Imbalance between pro- and antioxidant mechanisms in the lungs can compromise pulmonary functions, including blood oxygenation, host defense, and maintenance of an anti-inflammatory environment. Thus, tight regulatory control of reactive oxygen species is critical for proper lung function. Increasing evidence supports a role for the NADPH oxidase dual oxidase (Duox) as an important source for regulated H$_2$O$_2$ production in the respiratory tract epithelium. In this study Duox expression, function, and regulation were investigated in a fully differentiated, mucociliary airway epithelium model. Duox-mediated H$_2$O$_2$ generation was dependent on calcium flux, which was required for dissociation of the NADPH oxidase regulatory protein Noxa1 from plasma membrane-bound Duox. A functional Duox1-based oxidase was reconstituted in model cell lines to permit mutational analysis of Noxa1 and Duox1. Although the activation domain of Noxa1 was not required for Duox function, mutation of a proline-rich domain in the Duox C terminus, a potential interaction motif for the Noxa1 Src homology domain 3, caused up-regulation of basal and stimulated H$_2$O$_2$ production. Similarly, knockdown of Noxa1 in airway cells increased basal H$_2$O$_2$ generation. Our data indicate a novel, inhibitory function for Noxa1 in Duox regulation. This represents a new paradigm for control of NADPH oxidase activity, where second messenger-promoted conformational change of the Nox structure promotes oxidase activation by relieving constraint induced by regulatory components.

Inflammatory conditions often give rise to the generation of reactive oxygen species (ROS) and reactive nitrogen species, and many lung pathologies are linked to an imbalance in redox regulation of the airway. Pulmonary oxidant stress is associated with disease states, including acute respiratory distress syndrome, hyperoxia, ischemia-reperfusion, sepsis, chronic obstructive pulmonary disease, and asthma. On the other hand, ROS are important second messengers in various biological processes such as cellular signaling, cell proliferation, and apoptosis (1) and play a pivotal role in host defense against microbial infection (2, 3). In phagocytic cells, the Nox2 (gp91$^{phox}$)-based NADPH oxidase constitutes the main source for ROS generation in response to pathogens. This oxidase consists of at least six components as follows: Nox2, p22$^{phox}$, p67$^{phox}$, p47$^{phox}$, p40$^{phox}$, and the GTPase Rac. In resting cells, p67$^{phox}$, p47$^{phox}$, and p40$^{phox}$ exist in the cytosol as a complex, whereas p22$^{phox}$ and Nox2 form the heterodimer cytochrome b$_{558}$, which is located in the plasma membrane (4). Upon activation, multiple phosphorylations and conformational changes occur, permitting translocation of the cytosolic proteins and association with the Nox2-p22$^{phox}$ complex. This active oxidase complex then catalyzes the one-electron reduction of oxygen to O$_2^-$ at the expense of NADPH (5).

Nonphagocytic cells can also produce ROS, albeit usually at much lower levels when compared with the output of the phagocyte oxidase (6, 7). Many different ROS sources are implicated in these cell types, including the mitochondrial electron transport chain and NADPH oxidases (Nox). Six Nox homologs with restricted tissue expression profiles have been identified. The Nox family is now comprised of Nox1–4, whose features resemble the phagocyte gp91$^{phox}$ (now Nox2), and Nox5, which possesses four EF hands. The additional members, Duox1 and Duox2, are characterized by an N-terminal extracellular peroxidase homology domain, followed by a transmembrane segment and an EF hand-containing cytosolic region, which connects to a Nox2 homology structure. Unlike their Nox homologs, mature Duox enzymes release hydrogen peroxide (H$_2$O$_2$) without forming a detectable amount of superoxide. This distinct feature of Duox may be caused by the rapid conversion of superoxide via intramolecular dismutation (8).

In mammals, Duox1 and Duox2 were first characterized in the thyroid as essential H$_2$O$_2$-generating enzymes (9, 10). At the apical surface of the thyrocytes, H$_2$O$_2$ is the final electron acceptor for the thyroperoxidase-catalyzed biosynthesis of thyroid hormone (11). The critical role of Duox in thyroid hormone synthesis has been confirmed by the development of congenital hypothyroidism in patients with inactivating Duox2 mutations (12, 13). Duox is also highly expressed in the respiratory tract, in epithelial cells of exocrine glands, and in the mucosa (8, 14). A contribution of Duox to the host innate immune response was recently suggested in Drosophila melae-
nogaster and by in vitro studies, where H$_2$O$_2$ generated by Duox might be used by lactoperoxidase to oxidize thiocyanate anions present in the air-surface liquid to antimicrobial hypothiocyanite (3, 15–17). However, persistently elevated ROS can exert deleterious effects as observed in various airway diseases (18), indicating that ROS production has to be tightly regulated to ensure its targeted and limited generation.

