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Redox-Dependent Expression of Cyclin D1 and Cell Proliferation by Nox1 in Mouse Lung Epithelial Cells

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ABSTRACT

NADPH oxidases produce reactive oxygen species (ROS) that serve as co-stimulatory signals for cell proliferation. In mouse lung epithelial cells that express Nox1, Nox2, Nox4, p22^{phox}, p47^{phox}, p67^{phox}, and Noxo1, overexpression of Nox1 delayed cell cycle withdrawal by maintaining AP-1-dependent expression of cyclin D1 in low serum conditions. In cycling cells, the effects of Nox1 were dose dependent: levels of Nox1 that induced 3- to 10-fold increases in ROS promoted phosphorylation of ERK1/2 and expression of cyclin D1, whereas expression of Nox1 with Noxo1 and Noxa1 (or expression of Nox4 alone) that induced substantial increases in intracellular ROS inhibited cyclin D1 and proliferation. Catalase reversed the effects of Nox1 on cyclin D1 and cell proliferation. Diphenylene iodonium, an inhibitor of NADPH oxidase activity, did not affect dose-dependent responses of ERK1/2 or Akt to serum, but markedly inhibited the sequential expression of c-Fos and Fra-1 required for induction of cyclin D1 during cell cycle re-entry. These results indicate that Nox1 stimulates cell proliferation in actively cycling cells by reducing the requirement for growth factors to maintain expression of cyclin D1, whereas during cell cycle re-entry, NADPH oxidase activity is required for transcriptional activation of Fos family genes during the immediate early gene response. *Antioxid. Redox Signal.* 8, 1447–1460.

INTRODUCTION

Reactive oxygen and nitrogen species (ROS/RNS) have properties required for effective mediators of cell signaling: their levels fluctuate in response to a variety of extracellular and intracellular stimuli, they are diffusible in and between cells, and they interact with specific molecular targets (11–14). Depending on concentration, exogenous sources of ROS/RNS may increase cell proliferation, induce cell cycle arrest, or induce cell death, either through necrotic or apoptotic mechanisms (22, 37). Endogenous sources of ROS have been recognized as important mitogenic signaling molecules (15, 22, 43), and have been implicated in cell growth, transformation, and tumorigenicity (4). Growth factors induce the production of ROS-involved mitogenic signaling from multiple sources, including mitochondria, metabolic enzymes such as 5-lipoxygenase, and NADPH oxidase complexes.

In professional phagocytes, the gp91*phox* NADPH oxidase complex is a regulated source of ROS that serve as antimicrobial agents in host defense (18, 30). Homologs of gp91*phox* (also known as Nox2) that include Nox1, Nox3, Nox4, and Nox5 have been identified, and recent studies indicate that generation of H_2O_2 by gp91*phox* and related NADPH oxidases occurs in tissues and cells other than phagocytes, including aortic adventitia, kidney, liver, vascular smooth muscle cells, and fibroblasts (18, 30). These enzymes generate superoxide, which is rapidly converted to H_2O_2 either spontaneously or via superoxide dismutase (SOD). Nox1, a 65 kDa protein that shows 58% homology with Nox2, has been studied in regard to a role in cell growth and tumorigenesis (2,

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47). Human tumors produce large amounts of H_2O_2 (49), ROS promote tumor progression (46), and expression of Nox1 is required for the production of ROS and rapid tumor growth in response to activated Ras (39), a finding in agreement with studies that show antioxidants impede mitogenesis in Ras-transformed cells (28). Tumorigenicity in Nox1-expressing cells is reversed by co-expression of catalase (2), indicating that hydrogen peroxide (H_2O_2) regulates pathways promoting unrestrained cell proliferation. Further, antioxidants have been shown to inhibit growth factor induced ERK-activation and DNA synthesis in arterial smooth muscle cells (48), growth factor-induced c-Fos expression in chondrocytes (33), PMA-induced cyclin D1 expression and DNA synthesis in a subclone of NIH3T3 cells (27), and androgenic regulation of oxidative stress by NAD(P)H oxidases in rat prostate (51).

While the mechanism of regulation of the Nox2 oxidase complex of phagocytes by the small GTPase Rac2 (and in some cases Rac1), is well described (18, 30), the regulation of nonphagocytic oxidases is still incompletely understood. Recently the sequential activation of phosphatidylinositol-3 kinase (PI-3 kinase), the guanine nucleotide exchange factor β-Pix and Rac1 were shown to activate Nox1 oxidase activity (40), supporting a role for Nox1 in the reversible inactivation by oxidation of the tumor suppressor protein PTEN, a phosphatase that negatively regulates the PI-3 kinase/Akt pathway (31, 32). Noxo1 and Noxa1, novel assembly and activator proteins that potentiate superoxide production by Nox1, have been described (3, 9, 17, 50), but a role for these factors in mitogenic signaling has not yet been reported.

In general, ROS accentuate signaling through mitogenic signaling pathways that converge on transcription factors such as AP-1, NF-κB, Sp1, and Ets that control of expression of cyclin D1 (7), suggesting cyclin D1 may be an important mediator linking ROS and cell growth. Previously we examined the dose-dependent effects of reactive nitrogen species (RNS), ROS, and asbestos on cell cycle re-entry in serum-stimulated mouse lung epithelial (C10) cells, which require activation of both PI-3 kinase/Akt and extracellular signal-regulated kinase (ERK1,2) pathways for expression of cyclin D1 (8, 54, 55). These studies showed that ROS, RNS, and asbestos have dose-dependent effects on cell cycle progression by modulating the expression of cyclin D1. For example, in serum-stimulated C10 cells treated with crocidolite asbestos, those cells that expressed cycle D1 were able to continue on to S phase in the presence of continued exposure, whereas those that did not were unable to continue progression through the cycle (55). Based on such findings, we have proposed that expression of cyclin D1 represents successful resolution of redox-dependent signaling pathways that mediate the G0 to G1 transition of the cell cycle (7).

Here we have used expression of the NADPH oxidases Nox1 and Nox4 to influence the production of ROS in C10 cells, and show that constitutive production of low levels of intracellular ROS by Nox1 in cycling cells reduces the requirement for serum for maintenance of ERK1/2 phosphorylation and expression of cyclin D1. Unexpectedly, an inhibitor of NADPH oxidase activity did not block activation of ERK1/2 or Akt in serum-stimulated C10 cells, but inhibited the sequential expression of c-Fos and Fra-1 required for expression of cyclin D1 during cell cycle re-entry.

MATERIALS AND METHODS

Cell culture

Murine type II alveolar C10 cells (36) and Nox1-expressing C10 cells were maintained in CMRL medium supplemented with 10% fetal bovine serum (FBS) containing 100 units/ml penicillin and 100 μg/ml streptomycin.

Plasmids constructs and generation of stably transfected cell lines

The following expression vectors were used in transfection experiments: human Nox1 cDNA in pcDNA3, human catalase cDNA in pZeoSV, human Nox4 cDNA in pcDNA3.1, NOXO1-β cDNA in pcDNA3, and NOXA1-β in pCMV-Sport (kind gifts from G. Cheng, R. Arnold and D. Lambeth, Emory University, Atlanta, GA). C10 cells were transfected using Lipofectamine 2000 (Invitrogen, Fredrick, MD) with pcDNA3 vector alone or with vector encoding Nox1 and colonies of stably transfected cells were recovered by selection in 200 μg G418/ml. Clonal vector control cells (C10-vector) or Nox1-expressing C10 cells (C10-Nox1#A2 and C10-Nox1#B3) used in this study were identified by RT–PCR as shown in Fig. 1. Stably transfected C10 cells were maintained in media with 200 μg/ml G418 (Invitrogen) Selected Nox1-expressing cell clones were then transfected with human catalase cDNA in pZeoSV, and stable derivatives (C10-vector-catalase, C10-Nox1#A2-catalase6, and C10-Nox1#B3-catalase9) were identified by selection in hygromycin and immunoblotting for human catalase. In transient transfection studies, cell lines indicated in the text were transfected with reporter or expression plasmids using Lipofectamine, as described previously (8). Transfection efficiency was determined in each experiment by transfecting cells with a pCMV-GFP expression vector and analyzing GFP expression by either flow cytometry or fluorescence microscopy; transfection efficiency after 24 h ranged from 75% to 90% of the population (data not shown).

