An NAD(P)H oxidase regulates growth and transcription in melanoma cells

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reduction of membrane ubiquinone to ubiquinol, with subsequent generation of O_2 from molecular O_2. However, dicumarol also inhibited growth of H596 non-small cell lung cancer cells (11). H596 cells express a mutant NQO protein and have elevated mRNA for NQO but no detectable enzymatic activity (50). We therefore repeated our original studies, performing reverse transcriptase-polymerase chain reaction (RT-PCR) under different conditions (11). We found that dicumarol inhibits O_2 generation by isolated plasma membranes lacking a cytosolic source of NQO. M1619, other malignant melanomas, and normal human epidermal melanocytes express mRNA for the p22^{phox} membrane subunit of the NAD(P)H oxidase, and melanomas express gp91^{phox}. Melanomas and melanocytes additionally express the gp91^{phox} homolog NOX4, which has been recently found in renal tubular epithelial cells and renal cell carcinomas (44), glioblastomas (12), and Caco colon cancer cells (12). Finally, membrane O_2 generation and melanoma proliferation are reduced by inhibitor strategies directed at the leucocyte NAD(P)H oxidase, raising the possibility that a form of this same enzyme system serves as a growth regulatory oxidase in malignant melanoma cells.

MATERIALS AND METHODS

Materials. Human malignant cell lines were obtained from American Type Culture Collection (Rockville, MD). Human epidermal melanocytes, medium 154, and human melanocyte growth supplement (HMGS) were purchased from Cascade Biologics (Portland, OR). RPMI medium 1640, HEPEs, antibiotic-antimycotic (10,000 U/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin B), and trypsin-EDTA solution were purchased from the GIBCO-BRL division of Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). The intracellular oxidant sensitive probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Electrophoretic mobility shift assay (EMSA) supplies were purchased from Promega (Madison, WI). Supershift antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit phospho-specific antibodies for inhibitor of NF-κB (IκBα) phosphorylated at serine 32 were purchased from New England Biolabs (Beverly, MA). Protease inhibitors were from Sigma Chemical (St. Louis, MO). All other materials were purchased from Sigma Chemical unless otherwise specified.

Culture of malignant cell lines and cell culture treatments. Malignant melanoma cell lines were cultured and passaged as previously described (11). Human epidermal melanocytes were cultured in medium 154 supplemented with HMGS according to the supplier's instructions and passed with 0.05% trypsin and 0.53 mmol/l EDTA. Growth rates of melanocytes and M1619 melanoma cells were compared by measuring proliferation (as described in Measurement of proliferation in cell cultures) every 24 h for 72 h. Intracellular generation of ROS by melanocytes or M1619 cells was measured by oxidation of DCFH-DA to DCFH which has been recently found in renal tubular epithelial cells and renal cell carcinomas (44), glioblastomas (12), and Caco colon cancer cells (12). Finally, membrane O_2 generation and melanoma proliferation are reduced by inhibitor strategies directed at the leucocyte NAD(P)H oxidase, raising the possibility that a form of this same enzyme system serves as a growth regulatory oxidase in malignant melanoma cells.

The effect of NAD(P)H oxidase activity on transcriptional activation was studied by incubation of 70% confluent cultures with 0–50 µmol/l of the flavoprotein inhibitor diphenylene iodonium (DPI) for 24 h before measurement of DNA binding by EMSA, detection of constitutive NF-κB nuclear activation by immunohistochemical staining for the p65 component, or immunoassay of levels of active transcription factor component in nuclear protein.

Measurement of proliferation in cell cultures. Proliferation of cultured cells seeded into 24-well uncoated plastic plates (Costar) at 50,000 cells/well (except where indicated) was quantitated as previously described (11) by using a colorimetric method based on metabolic reduction of the soluble yellow tetrazolium dye 3-(4,5-dimethylthiazol)-2-y1-2,5-diphenyltetrazolium bromide (MTT) to its insoluble purple formazan by the action of mitochondrial succinyl dehydrogenase. For studies with a final cell density of less than about 40,000 cells/well, direct cell counts were performed on 10 random fields/well of Wright's-modified Geimsa-stained monolayers viewed at a ×100 magnification with a 0.91-cm² ocular grid.

Measurement of ROS generation by intact cells. Intracellular generation of ROS by M1619 cells or epidermal melanocytes was measured by oxidation of DCFH-DA to DCF (42). DCFH-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of H_2O_2, DCFH is oxidized to the highly fluorescent DCF. Approximately 1 × 10⁶ M1619 cells or human epidermal melanocytes were incubated in the dark for 10 min at 37°C with 50 µmol/l DCFH-DA, harvested, and resuspended in plain medium. Fluorescence was analyzed by using a FACScan (Becton Dickinson, Sunnyvale, CA) flow cytometer with excitation at 488 nm and emission at 530 nm.