The activation of Nox2, Nox1, and Nox3 relies on regulatory proteins such as the GTPase Rac and the proteins p47$\text{phox}$, p67$\text{phox}$, and their homologs Nox01 and Noxa1, respectively (19–24). Nox1 and p67$\text{phox}$ serve as activating regulatory components in Nox1- and Nox2-mediated superoxide generation (19–21). Of importance is their activation domain that is located adjacent to the GTP-Rac-binding tetra-trico-peptide repeat domain. Introducing an alanine for valine in position 204 of the p67$\text{phox}$ activation domain renders the Nox2-based NADPH oxidase inactive (25, 26). A similar change in Noxa1 inhibits superoxide production by Nox1. Little is known about the regulation of Duox enzymes in the lung. Although ROS production by Duox is dependent on calcium flux and seems not to require Rac, more in-depth analysis of Duox regulation has not been performed. We decided to investigate the molecular mechanisms regulating Duox in the context of primary human airway epithelial cells. Characterization of ROS generation in a pseudostratified mucociliary epithelial model indicates an important role for Duox as apical H$_2$O$_2$ source. We present evidence of a functional interaction between membrane-localized Duox and Noxa1, which is disrupted in the presence of calcium. Our data suggest that Noxa1 plays a vital role in stabilizing the inactive state of Duox in resting cells, possibly to prevent Duox-mediated ROS generation by transient, low level calcium flux.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Plasmids**—Polyclonal antibodies directed against c-Myc (A-14) and anti-RhoGDI (A-20) were from Santa Cruz Biotechnology, and actin (A2066) was from Sigma. The expression plasmid for hDuox1 in pcDNA3.1 was kindly provided by T. Leto. The Noxa1(V205A) and Noxa1(W436R) mutations were prepared by QuikChange site-directed mutagenesis kit (Agilent-Stratagene) and verified by sequencing. The full coding sequence of hDuoxA1 was amplified by PCR with Platinum$^\text{®}$ Pfx (Invitrogen) using total RNA of human airway epithelial cells in three dimensions were grown in serum-free medium (SABM, Sigma) supplemented with SAGM SingleQuots (Lonza) at 37 °C in a humidified 5% CO$_2$ incubator. For the differentiated lung epithelial model (three-dimensional), cells were detached with trypsin and seeded onto inserts (Costar Transwell-clear insert, 0.4 µm pore; Corning Costar) coated with human placenta collagen type V1 (15 µg/cm$^2$; Sigma). Airway epithelial cells in three dimensions were grown in serum-free culture medium (50% SABM, 50% Dulbecco’s modified Eagle’s medium high glucose) supplemented with defined growth factors in the SingleQuots and 20–50 nm retinoic acid, prepared freshly to induce differentiation. After 2–4 days in immersed culture conditions, cell culture was switched to an air-liquid interface system for 3 weeks. Differentiation was assessed by MUC5AC expression, mucin production, resistance, and permeability measurement and electron microscopy. NCI-H292, A549, HEK293, HeLa, and H661 cells were cultured in the appropriate media. Cells were preincubated with DIP (25 µM) or BAPTA/AM (0–0.75 µM; BD Biosciences) for 10 and 5 min, respectively, before exposure to stimuli. Cells were treated with ionomycin (2–3 µM, Sigma) for 30–60 min.

**Expression Analysis of Nox/Duox and NADPH Oxidase Components**—Total RNA was extracted from NCI-H292 cells, A549 cells, or SAEC cells grown in monolayer or on inserts by using RNAzol B reagent (TelTest, Inc.) according to the manufacturer’s protocol. Amplification by reverse transcription (RT)-PCR was performed. Briefly, cDNA was generated from 1 to 2 µg of extracted total RNA using Superscript II RNase H reverse transcriptase and oligo(dT) primers (Invitrogen) according to the supplier’s protocol. cDNAs were amplified by standard PCR using TaqDNA polymerase (Invitrogen), except for Noxa1 and Nox1 PCR, which were performed with the Advantage-GC 2 PCR kit (BD Biosciences). Sense and antisense primers were designed based on human sequences published in GenBank$^\text{TM}$. The primers used were as follows: 5’-CCATCGGACTACACGCGACGTG-3′ (forward primer) and 5’-GTAGCGACGTGACGGCAGCCG-3′ (reverse primer) for Nox1 cDNA; 5’-GCAGGACATCACCCCTGACTCTTC-3′ (forward primer) and 5’-CTGCCATCTACCAACGAGGACGTG-3′ (reverse primer) for Duox1 cDNA; 5’-ATGTTGCTTTCCAGCAGTGCCAGGAGCTG-3′ (forward primer) and 5’-CCATTCGAGTATTTTCATTTACACACG-3′ (reverse primer) for Nox1 cDNA; 5’-GCAGTGAGCTGCAGTCGCCGAC-3′ (reverse primer) for Duox1 cDNA and 5’-CAATCCCTTTGCTCTTACAGTCGAC-3′ (forward primer) and 5’-GGAGTACAGTGACAGTGAC-3′ (reverse primer) for Duox2 cDNA.
CAAAGGGGGTGAC-3’ (reverse primer) for Nox1 cDNA. RT-PCR products were resolved by electrophoresis on ethidium bromide-stained agarose gels and visualized by UV.

Small Interfering RNA (siRNA) Treatment—Double strand siRNA targeting hDuox was designed with BLOCK-it RNAi Designer (Invitrogen) to target both Duox1 and Duox2 (GenBank accession numbers AF213465 and AF267981, respectively). Predesigned hNoxa1 siRNA sets (NOXA1HSS145807, NOXA1HSS145808, and NOXA1HSS145809) were purchased from Invitrogen. The sequences of Duox siRNA 1 were (sense) GGACAGGGAGAACUGACUAUGGAAG and (antisense) UUCCCAUGUCAGUUCUUCCGUCC, and the sequences of Duox siRNA 2 were (sense) GCAUAAGUUAAGAGU- GUCAGGUGUA and (antisense) UAACACUGACACC-UCAAUCUAAUGC. Silencer Negative Control siRNA GC medium (Invitrogen) was used as control siRNA. siRNA transfection into cells was carried out by using Lipofectamine 2000. To optimize conditions, different concentrations of siRNA (1–100 nM) and various duration of RNAi treatment were applied. Two separate transfections of the same cell population with siRNA were performed before ROS generation was measured.