Cell growth assays

Cells were plated in 24-well tissue culture plates at 3×10^3 cells/well in media with various concentrations of FBS, as indicated. At selected times, cells were trypsinized, resuspended in medium supplemented with 10% FBS, and counted with a hemocytometer.

RT–PCR

TaqMan assays (Applied Biosystems, Foster City, CA) were used to examine expression of mRNAs for oxidase components in C10 cells. RNA was extracted using RNeasy mini kit protocol (Qiagen, Valencia, CA) and treated with DNase I on the column before elution. First strand cDNA was synthesized for 2 h at 42°C using Superscript II RT (Invitrogen): the reaction mix contained 1 mg of total RNA, 500 ng of oligo dT primers, 10 m*M* dNTPs, 1x first strand buffer, 0.1 *M* DTT, 40 units of RNase-out, and 50 units of Superscript II reverse transcriptase. The mixture was incubated for 2 h at 42°C. After inactivation at 70°C for 15 min, 50 ng of product was used for quantitative PCR using an ABI 7700 (Applied Biosystems) sequence detection system. The results are expressed in 1/ct (inverse of the number of cycles). The follow-

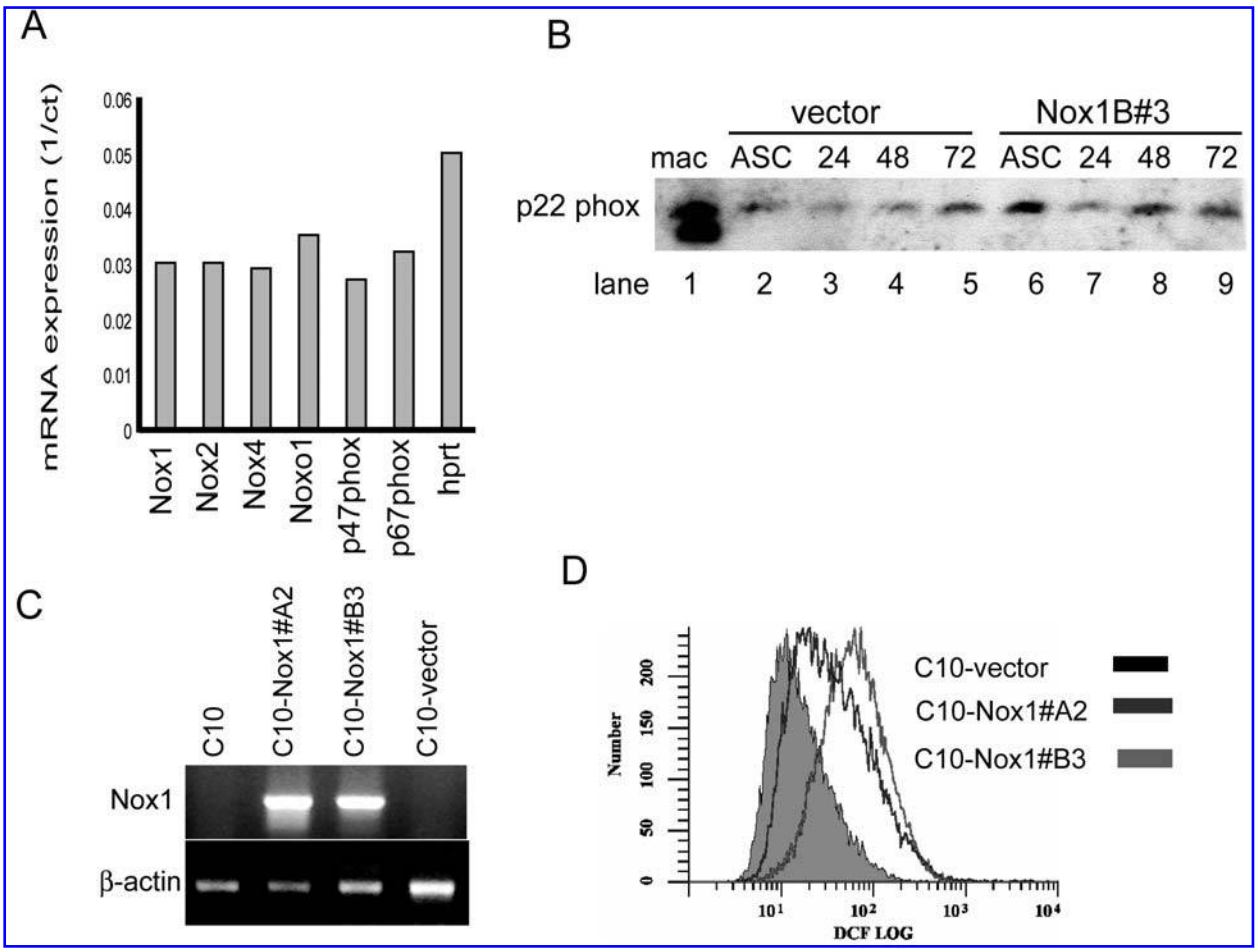

FIG. 1. Generation of mouse lung epithelial cells that stably express Nox1. (**A**) Expression of NADPH oxidase components in C10 cells. Quantitative RT–PCR was used to assay C10 cells for mRNAs encoding NADPH oxidase components, with HPRT as control. mRNA levels are expressed as 1/CT. No signal was obtained for Nox3, Noxa1, or $p40^{phox}$. (**B**) Expression of $p22^{phox}$ in C10 and Nox1#B3 cells. Equal quantities of total cell extracts were assessed for expression of $p22^{phox}$ by immunoblotting, with mouse macrophage cell extract as a control (mac, *lane 1*). (**C**) C10 cells were transfected with a plasmid expression vector for human Nox1 or with empty vector alone. Parental C10 cells and the indicated stable cell clones were analyzed for Nox1 expression by standard RT–PCR. Products were resolved by agarose gel electrophoresis and stained with ethidium bromide; β-actin was used as a control mRNA. (**D**) Production of intracellular H_2O_2 by Nox1 expressing C10 cells was assessed by staining live cells with 2 μ*M* dichlorofluorescin diacetate (DCF-HA) and quantifying mean fluorescence by flow cytometry. The Nox1#B3 and Nox1#A2 C10 cell lines were representative of clones expressing Nox1, the majority of which displayed two- to 10-fold increases in mean DCF fluorescence.

ing Assay-on-Demand primers (Applied Biosystems) were used: Nox1 Mm00549170_m1; Nox2 Mm00432775_m1; Nox3 Mm01339126_m1; Nox4 Mm0079246_m1; $p47^{phox}$ (Ncf1) Mm00447921_m1; $p67^{phox}$ (Ncf2) Mm00726636_s1; Noxo1 Mm00546832_g1, and Noxa1 Mm00549170-m1. For detection of Nox1 mRNA in stably transfected cells, total cellular RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instruction, reverse transcription was performed with using an Advantage RT-for-PCR kit (Clonetech, Mountain View, CA), and PCR was performed for 25 cycles with the primer pair: 5′-TAT TCG AGC AGC AGG GGA CTG GAC-3′ and 5′-CTC TGT CAA AGT TTA ATG CTG CAT GAC CA-3′.

Measurement of intracellular ROS

To assess the levels of intracellular ROS, flow cytometry of cells stained with dichlorofluorescin diacetate (DCF-HA, Molecular Probes, Eugene, OR) was used as described previously (54). Briefly, cells were plated in 100 mm dishes at 5×10^6 cells/ well, treated as described in the text, and then trypsinized, washed with Hanks balanced salt solution (HBSS, Invitrogen) containing 5% FBS, and then resuspended in 5% FBS in HBSS. Five $\times$ 10^5 cells per ml then were incubated in 2 μ*M* DCF-HA for 1 h in the dark at room temperature and then analyzed by flow cytometry.

Cell cycle analysis

Cells grown in different serum conditions were harvested at the indicated time-points and analyzed for cell cycle distribution by staining with propidium iodide and flow cytometry, as described previously (8, 54).