Measurement of ROS generation by cell membranes. The method of Pagano et al. (38), with centrifugation speeds modified according to the work of Mohazzab-H and Wolin (36), was used to prepare membranes for measurement of ROS. M1619 cells from six near-confluent T-75 flasks were harvested with cell dissociation solution (Sigma), washed once with ice-cold Dulbecco's phosphate-buffered saline (DPBS), and centrifuged for 5 min at 675 g. The pellet was resuspended in 500 µl of ice-cold Tris-sucrose buffer (pH 7.1; composed of (in mmol/l) 10 Trizma base, 340 sucrose, 1 phenylmethylsulfonyl fluoride, 1 EDTA, and 10 µg/ml protease inhibitor cocktail (Sigma)) and sonicated by four 15-s bursts. The cell sonicate was centrifuged at 1,475 g for 15 min in an Eppendorf microtuge to remove nuclei and unbroken cells. The supernatant was then centrifuged at 29,000 g for 4°C for 15 min in a Beckman Optima TL ultracentrifuge. The pellet was discarded, and the supernatant was further centrifuged at 100,000 g and 4°C for 75 min. The pellet was resuspended in 100 µl of Tris-sucrose buffer and stored at −80°C. Supernatant from the last centrifugation was also saved as a representative of lactate dehydrogenase-containing soluble elements of cytoplasm (36). Generation of ROS was measured by superoxide dismutase (SOD)-inhibitable lucigenin chemiluminescence, as recently reported (38, 56), in 500 µl of 50 mmol/l phosphate buffer (pH 7.0) containing 1 mmol/l EGTA, 150 mmol/l sucrose, 5 µmol/l lucigenin, 15 µg of cell membrane protein, 50 µg of cytosolic protein, and 100 µmol/l NADH or NADPH as substrate. Chemiluminescence (in arbitrary light units) was measured by using a Turner model 20/20D luminometer (Turner Designs, Sunnyvale, CA) at 30-s intervals for 5 min with and without addition of 300 units of SOD to determine dependence of light generation on O_2 generation. The signal was expressed as the sum of all measurements after subtraction of the buffer blank (45). DPI (50 µmol/l) and phenylarsine...
oxide (1 μmol/l) were added to determine dependence of light
generation on flavoprotein- and gp91phox- or NOX-containing
NADPH oxidase enzymatic activity, respectively.

RT-PCR detection of NAD(P)H oxidase components. To probe
for the presence of p22phox, gp91phox, p47phox and
p67phox components of the putative analog of neutrophil
NADPH oxidase and the newly described gp91phox homo-
lologs NOX1 (2, 4, 48) and NOX4 (12, 17, 30, 45), we
performed semiquantitative RT-PCR, as recently described
(10), on triplicate near-confluent cultures of proliferating
groups in 25-mm plastic dishes. Cell monolayers were
washed twice with DPBS and lysed with 4 mol/l guanidine
thiocyanate, 25 mmol/l sodium citrate, and 0.5%
N-lauroyl-
sarcosine. After scraping, lysates were sheared with four
passes through a pipette. RNA was extracted by the phenol-
chloroform method (13) and quantitated spectrophotometri-
cally at 260 and 280 nm. RNA (2 μg) was reverse transcribed
by using 200 units of M-MLV reverse transcriptase (Promega)
in a reaction mixture containing 1 mmol/l dATP, dCTP,
dGTP, and dTTP, 40 units of RNase inhibitor, 25 μmol/l
random hexamers, 5 mmol/l MgCl2, 500 mmol/l KCl, and 100
mmol/l Tris-HCl (pH 8.3) in a total volume of 50 μl. The
resultant cDNA was PCR amplified for glyceraldehyde-3-
phosphate dehydrogenase (GAPDH), p22phox, gp91phox, p47phox,
p67phox, NOX1, and NOX4 by using human gene-specific sense
and antisense primers based on sequences published in Gen-
Bank: GAPDH-5′, ACCACATTGGAAGAGCTTG; GAPDH-3′,
CTCGATGTGCCAGCCGATG; p22phox-5′, ATGGGCACTG-
GGGACAGAAGCACATG; p22phox-3′, GATGGTGGCTTCTCAGAT-
CTGGGCC; gp91phox-5′, AGGTGCACTATCCTGTTTG-
TG; gp91phox-3′, AAAGGCCCCATCAAGGCGGTATTTGATG;
NOX1-5′, CTGGGTTGTTAACCAGGT; NOX1-3′, ACC-
ATGGGCACTATCCCTAG; NOX4-5′, TACACCAAGGAC-
AGATTCCAT; NOX4-3′, CCGGAGGGGGTTGATCTCTAAA;
p47phox-5′, ACCAGGCCAGTAGCTG; p47phox-3′, AGA-
TGCCTGAGTCGTCT; p67phox-5′, CGAGGGACACCT-
GATAAG; and p67phox-3′, CATGGGCAACTGAGCTCA. PCR
was carried out on a Perkin-Elmer DNA thermal cycler 480.
Except where indicated, amplification was carried out for 30 cycles
for GAPDH, 92 cycles for p22phox, 94 cycles for NOX4, and 36
cycles for all other primers at 55°C for 1 min, 58°C for 1 min,
and 72°C for 2 min, followed by an extension step at 72°C for 10 min.
PCR-amplified DNA was separated on 1.2% agarose gel, stained
with ethidium bromide, and visualized and photographed under
ultraviolet light. PCR products from defined bands were purified
with QiAQuick gel extraction kits (Qiagen, Chatsworth, CA) and
sequenced automatically with an ABI Prism 310 genetic analyzer
(Applied Biosystems, Foster City, CA) by using the same respec-
tive primers for sequencing as for PCR.

Immunofluorescence assay for p22phox and gp91phox. IκBα,
phosphorylated IκBα, and 65 component of NF-κB. To measure protein
expression of p22phox and gp91phox in the cytosol and in the
100,000 g plasma membrane fraction of M1619 cells, we
performed immunofluoressays as described earlier (10, 11) using
previously described rabbit polyclonal antibodies prepared
against whole human p22phox (R3179) (24) and gp91phox
COOH-terminal peptide (R2089) (40) at dilutions of 1:1,000.
To assess nuclear translocation of the cytosolic transcription
factor NF-κB, we similarly immunostained the p65 NF-κB
component in nuclear protein that was isolated as outlined below.
For immunofluorosass of the NF-κB inhibitor IκBα or for
IκBα phosphorylated at serine 32, we followed the same
procedure previously reported (11), with cells lysed in boiling
buffer to which 50 mM dithiothreitol had been added as a
reducing agent.

