Reactive Oxygen Species-generating Mitochondrial DNA Mutation Up-regulates Hypoxia-inducible Factor-1α Gene Transcription via Phosphatidylinositol 3-Kinase-Akt/Protein Kinase C/Histone Deacetylase Pathway*

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Lewis lung carcinoma-derived high metastatic A11 cells constitutively overexpress hypoxia-inducible factor (HIF)-1α mRNA compared with low metastatic P29 cells. Because A11 cells exclusively possess a G13997A mutation in the mitochondrial NADH dehydrogenase subunit 6 (ND6) gene, we addressed here a causal relationship between the ND6 mutation and the activation of HIF-1α transcription, and we investigated the potential mechanism. Using trans-mitochondrial cybrids between A11 and P29 cells, we found that the ND6 mutation was directly involved in HIF-1α mRNA overexpression. Stimulation of HIF-1α transcription by the ND6 mutation was mediated by overproduction of reactive oxygen species (ROS) and subsequent activation of phosphatidylinositol 3-kinase (PI3K)-Akt and protein kinase C (PKC) signaling pathways. The up-regulation of HIF-1α transcription was abolished by mithramycin A, an Sp1 inhibitor, but luciferase reporter and chromatin immunoprecipitation assays indicated that Sp1 was necessary but not sufficient for HIF-1α mRNA overexpression in A11 cells. On the other hand, trichostatin A, a histone deacetylase (HDAC) inhibitor, markedly suppressed HIF-1α transcription in A11 cells. In accordance with this, HDAC activity was high in A11 cells but low in P29 cells and in A11 cells treated with the ROS scavenger 3-mercaptopropionic acid. The PI3K inhibitor LY294002, and the PKC inhibitor Ro31-8220. These results suggest that the ROS-generating ND6 mutation increases HIF-1α transcription via the PI3K-Akt/PKC/HDAC pathway, leading to HIF-1α protein accumulation in hypoxic tumor cells.

Somatic mutations in mitochondrial DNA (mtDNA) have been shown to accumulate in cancer cells and proposed to contribute to the progression of cancers of a variety of tissue origins. Mitochondria are the key regulators of the oxidative phosphorylation system that is composed of five complexes (I–V). Some somatic mtDNA mutations are envisioned as inhibiting the electron transport chain, resulting in a marked increase in mitochondrial reactive oxygen species (ROS)3 production (1). Actually, for example, a heteroplasmic frameshift mtDNA mutation in the NADH dehydrogenase subunit 5 (ND5) gene and a deletion mutant of cytochrome B (CYTB) gene promote ROS generation (2, 3). In addition, we have recently reported that a missense mutation in the ND6 gene causes the reduction of complex I activity, ROS overproduction, and increased metastatic potential of Lewis lung carcinoma cells (4).

Hypoxia is a common characteristic of locally advanced solid tumors. Hypoxic tumor cells activate many genes, including those related to cell survival, glycolysis, and angiogenesis, and invasion and metastasis to adapt to and escape from the microenvironment (5, 6). The oxygen-sensing mechanisms have been studied extensively and revealed hypoxia-inducible factors (HIFs) as the key regulatory transcription factors that are composed of HIF-α subunit and HIF-β/ARNT subunit. Under normoxic conditions, the α subunit (HIF-1α) is hydroxylated at Pro402 and Pro564 by specific Fe2+–oxoglutarate, and oxygen-dependent prolyl hydroxylases, recognized and ubiquitinylated by an E3 ubiquitin ligase complex consisting of the tumor suppressor VHL (von Hippel-Lindau), elongin B and elongin C, and rapidly degraded through the ubiquitin–proteasome pathway, whereas the β subunit of HIF-1 (HIF-1β) is constitutively expressed. Under hypoxic conditions, HIF-1α protein is stabilized, allowing its nuclear translocation and dimerization with HIF-1β. In the nucleus, HIF binds to the hypoxia response element of hypoxia-inducible genes, including vascular endothe-

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.
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3 The abbreviations used are: ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; DBPS, Dulbecco’s phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FACs, fluorescence-activated cell sorter; HDAC, histone deacetylase; HIF-1α, hypoxia-inducible factor-1α; INK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; ND6, NADH dehydrogenase subunit 6; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TSA, trichostatin A; VEGF, vascular endothelial growth factor.
mtDNA Mutations Control HIF-1α Transcription