Measurement of H$_2$O$_2$ Release—H$_2$O$_2$ generation was determined by measuring the oxidation of homovanillic acid (HVA) into its fluorescent derivative in the presence of horseradish peroxidase as described previously (27). The activity is monitored by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity is monitored by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity is determined by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity is determined by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity is determined by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity is determined by measuring the oxidation of homovanillic acid (HVA) obtained by incubating increasing amounts of H$_2$O$_2$ peroxidase as described previously (27). The activity was estimated to be 0.3–1 μM.

Immunofluorescence—SAEC, NCI-292, and A459 cells were seeded on a glass coverslips coated with human placenta collagen type VI (10 μg/ml), HEK293, and HeLa cells on polylysine or fibronectin. At 60% confluence or after transfection (siRNA or overexpression), the cells were fixed with 4% paraformaldehyde for 10 min. After permeabilization with 0.5% Triton X-100 and blocking with 5% bovine serum albumin in phosphate-buffered saline, coverslips were incubated for 2 h at room temperature with either anti-Duox or anti-Noxa1 antibody in 2% bovine serum albumin/phosphate-buffered saline. Rabbit preimmune sera were used to verify the specificity of the staining. The secondary antibodies were anti-rabbit IgG conjugated with Alexa Fluor 488 or 568 (Invitrogen). Coverslips were mounted and examined with indirect immunofluorescence (Olympus IX70) and confocal microscopy (×6, MRC 2010; Bio-Rad). All images are representative of several independent experiments recording multiple cells in different sections of the coverslip.

Cell Fractionation—With or without stimulation, cells were harvested and resuspended in relaxation buffer (KCl 100 mM, NaCl 3 mM, MgCl$_2$ 3.5 mM, EGTA 1 mM, Heps 10 mM, PIPES 0.5 mM, pH 7.4). The cells were then disrupted by two 15-s cycles of sonication at 4°C using a microprobe sonicator (Microson XL). Unbroken cells and nuclei were pelleted by centrifugation at 600 × g for 10 min at 4°C. The supernatant (S1) was then centrifuged at 100,000 × g for 30 min at 4°C in an Optima TLX ultracentrifuge with a TLA 100.3 rotor (Beckman Instruments Inc., Palo Alto, CA). The high speed supernatant (S2) represented the soluble cytosolic fraction. The pellet (P2) was resuspended in relaxation buffer with vigorous mixing, and this sample was again centrifuged for 15 min at 100,000 × g at 4°C. The final supernatant (S3) represented a wash fraction. The final pellet (P3), representing the membrane fraction, was resuspended in relaxation buffer. An equivalent of 100–200 μg of protein was resolved by gel electrophoresis, and the membranes were probes for Noxa1, Duox, actin, RhoGDI, HSP72, or epidermal growth factor receptor.

Pulldown Assay—Recombinant GST, GST-Duox1-Δ1, GST-Duox1-Δ2, GST-Duox1M-Δ2, GST-Nox1-Δ, and GST-Nox2-Δ bound to Sepharose beads were incubated with purified, recombinant Noxa1 for 3 h at 4°C. The beads were pelleted and washed three times with phosphate-buffered saline. Proteins were resuspended in Laemmli buffer and boiled 5 min to elute proteins from the beads. The amount of Noxa1 and GST fusion proteins contained in the eluates was evaluated by electrophoresis using anti-Noxa1 and anti-GST antibodies and quantified by densitometry performed with ImageJ software. The quantity of Noxa1 bound to the different GST-coupled peptide fragments was normalized by the ratio Noxa1/GST density versus the background represented by GST beads alone using values from four different experiments. For GST-Nox1-Δ and GST-Duox1M-Δ2, two experiments were performed.

Transfection—HEK293 cells were seeded in 6-well plates for 24 h prior to transfection, which was performed using Lipofectamine 2000 according to the manufacturer’s protocol. In studies expressing mutant proteins, equal amounts of mutant proteins were used for transfection.
plasmid were used in place of wild type plasmid. Twenty four hours after transfection, HEK293 cells were assayed for ROS release (with or without ionomycin stimulation) using the HVA assay, as described above.

Statistical Analysis—Experiments were evaluated with the Student’s t test. p < 0.05 was considered statistically significant. The symbol * indicates a value of p < 0.05; ** indicates p < 0.01, and *** indicates p < 0.001.

RESULTS

Expression of Oxidase Components in Human Airway Epithelial Cells—Several NADPH oxidases have been implicated in ROS production in the airways. The calcium-activated oxidases Duox1 and Duox2 were linked to H2O2 generation in lung epithelial cells (16, 17). Other reports connected the Nox1/Noxo1 system as well as Nox3 to tumor necrosis factor receptor or Toll-like receptor4 signaling in ROS-mediated lung alterations (29–31). RT-PCR and immunoblotting were performed to assess expression of NADPH oxidases and oxidase-regulatory components in primary human small airway cells (SAEC) and in the lung cancer cell lines NCI-H292 and A549. Duox1 and Duox2 message and protein were expressed in SAEC and NCI-H292 cells, whereas A549 cells showed faint transcripts for Duox2 only, and expressed no detectable Duox protein by immunoblot analysis. SAEC cell lysates showed a 180-kDa band representing mature Duox (Fig. 1A, see also Fig. 1C) together with a sporadic appearance of an unidentified, faster migrating band that disappeared upon airway cell differentiation and upon Duox RNAi. Comparison of SAEC cells cultured in monolayer versus air-liquid interface (ALI) conditions on inserts (three-dimensional SAEC) indicated a 5–10-fold increase in Duox expression after differentiation (Fig. 1A). This result correlates well with the initial suggestion by Geiszt and co-workers (16) that Duox expression increases with cell differentiation. Other oxidases or certain oxidase regulatory components were absent from the cell types used in this study (supplemental Fig. 1A and data not shown).