Transfection protocol for p22phox and NOX4 sense and
antisense treatment of M1619 cells. To transfect antisense
oligonucleotides for p22phox. M1619 cells were culture in
six-well plates at a density of 20,000 cells/well and grown in
RPMI 1640 containing 10% FBS. After 24 h, wells were
washed once with DPBS, and 800 μl of RPMI 1640 (serum
and antibiotic free) were added to each well. Previously
reported (33) p22phox sense (5′-GGTCCTCCACCTGAGGCC-
GATC-3′) or antisense (5′-GATCTGCCCCATGGTGAC-
GACC-3′) oligonucleotides (2 μg) were mixed with 5 μl of
Lipofectase reagent (Life Technologies) and 200 μl of serum-
and antibiotic-free RPMI 1640 at room temperature for 15
min. This mixture was then added to each well, and cells were
incubated at 37°C. After 6 h the transfection mixture was
gently removed and replaced with 2.5 ml of RPMI 1640
containing 10% FBS. Cells were incubated an additional 48 h
before staining with hematotoxylin and eosin for photography
or quantitation of growth with the MTT assay. M1619 cells
were transfected with NOX4 sense (5′-CGGAGGATCTTCT-
TGCGG-3′) or antisense (5′-AGCTCCTCCACGAGACAA-
CGTT-3′) oligonucleotides based on gene-specific unique se-
quences published in GenBank (accession no. NM 016931).
The transfection protocol was identical to that used for
p22phox oligonucleotides, except that 10 μl of Lipofectin
reagent (Life Technologies) were used instead, and cells were
photographed with a phase-contrast microscope and a green
filter.

EMSAs. To assess DNA binding of NF-κB or the cAMP
response element (CRE) family of binding proteins, nuclear
protein was isolated and EMSAs were performed as previ-
ously reported (11). The consensus binding oligonucleotides
5′-AGTTGAGGGGACCTTTCCCCAGGC-3′ and 3′-TCAA-
CTCCCCTGAAAGGGTCGG-5′ for the p50 component of
NF-κB, 5′-AGAGATTGCTGAAGGCTGAGCATAG-3′ and
3′-TCTGATGGGAGCTCCCTTCCTAGGCGTTCAG-5′ for CRE-5
CGT/GATGGAGCTCGGGAAG-3′ and 5′-GGGCTAGCACT-
TTCTCCTAGAATAA-3′ and 5′-CCATCGTTCGACTCCTGAC-
CTGTT-5′ for AP-1 and 5′-GATCTGGACTCCACAAAT-
CCAGTCTGACCTGCT-5′ for OCT-1 were used in binding reactions after end-labeling
by phosphorylation with [γ-32P]ATP and T4 polynucleotide
kinase. Competition experiments were performed with 10×
respective unlabeled wild-type oligonucleotide sequences,
and supershift experiments were carried out by incubating
the binding reaction with 1 μg of supershift antibody.

Immunohistochemical localization of NF-κB. Constitutive
activation of NF-κB was also studied by qualitatively assess-
ing nuclear localization of the p65 component by immunohis-
tochemical staining, as described previously (11).

Transduction protocols for IκBα gene transfer. To repress
activation of NF-κB, cells were transduced with adenoviral
(Ad serotype 5) vectors that were E1A/E1B-deleted and ex-
pressed a superrepressor of NF-κB (AdIxBoSR; 2 × 1011
plaque forming units/ml) under the regulation of the cyto-
megalovirus (CMV) immediate-early promoter region (6) or
expressed the CMV immediate-early promoter region alone
(AdCMV-3; 2.05 × 1011 plaque forming units/ml, control
vector). These adenoviral vectors were constructed in the
Vector Core Laboratory [Gene Therapy Center, University of
North Carolina (UNC), Chapel Hill, NC] and were generous
gifts, respectively, from Dr. Albert S. Baldwin of the UNC
Greene Comprehensive Cancer Center and Dr. Andrew
Ghio of the U.S. Environmental Protection Agency (UNC
Human Health Effects Center). Transduction was performed
by using previously published protocols (6). M1619 cells were
seeded onto 24-well plates at a density of 25,000 cells/well
and grown for 6 h in RPMI 1640 with 10% FBS. Medium was
replaced and washed with 200 μl of complete medium con-

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into the extracellular medium (11). Because NF-
 activation, interleukin-8 and GRO-α secretion, O_2^- release, and melanoma proliferation were all reduced by the anticoagulant vitamin K antagonist dicumarol, which blocks cytosolic NQO1, we had postulated that M1619 cells generate ROS via the reductive action of NQO on membrane ubiquinone, with subsequent one-electron transfer to O_2 to form O_2^- (11). M1619 melanoma cells oxidized DCFH to DCF (Fig. 1), suggesting intracellular generation of H_2O_2 (42). Pretreatment of cells with the flavoprotein inhibitor DPI or dicumarol inhibited intracellular oxidation of DCFH to DCF (Fig. 1). Proliferating epidermal melanocytes also oxidized DCFH to DCF, DCFH oxidation was inhibited by both DPI and dicumarol, and melanocyte proliferation was significantly reduced by addition of catalase to the culture medium (data not shown). Thus, during proliferation, melanomas and melanocytes produce intracellular ROS, oxidant generation is blocked by DPI and dicumarol, and growth is disrupted by antioxidant strategies, suggesting that a signaling oxidase may play a role in regulating cell proliferation.