Elevated HIF-1α protein levels are commonly observed in many tumor tissues and associated with increased angiogenesis, resistance to apoptosis and chemotherapies, and poor patient prognosis (6, 7). Hypoxia generated by aberrant vasculature formation and high interstitial pressure is undoubtedly a major factor, but other factors such as activation of HIF-1α gene transcription may also play a role in up-regulation of HIF-1α protein in tumor tissues. Actually, we and others have reported the up-regulation of HIF-1α mRNA in some tumor types (8–10). Although the precise mechanism of HIF-1α gene activation is largely unknown, an increase in gene dosage is reported as one of the mechanisms of constitutive up-regulation of HIF-1α mRNA expression (9, 10).

ROS are the physiological mediators to stabilize and increase the transcriptional activity of HIF-1α protein. Incubation of cells with H2O2 or an oxidative stressor leads to the stabilization of HIF-1α protein and activation of HIF target genes under normoxic conditions (11). Conversely, treatment of cells with antioxidants such as N-acetylcysteine and glutathione attenuates HIF-1α protein accumulation and the expressions of HIF target genes in various cell types (11). HIF-1α protein levels increase under normoxia in response to growth factors, hormones, coagulation factors, cytokines, and vasoactive peptides, which also stimulate ROS generation (12, 13). Mitochondria-derived ROS produced by electron transport chain complex III are also reported to be able to stabilize HIF-1α protein under hypoxic conditions (14). Although the stabilization of HIF-1α protein by ROS has been highlighted, HIF-1α mRNA expression is also stimulated by ROS from NADPH oxidase (15).

So far, there are no reports of the involvement of mtDNA mutations in the activation of the HIF-1α gene. Given a high frequency of mtDNA mutation rate in tumor cells and ROS-mediated HIF-1α accumulation at both the protein and mRNA levels, we reasoned that mtDNA mutations could be a cause of HIF-1α transcriptional activation. In the present study, we addressed this issue and investigated the potential mechanism. We report here that certain ROS-generating mtDNA mutations can stabilize HIF-1α transcription via the phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C (PKC)/phosphatidylinositol 3-kinase (PI3K)/protein kinase C 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with a $^{32}$P-labeled mouse HIF-1$\alpha$ cDNA probe or a mouse VEGF cDNA probe (8), which was prepared by the random primer method. Filters were finally washed at 50 °C in 30 mM NaCl, 3% sodium citrate, and 0.1% SDS.

**SDS-PAGE and Western Blotting**—Total cell lysates were prepared by directly solubilizing cells in SDS sample buffer. For analyzes of phosphorylated proteins, cells were lysed in 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM Na$_2$VO$_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Roche Applied Science). Nuclear extracts were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. Proteins were resolved by SDS-PAGE under reducing conditions. Protein concentration was determined by the method of Bradford, University of Wisconsin Medical School (17). Variants of the promoter from nucleotide −150 to +93 (the transcriptional start site was defined as +1) inserted in the KpnI/SacI site of a luciferase reporter plasmid pGL2-basic, hereafter termed pGL2-HIFpro (−150/+93), was a generous gift of Dr. C. A. Bradfield, University of Wisconsin Medical School (17). Various truncated forms of the promoter were made by utilizing restriction enzyme recognition sites in the promoter and the vector (XbaI, KpnI/BbPl, and SacI) for making pGL2-HIFpro (−1422/+93), pGL2-HIFpro (−293/+93), and pGL2-HIFpro (−150/+93), respectively or by PCR using the 5′ primers carrying the KpnI site at the 5′ end and the 3′ primer carrying SacI site at the 3′ end, 5′-GGAGGCTCCCGCTCGGTTCC-3′. The 5′ primers are: 5′-GAGGTACCTAAGTCTGAGTGTGTA-3′ for pGL2-HIFpro (−1048/+93), 5′-GAGGTACC AAGCTGGAGAGGAC-3′ for pGL2-HIFpro (−668/+93), 5′-GAGGTACCTTCCCTCCTCGGC-3′ for pGL2-HIFpro (−101/+93), and 5′-GAGGTACC TTTCCCTCGGTC-3′ for pGL2-HIFpro (−38/+93). The amplified PCR products were inserted into the KpnI/SacI site of a pGL2-basic vector. A pGL2-HIFpro (−150/+93) mutant plasmid that harbors a mutation in the putative Sp1 binding site was prepared by using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The sense and the antisense primers used were 5′-CCGCCCTGGCCAGGCCTGCGTCG-3′ and 5′-GCAGGCGAGGGTTCGGCGGCGCCCG-3′, respectively.