Immunoblotting

Immunoblotting was performed with total cell lysates, as described previously (34, 35). Briefly, cells were rinsed with PBS, lysed in 2X SDS sample buffer, and equal amounts of soluble protein were resolved by electrophoresis on 8% or 10% SDS-polyacrylamide gels. Proteins were then transferred to Immobilon-P membranes (Millipore, Billerica, MA) by electroblotting. Blots were blocked in 4% nonfat dry milk or 4% BSA fraction V in Tris-buffered saline. Blots were then probed with indicated primary antibodies, and then with the appropriate horseradish peroxidase-coupled secondary antibody (Amersham, Biosciences, Piscataway, NJ). Signals were detected by ECL system (Amersham). Primary antibodies were as follows: $p22^{phox}$: Santa Cruz SC-20781; cyclin D1: Santa Cruz SC-20044; pERK: Cell Signaling 9101S (Danvers, MA); total ERK: Cell Signaling 9102; Fra-1: Santa Cruz Sc-605: c-Fos: Santa Cruz SC-7202; pAkt: Cell Signaling 9271; Akt: Cell Signaling 9272: secondary rabbit HRP-conjugated: Amersham NA934V; secondary mouse HRP-conjugated: Amersham NA931V.

Cyclin D1 reporter gene assays

To construct the -3620 mCD1LUC reporter plasmid for the mouse cyclin D1 promoter, a 3753 bp fragment of the cyclin D1 promoter was isolated from a bacterial artificial chromosome (BAC 43C04) from the RPCI23 C57 Bl/6 mouse genomic library (Roswell Park Cancer Institute, Buffalo, NY) and subcloned into the pGL3 Basic luciferase reporter plasmid (Promega, Madison, WI) using standard molecular techniques. The remaining cyclin D1 luciferase reporter genes were a gift from R. Pestell (Lombardi Cancer Center, Georgetown University, Washington DC), and were used as described previously (8). Briefly, cells were plated into 100-mm tissue culture dishes at 60%–75% confluence and incubated in CMRL medium with 10% FBS overnight. Cells then were transiently transfected with either a cyclin D1 luciferase reporter construct or control vector, with or without the indicated expression vectors for NADPH oxidase components, using Lipofeactamine, as above. Under these conditions, the co-transfection efficiency was >90%. After 6 h, the culture medium was replaced with fresh CMRL with 10% FBS. The following day, transfected cells were trypsinized, plated in 24-well tissue culture plates, and treated as indicated. At the indicated time-points, culture wells were washed once with PBS, cells were lysed and luciferase activity was determined using a luciferase assay system (Promega). Luciferase activity was quantified using a Lumat LB 9507 luminometer (Berthold Industries, Oakridge, TN) and normalized to protein concentration as determined by BioRad Protein assays (BioRad, Hercules, CA) to determine the relative luciferase activity (RLU)/μg of protein lysate. To standardize transfection conditions, cyclin D1 promoter activity was normalized for transfection efficiency by co-transfecting with a cDNA encoding β-galactosidase (50 ng/100-mm dish). β-Galactosidase activity was assessed by colorimetric assay using o-nitrophenyl-β-D-galactoside as substrate (35). Transfection efficiency as assessed by β-galactosidase staining was found to be ˜70% (data not shown).

STATISTICAL ANALYSIS

Results are expressed as the mean ± the standard error of the mean (SEM) of at least three independent experiments. Statistical significance of difference between test groups was assessed by one-way ANOVA followed by Scheffe's test (post hoc). Statistical significance was defined at $p < 0.05$.

RESULTS

Expression of NADPH oxidase components in lung epithelial cells

Using quantitative RT–PCR and immunoblotting, the expression of NADPH oxidase components was examined in C10 cells, a mouse lung type II epithelial cell line. As compared to the control mRNA for HPRT, RT–PCR indicated that C10 cells express low but reproducible levels of mRNA for Nox1, Nox2, Nox4, $p47^{phox}$, the $p47^{phox}$ homolog Noxo1, and $p67^{phox}$, but undetectable levels of mRNA for Nox3, $p40^{phox}$, or the $p67^{phox}$ homolog Noxa1 (Fig. 1A). Based on previous studies (18, 30), these results suggest that C10 cells utilize Noxo1 or $p47^{phox}$ as the organizer and $p67^{phox}$ as the activator for Nox1-dependent oxidase activity. Nox4 may require no factors other than $p22^{phox}$ for activity (30). Using cell extracts, immunoblotting showed mouse macrophages express two proteins of ~22–23 kd that react with an antibody specific for $p22^{phox}$, an assembly factor essential for the activity of Nox complexes (Fig. 1B, lane1). C10 cells express only the slower migrating form of $p22^{phox}$ (Fig. 1B). Expression of $p22^{phox}$ increased in serum-stimulated C10 cells by 72 h (Fig. 1B, lanes 2–5). In the C10 cell line Nox1B#3 (see below), higher levels of $p22^{phox}$ were observed in asynchronous cells, and these levels were reduced by serum deprivation and increased by serum stimulation (Fig. 1B, lanes 6–9). Together these data show that C10 cells express multiple components of the Nox enzyme system.

Effects of expression of Nox1 expression on ROS production and growth rate

To test the effects of Nox1 on cell growth, clones of C10 cells expressing human Nox1 from a plasmid vector were generated by stable transfection. As shown in Fig. 1C, cell clones Nox1#B3 and Nox1#A2 expressed higher levels of Nox1 mRNA, as compared to parental C10 cells or the vector transfected controls (C10-vector). Quantitative RT–PCR indicates that the human Nox1 mRNA in the stably transfected cell lines was expressed at about 10-fold higher levels than the endogenous mouse Nox1 mRNA (data not shown). ROS production by these cell lines was assessed by DCF fluorescence and flow cytometry (Fig. 1D). Nox1#B3 and Nox1#A2 showed reproducible two- to eightfold increases in mean DCF fluorescence compared with either C10 cells (data not shown)

or the vector control cell line (Fig. 1D). Nox1#B3 cells consistently displayed larger increases in DCF fluorescence than did Nox1#A2 cells (Fig. 1D). As observed for other cell types previously (39, 47), C10 cells expressing Nox1 had altered morphology, with decreased adhesion to plastic culture dishes, and an altered actin cytoskeleton (data not shown).

The effects of stable expression of Nox1 on cell growth rates, growth to saturation, and requirements for serum for proliferation were determined. C10 vector controls and Nox1-expressing cells were plated in CMRL medium with 10% FBS and cell numbers were determined over a 5-day period (Fig. 2A). In medium with 10% FBS, Nox1#B3 and Nox1#A2 cells did not show a distinct growth advantage until ~3 days after plating, but by 5 days displayed about a 40% increase in cell number as compared with vector controls (Fig. 2A). To determine if expression of Nox1 reduced the requirement for serum growth factors, Nox1#A2, Nox1#B3, and vector control cells were incubated in medium containing either 0, 0.2, 2, or 5% FBS for 72 h. C10-vector control cells did not proliferate in medium with less than 2% FBS, whereas Nox1#B3 and Nox1#A2 cells proliferated under low serum conditions (Fig. 2B). Moreover, Nox1 expressing cells continued to proliferate for 2–3 days when plated in medium with 0.2% serum, while vector controls showed no significant increase in cell number over a 5-day time course (Fig. 2C). Nox1 expressing cells did not proliferate indefinitely in the absence of serum, and ceased growth entirely after ~5 days in serum-free medium (Fig. 2C, and data not shown).

Nox1 increases cyclin D1 expression

In serum-stimulated C10 cells, expression of cyclin D1 serves as a marker for activation of signaling pathways linked to proliferation (8, 54, 55). Since Nox1-expressing cells showed increased growth rate, we examined the effect of Nox1 on cyclin D1 expression and cyclin D1 reporter gene activity. Vector controls and Nox1#A2 and Nox1#B3 cells were plated in various concentrations of FBS and cyclin D1 levels were assessed after 72 h (Fig. 3A). As for parental C10 cells, the vector control cell line showed dose-dependent effects of serum on maintenance of cyclin D1 expression (Fig. 3A, lanes 1–4). Nox1-expressing Nox1#A2 and Nox1#B3 cells, and particularly Nox1#B3 cells, expressed higher levels of cyclin D1 at lower serum concentrations (Fig. 3A, lanes 5–12). To determine if this effect occurred at the level of transcription, test cell lines were transiently transfected with a mouse cyclin D1 luciferase reporter construct, and then incubated in medium containing either 5% FBS (asynchronous) or 0.2% FBS for 24–72 h. The activity of the cyclin D1 reporter gene was about two- to threefold higher under low serum conditions in Nox1#B3 and Nox1#A2 cells as compared to vector controls for the first 48 h in low serum conditions. By 72 h, the magnitude of this difference was statistically insignificant (Fig. 3B). As expected from the growth rates (Fig. 2A), reporter gene activity was also significantly higher in cultures propagated in media with 5% FBS for 72 h (Fig. 3B).

