX4 in kidney under oxidative damage such as hypoxia/reoxygenation and ischemia/reperfusion injury. It is also possible that NOX4 may function as a proton channel; an alternatively spliced form of NOX1/Mox1 and NOX2/gp91^{phox} in the active phagocyte oxidase complex are likely involved in a proton channel (27). This possibility is currently under investigation in our laboratory.

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[3] J. Kuroda, A. Shiose, and H. Sumimoto, manuscript in preparation.

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A Novel Superoxide-producing NAD(P)H Oxidase in Kidney

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