**Luciferase Reporter Assay**—Transient transfection of the luciferase reporter constructs harboring the HIF-1$\alpha$ promoter sequence was carried out using Lipofectamine Plus (Invitrogen). As a control for transfection efficiency, pRL-TK vector (Promega, Madison, WI) was cotransfected with test plasmids. pGL2-control vector (Promega) was used as a positive control. Luciferase activity in cell extracts was assayed 45 h after transfection according to the Dual-Luciferase reporter assay system protocols (Promega) using a luminometer (model TD-20/20; Turner Designs, Sunnyvale, CA).

**Electrophoretic Mobility Shift Assay (EMSA)**—The nuclear protein fractions for EMSA were prepared as described above. Consensus Sp1 (wtSp1, 5′-ATTCTGATGCGGCGGGCGGAGCG-3′) and mutant Sp1 (mutSp1, 5′-ATTCTGATGCTCGGCGGGCAGCGAGCGGAGCG-3′) double-stranded oligonucleotides were purchased from Santa Cruz Biotechnology. The HIF-1$\alpha$ gene promoter-specific double-stranded oligonucleotide probe (positions −72 to −48) (wtHIFpro-Sp1) or its mutant form (mutHIFpro-Sp1) was prepared by annealing the sense 5′-CCGCTCGCCCTTTGCCCAGCCCTCGTGG-3′ and the antisense 5′-CAGGGCGGGCAAGGGCGAGGGCGG-3′ oligonucleotides or the sense 5′-CGGCTGGGCGCACGGCGGAC-3′ and the antisense 5′-CAGGGTTGGCAGGCGGCGAGGGCGG-3′, respectively. The probes were labeled using $\gamma$-[32P]ATP (Amersham Biosciences) and MEGALABELTM kit (TaKaRa Bio). Five micrograms of nuclear protein, $^{32}$P-labeled double-stranded probe (5000 cpm), 1 μg of poly(dI-dC), and 17 μl of binding buffer (20 mM Hepes (pH 7.9), 50 mM NaCl, 5% glycerol, 0.1 mM dithiothreitol) were mixed in a total volume of 20 μl. In competition assays, a 50-fold molar excess amount of unlabeled competitors was included in the reaction mixture. The mixture was incubated at room temperature for 30 min, then loaded on a 5% polyacrylamide gel in TGE buffer (50 mM Tris-HCl (pH 8.5), 380 mM glycine, 2 mM EDTA), and subjected to electrophoresis at 4 °C. The gel was dried and exposed to x-ray film at −70 °C. A supershift assay was performed using 10 μg of specific goat polyclonal anti-Sp1 or anti-Sp3 antibody.

**Chromatin Immunoprecipitation Assay**—Cells were fixed with 1% formaldehyde for 10 min at 37 °C, and the reaction was quenched by adding glycine to a final concentration of 125 mM. The cells were washed with DPBS containing 1 mM PMSF; centrifuged; swelled in 5 mM Hepes (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 0.5 mM PMSE, 100 ng/ml leupeptin, 100 ng/ml aprotinin; incubated for 10 min on ice; and then lysed with a Dounce homogenizer. Nuclei were collected by centrifugation and resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5 mM PMSE, 100 ng/ml leupeptin, 100 ng/ml aprotinin). The nuclei were sonicated on ice to an average length of 500 to 1000 bp and then centrifuged at 10,000 × g for 15 min at 4 °C. The chromatin solution was diluted 10-fold in chromatin immunoprecipitation dilution buffer (500 mM Tris-HCl (pH 8.0), 1670 mM NaCl, 11% Triton X-100, 1.1% sodium deoxycholate, 10 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin), precleared by the addition of protein A-Sepharose beads for 1 h at 4 °C. Precleared chromatin solution was incubated with 5 μg of anti-Sp1 antibody, anti-Sp3 antibody, or anti-acetylhistone H4 antibodies at 4 °C for 13 h. Normal rabbit IgG served as a control. Protein-DNA complexes were immunoprecipitated by protein A beads, and the cross-links were reversed by heating to 65 °C for 5 h. The DNA was recovered by phenol/chloroform extraction and precipitated by ethanol. Then, the association of Sp1, Sp3, and acetylated histone H4 with the Sp1/Sp3 recognition site in the HIF-1$\alpha$ promoter was examined by hot-start PCR.
**RESULTS**

