Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages

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Kuncewicz, Teresa, Priya Balakrishnan, Mark B. Snuggs, and Bruce C. Kone. Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. Am J Physiol Renal Physiol 281: F326–F336, 2001.—Nitric oxide synthase-2 (NOS2) is responsible for high-output nitric oxide production important in renal inflammation and injury. Using a yeast two-hybrid assay, we identified Rac2, a Rho GTPase member, as a NOS2-interacting protein. NOS2 and Rac2 proteins coinmunoprecipitated from activated RAW 264.7 macrophages. The two proteins colocalized in an intracellular compartment of these cells. Glutathione-S-transferase (GST) pull-down assays revealed that both Rac1 and Rac2 associated with GST-NOS2 and that the NOS2 oxygenase domain was necessary and sufficient for the interaction. [35S]methionine-labeled NOS2 interacted directly with GST-Rac2 in the absence of GTP, calmodulin, or NOS2 substrates or cofactors. Stable overexpression of Rac2 in RAW 264.7 cells augmented LPS-induced nitrite generation (∼60%) and NOS2 activity (∼45%) without measurably affecting NOS2 protein abundance and led to a redistribution of NOS2 to a high-speed Triton X-100-insoluble fraction. We conclude that Rac1 and Rac2 physically interact with NOS2 in activated macrophages and that the interaction with Rac2 correlates with a posttranslational stimulation of NOS2 activity and likely its spatial redistribution within the cell.

NOS2 substrates or cofactors. Stable overexpression of Rac2 in RAW 264.7 cells augmented LPS-induced nitrite generation (∼60%) and NOS2 activity (∼45%) without measurably affecting NOS2 protein abundance and led to a redistribution of NOS2 to a high-speed Triton X-100-insoluble fraction. We conclude that Rac1 and Rac2 physically interact with NOS2 in activated macrophages and that the interaction with Rac2 correlates with a posttranslational stimulation of NOS2 activity and likely its spatial redistribution within the cell.

The free radical nitric oxide (NO) plays critical roles in numerous physiological and pathophysiological processes. In the kidney, NOS2-derived NO appears to play important roles in the evolution of ischemic acute renal failure, glomerulonephritis, and acute tubulointerstitial nephritis (see Ref. 22 for review). NO is synthesized from L-arginine by the family of NO synthases (NOS). The three types of NOS share a bidomain structure consisting of an NH2-terminal oxygenase domain, which binds heme, tetrahydrobiopterin, and L-arginine, and a COOH-terminal reductase domain that binds FAD, flavin adenine mononucleotide (FMN), and NADPH (40). NOS1 and NOS3 are basally expressed in selected tissues and are principally activated by intracellular Ca2+ transients that promote calmodulin binding sufficiently to produce low amounts of NO for signaling purposes (4). In contrast, expression of NOS2 generally requires provocation with immunomodulatory agents, such as bacterial lipopolysaccharide (LPS) and/or certain cytokines that activate de novo synthesis of the enzyme. Once formed, NOS2 sustains catalysis because calmodulin is tightly bound even at resting levels of intracellular Ca2+. In humans, NOS2 expression is most readily detected in monocytes or macrophages from patients with infectious and/or inflammatory diseases (31, 49). The large amounts of NO generated by NOS2 have been implicated in inflammation, host defense, tissue injury, and the profound hypotension accompanying sepsis (22, 27).

Because of its importance in cell injury and inflammation, the mechanisms governing the biosynthesis and function of NOS2 have been the subject of intense investigation. A complex array of transcriptional and posttranscriptional regulatory controls on NOS2 expression and activity have been described, including influences on transactivation of the NOS2 gene, NOS2 mRNA stability, substrate and cofactor binding and availability, and dimerization (27). For NOS1 and NOS3, interactions with heterologous proteins in addition to calmodulin have been shown to influence the activity or intracellular distribution of these isoenzymes. For example, a 10-kDa peptide, protein inhibitor of neuronal NOS (PIN), was shown to bind and inhibit the activity of NOS1 in vitro (18). Similarly, caveolins-1 and -3 interact directly with NOS1 and NOS3 (19) and inhibit NOS3 activity (19). NOS1 has been shown to interact physically with several proteins bearing PDZ domains that influence NOS1 targeting to discrete membrane domains of excitatory tissues (5, 6). In the case of NOS2, thus far kalirin (34) and NOS-associated protein-110 (35) have been bound to interact with NOS2 and inhibit its activity.

In the present study, we determined that Rac1 and Rac2, members of the Rho GTPase family, physically interact with NOS2. The Rac isoforms play central roles in several important cellular functions including cytoskeletal reorganization, gene expression, and assembly and activation of the phagocytic and nonphago-
cyelic NADPH oxidase. We further demonstrate that the point of interaction for Rac2 is the NOS2 oxygenase domain and that overexpression of Rac2 augments NO production in immune-activated murine RAW 264.7 macrophage cells. This remarkable convergence of two pluriotent signaling pathways reveals the unexpected complexity of NOS2 regulation, identifies a new target protein for Rac1 and Rac2, and suggests the possibility of coordinate regulation of the NADPH oxidase and NO production in phagocytes.