Malignant melanoma cell membranes produce ROS. To further study the source of O_2^- generation, we prepared plasma membrane and cytosolic fractions from proliferating M1619 melanoma cells and studied their respective abilities to support SOD-inhibitable lucigenin chemiluminescence. Plasma membranes alone significantly increased SOD-inhibitable lucigenin chemiluminescence without addition of cell cytosol (Fig. 2). When lucigenin was used at a concentration of 5 μmol/l to prevent artifactual redox cycling (32, 46), the preferred substrate for generation of O_2^- was NADPH rather than NADH (Fig. 2, M + NADPH vs. M + NADH). Cytosol from M1619 cells did not support chemiluminescence (Fig. 2, M + C), mitigating against

**RESULTS**

**Malignant melanoma cells produce intracellular ROS.** We have previously reported that proliferation of M1619 malignant melanoma cells is strongly inhibited by a number of antioxidants, including the H_2O_2 scavenger catalase, the sulfhydryl donor N-acetylcysteine, and the glutathione peroxidase mimetic ebsealen, suggesting the importance of oxidants in regulating growth of this cell line (11). We have also previously demonstrated that cultured melanoma cells release O_2^- into the extracellular medium (11). Because NF-κB

Fig. 1. Generation of intracellular reactive oxygen species (ROS) in melanomas is inhibited by diphenylene iodonium (DPI) and dicumarol. Intracellular production of ROS by M1619 cells was measured using oxidation of 2',7'-dichlorofluorescin diacetate (DCFH-DA) to 2',7'-dichlorofluorescein (DCF) (42). DCFH-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of H_2O_2, DCFH is oxidized to the highly fluorescent DCF. Approximately 1 × 10^6 M1619 cells were incubated in the dark for 10 min at 37°C with 50 μmol/l DCFH-DA, harvested, and resuspended in plain medium. Fluorescence was analyzed in ∼10,000 cells each with a FACScan (Becton Dickinson, Sunnyvale, CA) flow cytometer with excitation at 488 nm and emission at 530 nm. Compared with spontaneous fluorescence of cells without DCFH treatment, M1619 melanoma cells oxidized DCFH to DCF. Pretreatment of cells for 15 min with DPI (50 μmol/l) or dicumarol (250 μmol/l) reduced DCFH oxidation to DCF. Treatment with DPI or dicumarol in the absence of DCFH did not result in significant cellular fluorescence within the monitored spectrum (data not shown). Mean fluorescence is 754 for untreated, 408 for DPI, and 396 for dicumarol; median fluorescence is 685 for untreated, 355 for DPI, and 359 for dicumarol.

![Fig. 1](http://ajpcell.physiology.org/)  

**Fig. 2.** Plasma membranes from malignant melanoma cells generate ROS. To further study the source of O_2^- generation, we prepared plasma membrane and cytosolic fractions from proliferating M1619 melanoma cells and studied their respective abilities to support SOD-inhibitable lucigenin chemiluminescence. Plasma membranes alone significantly increased SOD-inhibitable lucigenin chemiluminescence without addition of cell cytosol (Fig. 2). When lucigenin was used at a concentration of 5 μmol/l to prevent artifactual redox cycling (32, 46), the preferred substrate for generation of O_2^- was NADPH rather than NADH (Fig. 2, M + NADPH vs. M + NADH). Cytosol from M1619 cells did not support chemiluminescence (Fig. 2, M + C), mitigating against...
the cytosolic enzyme NQO1 as the source driving $O_2^-$ generation in this cell line (11). Also, addition of cytosol to membranes and NADPH did not increase light emission. Chemiluminescence was significantly inhibited by SOD and by addition of the NADPH oxidase inhibitor phenylarsine oxide (31) and the flavoprotein inhibitor DPI. This suggests that light emission is the result of $O_2^-$ generated by a membrane NAD(P)H oxidase.

Both phenylarsine oxide (10–50 nmol/l) and DPI (10–50 μmol/l) also significantly inhibited M1619 malignant melanoma cell growth (96.2 ± 0.6% and 86.0 ± 0.5% inhibition after 48 h, respectively, at the highest dose of each; $P < 0.001$), but melanoma growth was unaffected by inhibitors of the other flavoprotein oxidases xanthine oxidase (allopurinol, 1 mmol/l) or nitric oxide synthetase ($N^\omega$-nitro-l-arginine, 100 μmol/l) (data not shown). Chemiluminescence generation was likewise reduced by direct addition of dicumarol to the membrane preparation in the absence of cytosol. This raises the possibility dicumarol inhibits plasma membrane NAD(P)H oxidase activity, perhaps by disrupting substrate binding in a fashion similar to the mechanism by which it inhibits cytosolic NQO1 (34).