The AP-1 site at −953 in the human cyclin D1 promoter (and at -902 in the mouse promoter) is necessary (but not sufficient) for D1 reporter gene activity in serum-stimulated C10 cells (8). C10 vector controls and Nox1#A2 cells were transfected with reporter plasmids with (CD-963) or without (CD963AP-1) the AP-1 site, and luciferase activity was examined in log phase cultures and cells incubated in 0.2% FBS for 48 h. When normalized to C10 vector controls, luciferase

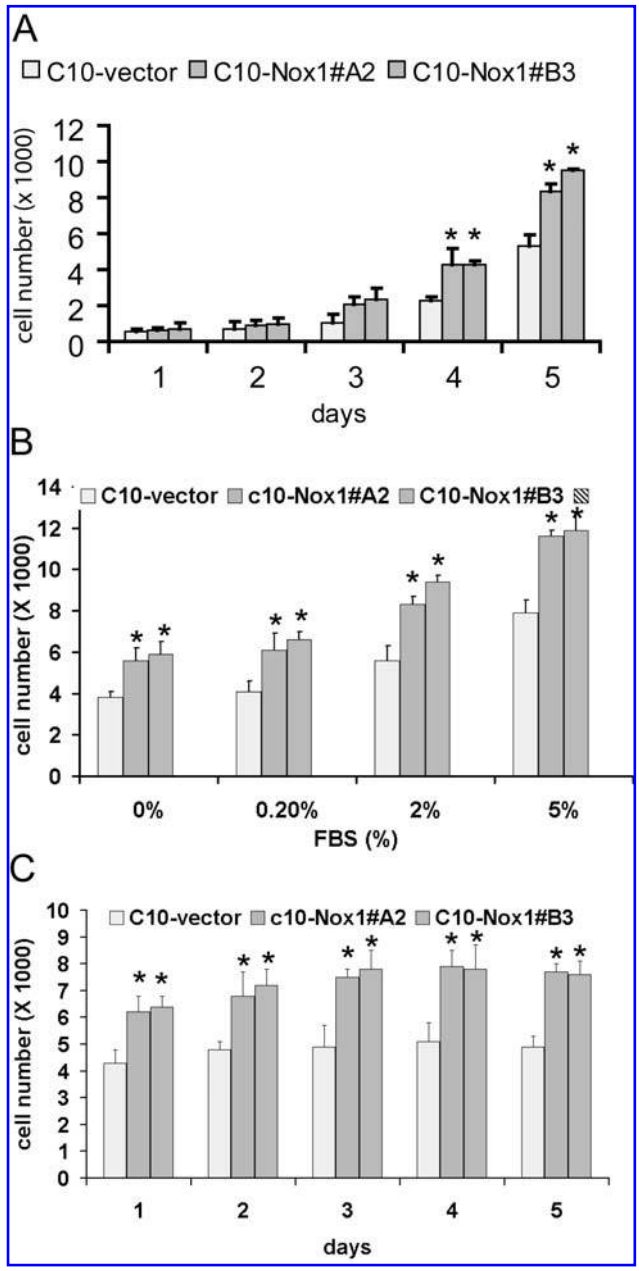

FIG. 2. Expression of Nox1 increases the proliferation potential of C10 cells. (**A**) The indicated cell lines were cultured in medium with 10% FBS, and cell counts were performed daily for 5 days. Both Nox1#A2 and Nox1#B3 cells grew to higher saturation density than did C10 vector control cells. (**B**) The indicated cell lines were cultured in media with varying concentrations of FBS for 72 h, and the number of cells per well was determined as before. (**C**) The indicated cell lines were plated directly in medium with 0.2% FBS, and cell number was determined daily for 5 days. Data shown in **A** through **C** are representative of three independent experiments; *asterisks* indicate increases in cell number that were statistically different from vector controls ($p < 0.05$).

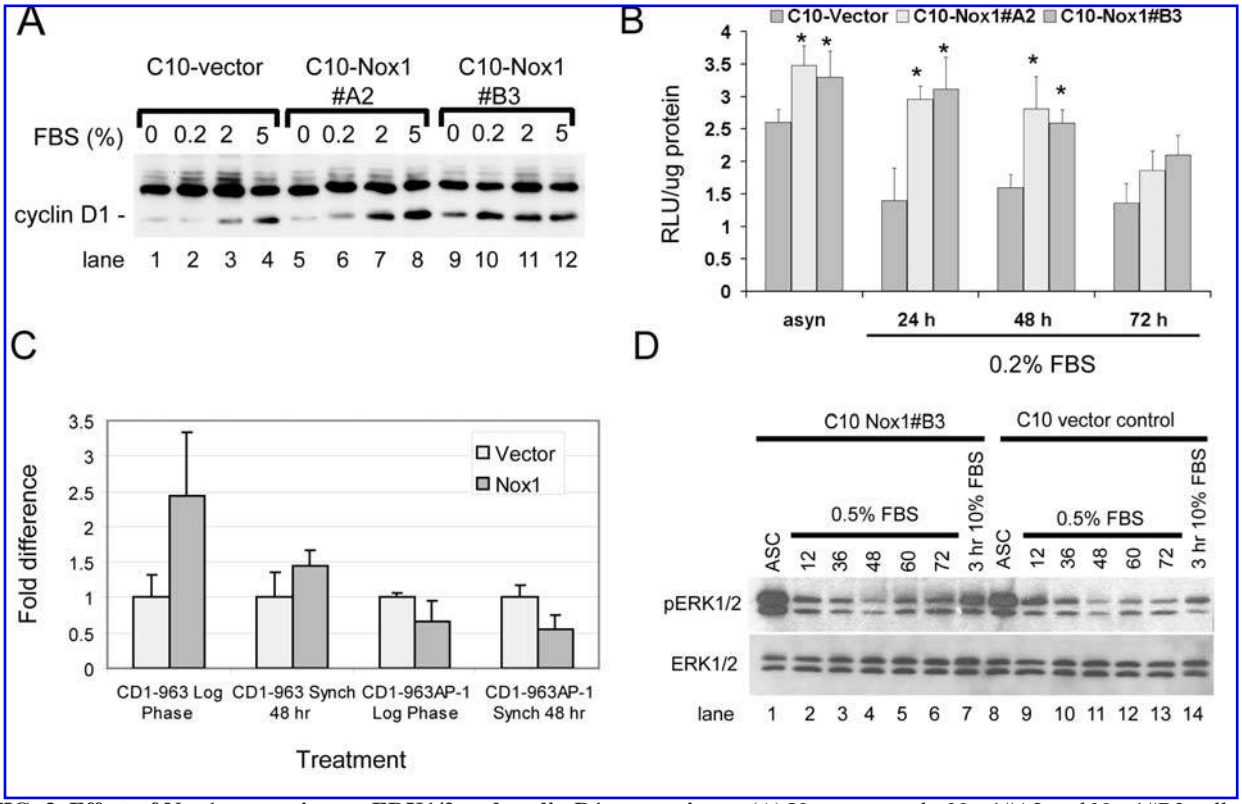

FIG. 3. Effect of Nox1 expression on ERK1/2 and cyclin D1 expression. (**A**) Vector controls, Nox1#A2 and Nox1#B3 cells were incubated in medium with the indicated concentration of FBS for 72 h, and cyclin D1 levels were assessed by immunoblotting. (**B**) Cells expressing Nox1 and vector controls were transiently transfected with a cyclin D1 luciferase reporter construct or empty vector, with co-transfection of a β-galactosidase expression vector as a control for transfection efficiency. Cells were then incubated under normal conditions with 10% FBS for 72 h (asyn) or in 0.2% FBS for 1–3 days. At the indicated times, cell extracts were prepared and luciferase and β-galactosidase activities were measured; luciferase activities are expressed as relative light units (RLU) per μg cell extract and were normalized for transfection efficiency using β-galactosidase levels. Data represent the mean ± S.E. for three independent experiments. (**C**) Vector control or Nox1#A2 cells were transfected with luciferase reporter plasmids containing the wild-type human cyclin D1 promoter (CD1-963) or a reporter plasmid lacking the AP-1 site at −952 (CD1-963AP-1). Luciferase activity was measured in log phase cells after 24 h or in cells incubated in medium with 0.2% FBS for 48 h. (**D**) Nox1#B3 and C10 vector controls were assayed by immunoblotting for levels of phospho-ERK (*top panel*) or total ERK (*bottom panel*) in extracts from asynchronous cultures propagated in medium with 10% FBS (*asc*) or in cells cultured in medium with 0.5% FBS for the indicated periods of time. After 72 h in medium with low serum, cells were then stimulated with medium containing 10% FBS (*lanes 7* and *14*).

activity from the wild-type promoter was ~2.5-fold higher in log phase C10-Nox1#A2 cells, and this difference in activity was maintained in low serum conditions (Fig. 3C). In contrast, either in asynchronous cells, or in cells deprived of serum for 48 h, Nox1 did not increase luciferase expression from a promoter lacking the AP-1 site above that seen in control cells (Fig. 3C). Thus, as for C10 cells re-entering the cell cycle (7), the AP-1 site is required for upregulation of cyclin D1 in response to continuous expression of Nox1.