Duox-mediated ROS Generation—As a physiological stimulus for Duox activation remains unidentified, the Ca2+ ionophore ionomycin was added to SAEC or NCI-H292 cells for 30–60 min to stimulate calcium influx. In resting conditions H2O2 release was not detected, whereas exposure to ionomycin led to substantial H2O2 production. Duox expression levels correlated with the extent of H2O2 production, with ~5-fold higher ROS production in differentiated SAEC (three-dimensional SAEC) than in SAEC cultured in monolayer (Fig. 1B). ATP and thapsigargin, two other stimuli known to increase calcium availability in cells, also led to a substantial increase in H2O2 generation in differentiated SAEC (data not shown). Similar results were obtained with Duox-expressing primary human bronchial epithelial cells and an immortalized SAEC cell line (data not shown).

By using ALI-cultured SAEC, the location of Duox-mediated H2O2 production was probed by treatment of the upper and lower chamber of the inserts with ionomycin. H2O2 production was only detected on the apical side of ionomycin-stimulated cells. This ROS production was abolished after pretreatment with diphenylene iodonium (DPI), an irreversible flavoenzyme inhibitor.

**FIGURE 1. Duox is the source for calcium-dependent ROS generation in airway epithelial cells.** A, presence of Duox1 and Duox2 was evaluated in the indicated cell types by RT-PCR and immunoblotting (IB). Actin served as loading control. Three-dimensional SAEC (SAEC 3D) represents SAEC grown in ALI conditions on inserts. Duox protein expression in two-dimensional (2D) culture versus three-dimensional (3D) culture was compared using increasing amounts of total cell lysate. B, H2O2 production in several cell types and culture systems was determined using HVA assays. Cells were stimulated with 3 μM ionomycin for 30–45 min. ROS generation was also analyzed in both compartments of three-dimensional SAEC (3D) grown in ALI culture (basolateral, apical). Cells were left untreated or pretreated with DPI (25 μM) for 10 min prior to ionomycin stimulation. Data represent means ± S.D. of one representative experiment with 10–30 comparable experiments performed in total in comparison with unstimulated cells; **, p < 0.01. C and D, NCI-H292 cells and SAEC cells were transfected twice with Duox 1 siRNA, Duox 2 siRNA, or with control siRNA (5 nM). C, expression levels of Duox were analyzed at the 96-h time point by immunoblotting. Ctrl, control. D, H2O2 production after ionomycin stimulation was determined using HVA assay and is expressed as percentage of H2O2 production in Duox siRNA- or control siRNA-treated cells versus control cells (100%). Values are representative of four (Duox#1 siRNA) or two (Duox#2 siRNA) independent experiments performed in duplicate.
H2O2 generation in primary human lung epithelial cells and that Duox proteins are the main source for calcium-stimulated ROS production was inhibited by 90–100% (Fig. 2B). This correlates well with the beginning of H2O2 production. Duox-mediated ROS generation was an event following Noxa1 translocation rather than preceding it, because pretreatment of cells with DPI did not affect stimulation-dependent Noxa1 movement to the cytosol (data not shown).

**Duox Regulation by Noxa1**

**FIGURE 2. Noxa1 translocates from the plasma membrane to the cytosol after ionomycin stimulation.**

A, expression of Noxa1 in the indicated cell types was evaluated by RT-PCR and Western blot, respectively. IB, immunoblot. B–C, NCI-H292 and SAEC cells were incubated in the presence (+) or absence (−) of ionomycin (3 μM) for 15 min. 3D, three-dimensional. B, immunofluorescence of Noxa1 (anti-Noxa1 antibody, red) with arrows indicating plasma membrane localization. C, Noxa1 localization after cell fractionation visualized by immunoblotting, as described under “Experimental Procedures.” D, Duox localization in lung epithelial cells by immunofluorescence imaging using anti-Duox antibody (green) and 4’,6-diamidino-2-phenylindole (blue). SAEC cells were exposed for a longer (left panel) or a shorter time (right panel) to differentiate plasma membrane staining from perinuclear staining of Duox. Duox antibody specificity was verified by transfecting SAEC cells with control siRNA or Duox 1 siRNA for 72 h. A549 cells show low level staining of the perinuclear region. E, localization of Duox was analyzed by cell fractionation followed by immunoblotting with anti-Duox. Immunoblotting for cytosolic RhoGDI was used to confirm the purity of the membrane fraction.

The contribution of Duox proteins to ROS generation by airway cells was probed with RNAi. Two different siRNAs were designed to regions identical in Duox1 and Duox2, thus targeting both Duox homologs at the same time. Time courses for both Duox1 and Duox2 mRNA and protein expression were performed in SAEC and NCI-H292 cells (Fig. 1C and supplemental Fig. 1B). Immunoblotting confirmed depletion of Duox1 and Duox2 after 96 h of RNAi treatment, which did not affect cell viability. After 96 h of treatment with Duox siRNA ROS production was inhibited by 90–100% (Fig. 1D), whereas control siRNA had no effect. These data indicate that Duox proteins are the main source for calcium-stimulated H2O2 generation in primary human lung epithelial cells and H292 cells.