**Malignant melanoma cells and melanocytes express NAD(P)H oxidase components that are necessary for proliferation.** The best-described membrane NAD(P)H oxidase is that of the neutrophil. To determine whether components of this NAD(P)H oxidase or its reported homologs are also expressed in melanomas and non-malignant melanocytes, we performed RT-PCR on RNA extracted from proliferating cells stimulated by 10% FBS (melanomas) or HMGS (melanocytes). M1619 melanoma cells (Fig. 3 A, M1619 melanoma, lane 1) and other malignant melanomas (Table 1) strongly

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**Fig. 3.** Proliferating malignant melanoma cells express p22phox, gp91phox, NOX4, and p67phox. A: RT-PCR performed on nearly confluent M1619 melanoma cells and normal human epidermal melanocytes. Gels represent RT-PCR at 36 cycles. M1619 cells strongly expressed p22phox (lane 2; first detected at 32 cycles). The 252-base pair PCR product obtained has been sequenced and is identical to bases 372–1158 of the reported human mRNA sequence (accession no. NM 016931). No NOX1 homolog was found in either melanomas or melanocytes (lane 6, 36 cycles). The p67phox cytosolic component was also detected in melanomas (lane 5, 36 cycles). The 727-base pair product was sequenced and corresponded to bases 630–1158 of the reported human mRNA sequence (accession no. NM 000397). Normal melanocytes expressed p22phox but not gp91phox. Surprisingly, M1619 melanoma cells strongly and melanocytes modestly expressed the gp91phox homolog NOX4 (lane 7), detected with the primers NOX4-5’ TAACCAGGGCCAGATC TACCT and NOX4-3’ CCGG GAGGTTG GGTATC TAA. The 564-base pair product corresponded to bases 197–741 of the reported human mRNA sequence (accession no. NM 016931). No NOX1 homolog was found in either melanomas or melanocytes (lane 6, 36 cycles). The p67phox cytosolic component was also detected in melanomas (lane 5, 36 cycles). The 727-base pair product has sequences identical to bases 556–1283 of the reported human mRNA sequence (accession no. BC 001606), and faint PCR product was found in melanomas for p47phox (lane 4, 36 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown in lane 1. PCR products were compared with that from an equal amount of mRNA from human polymorphonuclear neutrophils (PMNs; lanes 2–5) or Caco colon carcinoma cells (lanes 6 and 7), respectively, as shown. RT-PCR was performed using human gene-specific sense and antisense primers based on sequences and conducted as detailed in text and in Table 1. B: immunoassays of M1619 cells for p22phox and gp91phox. The 100,000 g membrane fraction from triplicate preparations demonstrated clear evidence of full-size p22phox and gp91phox. A second lighter band in immunoassays for gp91phox may represent unglycosylated protein. C: malignant melanoma cells express NOX4. RT-PCR was performed for 34 cycles using the gene-specific primers as described above. NOX4 was strongly expressed in the malignant melanoma cell lines M1619 (lane 1), M1585 (lane 2), RLW1495 (lane 3), CMC 9212 (lane 4), and RLW 537 (lane 5).
Table 1. RT-PCR expression of NAD(P)H oxidase components in human normal and malignant cell lines

<table>
<thead>
<tr>
<th>No. of PCR cycles</th>
<th>GAPDH</th>
<th>p22</th>
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<th>p47</th>
<th>p67</th>
<th>NOX1</th>
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**RT-PCR** was performed using human gene-specific sense and antisense primers based on sequences published in GenBank: GAPDH-5'-ACCACATGGGAGGCTTGGAAGGGCTTGCGAAGAACGATCG, p22phox-3'-ATGGACGCGGTACGAGCAGCAGCGACATG, gp91phox-5'-TGGATGACGCGGTAGCCCGAGCAAGCTG, p22phox-3'-AACGGGCGGCGGGACGCTG, gp91phox-5'-AACGGGCGGCGGGACGCTG, gp91phox-5'-ACCAGCCCGACCTATCGTTGAC, p47phox-3'-AGTACCGCTGACGCGCTGTCCT, p47phox-5'-CGAGGGAACACGCTGATAGA, p67phox-3'-CATGGGACACGGGAGATAGA. PCR was carried out for 30 cycles for GAPDH, 32 cycles for p22phox, and 36 cycles for all other primers, with amplification at 95°C for 1 min, 58°C for 2 min, followed by an extension step at 72°C for 10 min. PCR-amplified DNA was separated on 1.2% agarose gel, stained with ethidium bromide, and visualized and photographed under ultraviolet light.

expressed the α-subunit of cytochrome b558, p22phox, initially detected at 32 cycles. The 252-base pair PCR product was sought and is identical to bases 221–372 of the reported human mRNA sequence (accession no. XM 008040). The cytochrome- and flavin-bearing β-subunit of the cytochrome, gp91phox, was also present in malignant melanomas (Fig. 3A, M1619 melanoma, lane 3, and Table 1), but only after 36 cycles. This 527-base pair product was sequenced and corresponds to bases 630–1158 of the reported human mRNA sequence (accession no. NM 000397). By immunoassay, both p22phox and gp91phox were easily detectable in the 100,000 g plasma membrane fraction of M1619 melanoma cells (Fig. 3B). No evidence was found for the NOX1 homolog of gp91phox (Fig. 3A, M1619 melanoma, lane 6). However, NOX4 was easily detectable at 34 cycles in M1619 and other malignant melanoma cells (Fig. 3A, M1619 melanoma, lane 7, and 3C, lanes 1–5) as a 564-base pair product, with sequences corresponding to bases 197–741 of the reported human mRNA sequence (accession no. NM 016931). Melanocytes also expressed p22phox (Fig. 3A, melanocytes, lane 2) and NOX4 (Fig. 3A, melanocytes, lane 7) but did not contain mRNA for gp91phox (Fig. 3A, melanocytes, lane 3). The p67phox cytosolic component was observed in M1619 cells (Fig. 3A, M1619 melanoma, lane 5) as a 747-base pair product with sequences identical from bases 556–1283 of the reported human mRNA sequence (accession no. BC 001606) and was also observed in two other malignant melanoma cell lines (Table 1). Faint PCR product was also detected in M1619 cells for the p47phox cytosolic component of the leukocyte NADPH oxidase (Fig. 3A, M1619 melanoma, lane 4), but this product was not sufficiently well expressed to be sequenced. Neither p67phox nor p47phox was found in epidermal melanocytes. Thus three known membrane components (p22phox and two possible partners, gp91phox and NOX4) and the p67phox cytosolic component of the NAD(P)H oxidase are present in proliferating M1619 and other melanoma cells, and p47phox may be expressed at low levels. In contrast, when proliferating, normal melanocytes expressed only p22phox and NOX4.