The functional consequences of enhanced cyclin D1 expression were also evident in Nox1 expressing cells, for phosphorylation of the CDK4/cyclin D1 substrate pRB was elevated after 24, 48, and 72 h in medium containing 0.2% FBS, and cells expressing Nox1 also showed increased numbers of cells in S phase under low serum conditions (data not shown).

Expression of cyclin D1 in serum-stimulated C10 cells requires activation of the ERK1/2 and PI-3 kinase/Akt pathways (8). While expression of Nox1 had no effect on phospho-Akt levels (data not shown), Nox1 did affect the timing and degree of phosphorylation of ERK1/2. In asynchronous cultures grown in medium with 10% FBS, the levels of phospho-ERK1/2 in vector controls and Nox1#B3 cells were comparable (Fig. 3D, compare lanes 1 and 8). During serum withdrawal, vector control cells showed diminished levels in phospho-ERK1/2 over a 72 h time course (lanes 9–13). In response to low serum, phospho-ERK1/2 levels in Nox1#B3 cells dropped by 48 h, and then began increasing (Fig. 3D, lanes 2–6). When these cultures then were stimulated with medium containing 10% FBS for 3 h, phospho-ERK1/2 levels in Nox1#B3 cells increased markedly, as compared to vector controls (Fig. 3D, compare lanes 7 and 14). Together these data are consistent with previous reports that ROS produced by Nox1 promotes cell proliferation by stimulating mitogenic signaling through ERK1/2 (39).

Sustained expression of cyclin D1 is dependent upon production of H_2O_2

To determine if the effects of Nox1 on cyclin D1 expression were due to increased production of H_2O_2, the C10 vector control, C10-Nox1#A2 and C10-Nox1#B3 cell lines were stably transfected with a plasmid vector expressing human catalase. Human catalase migrates slightly slower than mouse catalase on denaturing SDS polyacrylamide gels, facilitating identification of stable derivatives of C10 vector control, C10-Nox1#A2 and C10-Nox1#B3 cells that express human catalase (Fig. 4A). Expression of human catalase had no effect on cyclin D1 levels in asynchronous cells propagated in 10% FBS (Fig. 4B, lanes 1–3), but obviated the ability of C10-Nox1#B3 cells to maintain cyclin D1 protein levels above that of control cells in 0.2% serum over a 72 h time course (Fig. 4B, compare lane 11 to lanes 10 and 12). Stable expression of catalase, but not empty vector, also prevented increased cyclin D1 reporter gene activity in low serum conditions as compared to that of vector controls. As shown in Fig. 4C, in low serum conditions, cyclin D1 reporter gene activity remained elevated for at least 48 h in C10-Nox1#B3 cells, whereas expression of catalase in this cell line reduced activity to control levels after 24 or 48 h in low serum conditions. By 72 h in 0.2% serum, there were no significant differences between the three test cell lines, though Nox1#B3 cells expressed slightly more luciferase activity as before (see Fig. 3B).

Co-expression of Nox1 co-activators and dose-dependent effects of ROS

Recently Noxo1 and Noxa1, novel coactivators that substantially increase superoxide production by Nox1, were identified and cloned (see Introduction). To investigate the effect of these accessory factors on ROS production by Nox1, Nox1#B3 cells were co-transfected with equivalent amounts of expression vectors for Noxo1 and Noxa1, and ROS production was assessed by DCF fluorescence. Transfection of Nox1#B3 cells with Noxo1 and Noxa1 resulted in dose-dependent increases in ROS production as measured by DCF fluorescence (Fig. 5A). Transfection of C10-vector controls with Noxo1 and Noxa1 resulted in an insignificant increase in DCF fluorescence (Fig. 5B). Enhanced DCF fluorescence induced by expression of Noxo1 and Noxa1 in C10 cells expressing Nox1 was dependent upon production of H_2O_2, as co-expression of catalase reduced DCF fluorescence to control levels (Fig. 5C). The effects of Noxo1 and Noxa1 on H_2O_2 production by Nox1 were specific, for these factors did not enhance production of ROS by Nox4 (Fig. 5D). When compared to cells stably expressing Nox1 (Fig. 2B), Nox1-expressing cells co-transfected with 1 μg of vector for Noxo1 and Noxa1, or C10 cells transfected with Nox4, displayed a highly asymmetrical flow cytometry histogram of DCF fluorescence, with relatively few cells in the upper ranges of DCF fluorescence (Fig. 5D).

The flow cytometry results suggested that high levels of ROS produced under these conditions may impede, rather than promote, cell proliferation or survival. To test this effect, C10 cells were transiently co-transfected with the cyclin D1 luciferase reporter gene and increasing amounts of expression vectors for Noxo1, Noxa1, and Nox1, with each transfec-

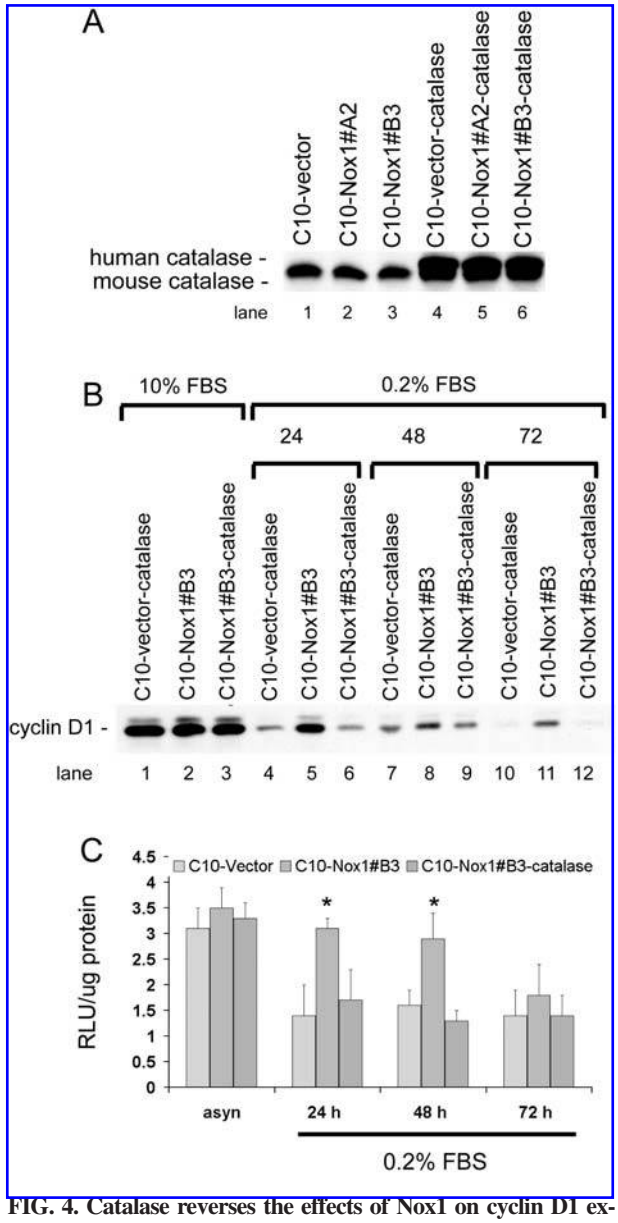

FIG. 4. Catalase reverses the effects of Nox1 on cyclin D1 expression. (**A**) C10-vector, C10-Nox1#A1, and C10-Nox1#B3 cells were transected with a plasmid expression vector for human catalase and cell lines were examined for catalase expression by immunoblotting with an antibody that recognizes both the human (*upper band*) and mouse (*lower band*) catalase. (**B**) C10 vector controls, C10-Nox1#B3, and C10-Nox1#B3 cells stably expressing catalase were incubated in medium with 10% FBS for 48 h (*lanes 1–3*), or in medium with 0.2% FBS for 1–3 days (*lanes 4–12*). Total cell lysates were prepared and examined for cyclin D1 expression by immunoblotting. (**C**) C10 vector controls, C10-Nox1#B3, and C10-Nox1#B3 cells stably expressing catalase were transfected with a mouse cyclin D1 luciferase reporter construct as before. Cells were incubated in medium with 10% FBS for 48 h (asyn) or 0.2% FBS for 1–3 days. At the indicated times, cell extracts were prepared and luciferase activity was determined as before. *Asterisks* indicate values that are statistically different from vector controls; *double asterisks* indicate values in catalase expressing cells that are statistically different from matched cell lines that express Nox1 alone. Data shown are representative of three independent experiments.

