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Cloning and characterization of human inducible nitric oxide synthase splice variants: A domain, encoded by exons 8 and 9, is critical for dimerization

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ABSTRACT **The inducible nitric oxide synthase (iNOS) contains an amino-terminal oxygenase domain, a carboxy-terminal reductase domain, and an intervening calmodulin-binding region. For the synthesis of nitric oxide (NO), iNOS is active as a homodimer. The human iNOS mRNA is subject to alternative splicing, including deletion of exons 8 and 9 that encode amino acids 242–335 of the oxygenase domain. In this study, $iNOS_{8-9}$ and full-length iNOS ($iNOS_{FL}$) were cloned from bronchial epithelial cells. Expression of $iNOS_{8-9}$ in 293 cell line resulted in generation of $iNOS_{8-9}$ mRNA and protein but did not lead to NO production. In contrast to $iNOS_{FL}$, $iNOS_{8-9}$ did not form dimers. Similar to $iNOS_{FL}$, $iNOS_{8-9}$ exhibited NADPH-diaphorase activity and contained tightly bound calmodulin, indicating that the reductase and calmodulin-binding domains were functional. To identify sequences in exons 8 and 9 that are critical for dimerization, $iNOS_{FL}$ was used to construct 12 mutants, each with deletion of eight residues in the region encoded by exons 8 and 9. In addition, two "control" iNOS deletion mutants were synthesized, lacking either residues 45–52 of the oxygenase domain or residues 1131–1138 of the reductase domain. Whereas both control deletion mutants generated NO and formed dimers, none of the 12 other mutants formed dimers or generated NO. The region encoded by exons 8 and 9 is critical for iNOS dimer formation and NO production but not for reductase activity. This region could be a potential target for therapeutic interventions aimed at inhibiting iNOS dimerization and hence NO synthesis.**

Nitric oxide (NO), an important signaling and cytotoxic molecule, is synthesized from L-arginine by isoforms of NO synthase (NOS), the constitutive endothelial and neuronal and the high-output inducible enzyme (1). The latter form, termed iNOS, is widely expressed in diverse cell types under transcriptional regulation by inflammatory mediators (1, 2). iNOS has been implicated in the pathogenesis of many diseases, including Alzheimer's disease, pulmonary tuberculosis, asthma, lung cancer, bacterial pneumonia, inflammatory bowel disease, arthritis, and septic shock (1, 3).

The human iNOS gene, containing 26 exons, encodes a protein of 131 kDa (4–6). Human iNOS, like all NOSs, has three domains: (*i*) an amino-terminal oxygenase domain (residues 1–504) that binds heme, tetrahydrobiopterin (H_4B) and L-arginine; (*ii*) a carboxy-terminal reductase domain (residues 537-1153) that binds FMN, FAD, and NADPH; and (*iii*) an intervening calmodulin (CaM)-binding domain (residues 505–536) that regulates electron transfer between the oxygenase and reductase domains (5–9). Whereas in the constitutive isoforms, CaM binds to the protein in response to an increase in calcium, CaM is coupled tightly to iNOS at basal calcium levels (10). For the synthesis of NO, iNOS is active as a homodimer in which the subunits align in a head-to-head manner, with the oxygenase domains forming a dimer and the reductase domains existing as independent monomeric extensions (7). For the regulation of NO synthesis, posttranslational subunit dimerization represents a potentially critical locus for therapeutic interventions.

Recently, it has been demonstrated that human iNOS is regulated, in part, by alternative mRNA splicing including deletion of exons 8 and 9 (11, 12). Exons 8 and 9 encode residues 242–335 of human iNOS (4). This region, although highly conserved among NOSs, has not been implicated in binding of iNOS cofactors or substrates. The studies reported here were aimed at the molecular cloning and functional characterization of the human iNOS splice variant with deletion of exons 8 and 9 ($iNOS_{8-9}$) from bronchial epithelium of normal volunteers. They revealed that the domain encoded by exons 8 and 9 is critical for iNOS dimer formation and hence for NO synthesis.