**Noxa1 Translocates from the Membrane to the Cytosol upon Calcium Influx**—The oxidase regulatory component Noxa1 was detected in cell lysates of lung epithelial cells or lung cancer cells (Fig. 2A). In resting cells immunostaining of endogenous Noxa1 showed localization at the plasma membrane on intracellular membrane structures and in the nucleus (Fig. 2B). After ionomycin stimulation for 5–15 min, the majority of the Noxa1 staining at the membrane edges disappeared in SAEC and NCI-H292 cells (Fig. 2B). Translocation of Noxa1 from intracellular membranes to cytosolic fractions was difficult to visualize in immunofluorescence, but it was clearly detected when cellular fractionation was used. These experiments demonstrated that a significant fraction of the membrane-bound Noxa1 protein translocated to the cytosol after ionomycin stimulation of SAEC or H292 cells (Fig. 2C). Noxa1 depletion from the plasma membrane already occurred at the earliest stages of ionomycin stimulation (5 min) and seemed to coincide with the beginning of H2O2 production. Duox-mediated ROS generation was an event following Noxa1 translocation rather than preceding it, because pretreatment of cells with DPI did not affect stimulation-dependent Noxa1 movement to the cytosol (data not shown).

**Duox Is Localized on the Plasma Membrane and on Intracellular Membranes**—Duox localization was confirmed by immunofluorescence and cell fractionation in human lung epithelial cells (Fig. 2, D and E). Confocal images showed Duox protein at the plasma membrane and on intracellular membranecellar membrane structures and in the nucleus (Fig. 2B). After ionomycin stimulation for 5–15 min, the majority of the Noxa1 staining at the membrane edges disappeared in SAEC and NCI-H292 cells (Fig. 2B). Translocation of Noxa1 from intracellular membranes to cytosolic fractions was difficult to visualize in immunofluorescence, but it was clearly detected when cellular fractionation was used. These experiments demonstrated that a significant fraction of the membrane-bound Noxa1 protein translocated to the cytosol after ionomycin stimulation of SAEC or H292 cells (Fig. 2C). Noxa1 depletion from the plasma membrane already occurred at the earliest stages of ionomycin stimulation (5 min) and seemed to coincide with the beginning of H2O2 production. Duox-mediated ROS generation was an event following Noxa1 translocation rather than preceding it, because pretreatment of cells with DPI did not affect stimulation-dependent Noxa1 movement to the cytosol (data not shown).

**Duox Regulation by Noxa1**—The shared plasma membrane localization of Duox and Noxa1 in resting cells prompted us to determine complex formation of these proteins. Overlapping migration of Noxa1 protein and the upper IgG band did not permit immunodetection of Noxa1 in Noxa1 immunoprecipitates. Thus, we performed a control experiment to validate
Duox Regulation by Noxa1

FIGURE 3. The association of Noxa1 and Duox is calcium-dependent. A, immunoprecipitation of transfected Myc-Noxa1 or empty vector control with anti-Noxa1 antibody in HEK293 cell lysates followed by immunoblot (IB) with anti-Myc antibody. Noxa1 expression in total cell lysates is shown using anti-Noxa1 antibody. B and E, co-immunoprecipitation of Noxa1 with Duox was performed using protein extracts preincubated without (B) or with (E) calcium (see “Experimental Procedures” for details). After Noxa1 immunoprecipitation (IP), associated Duox (180 kDa) was detected by immunoblotting (IB). B, top panel shows co-immunoprecipitation using increasing amounts of anti-Noxa1 in lysates derived from NCI-H292 cells. As controls, Noxa1 immunoprecipitation was performed in lysates of NCI-H292 cells, which were left untreated or treated with Duox 1 siRNA (2nd panel, left), or in lysates derived from Duox-deficient A549 cells (2nd panel, right) versus lysates derived from untreated NCI-H292 cells. Experiments were repeated three times with similar results. C, Noxa1 localization at the plasma membrane is dependent on Duox. SAEC cells treated with Duox 1 siRNA or control siRNA were stained with anti-Noxa1 antibody. D, effect of various concentrations of BAPTA/AM (0.125 to 0.75 mM) on ionomycin-stimulated H2O2 production by NCI-H292 cells was evaluated by using the HVA assay. Data are representative of three independent experiments, and error bars are the standard deviation. The immunoblot shows inhibition of Noxa1 translocation to the cytosol, when NCI-H292 cells were pretreated for 5 min with BAPTA/AM (0.75 mM) before ionomycin stimulation. E, in vitro pulldown assay to determine direct interaction of recombinant, purified Noxa1 with purified GST, GST-Nox2Δ, GST-Duox1Δ1, and GST-Duox1Δ2 bound to beads. Both GST or GST peptides bound to beads and associated Noxa1 were detected by immunoblotting, and the intensity of these bands was quantified using ImageJ software. The graph shows the ratio of band intensity between Noxa1 and GST fusion protein bands set in relation to Noxa1 binding to GST alone. Error bars are the standard error of four independent experiments.

the Noxa1 antibody for immunoprecipitation. Noxa1 antibody immunoprecipitated Noxa1 from HEK293 cell lysates expressing Myc-Noxa1 as visualized with anti-Myc immunoblotting (Fig. 3A). To test association of endogenous proteins, Noxa1 was immunoprecipitated with increasing amounts of anti-Noxa1 antibody followed by immunoblotting for Duox. We detected increasing amounts of Duox in Noxa1 immunoprecipitates (180 kDa; Fig. 3B). Several controls were carried out to validate these data. Immunoprecipitation with anti-Noxa1 in lysates derived from Duox-deficient A549 cells versus Duox-containing NCI-H292 cells demonstrated the absence of the 180-kDa band in A549 cells (Fig. 3B). Similarly, Duox siRNA in NCI-H292 cells resulted in a significant decrease in recovered Duox after Noxa1 immunoprecipitation, when compared with control siRNA-treated cells (Fig. 3B). The presence of Noxa1 on the plasma membrane of airway cells was dependent on plasma membrane-bound Duox as demonstrated by immunofluorescence staining of SAEC cells treated with control or Duox siRNA (Fig. 3C). Some Noxa1 staining on intracellular membranes remained, indicating that either Duox knockdown was not complete or that Noxa1 may have a secondary membrane target.