**Activation of HIF-1α Gene Transcription in High Metastatic Cells**—We compared the expression level of HIF-1α mRNA between the low metastatic (P29 and P34) and the high metastatic (D6 and A11) cells originated from Lewis lung carcinoma. The results showed that D6 and A11 cells expressed a larger amount of HIF-1α mRNA than P29 and P34 cells (Fig. 1A). Hypoxia did not affect the expression level of the mRNA. One of the possible mechanisms of HIF-1α mRNA up-regulation in D6 and A11 cells may be the difference in HIF-1α mRNA stability in the cells. To test this possibility, we cultured P29 and A11 cells in the presence of actinomycin D for up to 9 h and examined the mRNA level at each time point (Fig. 1B). The results showed that the half-life of HIF-1α mRNA in A11 cells was nearly equal to that in P29 cells (~8 h) (Fig. 1C). Thus, the transcription of the HIF-1α gene was found to be more activated in A11 cells than in P29 cells.

Under normoxic conditions, HIF-1α protein was scarcely detected in both the low and the high metastatic cells. However, upon hypoxic exposure, HIF-1α protein level markedly increased in D6 and A11 cells compared with P29 and P34 cells (Fig. 1D). Accordingly, hypoxia enhanced the expression of VEGF in D6 and A11 cells more than in P29 and P34 cells at both the mRNA and protein levels (Fig. 1A, A, and E). Thus, the up-regulation of HIF-1α mRNA in D6 and A11 cells resulted in overexpression of HIF-1α under hypoxic conditions, leading to VEGF overexpression.

**ND6 Mutation Activates HIF-1α Transcription**—Sequencing of the ND6 gene revealed that D6 and A11 cells harbored mutations in the ND6 gene. The ND6 gene encodes the ND6 protein, which is a component of the mitochondrial respiratory chain. The ND6 gene expression was found to be up-regulated in A11 cells compared with D6 cells under hypoxic conditions. This up-regulation of ND6 gene expression was associated with the up-regulation of HIF-1α mRNA, leading to the increased expression of VEGF. Thus, the ND6 gene was found to be a target of the HIF-1α-mediated transcriptional activation in A11 cells.
a G13997A mutation, which changes evolutionally conserved proline 25 to leucine, whereas P29 and P34 cells did not (Fig. S1 and Table S1). To examine a causal relationship between the ND6 mutation and HIF-1α transcription, we examined HIF-1α mRNA levels in trans-mitochondrial cybrids, P29mtA11 and A11mP29 cells, which carry mtDNA from A11 and P29 cells and nuclear DNA from P29 and A11 cells, respectively. We used P29mtP29 and A11mtA11 cells as control cybrids. The results showed that the expression level of HIF-1α mRNA was higher in the cybrids with A11 mtDNA (P29mtA11 and A11mP29) compared with A11mtP29 and P29mtP29 cells (Fig. 2 and C). Furthermore, P29mtA11 and A11mtA11 cybrids showed enhanced angiogenesis in vivo (Fig. 2, D and E). These results indicate that the HIF-1α mRNA overexpression in A11 and D6 cells is attributed to the ND6 mutation.