MATERIALS AND METHODS

Isolation of Full-Length Human iNOS ($iNOS_{FL}$) and $iNOS_{8-9}$ Splice Variants cDNAs from Normal Human Bronchial Epithelial Cells. Informed consents, approved by the Institutional Review Board, were obtained from normal, nonsmoking volunteers. Bronchial epithelial cells were obtained by using a standard cytology brush and fiberoptic bronchoscopy (13). Total RNA was extracted from bronchial epithelial cells by the guanidinium thiocyanate-CsCl gradient method. cDNA was transcribed from RNA by using Molony murine leukemia virus RNase H^- reverse transcriptase and oligo (dT) 12–18 primer (Life Technologies, Gaithersburg, MD). Full-length human iNOS cDNAs were generated by using "long" PCR (r*Tth* DNA polymerase XL; Perkin–Elmer) (14). The primers HINO29 in exon 1 (5′-ATAACTTTGTAGCGAGTCGAAAACTG-3′) and INH10 in exon 26 (5′-GATTAAAGTAAAATGCAATTCATGTA-3′) were used to span the entire iNOS coding region in addition to 264 bp of the 5′-untranslated region and 477 bp of the 3′-untranslated region (4). The "hot start" technique was employed by using *TaqStart* antibody (CLONTECH), and amplification was carried out for 35 cycles of 95°C/30 s, 55°C/30 s, and 72°C/5 min. By using PCR amplification products, a human bronchial epithelium

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Abbreviations: NOS, nitric oxide synthase; iNOS, inducible NOS; $iNOS_{FL}$, full-length iNOS; $iNOS_{8-9}$, iNOS with deletion of exons 8 and 9; CaM, calmodulin; H_4B, tetrahydrobiopterin; HEK, human embryonic kidney.

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iNOS-specific cDNA library was constructed in pBluescript II SK M13 (+) phagemid (Stratagene). Phagemid inserts were subjected to sequence analysis by using double-stranded DNA cycle sequencing (Life Technologies).

Cell Culture. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection), were cultured at 37°C in 5% CO_2 in IMEM medium (Biofluids, Rockville, MD) supplemented with 2 mM glutamine, 25 μg/ml gentamycin, and 10% heat-inactivated, filtered (40-nm filter) fetal bovine serum (HyClone).

Transfection and Cell Lysis. $iNOS_{FL}$ or $iNOS_{8-9-}$ cDNA was inserted into the expression vector pRc/CMV (Invitrogen) under the control of the cytomegalovirus promoter. Cationic lipid-mediated transient transfection was done in 100-mm diameter tissue culture plates by using 8 μg of the desired DNA and 48 μl of Lipofectamine (Life Technologies) following the manufacturer's instructions. After 23 h, medium was collected for nitrite measurements. After gentle rinsing with PBS, the cell layer was lysed on ice for 45 min in 40 mM 1,3-bis[tris(hydroxymethyl)methylamino] propane buffer (pH 7.7), 150 mM NaCl, and 10% glycerol with 25 mM of the detergent sodium taurocholate and in the presence of protease inhibitors [phenylmethylsulfonyl fluoride (1 mM), pepstatin A (10 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml), phenanthroline (10 μg/ml), and benzamidine HCl (16 μg/ml)] (PharMingen, San Diego, CA). Lysates were centrifuged (16,000 $\times$ *g*, 5 min, 4°C) and supernatants were stored at −80°C. Total protein concentrations were determined by bicinchoninic acid reagent (Pierce) using BSA as a standard. To produce an HEK 293 cell line with stable expression of human iNOS, transfection was done by using the calcium phosphate method with positive colony selection at 600 μg/ml of G418 (Life Technologies).

Northern Analysis. Total RNA (10 μg/lane), extracted from transfected HEK 293 cells by the guanidinium thiocyanate-CsCl gradient method, was subjected to gel electrophoresis by using 1.1% formaldehyde-agarose and was transferred to nylon membranes that were hybridized with a ^{32}P-labeled 0.582-kb fragment of human iNOS (exons 1–4).

Antibodies. 1E8-B8 (IgG1 κ) and 21C10–1D10 (IgG2b κ) (Research and Diagnostic Antibodies, Berkeley, CA) are two mAbs raised against purified recombinant human iNOS and are specific for the iNOS isoform (3). They were used as culture supernatants following manufacturer's instructions. Anti-iNOS rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), raised against intact iNOS purified from RAW 264.7 cells (2), was used as an IgG-purified fraction. M2 is a FLAG epitope-specific mAb (Eastman Kodak).

Low-Temperature (Partially Denaturing) SDS/PAGE/Western Analysis. Lysates (50 μg) from transfected HEK 293 cells were incubated at 37°C for 30 min in the presence of 2 mM L-arginine and 0.1 mM H_4B. Lysates then were mixed with one-third volume of 4X Laemmli sample buffer stock solution (200 mM Tris·HCl, pH 6.8/8% SDS/0.004% bromophenol blue/40% glycerol/400 mM DTT), and immediately were subjected to SDS/PAGE at 4°C (15–17). Precast 4% gels (NOVEX, San Diego) were used, and electrophoresis was performed at a constant 200 V. Temperature throughout electrophoresis was controlled thermostatically by circulating the running buffer through a high-efficiency heat exchanger at a high flow rate (ThermoFlow System; NOVEX) connected to an external recirculating chiller (Isotemp 1016; Fisher Scientific). Prestained molecular mass standards (Bio-Rad) were used. After SDS/PAGE, proteins were transferred to nitrocellulose membranes (0.2 μm pore size; Schleicher & Schuell) by using a semi-dry transfer method (TRANS-BLOT SD; Bio-Rad). The membrane was blocked in 5% goat milk in PBS/0.1% Tween 20 and incubated with 1E8-B8 mAb. A goat anti-mouse IgG conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) was used as a secondary antibody. An enhanced chemiluminescence system was used for detection (Amersham).