MATERIALS AND METHODS

Reagents. L-α-Glutamine, heat-inactivated FBS, penicillin-streptomycin, DMEM, and LipofectAMINE PLUS reagent were from Life Technologies. DMEM lacking phenol red, LPS from Escherichia coli O111:B4 and protease inhibitor cocktail were from Sigma. Glutathione-Sepharose 4B beads and RediPack columns, pGEX-3X, pGEX-5X, factor Xa, ECL reagents, and recombinant glutathione-S-transferase (GST) were from Amersham Pharmacia Biotech (Piscataway, NJ). Radiochemicals were purchased from Amersham. RNAzol II was from Tel-TEST “B,” The MATCHMAKER Two-Hybrid System 2, the Mouse Kidney MATCHMAKER cDNA library, the GAL4-activation domain hybrid cloning vector pGAD10, and pAS2–1 (GAL4-DNA binding domain hybrid cloning vector) were purchased from Clontech. Zeocin and pcDNA3.1/Zeo were acquired from Invitrogen. Mouse monoclonal anti-NOS2 antibody, anti-nitrotyrosine antibody, and an anti-nitrotyrosine-positive control were obtained from Transduction Laboratories. N2-monomethyl-l-arginine (l-NMMA) was purchased from Alexis Biochemicals. Mouse monoclonal anti-nitrotyrosine antibody was from Zymed (San Francisco, CA). Rabbit polyclonal IgGs specific for Rac1 and for Rac2 and protein A/G PLUS-agarose were purchased from Santa Cruz Biotechnologies. Oligonucleotides were custom synthesized by Genosys, the BCA protein assay kit was from Pierce Chemical, and the LipofectAMINE PLUS reagent was purchased from Promega.

Plasmids and constructs. A fusion of a segment of murine NOS2 with the GAL4-DNA binding domain of pAS2–1 was constructed by subcloning an NcoI-NcoI fragment of the NOS2 cDNA (encoding M352-H498) into these restriction sites of pAS2–1 to yield the recombinant molecule pAS2-NOS2825–934. An expression plasmid for full-length NOS2 cloned into pCDNA3.1 (Neo) (Invitrogen) has been previously described (23). To generate fusions with GST, cDNA inserts encoding murine Rac2, human Rac1 (a gift from Dr. J. David Lambeth, Emory University), or various portions of murine NOS2 (M1-L1144, M1-E498, K499-L1144) were PCR amplified from cloned cDNAs or, in the case of Rac2, cDNA prepared from LPS-treated RAW 264.7 cells. The cDNAs were subcloned into convenient restriction sites in pGEX-3X or pGEX-5X vectors to maintain appropriate reading frames. For construction of a Rac2 expression plasmid, the entire coding region of Rac2 (nucleotides 35–614, GenBank Accession No. X53247) was PCR amplified from RAW 264.7 cell cDNA by using primers based on the published sequence. The Rac2 cDNA was subcloned into the BamH1 and EcoR1 sites of the mammalian expression vector pcDNA3.1/Zeoc to create pcDNA3.1/Rac2-Zeo. All constructs were analyzed by DNA sequencing to confirm their authenticity and reading frames within the vectors.

Yeast two-hybrid assay. The MATCHMAKER Two-Hybrid System 2 and the mouse kidney MATCHMAKER cDNA library were used to identify proteins interacting with NOS2. Saccharomyces cerevisiae strain CG-1945, which is auxotrophic for tryptophan and leucine and contains lacZ and HIS3 reporter genes, was grown at 30°C in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or minimal synthetic dropout (SD) medium (0.5% yeast nitrogen base without amino acids, 2% glucose, and 1% desired amino acid dropout solution). pAS2-NOS2825–934 and pGAD10-kidney cDNA library fusions were introduced into the 1945 yeast reporter strain by the lithium acetate transformation procedure (2). Cotransformants were grown on SD plates containing 5 mM 3-amino-1,2,4-triazole (to reduce background from “leaky” HIS3 expression) and lacking histidine, tryptophan, and leucine to select for cotransformants in which the pAS2–1- and pGAD10-hybrid proteins interact. The resultant colonies were then screened for β-galactosidase activity by a colony lift filter assay (2). Insert cDNAs of clones demonstrating activation of the HIS3 and lacZ marker genes were amplified by PCR of total yeast DNA by using flanking primer sequences derived from pGAD10. Selected clones were then sequenced on both strands by using an automated method. In control experiments, the GAL4-DNA binding domain and GAL4-activation domain fusion constructs were confirmed not to activate reporter gene transcription by themselves, and GAL4-activation domain fusion constructs were confirmed not to activate transcription when combined with the unrelated pLAM 5-1, which encodes a GAL4-DNA binding domain-human lamin C hybrid in pAS2–1.

Coimmunoprecipitation. RAW 264.7 cells were incubated with basal medium or stimulated with LPS for 16 h to induce NOS2 expression. Lysates of the cells were prepared with ice-cold RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 3% protease inhibitor cocktail). One milliliter of lysate was preclarified with 10 µl normal rabbit serum together with 20 µl protein A/G PLUS-agarose for 1 h at 4°C. The lysates were centrifuged at 1,500 rpm at 4°C for 5 min to remove insoluble material. Two hundred and fifty microliters of the resulting supernatant were subjected to immunoprecipitation with anti-NOS2 mouse monoclonal antibody (2.5 µg; or with an equal amount of mouse IgG) or with rabbit polyclonal anti-Rac2 (1 µg; or with an equal amount of rabbit IgG) for 1 h at 4°C together with 20 µl protein A/G PLUS-agarose for 2 h at 4°C. The immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4°C, and washed four times with 1 ml RIPA buffer. Proteins precipitated by anti-NOS2 or mouse IgG were separated by SDS-PAGE, electroblotted to Hybond-ECL membranes, and immunoblotted with anti-Rac2. Proteins precipitated by anti-Rac2 or rabbit IgG were immunoblotted with anti-NOS2. Bound antibody was visualized by the ECL chemiluminescent detection system, using sheep anti-mouse (for NOS2 and mouse IgG) or goat anti-rabbit (for Rac2 and rabbit IgG) IgG-peroxidase conjugate and autoradiography. The relative amount of NOS2 and Rac2 coprecipitated was grossly estimated by running 50- and 100-µg aliquots of the total lysate (not subjected to the immunoprecipitation procedure) on the final immunoblot of the coprecipitates and comparing the relative abundance of the bands.