Disruption of the Noxa1-Duox Association by Calcium Elevation—Duox requires the presence of micromolar concentrations of calcium to generate ROS (8). Indeed, when calcium was omitted from buffers used for ROS detection, H2O2 was not produced upon ionomycin stimulation (data not shown). Similarly, when cells were pretreated with the calcium chelator BAPTA/AM, H2O2 production was inhibited (Fig. 3D). In these conditions, translocation of Noxa1 from the membrane to the cytosol did not occur (see immunoblot Fig. 3D). This suggests that calcium elevation is, at least in part, responsible for the translocation of Noxa1. To substantiate this hypothesis, we investigated if the interaction between Duox and Noxa1 was calcium-dependent. Resting NCI-H292 cells were lysed in the presence of 5 mM EGTA with or without the addition of 5–10 mM CaCl2 before immunoprecipitation of Noxa1 was performed. As shown in Fig. 3E, an increase in free calcium to ~0.3–1 μM free Ca2+ substantially decreased the association of Duox and Noxa1 in vitro.

Noxa1 Binds to the Duox1 C Terminus in Vitro—To determine whether the association between Noxa1 and Duox is direct or mediated by another protein, protein fragments encompassing intracellular domains of Duox1 were generated. GST fusion proteins, including a fragment containing the calcium-binding EF hands (GST-Duox1Δ1) or encompassing the C terminus (GST-Duox1Δ2), were generated. GST protein and a C-terminal fragment of Nox1 served as positive control, whereas a C-terminal fragment of Nox2 was prepared as a negative control (supplemental Fig. 3A). These fragments were
tested for their ability to interact with purified, recombinant Noxa1 in in vitro pulldown assays. Although we noticed some unspecific binding of Noxa1 to the beads, including the GST control beads, Noxa1 binding to the C terminus of Duox1 (GST-DuoxΔ2) was 8–10 times higher than the GST background values (Fig. 3F). By determining the ratio of GST fusion proteins bound to the beads versus Noxa1 binding to these proteins, the Nox2 C terminus and the EF hand-containing fragment of Duox1 exhibited clearly less binding of Noxa1 than the Duox1 C terminus. As anticipated, the C terminus of Nox1 bound to Noxa1 in the pulldown assay (supplemental Fig. 3). These results indicate that Noxa1 binds directly to the Duox1 C terminus in vitro.

**Noxa1 Inhibits Duox-mediated ROS Generation in Resting Cells**

The maturation factor DuoxA1 is required for Duox expression in heterologous cell systems (32). Cotransfection of Duox1 and Myc-tagged DuoxA1 in HEK293, HeLa, and H661 cells generated calcium-inducible ROS (Fig. 4A). In fact, coexpression of Duox1 and Myc-DuoxA1 generated also basal, unstimulated H2O2 in HEK293 and HeLa cells. This was not observed in H661 cells, which are deficient in Duox and DuoxA proteins, but express Noxa1 (see Fig. 5C). We assessed if Noxa1 expression alters the basal and stimulated H2O2 production by Duox1. Duox1 and DuoxA1 were co-transfected without or with Noxa1 into HEK293 cells, followed by HVA assay and immunoblotting (Fig. 4B, C). As expression levels did not change significantly between the different conditions, ROS production by Duox1/DuoxA1 was set to 100%. An inhibition of ~40–50% of the basal H2O2 production was observed in the presence of Noxa1. Only a slight inhibitory effect of Noxa1 on ionomycin-stimulated H2O2 production was detected (Fig. 4B). This suggests that the association of Noxa1 with Duox1 acts as a negative Duox1 regulator, keeping the oxidase inactive in resting cells via complex formation. Release of Noxa1 from Duox1 was observed in transfected HEK293 cells, showing loss of Noxa1 from the membrane after ionomycin stimulation. Although, in contrast to endogenous Noxa1, overexpressed Noxa1 was partially bound to the plasma membrane in the absence of Duox1, this fraction of Noxa1 did not translocate to the cytosol after ionomycin treatment (data not shown).

The importance of the Noxa1 activation domain on Duox-mediated H2O2 generation was tested. Transfection of Noxa1 or Noxa1(V205A) inhibited equally well basal ROS generation by Duox1 (Fig. 4B and C). Thus, the activation domain of Noxa1 is not required for Duox function. We then set out to

**FIGURE 4. Noxa1 inhibits basal activity of Duox1.** HEK293 cells were transfected with different combinations of DuoxA1, Duox1wt, and/or Noxa1wt or Noxa1 V205A as indicated. 24 h after transfection, cells were incubated 30 min in HVA solution with or without ionomycin to measure ROS generation (A and B, comparison with cells transfected with Duox1 alone; **, p < 0.01, and ***, p < 0.001; or with cells transfected with DuoxA1 and Duox1 without Noxa1; **, p < 0.01) or were harvested and lysed in Laemmli buffer for immunoblotting (C, EV, empty vector. D and E, down-regulation of endogenous Noxa1 in SAEC cells. Cells were transfected with three different Noxa1 siRNAs or control siRNA. D, 24 and 48 h after the transfection, Noxa1 mRNA levels were evaluated by RT-PCR. The appropriate cycle number for the linear range of Noxa1 PCR was tested. E, expression levels of Noxa1 were analyzed 48 h after siRNA treatment by Western blotting (lower panel), and basal H2O2 production was determined using HVA assay without stimulation of cells (upper graph). Data are representative of three independent experiments, and error bars are the standard deviation, n = 3; comparison with cells transfected with control siRNA; *, p < 0.05.**
evaluate the effect of Noxa1 knockdown on basal ROS production by primary airway cells. siRNA treatment decreased Noxa1 protein 50–60%. Noxa1 RNAi for more than 36–48 h or at higher concentration was not well tolerated by SAEC. Knockdown of Noxa1 augmented basal H₂O₂ production, but it did not alter significantly ionomycin-stimulated ROS generation (Fig. 4, D and E, and data not shown).