ROS Are Involved in HIF-1α Transcriptional Activation by the ND6 Mutation—It is possible that mitochondrial ROS production caused by the ND6 mutation mediates the activation of HIF-1α transcription. To examine this possibility, we measured the intracellular ROS level. Fluorescence-activated cell sorter (FACS) analysis and confocal images showed that D6 and A11 cells produced a larger amount of ROS than P29 and P34 cells (Fig. 3). In addition, the cybrids with mtDNA from A11 cells...
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Addition of H2O2 stimulated the expression of HIF-1α mRNA in P29 cells (Fig. 4C).

PI3K-Akt and PKC Signaling Pathways Are Involved in Mitochondrial ROS-mediated HIF-1α Overexpression—We next investigated signaling pathways of the mitochondrial ROS-mediated HIF-1α gene activation. For this, we treated P29 and A11 cells with PD98059, a MEK1 inhibitor, SB203580, a p38 MAP kinase inhibitor, SP600125, a JNK inhibitor, and LY294002, a PI3K inhibitor. As shown in Fig. 5A, LY294002 significantly inhibited HIF-1α mRNA expression in A11 cells in a dose-dependent manner, whereas PD98059, SB203580, and SP600125 did not, suggesting the involvement of the PI3K-Akt pathway in the mitochondrial ROS-mediated HIF-1α gene transcription. None of these inhibitors significantly suppressed HIF-1α mRNA expression in P29 cells, implying that the PI3K-Akt pathway does not contribute to the basal expression level of HIF-1α mRNA. In accordance with the data, Akt was highly activated, indicated by its protein phosphorylation, in D6 and A11 cells but not in P29 and P34 cells, and there was no consistent difference in the phosphorylation level of p38 MAP kinase, p44/42 MAP kinase, or JNK (Fig. 5B). Akt was also highly activated in P29mtA11 and A11mtA11 hybrids, but not in P29mtP29 and A11mtP29 hybrids (Fig. 5C). H2O2 strongly induced Akt activation in P29 cells in a time-dependent manner (Fig. 5D). Moreover, ebseleine inhibited Akt phosphorylation in A11 cells (Fig. 5E). Thus, Akt phosphorylation is linked to the level of HIF-1α mRNA. In addition, Ro31-8220, a pan-specific PKC inhibitor, markedly inhibited HIF-1α mRNA expression in A11 cells, but only slightly in P29 cells, suggesting the involvement of PKC in the ROS-mediated HIF-1α mRNA expression (Fig. 5F). On the other hand, rapamycin, an mTOR inhibitor, sulfasazarine, an NF-κB inhibitor, and curcumin, an AP-1 inhibitor, did not significantly inhibit HIF-1α mRNA expression in A11 cells (Fig. S3). Collectively, these data indicate that the PI3K-Akt and PKC pathways are involved in the ROS-mediated HIF-1α transcriptional activation in A11 cells.

Sp1 Is Necessary but Not Sufficient for ROS-mediated HIF-1α Gene Activation—To gain further insight into the underlying mechanisms of HIF-1α gene activation in the high metastatic cell lines, we treated the cells with mithramycin A, an Sp1 inhibitor. The results showed that it significantly suppressed the expression of HIF-1α mRNA in D6 and A11 cells but not in P29 and P34 cells (Fig. 6A), suggesting the involvement of Sp1 in the ROS-mediated HIF-1α mRNA overexpression. To examine which region of the HIF-1α promoter is responsible for the activated transcription of the gene in A11 cells, we constructed luciferase reporter plasmids harboring the full-length (−1958/+93) and a series of truncated promoters (Fig. 6B). We transiently transfected them into A11 cells and examined their activities. The results showed that deletion of the region from −1958 to −101 effectively reduced the promoter activity compared with the full-length promoter, whereas deletion from −1958 to −150 did not significantly reduce the activity. Deletion of the region from −1958 to −38 abrogated the promoter activity, indicating that an important sequence for the promoter activity resides in the region from −149 to −38. Sequence analysis of this region using a computer software (TFSEARCH, Papia system) revealed a putative Sp1 binding

(P29mtA11 and A11mtA11) overproduced ROS compared with the cybrids with mtDNA from P29 cells (P29mtP29 and A11mtA11) (Fig. S2). Thus, the ND6 mutation is correlated well with both ROS overproduction and HIF-1α mRNA up-regulation.