SDS/PAGE/Western Analysis (Fully Denaturing Conditions). SDS/PAGE/Western was performed as above except that prior incubation of samples with L-arginine and 0.1 mM H_4B was omitted; samples were boiled in the presence of Laemmli sample buffer for 5 min and electrophoresis was performed at room temperature.

NOS Activity. NOS activity was determined for intact cells and their lysates. For intact cells, 400 μl of culture medium was mixed with 400 μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in water) for 10 min at room temperature, and absorbance at 543 nm was recorded (18). Serial dilutions of sodium nitrite were used as standards. For enzyme assays, transfected cell lysates (100 μg) were mixed in 100 μl reaction volume of 30 mM Tris·HCl (pH 7.9), 2 mM L-arginine, 2 mM NADPH, 4 μM FAD, 4 μM FMN, 4 μM H_4B, and 3 mM DTT. After incubation at 37°C for 3 h, 10 μl of lactic dehydrogenase (Sigma; 1:25 dilution in 500 mM sodium pyruvate) were added, followed 15 min later by Griess reagent. Nitrite then was assayed at 540 nm in a microplate reader (17, 19).

Gel Permeation Chromatography. Transfected HEK 293 cells were lysed by sonication in elution buffer {40 mM 1,3-bis[tris(hydroxymethyl)methylamino] propane (pH 7.7), containing 3 mM DTT, 1 mM L-arginine, 4 μM H_4B, 4 μM FAD, 10% glycerol, 150 mM NaCl, and antiproteases}. Size exclusion chromatography was carried out at 4°C by using a Pharmacia Superdex 200 HR 10/30 with exclusion limit of 1.3×10^6 M_r and a fast performance liquid chromatography (FPLC) system (Pharmacia). The column was equilibrated at 0.5 ml/min with elution buffer. Equal amounts of protein (0.4–0.6 mg) from cell lysates were injected in 100-μl sample volume, and the protein in the column effluent was monitored at 280 nm by using a flow-through detector. Collected fractions were stored immediately at −80°C for further analysis. The column was calibrated with the following M_r standards (Pharmacia): thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000), albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700) (19).

Immunoprecipitation. Lysates of transfected HEK 293 cells (1–1.5 mg) were incubated with anti-iNOS polyclonal antibody. After 90 min at 4°C, protein A-Sepharose beads (Pharmacia) were added to the samples. After further incubation for 1 h at 4°C, beads were washed three times in lysis buffer. For Western analysis, immunoprecipitated proteins were eluted by boiling for 5 min in Laemmli sample buffer and analyzed by SDS/PAGE, followed by immunoblotting (17).

NADPH-Diaphorase Activity. NADPH-diaphorase activity was assayed by measuring the reduction of 0.5 mM nitro-blue tetrazolium with 1 mM NADPH in 100 μl of 50 mM Tris·HCl (pH 8.0). After incubation at 37°C for 8 min, the reaction was stopped with 100 μl of 100 mM sulfuric acid, and the absorbance was determined at 570 nm in a microplate reader (20).

Calmodulin Binding. Immunoprecipitated proteins, eluted by boiling in Laemmli buffer, were analyzed by SDS/PAGE followed by immunoblotting with a mAb against CaM (Upstate Biotechnology) (10, 17, 21).

Mutagenesis. Site-specific oligonucleotide-directed mutagenesis was performed using the QuikChange mutagenesis system (Stratagene). All products were confirmed by DNA sequence analysis.