The Duox C-terminal PXXP Motif Is Involved in Noxa1 Binding—The Duox1 C terminus, the site of Duox1-Noxa1 association in vitro (Fig. 3F), contains a proline-rich motif, which represents a putative Noxa1 SH3 domain-binding site. We generated a Duox1 mutant in which proline 1497 was replaced by alanine and compared Duox wild type and Duox mutant for their ability to support basal and stimulated H₂O₂. Co-transfection of Noxa1 did not alter basal and stimulated H₂O₂ production by mutant Duox1(P1497A) (Duox1M) (Fig. 5, A and B). Unexpectedly, the Duox1(P1497A) mutant displayed increased ROS production, suggesting that this mutation leads to a conformational change in the Duox1 C terminus and/or altered FAD/NADPH binding. This substantially higher H₂O₂ production by Duox1M was observed in three different cell types (Fig. 5C and data not shown).

Mutation of the Duox1 PXXP site may abolish binding of Noxa1 to Duox1. When overexpression of DuoxA1, Duox1, or Duox1M and Noxa1 was carried out in HEK293 cells, expression levels of Duox1 and Duox1M were comparable (Fig. 5D). Immunoprecipitation with anti-Noxa1 antibody revealed that the Duox1 mutant can still bind to Noxa1, albeit with less efficiency (Fig. 5D). Similar results were obtained in in vitro pulldown experiments using the C termini of Duox1 or Duox1M (supplemental Fig. 3B). Additional mutation of the Noxa1 SH3 domain was carried out for co-expression experiments of Noxa1(W436R) and Noxa1wt with Duox1wt or Duox1(P1497A). Noxa1 immunoprecipitation revealed that the association between wild type and mutant Noxa1 and the Duox1 mutant (Duox1M) was perturbed. On the other hand, we did not observe a significant change in Noxa1 or Noxa1 mutant association with wild type Duox1. This suggests that the interaction between the Duox C-terminal PXXP motif and the Noxa1 SH3 domain alone is not sufficient to convey high affinity Noxa1 binding in vitro. A second binding site or another modification on Duox or Noxa1 seems to be required for the
Duox1-Noxa1 interaction at the cell membrane and for release of Noxa1 into the cytosol. This scenario would be reminiscent of our recent observations regarding Noxa1 dissociation from the membrane-bound Nox1 complex, which was facilitated by protein kinase A and 14-3-3 protein (33).

**DISCUSSION**

Inflammatory cells such as alveolar macrophages or activated neutrophils are considered the major source for ROS in the lung. Lately, however, cells of the pulmonary endothelium and epithelium are also recognized as important ROS generators. The discovery of the NADPH oxidases Duox, which reside in the airway epithelium and thyroid, stimulated new studies elucidating how pulmonary ROS are produced and how these enzymes might be regulated. To analyze and understand the complex and varied functions of human airway epithelia in a controlled setting and in the absence of other cell types, an *in vitro* differentiation model was used in this study. This three-dimensional model system, which uses primary human bronchial or small airway cells, creates a mucus-coated, pseudostratified epithelial airway after 3–4 weeks of continuous culture (34–36). Several markers for differentiation, biophysical measurements, and electron microscopy were used to ensure the presence of the phenotypic features described for a well differentiated airway epithelium (data not shown). When we examined the expression of the two Duox isoforms, Duox1 and Duox2, it became clear that the differentiation process leads to highly increased expression of Duox protein. It is presently not feasible to distinguish between Duox1- or Duox2-dependent functions, because available antibodies, including ours, cannot differentiate between the Duox isoforms. Elevated Duox expression caused, as expected, 5-fold higher output of \( \text{H}_2\text{O}_2 \) after stimulation with \( \text{Ca}^{2+} \) ionophore. Confirming earlier suggestions (16, 17), \( \text{H}_2\text{O}_2 \) generation was confined to the apical surface of the epithelial model. Knockdown studies in two different cell types determined Duox as the source of calcium-stimulated \( \text{H}_2\text{O}_2 \) generation in airway cells. It is important to note that we could not detect functional Duox in the lung cancer cell line A549, which is widely used as airway type II cell model.

Screening of human primary lung epithelial cells revealed the presence of the NADPH oxidase component Noxa1, whereas many of the other previously described oxidase proteins were missing. Noxa1 exhibits only \( \sim 30\% \) identity with the NADPH oxidase component p67phox but presents very similar domain architecture (19–21). In contrast to the role of p67phox in the Nox2-based phagocyte oxidase, Noxa1 seems to fulfill different functions in epithelial cells. The Noxa1 SH3 domain was not required for phorbol ester-induced Nox1-dependent ROS generation (37) and decreased Nox0-stimulated Nox3 activity by \( \sim 50\% \) (23, 38). Here we show that endogenous Noxa1 is membrane-bound in primary lung epithelial cells and associates with Duox via its SH3 domain. Upon calcium influx Noxa1 dissociates from the membrane compartment and translocates to the cytosol. Calcium entry, but not ROS production, was required for Noxa1 translocation, indicating that dissociation was an early step in the Duox activation process. This suggests that Noxa1 may act as an inhibitory adaptor rather than an activator of Duox activity.