In vitro translation. NOS2 was transcribed and translated from pCDNA3.1/Neo-NOS2 in the presence of [35S]methionine by using T7 RNA polymerase and a troponin T-coupled reticulocyte lysate kit (Promega) by methods previously described (24).

GST pull-down assays. Three GST-fusion proteins, constructed to contain full-length NOS2 (M1-L1144), the isolated oxygenase domain (M1-E498), or the isolated reductase domain (K499-L1144), were purified from sonicates of isopropyl β-d-thiogalactoside-induced HB101 bacterial cells according
to the manufacturer’s (Amersham Pharmacia Biotech) instructions. For in vitro binding assays using cell lysates, ~4 μg of GST-fusion protein constructs were added to RediPack glutathione-Sepharose 4B columns. The columns were washed with 20 ml ice-cold PBS. Two hundred microliters of total cell lysates of LPS-treated RAW 264.7 cells prepared in RIPA buffer as above were used as a source of NOS2 protein and added to the column, followed by incubation with continuous rotation for 90 min at 4°C. The columns were then washed with 30 ml of ice-cold PBS containing 0.1% Tween followed by a final 3−ml wash of PBS, with eluates collected after each wash for analysis by SDS-PAGE. Factor Xa (50 μl in 450 μl of PBS containing 0.5% SDS) was added to the column for an overnight incubation at room temperature to cleave the bait and prey proteins from the GST tag. The final washes and the factor Xa-cleaved eluates were analyzed by SDS-PAGE and immuno blotting with anti-Rac1 or anti-Rac2 antibodies.

Studies of the direct nature and nucleotide dependence of the NOS2-Rac2 interaction were performed following the method of Spaargaren and Robb (39). Approximate amounts of GST, GST-Rac1, or GST-Rac2 were coupled to 0.1 ml glutathione-Sepharose 4B beads for 1 h at 4°C in nucleotide binding buffer [NBB; 20 mM Tris-HCl, pH 7.5/100 mM NaCl/1 mM dithiothreitol/0.1% Triton X-100/1 mM protease inhibitor cocktail] containing 10 mM EDTA overnight at 4°C to achieve nucleotide depletion. The amount of fusion protein coupled to the beads was estimated by comparing an aliquot of the slurry containing the bound fusion proteins along with 1 or 5 μg purified GST on Coomassie brilliant blue-stained SDS-PAGE gels. After 3 washes in NBB, the proteins were loaded with either GTPγS or GDPβS (Sigma) by incubation in 0.5 mM nucleotide in NBB containing 10 mM MgCl2 for 1 h at room temperature. Fusion proteins to be maintained nucleotide free were incubated in NBB containing 10 mM EDTA. The protein-bead samples were washed three times and then resuspended in NBB and either 10 mM MgCl2 (for GTPγS- or GDPβS-loaded GTPases) or 10 mM EDTA (for nucleotide-free GTPases). GST or GST-Rac2 were incubated with 30 μl of [35S]methionine-labeled full-length NOS2 translation product for 1.5 h at 4°C. The samples were then washed four times in NBB. The samples were boiled in Laemmli sample buffer and analyzed by SDS-PAGE and fluorography.

Cell culture and transfection. The murine macrophage-like cell line RAW 264.7 was grown in complete medium (DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FBS) at 37°C in a humidified incubator with 5% CO2. LPS (5 or 10 μg/ml) with or without interferon (IFN)-γ (200 U/ml) was added to the cells as indicated in the text and figure legends. For nitrite assays, the factor Xa-cleaved was eluted from the resin supplied with the kit, and the GST-Rac2-Zeo (nucleotides 525–614 of Rac2 and nucleotides 951–1271 of pcDNA3.1/Zeo) that hybridizes to vector-derived, but not endogenous, Rac2. Six representative lines of the highest expressing RAW-Rac2 clones as well as the RAW-Zeo clones were maintained in zeocin-containing medium, frozen after one to three in vitro passages, and studied in further detail. Deconvoluted immunofluorescence microscopy. RAW 264.7, RAW-Zeo, and RAW-Rac2 cells grown on glass coverslips were stimulated with LPS for 16 h to induce NOS2 expression. The cells were fixed in buffered 3.7% formaldehyde for 20 min, permeabilized with PBS containing 0.5% Triton X-100, and blocked in PBS containing 10% goat serum at 37°C for 1 h. The cells were incubated for 1 h with anti-Rac2 and anti-NOS2 antibodies together with FITC-conjugated phallolidin diluted in PBS+10% normal goat serum at 37°C for 1 h. As a negative control, primary antibody was omitted from the immunostaining procedure. After 3 washes in PBS containing 0.05% Tween-20, the cells were incubated with Cy5-conjugated goat anti-rabbit IgG (Molecular Probes) and Texas red-conjugated goat anti-mouse IgG (Molecular Probes). The cells were incubated in PBS containing 10% normal goat serum and 0.05% Tween 20. After final washes in PBS containing 0.05% Tween, the cells were overlaid with Elvanol (DuPont) and mounted. The cells were then imaged using an Olympus IX70 inverted epifluorescence microscope. Data sets were acquired by using a mercury short-arc lamp and stored in digital format by using a cooled charge-coupled device (CCD) camera (Applied Precision, Delta Vision System). The data sets were then transferred to a Silicon Graphics workstation for deconvolution and three-dimensional reconstruction (9, 28). Delta Vision System SoftWoRx (Applied Precision, Issaquah, WA) software was used to deconvolve 0.1-μm optical sections before reconstruction. The data sets were then transferred to Imaris 3 (Bitplane, Zurich, Switzerland) for digital image restoration and shadowing. For each cell, a series of 30–50 individual optical sections of 100-nm thickness in the vertical axis was obtained. Colors were arbitrarily computer-assigned to individual fluoros (Cy5 = green, Texas red = red; FITC = blue).