RESULTS

Cloning and Expression of Human iNOS Splice Variants. By using long PCR and cDNA from bronchial epithelial cells

of normal volunteers, a human bronchial epithelium iNOS-specific cDNA library was generated. Screening of this library led to isolation of full-length clones for $iNOS_{8-9-}$ and for iNOS containing all 26 exons ($iNOS_{FL}$). Sequence analysis of $iNOS_{8-9-}$ cDNA was consistent with an in-frame deletion of amino acids 242 through 335. Northern analysis revealed that cells transfected with cDNAs encoding either $iNOS_{FL}$ or $iNOS_{8-9-}$ expressed iNOS mRNA (Fig. 1*A*). As expected, $iNOS_{8-9-}$ mRNA migrated slightly faster than that of $iNOS_{FL}$. Transfected cell lysates were incubated with L-arginine and H_4B and subjected to low-temperature SDS/PAGE followed by an iNOS immunoblot (16, 17). In $iNOS_{FL}$-transfected cells, a band that corresponded in molecular mass to an iNOS monomer was seen (marked "monomer") together with an additional, more slowly migrating band (marked "dimer"). In contrast, in lysates from $iNOS_{8-9-}$-transfected cells, only monomers that migrated slightly faster than $iNOS_{FL}$ monomers were detected (Fig. 1*B*). Similar to $iNOS_{FL}$, $iNOS_{8-9-}$ was detected by Western analysis in soluble and particulate fractions of lysates of transfected HEK 293 cells (data not shown). Nitrite accumulation in the culture medium of transfected cells (Fig. 2*A*) and the enzyme activity of cell lysates (Fig. 2*B*) indicated that only cells transfected with $iNOS_{FL}$ exhibited NOS activity; $iNOS_{8-9-}$ transfected cells, in that respect, did not differ from cells transfected with vector only.

NADPH-Diaphorase Activity. To evaluate whether $iNOS_{8-9-}$ retains NADPH-diaphorase activity, $iNOS_{FL}$ and $iNOS_{8-9-}$ were immunoprecipitated from transfected cells with anti-iNOS mAb 21C10–1D10. The presence of $iNOS_{FL}$ and $iNOS_{8-9-}$ in the immunoprecipitate was confirmed by immunoblotting with anti-iNOS mAb 1E8-B8 (Fig. 3*A*). Immunoprecipitates from cells transfected with $iNOS_{FL}$ or $iNOS_{8-9-}$ showed markedly greater NADPH-diaphorase activity than those from cells transfected with vector only (Fig. 3*B*).

Calmodulin Binding. By using anti-iNOS polyclonal antibody for immunoprecipitation, CaM was detected in immunoprecipitates of HEK 293 cells transfected with $iNOS_{FL}$ or $iNOS_{8-9-}$ but not with vector alone (Fig. 4), indicating that CaM coimmunoprecipitated with both $iNOS_{FL}$ and $iNOS_{8-9-}$ in the absence of exogenously added calcium.

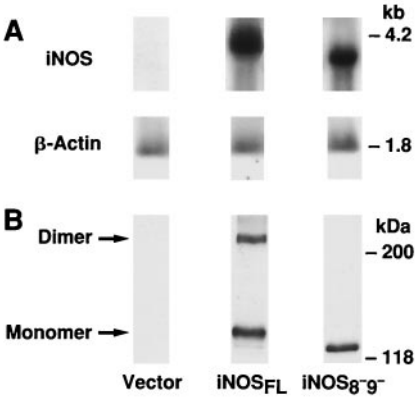

FIG. 1. Expression of human $iNOS_{FL}$ and $iNOS_{8-9-}$ splice variants. HEK 293 cells were transfected with plasmids containing vector-only, $iNOS_{FL}$ or $iNOS_{8-9-}$ cDNA and were analyzed 23 h later. (*A*) Total RNA prepared from HEK 293 cells was subjected to Northern analysis by using a ^{32}P-labeled 0.582-kb fragment of human iNOS (exons 1–4) as a probe. Human ^{32}P-labeled β-actin cDNA hybridization is shown as a control. (*B*) Lysates (50 μg) from transfected HEK 293 cells were incubated at 37°C for 30 min in the presence of 2 mM L-arginine and 0.1 mM H_4B and were subjected to SDS/PAGE in 4% gel at 4°C and immunoblotting with anti-human iNOS mAb 1E8-B8. Positions of monomer and dimer are indicated. Experiments were repeated three times with similar results.

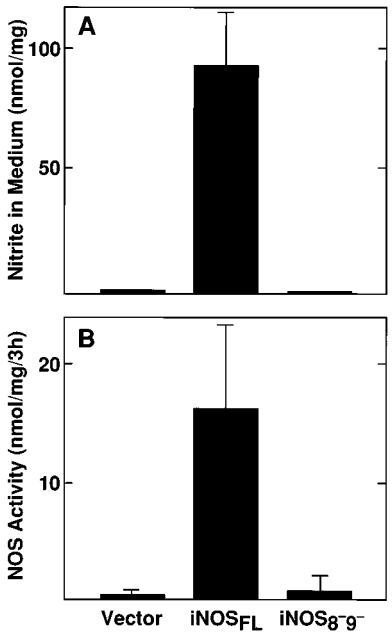

FIG. 2. Enzyme activity of $iNOS_{FL}$ and $iNOS_{8-9-}$ in transfected HEK 293 cells. (*A*) Nitrite accumulation (23 h) in the culture medium, after transfection with vector-only, $iNOS_{FL}$ or $iNOS_{8-9-}$, is expressed as nanomoles per milligram of total cell protein. (*B*) NOS activity in lysates of transfected cells expressed as nanomoles of nitrite per milligram of total cell protein per 3 h. Data are means ± SD of three independent experiments, each done in duplicate. Invisible error bars are too small to be drawn.