Recent identification of Duox maturation factors (32) such as DuoxA1 permits the processing of Duox1 and the recovery of a mature, active form of Duox1 on the plasma membrane of epithelial cell lines. We noticed that co-transfection of Duox1 with DuoxA1 triggered \( \text{H}_2\text{O}_2 \) production without prior stimulation in HEK293 cells. This ROS generation was substantially enhanced after stimulation with ionomycin. Basal ROS generation was not observed in primary human lung epithelial cells or Duox-deficient lung cancer cells (H661) where Duox was reintroduced via transfection. These cell types express Noxa1, whereas HEK293 or HeLa cells lack expression of this protein. This prompted us to investigate the role of Noxa1 in regulating ROS production by Duox in more detail. Co-transfection of Noxa1 together with Duox1 and DuoxA1 into HEK293 cells inhibited basal production of \( \text{H}_2\text{O}_2 \), but it did not affect calcium-stimulated ROS generation when a strong calcium flux inducer like ionomycin was used. Additionally, we observed a substantial increase of basal ROS generation when endogenous Noxa1 protein was knocked down by RNAi. These data support the idea that Noxa1 functions as a negative regulator of basal Duox stimulation, which could occur in the course of spontaneous calcium oscillations. In accord, mutational analysis revealed that the activation domain of Noxa1 was dispensable for Duox regulation.

Because mutation of a proline-rich motif in the C terminus of Duox1 diminished Noxa1-dependent inhibition of basal ROS generation, we propose that the PXXP domain of Duox is involved in the Duox regulation. The C terminus of both Duox isoforms is highly homologous, suggesting that Noxa1 may regulate Duox1 and Duox2. This scenario is reminiscent of studies suggesting calcium-mediated regulation of Nox5 via intramolecular interaction of the Nox5 N terminus with the Nox5 C terminus (39). In the case of Nox5, an increase of intracellular calcium leads to binding of \( \text{Ca}^{2+} \) to the EF hands in the N terminus. This may cause a conformational change, which then provides an interface for the interaction between the N terminus and the C terminus, leading to electron transport and oxidase activation. Although detailed *in vitro* studies with recombinant Duox might be necessary to deduce the Duox activation process, our data suggest an inhibitory mechanism via the Duox C terminus. This inhibition is relieved when \( \text{Ca}^{2+} \) binds to the two EF hands, causing Noxa1 to drop off. Most of the endogenous Noxa1 is membrane-bound in airway cells, whereas Noxa1 when expressed in model cell lines localizes to the membrane and the cytosol. Thus, the overexpression system does not exactly reflect the Duox-Noxa1 regulation in lung epithelial cells. This may account for the only partial effect of Duox1 P1497A or Noxa1 W436R mutations in abolishing Duox1-Noxa1 interaction. It is also likely that additional modifications on Duox or Noxa1 are required to keep Noxa1 in the cytosol during Duox1 activation and that other yet unknown proteins are involved in Noxa1 shuttling from the membrane to the cytosol.

Regions in close proximity to the Duox PXXP domain seem to be critical for regulation of oxidant production by Duox, because a point mutation in this region increased basal and
calcium-stimulated H$_2$O$_2$ generation significantly. This might reflect the importance of the Duox C terminus as control mechanism for Ca$^{2+}$ binding or for termination of the electron flow. Limited proteolysis of thyroid NADPH oxidase by a-chymotrypsin results in irreversible, calcium-independent H$_2$O$_2$ production (40). These data together with our observation support the idea that Duox activity might be regulated via the displacement or conformational change of an inhibitory domain. Although Nox5 and Duox regulation have some regulatory features in common, such as Ca$^{2+}$-mediated conformational change and activation by relieving inhibition, there seem to be also very distinctive differences. A role for Nox1 or any other regulatory protein has not been reported for Nox5. Duox and Nox5 are emerging as NADPH oxidases that use an inhibitory mechanism in the basal state, which represents a novel way of oxidase regulation. Because binding of calcium is also used as activation mechanisms for plant oxidases (41, 42), this model of activation may have broader relevance for Ca$^{2+}$-activated oxidases in general.

Acknowledgments—We gratefully acknowledge F. Miot, T. Leto, and G. Bokoch for reagents. We also thank K. Schreiber for excellent editorial and graphical assistance and Y. Pavlova for technical support.

REFERENCES
1. Burdon, R. H. (1995) Free Radic. Biol. Med. 18, 775–794
2. Babor, B. M. (1999) Blood 93, 1464–1476
3. Moskwa, P., Lorentzen, D., Eccoffon, K. J., Zabner, J., McCray, P. B. Jr., Nauseef, W. M., Dupuy, C., and Banfi, B. (2007) Ann. J. Respir. Crit. Care Med. 175, 174–183
4. Babor, B. M. (1995) Curr. Opin. Hematol. 2, 55–60
5. Yu, L., Quinn, M. T., Cross, A. R., and Dinauer, M. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7993–7998
6. Cross, A. R., and Jones, O. T. (1991) Biochim. Biophys. Acta 1057, 281–298
7. Finkel, T. (1999) J. Leukocyte Biol. 65, 337–340
8. Ameziane-El-Hassani, R., Morand, S., Boucher, J. L., Frapart, Y. M., Ameziane-El-Hassani, R., Morand, S., Boucher, J. L., Frapart, Y. M., Apos-