Nitrite assays. Nitrite, the stable metabolite of NO, was measured in culture supernatants by a modification of the Griess reaction as previously detailed (30). Standard curves were generated from sodium nitrite. Triplicate determinations were performed for each condition and represent a single observation (n). The data were normalized to protein content as determined by the BCA assay.

NOS activity assay. Total cell homogenates were prepared at 4°C in homogenization buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA). The NOS activity of the lysates was measured for 30 min at room temperature by the formation of L-[3H]citrulline from L-[3H]arginine (30 μM) by using the components of a NOSdetect Assay Kit. Parametric studies indicated that these conditions were within the linear range of the reaction. The reaction mixture contained 25 mM Tris-HCl (pH 7.4), 3 mM tetrahydrobiopterin, 25 nM calmodulin, 0.6 mM CaCl2, 1 μM FAD, 1 μM FMN, and 1 mM NADPH, with or without 1 mM Nω-nitro-L-arginine. L-citrulline was eluted from the resin supplied with the kit, and L-[3H]citrulline was quantified by liquid scintillation counting. The data were normalized to protein content as determined by the BCA assay.

Superoxide anion assays. Superoxide anion (O2−) produced from the RAW-Rac2 and RAW-Zeo cell lines was measured by using a LumiMax Superoxide Anion Detection Kit (Stratagene) according to the manufacturer’s protocol. The cells were incubated with basal medium or stimulated with LPS and IFN-γ for 16 h at 37°C. The cells were then collected, and...
10⁶ cells were pelleted and resuspended in 100 µl superoxide anion medium (Stratagene). Luminol and the proprietary enhancer were added to a final concentration of 100 and 125 µM, respectively. The luminescence, indicative of the superoxide anion produced, was measured in a Turner Systems 20/20 luminometer. The data were normalized to the protein content of the samples.

RT-PCR. Isolation of total RNA and RT-PCR for NOS1 and NOS3 was performed as described in previous work from our laboratory (29). For amplification of NOS1, primers corresponded to nucleotides 2462–2616 (forward) and 3007–3062 (reverse) of the rat sequence (3). NOS3 amplification was performed with primers directed to nucleotides 1889–1902 (forward) and 2594–2616 (reverse) of the murine sequence (13).

Triton X-100 fractionation. RAW-Rac2 and RAW-Zeo clonal cell lines grown on 100-mm² culture dishes and treated with vehicle or LPS for 16 h were washed twice with ice-cold PBS and lysed for 10 min on ice in lysis buffer [125 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10 mM NaF, 1% Triton X-100, protease inhibitor cocktail] or cytoskeleton-stabilizing buffer (20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 8% sucrose, 0.5% Triton X-100, protease inhibitor cocktail) (47). A “total lysate” sample was taken and, after boiling in Laemmli sample buffer, analyzed on Western blots. All fractionation steps were performed at 4°C. The remaining Triton X-100 lysate was centrifuged at 15,000 g for 10 min. The resulting pellet was washed once in lysis or cytoskeleton-stabilizing buffer, as appropriate, and taken as the “low-speed Triton X-100-insoluble fraction.” The supernatant was collected and centrifuged at 200,000 g for 100 min. The resulting pellet, which was washed once with lysis buffer or cytoskeleton-stabilizing buffer, as appropriate, represents the “high-speed Triton X-100-insoluble fraction,” whereas the supernatant represents the “high-speed soluble fraction.” For analysis by SDS-PAGE and Western blotting, all samples were boiled in Laemmli sample buffer.

Immunoblotting for nitrosylated proteins. Immunoblots of cell lysates were prepared from RAW-Zeo and RAW-Rac2 cells that had been treated with LPS+IFN-γ overnight as previously described (14). A commercially available positive control containing nitrated proteins was used. As an additional positive control, lysates of RAW 264.7 cells that had been exposed 20 times to exogenously applied 5 mM peroxynitrite (a gift from Drs. Yasu Irie and Ferid Murad) were used. The blots were probed with two different anti-nitrotyrosine antibodies and sheep anti-mouse or goat anti-rabbit IgG-peroxidase conjugate as appropriate.

RESULTS

NOS2 interacts with Rac1 and Rac2 in vivo. To identify proteins that physically interact with NOS2, a yeast two-hybrid screen was performed. A fusion gene, pAS2-NOS21235–1234, containing the GAL4-DNA binding domain of pAS2–1 fused to cDNA encoding M325-H34 of murine NOS2, was constructed. Yeast CG-1945 cells expressing this construct were used to screen a library of GAL4-activation domain-tagged mouse kidney cDNA. Of the ~3,000,000 clones screened, 9 transformants exhibited pAS2-NOS21235–1234-dependent activation of both the HIS3 and lacZ markers in CG-1945 cells. Sequence analysis of one of the nine pAS2-NOS21235–1234-derived clones revealed it to contain nucleotides 363–899 of murine Rac2 in-frame with the GAL 4-activation domain sequence. This sequence of Rac2 corresponds to the coding sequence beginning at codon 110 and extending into the 3’-untranslated region.