Size Analysis by Gel Permeation Chromatography. Lysates of transfected cells were subjected to size exclusion chromatography under conditions that maximize iNOS dimer formation (19). As expected, $iNOS_{FL}$ eluted mostly as a dimer with maximal NOS activity at ≈266 kDa (Fig. 5). Surprisingly, $iNOS_{8-9-}$ eluted more rapidly than $iNOS_{FL}$, consistent with a higher molecular mass than expected for a dimer; its peak NADPH-diaphorase activity corresponded to a molecular mass of ≈807 kDa (6–7 subunits) (Fig. 5). None of the fractions containing $iNOS_{8-9-}$ had detectable NOS enzymatic

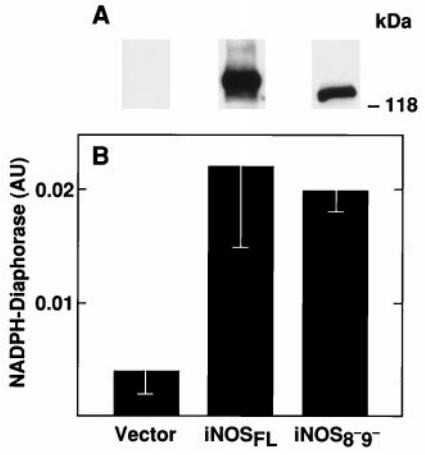

FIG. 3. NADPH-diaphorase activity of $iNOS_{FL}$ and $iNOS_{8-9-}$. Lysates from HEK 293 cells transfected with vector-only, $iNOS_{FL}$ or $iNOS_{8-9-}$ were immunoprecipitated with anti-human iNOS mAb 21C10–1D10 together with protein A-Sepharose. An aliquot of the immunoprecipitate was used for Western analysis with anti-human iNOS mAb 1E8-B8 (*A*). The remainder was evaluated for NADPH-diaphorase activity by measuring the reduction of nitro-blue tetrazolium with NADPH at 570 nm (*B*). Data are means ± SD of three independent experiments, each done in duplicate.

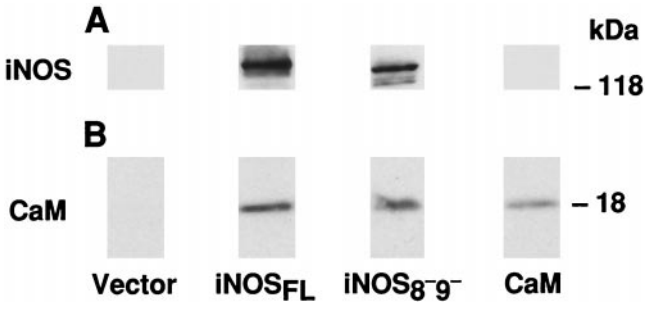

FIG. 4. Calmodulin binding by $iNOS_{FL}$ and $iNOS_{8-9-}$. Lysates from HEK 293 cells transfected with vector-only, $iNOS_{FL}$ or $iNOS_{8-9-}$ were immunoprecipitated with anti-iNOS polyclonal antibody and then were subjected to Western analysis with anti-human iNOS mAb 1E8-B8 (*A*) or with a mAb against calmodulin (*B*). The lane with CaM contains authentic calmodulin. Experiments were repeated three times with similar results.

activity (data not shown). Additionally, when cell lysis and gel permeation chromatography were performed in the presence of 25 mM sodium taurocholate (detergent), similar molecular masses were found for $iNOS_{FL}$ (265 kDa) and for $iNOS_{8-9-}$ (805 kDa). Similar results were obtained when samples of transfected cell lysates with varying total protein concentrations were used (data not shown).