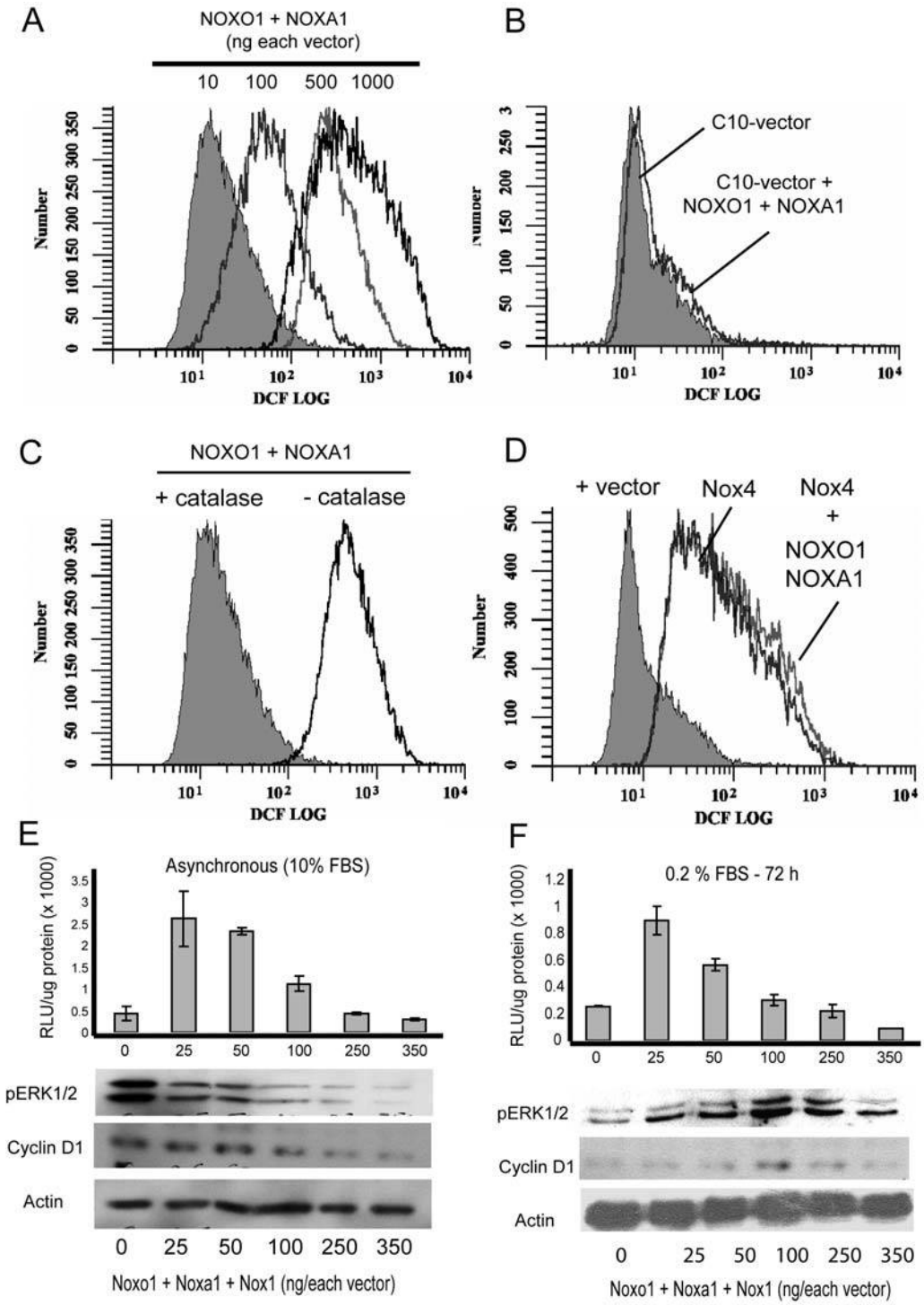

FIG. 5. Dose-dependent effects of Noxo1 and Noxa1 on ROS production. (**A**) C10-Nox1#B3 cells were co-transfected with the indicated amounts of plasmid expression vectors for the Nox1 co-activators Noxo1 and Noxa1, and mean DCF fluorescence was determined by flow cytometry. (**B**) C10-vector cells were transfected with 500 ng each of expression vectors for Noxo1 and Noxa1, and DCF fluorescence was determined by flow cytometry. (**C**) C10-Nox1#B3 cells were transfected with 500 ng each of expression plasmids for Noxo1 and Noxa1, with or without co-transfection with 1,000 ng of an expression vector for human catalase. After 24 h, cells were trypsinized and analyzed for DCF fluorescence as before. (**D**) C10 cells were transfected with 100 ng of an expression vector for Nox4, with or without co-transfection with expression vectors for Noxo1 and Noxa1 (500 ng each). After 24 h, cells were examined for DCF fluorescence by flow cytometry. (**E**) C10 cells were co-transfected with a cyclin D1 luciferase reporter plasmid and the indicated concentrations of Nox1, Noxo1, and Noxa1. Empty vector was used to ensure that the total amount of DNA in each transfection was identical. After 24 h, luciferase activity was determined, and the same cell extracts were assayed for levels of phospho-ERK, cyclin D1, and actin by immunoblotting. (**F**) C10 cells were transfected as in **E** and then incubated in medium with 02% FBS. After 72 h, extracts were prepared, luciferase activity was determined, and the same cell extracts were assayed for levels of phospho-ERK, cyclin D1, and actin by immunoblotting.as before.

tion containing the same total amount of plasmid DNA. In 10% FBS, asynchronous transfected C10 cells showed increased luciferase activity and cyclin D1 protein levels at low-to-moderate levels of expression of the oxidase components (Fig. 5E). Higher levels of expression of the Nox1 components inhibited reporter gene activity, expression of endogenous cyclin D1, and levels of phospho-ERK1/2 (Fig. 5E). After serum deprivation for 72 h, the luciferase reporter gene profile was very similar to that in asynchronous cells (Fig. 5F). In contrast, a biphasic response was observed for endogeneous levels of phospho-ERK1/2 and cyclin D1 in the same cells, with a peak of cyclin D1 expression in cells transfected with 100 ng of each Nox1, Noxo1 and Noxa1 (Fig. 5F).

A similar dose-dependent effect of the Noxo1 and Noxa1 expression vectors was observed in transfected Nox1#B3 cells, with modest increases in reporter gene activity at low levels (~1.8-fold) and inhibition at high levels (data not shown). For example, co-transfection with 1 μg each of Noxo1 and Noxa1 markedly increased DCF fluorescence in Nox1#B3 cells, and by 48 h after transfection, C10-Nox1#B3 rounded up and began to detach from the culture plate. Changes in cell morphology and adhesion also were evident in C10 cells transiently transfected with 1000 ng of expression vector for Nox4 alone (data not shown). Hence, high levels of expression of Nox1/Noxo1/Noxa1 or Nox4 causes C10 cells to round up and detach from the culture plate, and this occurred without inducing cell death (unpublished data). Because ROS are known to influence cell adhesion, the actin cytoskeleton, and cell migration (1, 10, 53), future studies will be required to understand how Nox expression levels affect pathways that control cell migration, adhesion, and the cytoskeleton.

We also examined the response of the cyclin D1 reporter gene in log phase C10-vector and Nox1#A2 cells to cells treated directly with H_2O_2. Although addition of 25 μ*M* H_2O_2 directly to culture medium induced a slight increase in luciferase activity in C10-vector cells after 24 h, this response was not statistically different from untreated cells, and higher levels of exogenous H_2O_2 inhibited activity (Fig. 6A). Immunoblotting for cyclin D1 protein in the same cells confirmed these results, showing H_2O_2 at concentrations >25 μ*M* inhibited expression of cyclin D1 (Fig. 6B). These results suggest that H_2O_2 added directly to culture medium as a bolus evokes cellular responses distinct from those elicited by continuous generation of intracellular H_2O_2.