To confirm the results of the two-hybrid experiments by an independent biochemical method and to establish the occurrence of the Rac2-NOS2 interaction in mammalian phagocytic cells, a coimmunoprecipitation strategy was used. RAW 264.7 macrophage cells were treated with LPS for 16 h to induce expression of NOS2. Cell lysates were then prepared and immunoprecipitated with either anti-Rac2 or anti-NOS2 antibodies. Proteins precipitated by anti-Rac2 were separated by gel electrophoresis, transferred to nylon membranes, and immunoblotted with anti-NOS2. In the reciprocal experiment, anti-NOS2 immunoprecipitates were immunoblotted with anti-Rac2. Coprecipitation of the two proteins was taken as evidence for a direct and stable interaction between them. Figure 1A shows the results of the coimmunoprecipitation experiments. Immunoblots of the anti-Rac2 immunoprecipitates with the anti-NOS2 antibody revealed the expected ~130 NOS2 monomer. Similarly, immunoblots of the anti-NOS2 immunoprecipitates probed with the anti-Rac2 antibody revealed the presence of the ~21 kD Rac2 protein. A gross comparison of the relative abundance of the immunoprecipitated proteins vs. the input amount revealed that the association appeared to be roughly stoichiometric in that ~20% of the total NOS2 and Rac2 was coimmunoprecipitated. Because some of the protein A/G PLUS-agarose is lost in the multiple washes during the immunoprecipitation procedure, this value probably underestimates the true amount of associated proteins. In both cases, immunoprecipitation with non-immune IgG failed to precipitate the Rac2 or NOS2 proteins (Fig. 1B). As an additional control, immunoprecipitation with the anti-Rac2 and blotting with anti-NOS2 failed to detect NOS2 in RAW 264.7 cells not pretreated with LPS (Fig. 1C).

These results confirmed the association of full-length Rac2 with NOS2 in LPS-stimulated RAW 264.7 cells. Because Rac2 bears ~94% structural identity to Rac1, we sought to determine whether interactions between Rac1 and NOS2 also occur in RAW 264.7 cells. Using a GST pull-down assay, lysates of LPS-treated RAW 264.7 cells were incubated with a GST-NOS2 fusion protein immobilized on glutathione-Sepharose 4B columns. After extensive washing of the columns, Rac1 and Rac2 were detected by immunoblotting of the factor Xa-cleaved samples (Fig. 1D). These results indicate that both Rac isoforms can associate with NOS2 in these cells.

The NOS2-Rac interaction in vitro is direct. GTP independent, and does not require calmodulin, NOS substrates, or cofactors. In the yeast two-hybrid system and coimmunoprecipitation assays, protein-protein interactions may be direct or mediated by a bridging endogenous protein. Therefore, Rac2 was produced and purified as a GST-fusion protein, and [35S]methionine-labeled NOS2 was translated in vitro to test their ability to bind directly in vitro in a GST pull-down assay conducted in the absence of calmodulin or NOS

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substrates or cofactors. In addition, to test the GTP
dependence of the interaction, the GST-Rac2 fusion
proteins were prebound with GDP_bS, the nonhydrolyz-
able GTP_gS, or were generated nominally free of nu-
cleotides and subjected to the pull-down assay. As
shown in Fig. 2, [35S]methionine-labeled NOS2 directly
interacted with GST-Rac2, but not GST alone, under
each of these conditions.

The NH2-terminal oxygenase domain of NOS2 is
necessary and sufficient to mediate the association of
NOS2 with Rac2. To determine the regions of NOS2 to
which Rac2 binds, GST-fusion proteins encoding the
isolated oxygenase and reductase domains of NOS2 as
well as full-length NOS2 were used in a GST pull-down
assay with lysates of LPS-treated RAW 264.7 cells. As
shown in Fig. 3, Rac2 in the lysates bound to the
GST-full-length NOS2 fusion protein (in agreement
with the experiment in Fig. 1B) and the GST-NOS2
oxynase domain fusion protein. However, no inter-
action with Rac2 was observed with the GST-NOS2
reductase domain fusion protein (Fig. 3). Because the
NOS2 oxygenase construct lacks the binding sites for
calmodulin and flavin nucleotides, this result indicates
that the latter are not required for the interaction with
Rac2. This result substantiates the findings presented
in Fig. 2. The fact that Rac2 did not interact with the
GST-NOS2 reductase domain fusion protein also dem-
strates specificity of the interactions observed with
the GST-full-length NOS2 fusion protein and the GST-
NOS2 oxygenase domain fusion protein.

Overexpression of Rac2 stimulates NOS2 activity. To
determine whether Rac2 might influence NOS2 ex-
pression or activity, we stably transfected RAW 264.7 cells with a Rac2 expression plasmid or the parent vector pcDNA3.1/Zeo. To confirm that the transfection procedure did not somehow result in de novo expression of NOS1 or NOS3 in the resultant cell lines, RT-PCR analysis for these isoforms was performed using isoform-specific primers and RNAs prepared from normal and LPS-treated wild-type RAW-Zeo cells, RAW-Rac2 cells, and mouse kidney (as a positive control). NOS1 and NOS3 transcripts were successfully amplified from mouse kidney RNA, but not from the cell lines (data not shown), indicating that NOS2 is the sole NOS isoform expressed in the transfected cell lines treated with LPS.