Coexpression of $iNOS_{FL}$ and $iNOS_{8-9-}$. HEK 293 cells stably transfected with human $iNOS_{FL}$ cDNA were transiently transfected with either vector alone or with $iNOS_{8-9-}$. There was no significant difference in NO generation, monitored by nitrite accumulation, between the two conditions (data not shown). Further, in similar experiments, a plasmid containing $iNOS_{8-9-}$ with a FLAG epitope tag fused to its carboxy terminus was expressed in the HEK 293 stably expressing $iNOS_{FL}$, and protein was monitored with anti-FLAG epitope M2 mAb. No heterodimers between $iNOS_{FL}$ and $iNOS_{8-9-}$ were detected on immunoblots of low-temperature SDS/PAGE, and no $iNOS_{FL}$ was detected by immunoblotting after immunoprecipitation of $iNOS_{8-9-}$ with M2 antibody (data not shown).

Characterization of iNOS Deletion Mutants. Human $iNOS_{8-9-}$ lacks amino acids 242 through 335. In an attempt to identify specific sequences in exons 8 and 9 that are critical for dimerization, human $iNOS_{FL}$ cDNA was used as a template to construct 12 deletion mutants, each with deletion of a sequence coding for 8 amino acids between iNOS amino acids 241 and 336. To validate the experimental procedure used, two additional control iNOS deletion mutants were synthesized lacking either amino acids 45–52 or 1131–1138. These control deletions were chosen from among the less conserved residues in the oxygenase and reductase domains, respectively. In transfected cells, both control deletion mutants generated NO and formed dimers detectable by immunoblotting after low-temperature SDS/PAGE (Fig. 6). In contrast, all 12 mutants with sequence deletions in exon 8 or 9 failed to form dimers or generate NO. Using transfected cell lysates, the inability of iNOS deletion mutants to form dimers was further confirmed by gel permeation chromatography for $iNOS_{\Delta 241-248}$, $iNOS_{\Delta 265-272}$, $iNOS_{\Delta 289-296}$, and $iNOS_{\Delta 313-320}$. All four iNOS deletion mutants tested had molecular masses higher than expected for dimers, consistent with multimers (6–7 subunits) and similar to those obtained for $iNOS_{8-9-}$ (data not shown).

DISCUSSION

In this study, a full-length human $iNOS_{8-9-}$ cDNA cloned from bronchial epithelial cells obtained from normal subjects was characterized functionally in HEK 293 cells, a mammalian cell line in which endogenous NOS genes are not expressed (17). A full-length human iNOS cDNA containing all 26 exons was cloned similarly and was included in all experiments as a control. $iNOS_{8-9-}$ did not generate NO, possibly because of its inability to assemble into a dimer, as monitored by SDS/PAGE done under partially denaturing conditions (16, 17). Using this method, $iNOS_{8-9-}$ migrated only as a monomer and could not form dimers even after incubation with L-arginine

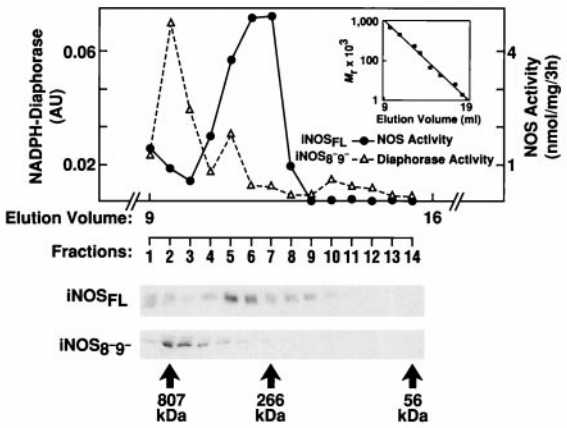

FIG. 5. Gel permeation chromatography of $iNOS_{FL}$ and $iNOS_{8-9-}$. Lysates of HEK 293 cells, transfected with $iNOS_{FL}$ or $iNOS_{8-9-}$, were subjected to fast performance liquid chromatography using a Superdex 200 gel permeation column in the presence of 1 mM L-arginine, 4 μM H_4B, 4 μM FAD, and 3 mM DTT. Calibration with M_r standards (13,700–699,000) is shown (*Inset*). Fractions eluted between 9 and 15.5 ml were assayed for NOS and NADPH-diaphorase activity and were examined by Western analysis with anti-human iNOS mAb 1E8-B8. Lysates of $iNOS_{FL}$-transfected cells exhibited NOS activity that peaked around 266 kDa, representing dimeric iNOS. Lysates of cells transfected with $iNOS_{8-9-}$ exhibited no detectable NOS activity (data not shown), and both its peak NADPH-diaphorase activity and NOS protein detection by immunoblotting were consistent with a molecular mass higher than expected for a dimer. Arrows indicate calculated molecular mass of the corresponding fractions. Experiments were repeated three times with similar results.