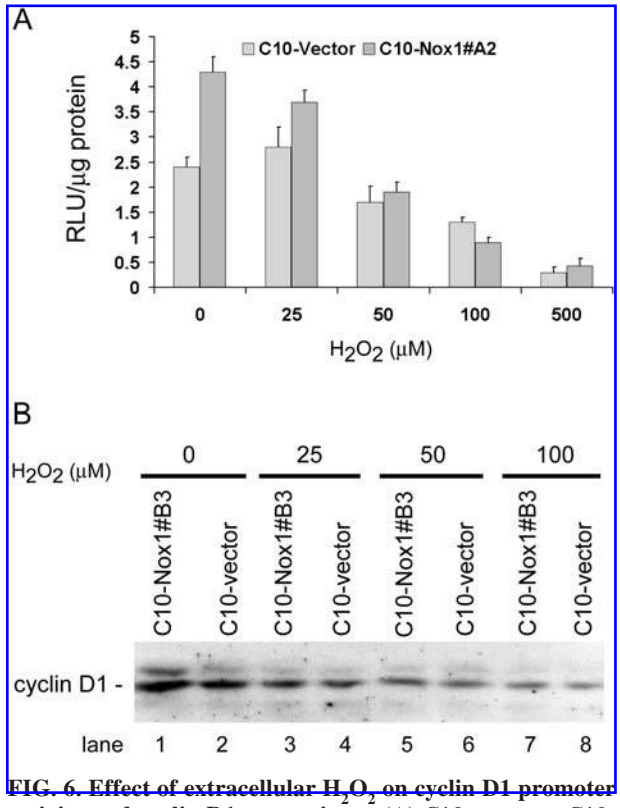

FIG. 6. Effect of extracellular H_2O_2 on cyclin D1 promoter activity and cyclin D1 expression. (**A**) C10-vector or C10-Nox1#A2 cells were transfected with the mouse cyclin D1 luciferase reporter construct or empty vector and treated with indicated concentrations of H_2O_2 in medium containing 5% FBS. After 24 h, cell lysates were prepared and examined for luciferase activity as before. (**B**) C10-vector or Nox1 expressing C10 cells were treated with indicated concentrations of H_2O_2. After 24 h, total cell extracts were prepared and analyzed for cyclin D1 expression by immunoblotting.

Nox activity and cell cycle re-entry

In actively cycling C10 cells, Nox1 promoted proliferation in C10 cells by sustaining expression of cyclin D1 during withdrawal of serum growth factors. Moreover, diphenylene iodonium (DPI), a general flavoprotein inhibitor, was cytostatic at 10 μ*M* in actively cycling C10 cells (data not shown). However, because actively cycling cells do not pass through the G0 phase of the cell cycle, we tested if Nox activity contributes to mitogenic signaling during the G0 to G1 transition by treating serum-stimulated C10 cells with DPI (Fig. 7). First, serum-starved C10 cells were preincubated with 5 μ*M* DPI for 45 min, and then the cells were stimulated with medium containing 2, 5, or 10% FBS plus 10 μ*M* DPI. Control cells were treated with medium with 2, 5, or 10% FBS alone. After 6 h, lysates were examined for phospho-ERK, phospho-Akt, and expression of Fra-1 and cyclin D1 by immunoblotting. As expected from previous studies (8, 55), FBS caused dose-dependent phosphorylation of Akt and ERK1/2 that correlated with increased expression of Fra-1 and cyclin D1 levels (Fig. 7A, lanes 1–6). DPI had virtually no effect on the levels of phospho-Akt or phospho-ERK1/2, but markedly inhibited expression of both Fra-1 and completely blocked expression of cyclin D1 in response to any concentration of FBS (Fig. 7A, lanes 6–10). DPI did not significantly alter the kinetics with which these phosphorylated proteins accumulated in response to 10% FBS (Fig. 7B), nor prevent reduction in the level of a portion of the phospho-ERK1/2 pool after 2 h, which is evident in changes in mobility of total ERK (Fig. 7B, compare lanes 3–6 to lanes 10–12). The increase in mobility of total ERK1/2 at these times may correspond to loss of nuclear phospho-ERK1/2 1–2 h after serum stimulation in C10 cells (55). In contrast, DPI markedly affected the levels of Fra-1 expressed in response to different concentrations of serum (Fig. 7A), and the amounts of both c-Fos and Fra-1 that accumulate over time in response to 10% FBS (Fig. 7B). Interestingly, DPI did not alter the

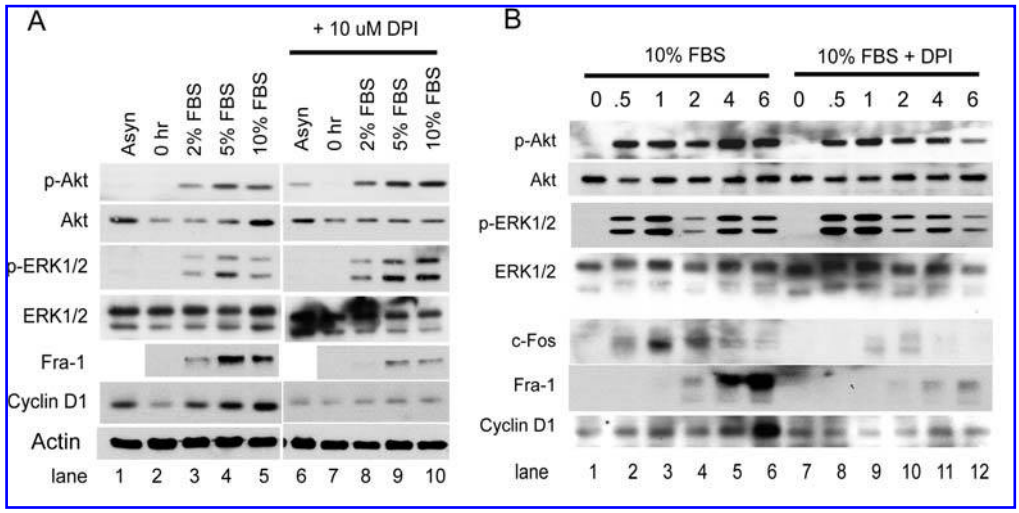

FIG. 7. DPI blocks expression of c-Fos and Fra-1 but not phosphorylation of ERK1/2 and Akt. (**A**) Synchronized C10 cells (0 time) were stimulated with medium containing 2, 5, or 10% FBS with or without DPI for 6 h, and cell extracts then were assayed for the indicated markers by immunoblotting. Extracts from asynchronous cells (Asyn) were used as controls. (**B**) Synchronized C10 cells were treated with medium containing 10% FBS or 10% FBS plus 10 μ*M* DPI. At the indicated times, extracts were prepared and examined for markers of mitogenic signaling and cell cycle progression using immunoblotting.

time at which c-Fos and Fra-1 were expressed, or affect reduction in mobility of c-Fos associated with its phosphorylation (Fig. 7B, lanes 9 and 10).

DISCUSSION

Mitogenesis in response to growth factors such as EGF, FGF, and GM-CSF requires the transient production of H_2O_2, which then acts as second messenger on downstream signaling pathways (11–15, 37, 46). The direct and indirect targets of H_2O_2 in mitogenic signaling include tyrosine and lipid phosphatases, MAPK kinases, small GTPases such as Rac and Ras, and transcription factors such as AP-1 and NF-κB, all of which regulate pathways that have been linked to expression of cyclin D1 (7). In actively cycling cells, the levels of cyclin D1 are regulated primarily posttranscriptionally, and respond to the levels of growth factors and activated Ras in the G2 phase of the cell cycle (21, 24, 25). If growth conditions are favorable in G2, cyclin D1 levels are maintained and cells continue cycling after the ensuing mitosis. If not, cells withdraw from the cycle and enter G0. Cyclin D1 thereby serves as a critical switch for both cell cycle re-entry from quiescence and entry into G1 from G2/M.