The RAW-Rac2 and RAW-Zeo cell lines were then examined for their ability to produce NO and NOS2 protein under basal conditions and in response to LPS. Under basal conditions, both cell lines produced comparable, but negligible, nitrite (Fig. 4A), indicating that overexpressed Rac2, in the absence of immune stimuli, cannot provoke NO generation. Figure 4A shows that LPS promoted, as expected, high-level nitrite production in the RAW-Zeo cells, but even greater (~60%) nitrite levels were observed in the RAW-Rac2 cells. Because nitrite production from the intact cells may be influenced by changes in the availability of NOS2 cofactors or substrate, the NOS2 activity of RAW-Zeo and RAW-Rac2 cell homogenates was measured by the L-[3H]arginine to L-[3H]citrulline conversion assay in the presence of fixed and saturating concentrations of cofactors and substrates. In response to LPS, the RAW-Rac2 cells exhibited NOS2 activity that was roughly 45% higher than that of the RAW-Zeo cells (Fig. 4B), in close agreement with the nitrite production of the intact cells. The enhanced LPS-induced nitrite production and NOS2 activity of the RAW-Rac2 cells appeared to be principally the result of a posttranslational event, because immunoblots of lysates prepared from the cells revealed NOS2 protein amounts comparable to that of the LPS-treated RAW-Zeo cells (Fig. 4C).

NOS2 and Rac2 colocalize in RAW 264.7 cells. To determine whether NOS2 and Rac2 colocalize in macrophages, the subcellular distribution of NOS2 and Rac2 was analyzed by immunofluorescence deconvolution microscopy (9, 28) of LPS-treated RAW 264.7 cells. Both NOS2 and Rac2 immunofluorescence were found in a perinuclear location (judged by DAPI staining of the nuclei; not shown) as well as in more peripheral cytoplasmic sites. An overlapping subcellular localization was observed between a pool of NOS2 and Rac2 (Fig. 5). In control experiments, no staining was evident in the absence of primary antibody for either NOS2 or Rac2 (not shown).

Overexpression of Rac2 promotes NOS2 translocation to a high-speed Triton X-100-insoluble fraction. Because Rac2 has been shown to influence the organization of the cytoskeleton and the spatial distribution of NADPH oxidase components in activated phagocytes, we hypothesized that overexpression of Rac2 might influence the subcellular distribution of NOS2 and thereby provide a molecular basis for observations of soluble and particulate forms of NOS2. As an initial...
approach to this question, we used Triton X-100 fractionation of lysates from RAW-Zeo and RAW-Rac2 cell lines to examine this possibility. This method has been commonly used to study Rac association with the components of the NADPH oxidase. Two separate buffer systems, lysis buffer and cytoskeleton-stabilizing buffer, were used. The results below were comparable regardless of the buffer system used to prepare the lysates. The low-speed Triton X-100-insoluble pellet has been reported to contain nuclei, large cytoskeletal networks, and caveolae. The high-speed Triton X-100-insoluble pellet contains submembranous cytoskeleton complexes and associated signaling molecules (12, 42), including Rac2 (10). In agreement with these studies, Rac2 was found in the high-speed Triton X-100-insoluble pellet (and the low-speed supernatant from which the high-speed fractions were derived) but not high-
speed supernatant in control and LPS-treated (16 h) RAW-Zeo and RAW-Rac2 cells (Fig. 6A). Rac2 was also consistently more abundant in the LPS-treated cells. NOS2 was undetectable under basal conditions in RAW-Zeo and RAW-Rac2 cell lines (Fig. 6A). After LPS treatment for 16 h, NOS2 was apparent in the low-speed Triton X-100-soluble fraction and both the high-speed Triton X-100-soluble and -insoluble fractions (Fig. 6B). Similar results were obtained in untransformed RAW 264.7 cells after LPS treatment (data not shown). In the LPS-treated RAW-Rac2 cells, however, NOS2 was detected exclusively in the low-speed Triton X-100-soluble fraction and the high-speed Triton X-100-insoluble fraction derived from it (Fig. 6B). These results indicate that overexpression of Rac2 promotes NOS2 translocation to and/or retention in the high-speed Triton X-100-insoluble fraction. However, deconvolution immunofluorescence microscopy, which has comparable resolution to confocal microscopy, did not demonstrate an unequivocal difference in the subcellular labeling of the NOS2 and Rac2 proteins in the LPS-treated RAW-Zeo and RAW-Rac2 cell lines (data not shown).

Effects of overexpression of Rac2 on superoxide anion production. To assess whether overexpression of Rac2 alters superoxide anion production in macrophages, a chemiluminescence assay was used to measure superoxide anion produced by resting or LPS+IFN-γ-stimulated RAW-Zeo and RAW-Rac2 cells. As shown in Table 1, RAW-Rac2 cells generated greater levels of superoxide anion than RAW-Zeo cells, suggesting, for the first time, that Rac2 overexpression drives superoxide production by the NADPH oxidase. LPS+IFN-γ treatment resulted in a significant reduction in the amount of superoxide anion produced by both groups of cell lines. Because LPS+IFN-γ treatment promotes NO production in these cells, and because NO chemically inactivates superoxide anion by forming peroxynitrite, we reasoned that the lower levels of superoxide anion in the activated RAW-Rac2 and RAW-Zeo cell lines might reflect a quenching of superoxide by NO. Accordingly, we assayed superoxide production in LPS+IFN-γ-treated cells when NOS2 was inhibited with L-NMMA. With NOS2 inhibited, superoxide anion production was enhanced in the LPS+IFN-γ-treated RAW-Zeo cells and to a far greater extent in the RAW-Rac2 cells (Table 1). If the L-NMMA-dependent component of superoxide anion production represents the superoxide anion inactivated by NO, this result suggests that the RAW-Rac2 cells form far greater levels of peroxynitrite than the RAW-Zeo cells after exposure to LPS+IFN-γ. Alternatively, high concentrations of NO peroxynitrite have been reported to inactivate the NADPH oxidase in polymorphonuclear leukocytes (26).