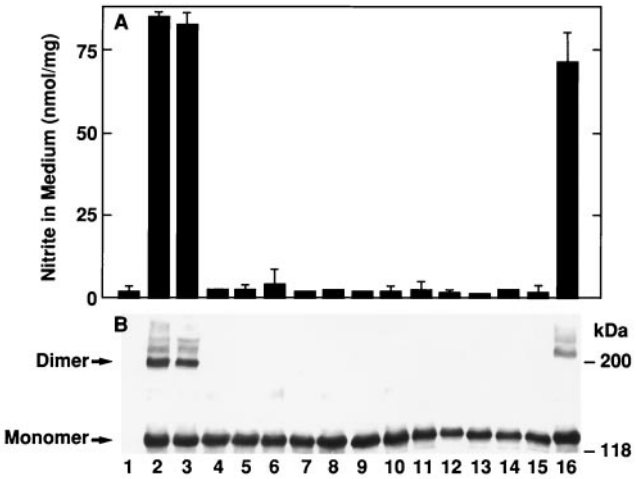

FIG. 6. Dimer formation and activity of $iNOS_{FL}$ and iNOS deletion mutants. HEK 293 cells were transfected with vector only (lane 1), $iNOS_{FL}$ (lane 2), or iNOS deletion mutants (Δ45–52, Δ241–248, Δ249–256, Δ257–264, Δ265–272, Δ273–280, Δ281–288, Δ289–296, Δ297–304, Δ305–312, Δ313–320, Δ321–328, Δ329–336, Δ1131–1138) depicted in lanes 3–16, respectively. NOS activity was assessed by nitrite accumulation (23 h) in the culture medium (*A*). Lysates (50 μg) were incubated at 37°C for 30 min in the presence of 2 mM L-arginine and 0.1 mM H_4B and were subjected to SDS/PAGE in 4% gel at 4°C followed by immunoblotting with anti-human iNOS mAb 1E8-B8 (*B*). Positions of monomer and dimer are indicated. Data represent three independent experiments. Nitrite data are means ± SD. Invisible error bars are too small to be drawn.

and H_4B at concentrations reported to maximize NOS dimer formation (7, 16, 19). Residues 242–335, encoded by exons 8 and 9, lie distal to the residue critical for heme binding (Cys^{200}) (17) and proximal to the critical residues for binding of L-arginine (Glu^{377}) (22) and H_4B (Gly^{456}, Ala^{459}) (23). Because dimerization is a prerequisite for binding of heme, L-arginine, and H_4B (7, 24), the impact of the lack of residues 242–335 in $iNOS_{8-9-}$ on their binding cannot be assessed. Because the oxygenase domain is responsible for maintaining the dimeric structure of iNOS (7), lack of amino acids 242–335 seems to render the domain inactive in that respect.

Recently, iNOS purified from human neutrophils was reported to be primarily membrane-associated (25). In transfected cell lysates, both $iNOS_{8-9-}$ and $iNOS_{FL}$ were recovered from both soluble and particulate fractions, suggesting that $iNOS_{8-9-}$ had a subcellular distribution similar to $iNOS_{FL}$. Sodium taurocholate was used to solubilize membrane-bound iNOS after our preliminary experiments showed it to be more effective in solubilizing and maintaining activity of human iNOS than other detergents {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (20 mM), Big 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (20 mM), Triton X-100 (1%), deoxycholate (0.8%), sodium cholate (40 mM), N-octyl-β-D-glucopyranoside (100 mM)}. In addition, the relatively low molecular mass of its micelles made it ideal for size analysis by gel permeation chromatography.

In addition to NO synthesis, all three types of NOS, through their reductase domain, exhibit an NADPH-diaphorase activity by transferring electrons derived from NADPH with nitroblue tetrazolium as an electron acceptor (20, 26). Like $iNOS_{FL}$, $iNOS_{8-9-}$ exhibited NADPH-diaphorase activity, indicating that its reductase domain was functionally active and independent of dimer formation. This finding confirms prior *in vitro* observations made with isolated proteolytically generated hemimers of iNOS that the reductase domain does not participate in subunit interactions and can exist and function independently (7, 24).

Calmodulin regulates NOS activity by a dual mechanism. In addition to its role in facilitating transfer of NADPH-derived electrons from the reductase domain to the oxygenase domain for NO synthesis (9), CaM activates the reductase domain directly by increasing the rate of electron transfer from NADPH to the flavin centers (27). Like $iNOS_{FL}$, $iNOS_{8-9-}$ had tightly bound CaM at basal cell Ca^{2+} concentration. Because $iNOS_{8-9-}$ does not synthesis NO, the function of CaM could be to enhance the reductase domain catalytic activity. The ability of $iNOS_{8-9-}$ to use NADPH and to bind CaM at trace levels of calcium implies an intact tertiary structure for the reductase domain and for the CaM-binding domain in the middle of the primary sequence.