To begin probing the role of individual NAD(P)H oxidase components in melanoma proliferation, we transfected sense and antisense oligonucleotides for p22phox mRNA into growing M1619 cells. A wide range of commercially available transfection reagents all produced some toxicity to the cells, including the transfection reagents we ultimately employed. Nevertheless, M1619 cells treated with p22phox antisense oligonucleotides had significantly slower growth subsequent to treatment than did identical cells transfected with sense oligonucleotides for p22phox (Fig. 4A). Melanoma cells transfected with NOX4 antisense oligonucleotides also had significantly slower growth subsequent to treatment than did cells transfected with sense oligonucleotides for NOX4 (Fig. 4B). Thus p22phox and
NOX4 appear to play roles in growth signaling for this melanoma cell line. Whether p22<sub>phox</sub> partners with gp91<sub>phox</sub> or its NOX4 homolog in forming the complete membrane portion of the growth regulatory NAD(P)H oxidase remains to be determined.

Using the same primers, we also detected expression of p22<sub>phox</sub>, gp91<sub>phox</sub>, and occasionally p67<sub>phox</sub> by other malignant cell lines, including small cell and non-small cell lung cancers and ovarian, breast, and prostate adenocarcinomas (Table 1). Prostate LnCap carcinoma expressed the NOX1 homolog. H82 small cell carcinoma strongly and H520 squamous cell lung cancer weakly expressed NOX4 (data not shown).

NF-κB is constitutively expressed in melanoma cells and may be regulated by the NAD(P)H oxidase. We have previously reported that NF-κB is constitutively
activated in M1619 melanoma cells (11). NF-κB is also constitutively activated in proliferating human epidermal melanocytes (35). The flavoprotein-dependent NAD(P)H oxidase inhibitor DPI reduced constitutive activation of NF-κB in melanoma cells as studied by NF-κB DNA binding activity (Fig. 5, A and B), immunohistochemically detectable p65 in nuclei (Fig. 5, C vs. D), and immunoassay of the p65 NF-κB component in nuclear protein (Fig. 5E). DPI treatment of melanoma cells also decreased phosphorylation of the NF-κB in-

Fig. 5. Nuclear factor (NF)-κB activation in melanomas is inhibited by the NAD(P)H oxidase inhibitor DPI. A: the flavoprotein-dependent NAD(P)H oxidase inhibitor DPI decreases NF-κB DNA binding. Near-confluent (70%) cultures of M1619 cells (n = 3 per group) were incubated overnight with or without 50 μmol/l DPI. Cells were then lysed, nuclear protein was isolated, and electrophoretic mobility shift assays (EMSAs) were performed with 32P-labeled NF-κB consensus oligonucleotides. The p65/p50-containing dimer is indicated by the arrow. Constitutive NF-κB DNA binding of melanoma cells was greatly reduced in DPI-treated cells (lanes 4–6), compared with cells incubated in growth medium alone (lanes 1–3). FBS, fetal bovine serum; CRE, cAMP responsive element. B: densitometry results of the p65-p50-containing bands from gels in A. *P < 0.001 compared with no DPI. C: constitutive nuclear translocation of NF-κB is demonstrated in M1619 cells by intense brown immunohistochemical staining for p65 in nuclei. Confluent cells were fixed in paraformaldehyde, permeabilized, stained with an antibody to the p65 component of NF-κB and a streptavidin-biotin-immunoperoxidase-based method outlined in text, viewed under light microscopy with a blue filter to enhance contrast, and photographed at ×400 magnification. D: DPI (50 μmol/l overnight) reduced constitutive nuclear translocation of NF-κB. Compared with the intense brown nuclear staining for p65 shown in C, DPI-treated M1619 cells demonstrate little anti-p65 brown staining in nuclei. Nucleoli are recognizable in DPI-treated cells (D) but are only occasionally visible in untreated control cells (C). E: DPI reduced immunoreactive p65 in nuclear protein. Near-confluent (75%) cultures of M1619 cells (n = 3 per group) were incubated overnight with (lanes 4–6) or without (lanes 1–3) 50 μmol/l DPI. Nuclear protein was isolated, and immunoassays were performed for the p65 component of NF-κB. F: DPI inhibited phosphorylation of the NF-κB inhibitor (IκBα). Near-confluent (75%) cultures of M1619 cells (n = 3 per group) were incubated overnight with (lanes 4–6) or without (lanes 1–3) 25 μmol/l DPI. Cells were lysed, and immunoassays were performed with a phospho-specific antibody for IκBα phosphorylated at serine 32 (IκBα-P).
hinator IκBα (Fig. 5B). Taken together, these findings and those reported previously (11) suggest that a flavoprotein-containing NAD(P)H oxidase may play a role in stimulating constitutive NF-κB transcriptional activity in these cells through generation of ROS.