High concentrations of peroxynitrite are known to nitrosylate target proteins. Protein nitrosylation, therefore, can be used as a surrogate marker for peroxynitrite generation in cells. Accordingly, we probed immunoblots of lysates prepared from control and LPS+IFN-γ-treated RAW-Zeo and RAW-Rac2 cells with antibodies directed against nitrotyrosine. As positive controls, we used a commercially available panel of nitrated proteins and lysates that had been prepared from RAW 264.7 cells repetitively treated with 5 mM peroxynitrite. No remarkable difference in the intensity or pattern of protein bands was observed between the RAW-Zeo and RAW-Rac2 cells under basal or induced conditions with either of the anti-nitrotyrosine antibodies (not shown).

**DISCUSSION**

In this study, we report several novel findings important to an understanding of NOS2 biology, Rac1 and Rac2 signaling targets, and macrophage function.

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**Table 1. Overexpression of Rac2 in RAW 264.7 cells promotes superoxide anion generation**

<table>
<thead>
<tr>
<th></th>
<th>Superoxide Anion Production, RLU/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAW-Zeo</td>
</tr>
<tr>
<td>Control</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>LPS + IFN-γ</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>LPS + IFN-γ + L-NMMA</td>
<td>2.6 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. RAW-Zeo and RAW-Rac2 cell lines were treated with vehicle or lipopolysaccharide (LPS)+interferon (IFN)-γ, or LPS+IFN-γ+0.1 mM Nω-monomethyl-L-arginine (L-NMMA) for 16 h. The cells were then collected and assayed for superoxide anion production by using a chemiluminescence method (n = 3). *P < 0.05 vs. RAW-Zeo condition. †P < 0.05 vs. RAW-Rac2 control. ‡P < 0.05 vs. LPS+IFN-γ-treated RAW-Rac2 cells. RLU, relative light units.
By demonstrating the physical interaction of NOS2 with Rac1 and Rac2 in activated murine macrophages, we provide new examples of heterologous proteins other than calmodulin that physically associate with NOS2 and identify NOS2 as a new target for the Rac isoforms. Our demonstration that overexpression of Rac2 augments NOS2-generated NO production and NOS activity in LPS-treated RAW 264.7 cells establishes not only the functional importance of the NOS2-Rac2 interaction but also suggests a novel posttranslational mechanism for controlling NO synthesis. In vitro binding studies showed that Rac2 binds to the oxygenase, but not the reductase domain of NOS2, that the interaction is direct, and that it can occur in vitro in the absence of calmodulin, heme, and NOS2 substrates and cofactors. Finally, our finding that NOS2 exclusively distributes to a high-speed Triton X-100-insoluble fraction in RAW 264.7 cells overexpressing Rac2 suggests that the interaction influences the spatial distribution of NOS2 in the cell, apparently in such a way that optimizes LPS-induced NO release. Given the diverse cell processes in which both NOS2 and the Rac isoforms participate, the physical association of these proteins in vivo suggests the possibility that the interaction may play important biological roles.

Rac2 is principally expressed in cells of myeloid origin, where it participates in the formation of the NADPH oxidase complex in phagocytic cells (8), and it is also believed to be a component of the NAD(P)H oxidase in vascular smooth muscle cells (33). Its close structural relative, Rac1, is more widely distributed among cell types and has been implicated in diverse cell processes, such as the organization of the actin cytoskeleton, protein kinase cascades, transcriptional activation, and cell cycle progression (15). The Rac proteins are believed to play a role in membrane ruffling and phagocytosis in macrophages. The NADPH oxidase of phagocytes generates superoxide anion and other reactive oxygen species such as H₂O₂, HOCI, and OH that are cytotoxic to invading pathogens (8). In resting cells, the phagocytic NADPH oxidase is composed of membrane-associated flavocytochrome b₅₅₉ and several cytosolic proteins, including p47фох, p67фох, p40фох, and Rac2 or, less commonly, Rac1. When the phagocytic NADPH oxidase is activated to generate superoxide anion, p47фох, p67фох, and Rac2 translocate to a detergent-insoluble subcellular fraction (10) and become stably associated with the plasma membrane (8). Similarly, we found that overexpression of Rac2 in LPS-treated RAW 264.7 cells promotes redistribution of NOS2 from high-speed Triton X-100-soluble and -insoluble fractions exclusively to the Triton X-100-insoluble fraction. Whether Rac2, through its physical association, ushers NOS2 from the high-speed Triton X-100-soluble compartment to the high-speed Triton X-100-insoluble compartment, facilitates its retention in the latter compartment, or acts indirectly to promote NOS2 translocation from one compartment to the other will require additional study.

In suggesting that the distribution of NOS2 within the cell might be regulated and that Rac2 is involved in this process, the present data extend earlier reports of soluble and particulate forms of NOS2 (16, 17, 38, 48). Hiki and co-workers (17) demonstrated nitrite-producing activity in the high-speed pellet of sonicated peritoneal macrophages from Calmette-Guerin bacillus-treated rats. A portion of the particulate activity was Triton X-100 insoluble. Hecker et al. (16), studying IFN-γ-treated J774.2 macrophage-like cells, recovered roughly one-half of the cGMP-elevating activity in a 200,000-g pellet, but the molecular identity and detergent solubility of the activity were not established. Schmidt et al. (38) found that roughly one-third of NOS activity in LPS and IFN-γ-treated RAW 264.7 cells distributed to a 105,000-g, KCl-washed pellet. The majority of the particulate activity remained insoluble in 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate. Finally, Vodovoz and co-workers (48), using activated primary macrophages, found approximately one-half of the NOS2 activity and immunoblotted NOS2 protein in a 100,000-g, KCl-washed pellet. Of this particulate activity, about one-half was Triton X-100 insoluble. Immunoelectron microscopy demonstrated NOS2 immunoreactivity in 50- to 80-nm vesicles that were not labeled by lysosomal or peroxisomal antibodies. Spatial restrictions of NOS2 within the cell presumably serve functional purposes. Because NO and O₂ are both produced by activated phagocytes, production of more toxic reactive oxygen and nitrogen species, such as peroxynitrite (ONOO⁻), is favored with the prospect of injury not only to pathogens but also to the host cell. Compartmentalizing NO production may represent a means for the cell to optimize microbial killing or inhibition of viral replication and minimize exposure of host cell components to these toxic molecules. Whether Rac2 ushers a pool of NOS2 to the NADPH oxidase remains to be clarified. Further studies will also be needed to determine whether the NOS2-Rac2 interaction alters the GTPase activity of the Rac proteins. Furthermore the biochemical composition and ultrastructural correlate of this compartment remain to be determined, because we did not discern a distinct difference in intracellular locale at the resolution of deconvolution immunofluorescence microscopy.