Under native conditions, $iNOS_{8-9-}$ is multimeric, as revealed by gel permeation chromatography. No $iNOS_{8-9-}$ monomers were detected, indicating that $iNOS_{8-9-}$ monomers seen on SDS/PAGE arose from multimer dissociation. It may be inferred further that an intact oxygenase domain is required for the noted stability of $iNOS_{FL}$ dimers on low temperature SDS/PAGE. Formation of multimers by $iNOS_{8-9-}$ and its inability to dimerize suggest that the lack of residues 242–335 perturbs the tertiary structure of the oxygenase domain necessary for forming a dimer interface and/or proper folding of this domain. So, $iNOS_{8-9-}$ could be an example of an iNOS protein in which the tertiary structure is perturbed in one part of the protein while the rest of the protein remains intact. A similar phenomenon was found with a carboxy-terminal iNOS mutant that exhibited normal dimerization and normal binding of heme and CaM, indicating that the amino-terminal oxygenase domain and the middle portion of the protein were intact functionally but could not synthesize NO because of its inability to bind NADPH, resulting from deviant tertiary structure of the carboxy-terminal reductase domain (28).

Because $iNOS_{8-9-}$ does not produce NO, it is not clear what biological function this splice variant may serve. Although endogenous $iNOS_{8-9-}$ mRNA constitutes a minor fraction of total iNOS, amounting to 38% in bronchial epithelium, it is distributed widely in human tissues and in various cell types (11). Further, its expression is regulated in a tissue-specific manner and is up-regulated in response to cytokines (11). A similar pattern of alternative splicing of mRNA with an in-frame deletion of 105 amino acids in the oxygenase domain has been reported for mouse and human neuronal NOS (29, 30) as well as for NOS cloned from *Drosophila* (31). The presence of a conserved pattern of alternative splicing in vertebrates and flies and among various NOSs suggests a common function for the resulting isoforms. Because $iNOS_{8-9-}$ retains reductase domain activity, its function could be to transfer electrons derived from NADPH to a yet unidentified electron acceptor. Alternatively, the function of $iNOS_{8-9-}$ could be to form heterodimers with $iNOS_{FL}$, thus modulating its function. In our transfected cells, however, we could not find evidence for heterodimer formation or for modulation of NO production when $iNOS_{8-9-}$ was coexpressed with $iNOS_{FL}$. These experiments, however, are constrained inherently by the degree of "co"-expression and the sensitivity of detection.

Human iNOS has 51% and 68% deduced amino acid sequence identity with endothelial and neuronal NOS, respectively (6). Residues 242–335 of human iNOS, encoded by exons 8 and 9, are conserved significantly, with 68% and 73% identity to endothelial and neuronal NOS, respectively. They have no sequence similarity to other known proteins or specific sequence motifs. To our knowledge, however, these residues have not been implicated in binding of any cofactors or substrates of iNOS, and no mutagenesis analysis of these residues has been reported. In an attempt to identify which of these residues are critical in maintaining an intact oxygenase domain and dimer formation, 12 deletion mutants of $iNOS_{FL}$, each lacking 8 of the residues 241–336, were characterized. In addition, two iNOS deletion mutants were used as controls, each lacking eight amino acids within less conserved regions (6, 31), either in the oxygenase or in the reductase domains. Inability to synthesize NO or to dimerize, together with the tendency for multimer formation, were seen only in mutants lacking residues encoded by exons 8 and 9, suggesting that these residues are critical for the formation of a structurally and/or functionally intact oxygenase domain that is required for dimer formation.

Recently, the crystal structures for mouse iNOS residues 115–498 of the oxygenase domain corresponding to residues 121–504 of human iNOS have been determined (8). They reveal an elongated, curved molecule with an unusual nonmodular, single-domain α–β fold that resembles a baseball catcher's mitt for the left hand. The structure reveals that the cognate residues for 242–335 of human iNOS are surprisingly closer to the active catalytic site than could be predicted from the primary sequence. Further, these residues partially contribute to form the "palm and fingers" of a baseball catcher's mitt through the formation of six-stranded β wings. Long helical hairpins cap both ends of the winged β sheet, and a long α helix runs lengthwise between these hairpins. In addition, an antiparallel β strand composed of three segments zigzags across the length of the β structure. These structures partially contribute, both to the distal pocket of heme site and to the proposed dimer interface. Although the crystal structures were reported only for part of mouse iNOS, they are consistent with the strategic structural/functional importance of residues 242–335 inferred from our data. Further studies will dissect the structural and functional elements in these residues that control dimer formation and hence NO synthesis. This information should be valuable in designing therapeutic agents to regulate NO production under physiological and pathological conditions.

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