**Inhibition of NF-κB does not impair melanoma proliferation.** Inhibition of NF-κB by antisense strategies reduces tumorigenicity of fibrosarcomas (20), and over-expression of the NF-κB inhibitor IκBα blocks tumor cell growth of Hodgkin’s disease (5), squamous cell lung cancer (6), squamous cell head and neck cancer (16), and breast cancer (47) cells. We therefore infected M1619 cells with an adenoviral vector encoding a superrepressor version of the NF-κB inhibitor IκBα (AdIκBαSR) to determine whether selectively inhibiting NF-κB could reduce M1619 melanoma cell proliferation. Infection with AdIκBαSR resulted in dose-related increases in IκBα expression (Fig. 6A). However, infection with even the highest dose (1 × 10⁷ colony forming units) of AdIκBαSR did not substantially reduce M1619 melanoma proliferation (Fig. 6B), especially compared with the profound growth inhibitory effect shown by antioxidants and NAD(P)H oxidase inhibitors in Fig. 1. Therefore, oxidant generation by a growth regulatory NAD(P)H oxidase must regulate proliferation through other signal transduction pathways.

**Inhibition of NAD(P)H oxidase in melanoma cells reduces DNA binding activity for CRE.** Another family of redox responsive transcription factors important for melanoma proliferation are activating transcription factor/CRE-binding (ATF/CREB) proteins that bind CRE. The transcription factor CREB and its associated family member ATF-1 promote tumor growth, metastasis, and survival through CRE-dependent gene expression (58), and expression of the dominant negative KCREB construct in melanoma cells decreases their tumorigenicity and metastatic potential in nude mice (23). We therefore explored whether inhibition of NAD(P)H oxidase in melanoma cells might reduce DNA binding of transcription factors to CRE. As reported previously for the MeWo melanoma cell line (23, 58), proliferating M1619 cells displayed prominent DNA binding activity for CRE, composed of ATF-1, ATF-2, and CREB-1 transcription factors (Fig. 7A). Treatment of proliferating M1619 cells overnight with DPI inhibits CRE-binding activity in a dose-dependent manner (Fig. 7, B and C) but does not change DNA binding activity of nuclear protein for the transcription factors AP-1 (Fig. 7D) or OCT-1 (Fig. 7E). Thus the growth regulatory NAD(P)H oxidase may signal melanoma proliferation in part through activation of CRE-mediated transcriptionally related events.

**DISCUSSION**

In this report we demonstrate evidence for a growth regulatory oxidase activity in human malignant melanoma cells. By using RT-PCR, we found clear evidence in M1619 melanoma cells for mRNA expression of the NAD(P)H oxidase components p22phox, gp91phox, the gp91phox homolog NOX4, p67phox, and possibly p47phox (Fig. 3, A and C). Expression of oxidase components is not necessarily a transforming event in melanomas, because p22phox and NOX4 were also expressed in normal melanocytes. However, several oxidase components appear critically important for malignant growth, because melanoma proliferation is reduced by transfection of antisense but not sense oligonucleotides p22phox (Fig. 4A) and NOX4 (Fig. 4B). Thus the NAD(P)H oxidase is a normal component of signaling machinery that may be parasitized to serve malignant proliferation.

The putative melanoma NAD(P)H oxidase shares some of the functional properties of the NAD(P)H oxidase of phagocytes but with important differences. Like the phagocyte oxidase, the initial enzyme product appears to be O₂ (Fig. 2). Also, the melanoma oxidase
activity appears to be localized in membranes rather than in the cytosol (Fig. 2), but whether it is found at the cell surface or at an endoplasmic location remains to be explored. Similar to what has been reported recently for vascular smooth muscle cells (46), the melanoma oxidase utilizes NADPH as its preferred substrate (Fig. 2). However, the exact functionally important components of the melanoma oxidase, and their potential interactions, remain to be settled. Because both are expressed, more work is needed to identify whether gp91 \textsubscript{phox} or NOX4 is the dominant functional homolog important for oxidase performance.

A similar dilemma exists in vascular smooth muscle cells, which express gp91 \textsubscript{phox}, NOX1, and NOX4 but

Fig. 7. NAD(P)H oxidase inhibition reduces DNA binding to the CRE but not to activator protein-1 (AP-1) or OCT-1. A: proliferating M1619 melanoma cells displayed prominent DNA binding activity (lanes 1 and 6) for the CRE. Supershift experiments with specific antibodies demonstrated that the CRE binding protein (CREB) family members activating transcription factor (ATF)-1 (lane 2), ATF-2 (lane 3), and CREB-1 (lane 4) contribute to DNA binding activity for CRE. A competition experiment is shown at right, in which addition of 10\textsuperscript{-5} molar excess unlabeled CRE (lane 7) but not AP-1 (lane 8) eliminated DNA binding activity for CRE in melanoma nuclear protein. B: DPI inhibited CRE DNA binding activity in M1619 cells in a dose-dependent manner. Near-confluent cultures of M1619 cells were treated overnight with DPI, and nuclear protein was harvested for EMSAs. Compared with that in untreated cells (lanes 1–3), treatment with DPI inhibits DNA binding of nuclear protein to CRE in a dose-dependent manner (lanes 4–6, 10 \textmu mol/l; lanes 7–9, 15 \textmu mol/l; lanes 10–12, 20 \textmu mol/l). C: densitometry results of EMSAs shown in B. *P < 0.001 vs. untreated cells. D: DPI treatment of M1619 cells did not inhibit DNA binding to the AP-1 oligonucleotide consensus sequence. Lanes 1–3, untreated cells; lanes 4–6, cells treated with 10 \textmu mol/l; lanes 7–9, cells treated with 15 \textmu mol/l; lanes 10–12, cells treated with 20 \textmu mol/l. E: DPI treatment of M1619 cells did not inhibit DNA binding to the OCT-1 oligonucleotide consensus sequence. Lanes 1–3, untreated cells; lanes 4–6, cells treated with 10 \textmu mol/l; lanes 7–9, cells treated with 15 \textmu mol/l; lanes 10–12, cells treated with 20 \textmu mol/l.
appear to employ only NOX1 as the functional homolog (30).