It is intriguing to speculate that the association of Rac2 with both the NADPH oxidase complex and NOS2 may place superoxide anion and NO production in close proximity to facilitate the formation of peroxynitrite for microbicidal effects or, in contrast, to promote the NO-mediated inactivation of the NADPH oxidase (26) to limit damage to host tissues. We found that Rac2-overexpressing macrophages produced more superoxide anion than did controls under basal conditions, suggesting that Rac2 may be rate limiting for NADPH oxidase activity. In addition the Rac2-overexpressing cells generated little superoxide anion when NOS2 was induced by LPS+IFN-γ but high amounts when NOS2 was inhibited with L-NMMA. These data suggest that the superoxide anion produced by the LPS+IFN-γ-treated macrophages rapidly reacts with NOS2-generated NO to form peroxynitrite. However,
because the relative amounts and patterns of proteins immunoreactive with two different anti-nitrotyrosine antibodies, an index of peroxynitrite formation, did not remarkably differ between the LPS+IFN-γ-treated Raw-Rac2 and the Raw-Zeo cells, it remains possible that NO inactivates the NADPH oxidase in these cells to limit superoxide anion production, as has been reported in other cell types (26).

The Rac proteins generally function as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound state. In most examples, the active GTP-bound Rac binds to target effector proteins that relay cell signals. We found that recombinant Rac2 bound to NOS2 in vitro in the GDP-bound state and in the nominal absence of guanine nucleotides (Fig. 2). GTP-independent interactions of Rac isoforms in vitro have also been reported for other targets, such as Sra-1 (20), type I phosphatidylinositol-4-phosphate 5-kinase (45), and diacylglycerol kinase (44). Although we did not observe a consistent difference in the amount of in vitro translated NOS2 bound to GTP- or GDP-bound GST-Rac2 (Fig. 2), it remains possible that GTP-GDP cycling may influence the kinetics or stability of the interaction in vivo.

The fact that NOS2 interacted with both Rac1 and Rac2 in the GST pull-down assays (Fig. 1B) suggests that NOS2 associates with regions common to the Rac isoforms. This common sequence region would include all but the extreme COOH termini of the Rac proteins. The identification of the minimal region(s) within the NOS2 oxygenase domain to which Rac1 and Rac2 binds will be important, because it will not only provide insights into NOS2 bioregulation but also may help to identify other targets for the Rac isoforms and related RhGTPases. Because the Rh GTPases participate in numerous cell processes, considerable effort has been made to identify molecular targets of these proteins. Several protein motifs recognized by Rh GTPases have been characterized in various target proteins, including the CRIB motif (7), REM-1 (36), and P-OH (41), but these motifs are absent from the oxygenase domain of NOS2. Rac2 has been shown to bind in vitro to the COOH terminus of p67PHOX (11) and tetra-cotid-repeat motifs in the NH2-terminal region of p67PHOX (21), but there is no significant homology between p67PHOX and NOS2. Similarly, other known Rac targets, such as PAK (43), Wiskott-Aldrich syndrome protein (37), POR1 (46), IQGAP1 (25), and specifically Rac1-associated protein (20), also share no sequence homology with NOS2. Thus the Rac binding motif in NOS2, once defined, will be novel. Further definition of the protein domains of NOS2 and Rac2 that interact will require additional studies, as will determination of whether other members of the NOS and Rh GTPase families can associate with one another. Future studies to investigate the functional and regulatory aspects of NOS2-Rac relationships should provide fresh insights into the cell biology of not only phagocytic cells but also the numerous tissues and cell types in which these proteins are expressed in physiological and pathophysiological states.

Biosynthesis of NOS2 is principally regulated at the level of gene transcription. The posttranslational control of NOS2 activity has received little investigative attention. Inferential evidence suggests that tyrosine phosphorylation of NOS2 in activated RAW 264.7 cells may increase NOS2 activity by a posttranslational mechanism. Exposure of activated RAW 264.7 cells to a tyrosine phosphatase inhibitor significantly increased the level of NOS2 tyrosine phosphorylation and NOS2 activity (32). The present report suggests a second example of a posttranslational mechanism that augments activity of the enzyme. The fact that NOS2 activity in the RAW-Rac2 cell homogenates, under conditions in which the concentrations of substrates and cofactors were fixed and saturating, was greater than that of RAW-Zeo cells suggests that Rac2 influences the activity of the enzyme via a direct mechanism rather than by increasing the availability of substrate or cofactors. This conclusion is supported by knowledge that intracellular levels of heme and tetrahydrobioppterin increase coincident with NOS2 induction in activated RAW 264.7 cells (1) and that further supplementation of these prosthetic groups or the substrate L-arginine does not increase NOS2 activity or dimerization in these cells (1). Further studies will be required to decipher the specific mechanism by which Rac2 enhances NOS2 activity.

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