In quiescent cells, growth factors induce a cascade of signaling pathways that culminate in expression of cyclin D1 at the G0 to G1 transition of the cell cycle, and cyclin-dependent kinase/cyclin D complexes drive passage through the G1 restriction point via phosphorylation of pRB (5). Our previous studies showed that in C10 cells the expression of cyclin D1 at the G0 to G1 transition is an EGF, ERK1/2 and AP-1-dependent process that is sensitive to environmental oxidants (8, 54, 55), suggesting control of cyclin D1 levels represents an important redox-dependent regulatory node in cell proliferation.

C10 cells express low, but reproducible levels of mRNA for multiple components of the Nox enzyme system (Fig. 1A and B). Moreover, DPI, an inhibitor of Nox activity, blocked proliferation of C10 cells. We therefore examined the role of Nox1 in the production of intracellular ROS, cell proliferation, and expression of cyclin D1 in C10 epithelial cells. DCF fluorescence, a semiquantitative assay for ROS production, showed that in stable cell lines, expression of Nox1 that caused an eightfold increase in ROS promoted cell proliferation (Fig. 2). In the presence of 10% FBS, expression of Nox1 had little effect on cell growth rates until 72 h in culture, a time when serum growth factors may be depleted (Fig. 2A). In concentrations of serum that limited the growth of C10 vector control cells, expression of Nox1 promoted proliferation (Fig. 2B), as well as expression of cyclin D1 (Fig. 3A). Reporter gene assays indicate that Nox1 acts on cyclin D1 transcription through AP-1 (Fig. 3C), and these observations were consistent with increased levels of phospho-ERK1/2 in serum-deprived and serum-stimulated Nox1-expressing cells (Fig. 3D). However, expression of Nox1 alone was not able to obviate the requirement for serum for proliferation of C10 cells, suggesting activation of multiple pathways may be necessary. For example, activated MEK stimulates expression of AP-1 independently of PI3-K, but PI3-K is required along with MEK signaling for DNA synthesis (52). Since C10 cells express $p47^{phox}$, and this cofactor is activated by phosphorylation by Akt (26), and inhibitors of PI3-K inhibit Rac-dependent activation of oxidase activity through the EGF receptor (44), it is not unlikely that signaling by PI3-K/Akt modulates Nox activity in C10 cells and in Nox1-expressing stable cell lines.

Our results in actively cycling C10 cells are consistent with previous studies that showed Nox1 promotes cell proliferation through an H_2O_2-dependent mechanism (2, 47). However, the effects of DPI on expression of cyclin D1 dur-

ing cell cycle re-entry were unexpected. In serum-stimulated C10 cells, c-Fos is transiently expressed, reaching maximal levels by 1 h, and then is rapidly degraded (8). Degradation of c-Fos is followed by recruitment of Fra-1 to chromatin and expression of cyclin D1. Oxidative stress during serum stimulation prolongs activation of ERK1/2 in the nucleus, which stabilizes c-Fos through phosphorylation on C-terminal threonine residues, and thereby prevents Fra-1 from gaining access to the nucleus and activating expression of cyclin D1. Termination of ERK signaling destabilizes c-Fos, permits Fra-1 to access chromatin, and restores expression of cyclin D1 (8).

Based on studies that suggest NADPH oxidases act as co-stimulatory signals in growth factor signaling by producing H_2O_2 (40), we expected that inhibition of Nox activity with DPI might affect the levels or timing of phosphorylation of Akt and ERK1/2, both of which are required for expression of cyclin D1 in C10 cells. DPI did not affect dose-dependent increases of phospho-Akt or phospho-ERK1/2 in response to serum (Fig. 7A), or significantly alter the kinetics with which these phosphorylated proteins accumulated in response to 10% FBS (Fig. 7B). In contrast, DPI markedly inhibited Fra-1 expression in response to different serum concentrations (Fig. 7A), and the accumulation of both c-Fos and Fra-1 over time in response to 10% FBS (Fig. 7B). Others have observed that DPI inhibits expression of c-Fos through the serum response element (45), and studies show that activation of a protein complex that resides on the Fos promoter composed of serum response factor and ternary complex factor Elk-1 requires ERK (29). While oxidative stress clearly affects the magnitude and duration of ERK1/2 signaling, DPI appeared to affect AP-1 gene expression without changing the levels of phospho-ERK and Akt detected by immunoblotting. Because phospho-ERK1/2 levels detected by immunoblotting correlate very well with ERK activity in immunoprecipitation kinase assays (data not shown), further work on the effects of DPI on subcellular trafficking of ERK1/2 will be required to understand if DPI blocks expression of c-Fos and Fra-1 through ERK1/2.

The demonstration that Nox1 promotes expression of cyclin D1 through AP-1 may be relevant to an emerging relationship between AP-1, Noxes, ROS production and proliferation in cancer. Human tumor cells produce large amounts of H_2O_2 (4, 49), and rapid tumor growth in response to activated Ras requires expression of Nox1, providing one mechanism for production of H_2O_2 in tumor cells (39). Moreover, Nox4 expression is elevated in melanomas, where it contributes to the regulation of growth and transcription (6). While the AP-1 family member c-Jun stimulates cell proliferation through cyclin D1, JunD slows cell growth and induces G1 arrest (38). Interestingly, JunD antagonizes transformation by Ras (41) and reduces tumor angiogenesis by limiting Ras-dependent production of ROS (19). Interestingly, mouse fibroblasts lacking JunD express three- to fivefold more Nox4 mRNA than wild-type cells (19).

While Nox1 and Nox4 have been implicated in signaling and cancer, the role of NADPH oxidases in tumorigenesis remains controversial. For example, suppression of Nox1 expression in HT29 colon cancer cells does not block proliferation (16). Because there are multiple sources of ROS in cells, including a variety of metabolic enzymes and mitochondria, we consider it likely that NADPH oxidase complexes have multiple functions in different nonphagocytic cell types, including host defense, regulation of cell adhesion and migration, and cell signaling. Compartmentalization of signaling may represent one mechanism for targeting ROS to specific pathways (20), as Nox1 and caveolin have been colocalized in punctate patches at the cell surface of vascular smooth muscle cells, as might be expected for signaling proteins, while Nox4 colocalizes with vinculin in cell adhesion complexes (23).

Little is known about the physiological function of Noxo1 and Noxa1 in mitogenesis, cell cycle arrest, or apoptosis. Noxo1 and Noxa1 are homologs of $p47^{phox}$ and $p67^{phox}$, respectively, proteins that regulate the assembly and activity of the phagocytic oxidase complex containing the NADPH oxidase $gp91^{phox}$ (18, 30). In contrast to $p47^{phox}$, which is recruited to the cell membrane when activated by the lipid products of PI-3 kinase, Noxo1 binds monophosphorylated phosphatidylinositols found in resting cells, and colocalizes with Nox1 in the cell membrane (9). Transient transfection of C10 cells with Nox1, Noxo1, and Noxa1 expression vectors induced dose-dependent responses in regard to phosphorylation of ERK1/2 and expression of cyclin D1, with high levels of expression inhibiting these endpoints (Fig. 5A and B). Similar dose-dependent effects of Noxo1 and Noxa1 were observed in Nox1-expressing Nox1#B3 cells (data not shown).

When expressed alone, Nox1 is able to activate transcription from an antioxidant response element that binds AP-1 through ERK without causing oxidation of glutathione or thioredoxin (20). Because the endogenous production of H_2O_2 in endothelial cells activates c-Jun N-terminal kinase and caspase 3, leading to apoptosis (42), we expected that high levels of endogenous H_2O_2 produced by Nox1/Noxo1/Noxa1 or Nox4 would induce apoptosis in C10 cells. However, in C10 cells we have yet to observe conditions of expression of Nox1/Noxo1/Noxa1 that cause apoptosis, and rather observe that high levels of expression cause cells to round up and detach from the culture plate. This is consistent with the effects of activated Ras on cell morphology (39). Although current models indicate that ROS act on mitogenic pathways through the modification of specific cysteine residues in protein phosphatases (13), it is not unlikely that ROS produced by Nox1 and Nox4 act on other signaling targets.

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ABBREVIATIONS

CMV, cytomegalovirus; DCF, 2′,7′-dichlorodihydrofluorocein diacetate; DMEM, Dulbecco's minimal essential medium; dNTP, deoxynucleotide triphosphate; DPI, diphenylene iodonium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; ERK1/2, extracellular signal-regulated kinase 1 and 2; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; Nox, NADPH oxidase; phox, phagocytic oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RTK, receptor tyrosine kinase.

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