In phagocytes, the NAD(P)H oxidase consists of two membrane proteins, gp91phox and p22phox, that bind a flavin adenine nucleotide (FAD) and form a cytochrome with a redox midpoint potential of −245 mV and a reduced-minus-oxidized difference spectrum of 558 (3). At least two and possibly three cytosolic proteins (p47phox, p67phox, and p40) are also essential, and several other cytosolic components participate, including the small GTPases Rac1 or Rac2. The oxidase is thought to contain all the factors necessary for transporting electrons from the donor substrate NADPH via FAD to generate \( \text{O}_2^- \) from molecular \( \text{O}_2 \). A similar oxidase has recently been reported to serve a signaling function in nonphagocytic cells, where it appears to share some of the components with its phagocyte cousin but with critical distinctions, including a delayed time course for activation and lower level of activity. Endothelial cells appear to express all the phagocyte oxidase components, including gp91phox, p22phox, p47phox, and p67phox (18, 25). In contrast, vascular smooth muscle cells express p22phox (19, 51), p47phox (19), and the unique homolog NOX1 (2, 4, 19, 48). Yet another gp91phox homolog, NOX4, has been described in renal tubular epithelial cells (17, 45), fetal tissue (12), placenta (12), and proliferating vascular smooth muscle (30). We now report NOX4 in normal human epidermal melanocytes and in malignant melanoma cells, where interference with its expression substantially inhibits malignant proliferation (Fig. 4B). These results differ from experiments in normal cells, where NOX4 transfection suppresses rather than enhances proliferation (17, 45). However, the finding of NOX4 in renal cell carcinomas (45), glioblastomas (12), Caco colon cancer cells (12), and now in malignant melanomas raises the possibility that this unique homolog might play an important redox signaling role necessary for malignant prosperity and progression.

A potentially large number of signal transduction and gene expression systems might be influenced by a growth regulatory NAD(P)H oxidase (2), among which is the redox-regulated transcription factor NF-\( \kappa \)B. We have previously shown that antioxidants reduce IkB phosphorylation and constitutive NF-\( \kappa \)B activation in malignant melanoma cells (11). We now demonstrate that the flavoprotein inhibitor DPI reduces IkB phosphorylation (Fig. 5P) and constitutive NF-\( \kappa \)B activation (Fig. 5, A–E), suggesting that ROS from an NAD(P)H oxidase contribute to constitutive NF-\( \kappa \)B activation. NF-\( \kappa \)B has been recently demonstrated to be important for malignant proliferation of a variety of cancers (5, 6, 9, 47, 55). Repression of NF-\( \kappa \)B interferes with normal and transformed cell proliferation (21, 27), and inhibition of NF-\( \kappa \)B by antisense strategies (20) or overexpression of the NF-\( \kappa \)B inhibitor IkB (5, 6, 16, 47) blocks tumor growth. In malignant melanomas NF-\( \kappa \)B is activated as a result of enhanced constitutive IkB kinase activity (44) and is thought to play a significant role in autocrine generation by melanomas of the chemokines MGSA-\( \alpha \)/GRO-\( \alpha \) and interleukin-8 (56). However, in contrast to results with other tumor types, we were unable to suppress growth of M1619 melanoma cells by expression of a superrepressor form of the NF-\( \kappa \)B inhibitor IkB (Fig. 6). Thus NF-\( \kappa \)B activation in this melanoma cell line may play a greater role in conferring resistance of the tumor to apoptosis, chemotherapy, and radiation through upregulating expression of antiapoptotic Bcl-2 family proteins (7, 52, 53). As shown by the significant inhibition by DPI of DNA binding to CRE (Fig. 7, B and C), an alternative group of transcription factors that could be redox regulated by the NAD(P)H oxidase is the ATF/CREB family (1, 29). Molecular disruption of ATF/CREB-mediated transcription has been previously shown to reduce proliferation, metastatic potential, and radiation resistance of malignant melanomas (14, 23, 41, 57, 58).

Others have previously shown the importance of \( \text{O}_2^- \) in mitogenic signaling (22, 26), and transfection with NOX1 transforms normal fibroblasts (48) and creates cell lines that are tumorigenic in athymic mice (2). The exact contribution of autocrine ROS toward the transformed, neoplastic condition is still unclear. However, based on its strategic role in melanomas and its presence in other malignant cell lines (12, 28, 45, 48, 54), a membrane NAD(P)H oxidase may be fundamentally important for growth signaling in a broad array of tumors. If so, NAD(P)H oxidase inhibitors might present a new strategy for cancer therapy, with coumarin analogs offering promise to this end. We had previously shown that dicumarol inhibits ferricytochrome c reduction, constitutive NF-\( \kappa \)B activation, and proliferation in melanomas (11). In this report we show that dicumarol inhibits lucigenin chemiluminescence by melanoma plasma membranes in an in vitro system lacking cytosolic components (Fig. 2), suggesting inhibition of membrane NAD(P)H oxidase activity. Other coumarins have previously been reported to block \( \text{O}_2^- \) by the neutrophil NADPH oxidase (8, 39). Furthermore, prolonged treatment with the coumarin warfarin has been recently shown to reduce subsequent risk of cancer (43). This beneficial effect of warfarin has been attributed to anticoagulation (43, 59), but an alternative possibility is that certain coumarins inhibit a growth regulatory NAD(P)H oxidase important for malignant cell growth.

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