Next, to gain evidence of a causal relationship between ROS and HIF-1α mRNA expression, we examined the effects of general antioxidants ebselene and PDTC, and antinymycin A, which inhibits electron transport pathway (18). FACS analysis showed that intracellular ROS level was low in ebselene- and in PDTC-treated cells whereas high in antinymycin A-treated cells compared with untreated cells, showing more distinct changes in A11 cells than in P29 cells (Fig. 4A). Ebselene and PDTC effectively suppressed the expression of HIF-1α mRNA in A11 cells, whereas antinymycin A increased the expression in both P29 and A11 cells (Fig. 4B). These results strongly suggest that the HIF-1α transcriptional activation is regulated by mitochondrial ROS. Supporting this, we found that exogenously
sequence (−60/−51). Mutation of this sequence TGCCCG-C CCC to TGCCCGAACC significantly reduced the promoter activity (Fig. 6B), demonstrating the importance of this sequence for the promoter activity.

To obtain direct evidence that Sp family members bind to this putative Sp1 binding sequence, we carried out EMSAs using wtHIFpro-Sp1 (−72/−48) as a DNA probe. As shown in Fig. 6C, these assays revealed three constitutive binding complexes (C1–C3) (lane 2) that were almost entirely Sp-dependent, as shown by competition with excess wtHIFpro-Sp1 or Sp1 consensus oligonucleotides (wtSp1) (lanes 3 and 5), but not with their mutant form mutHIFpro-Sp1 or mutSp1 (lanes 4 and 6). Addition of antibodies directed against either Sp1 or Sp3 induced a supershift and/or a significant reduction of Sp1/Sp3-dependent binding activities (lanes 7 and 8). Simultaneous addition of both antibodies led to a nearly complete supershift (lane 9). These data indicate that Sp1 and Sp3 proteins actually bind to the region proximal to the transcription initiation site.

We then compared the expression levels of Sp1 and Sp3 between the high and the low metastatic cell lines. However, we could not detect any difference (Fig. 6D). Also, the DNA binding activity of Sp1/Sp3, as demonstrated by EMSA analysis, did not correlate with the HIF-1α transcriptional level (Fig. 6E). Moreover, chromatin immunoprecipitation assays revealed that there were no differences in the binding of Sp1 and Sp3 to the Sp1/Sp3 binding site and the level of histone H4 acetylation around the site between P29 and A11 cells (Fig. 6F). These results indicate that Sp1 is necessary but not sufficient for explaining the higher expression of HIF-1α mRNA in the high metastatic cell lines.

Thus, the HDAC activity is positively correlated with the HIF-1α mRNA up-regulation.

DISCUSSION

The present study demonstrates that an ROS-generating mtDNA mutation in the ND6 gene leads to HIF-1α mRNA overexpression, resulting in marked up-regulation of HIF-1α protein and VEGF production levels under hypoxic conditions. This study also suggests the possibility for the first time that some of pathogenic mtDNA mutations can activate HIF-1α transcription.

mtDNA mutations are frequently observed in tumor cells and implicated to be a factor in the progression of tumors. mtDNA mutations in tumor cells include severe mutations such as insertion-deletion and chain termination mutations and mild missense mutations. The mutation in the ND6 gene found in A11 cells is a missense mutation that reduces complex I activity (4). This mutation was also found in the other high metastatic D6 cells but not in the low metastatic P29 or P34 cells. In both A11 and D6 cells, up-regulation of HIF-1α gene transcription was detected, suggesting a causal linkage between the ND6 mutation and HIF-1α transcription. In the present study, we used trans-mitochondrial cybrids to prove this linkage, and as expected, the cybrids carrying mtDNA from A11 cells overexpressed HIF-1α mRNA, despite the source of nuclear DNA.

Several lines of evidence supported that ROS caused by the ND6 mutation primarily mediates HIF-1α transcription. First, the cells carrying A11 mtDNA overproduced ROS. Second, ebselene and PDTC reduced the intracellular ROS level and concomitantly abolished HIF-1α transcription. Third, antimi-
cin A that inhibits the function of complex III, thereby generating large quantities of superoxide radicals, increased the expression of HIF-1α mRNA in both P29 and A11 cells. Fourth, exogenous H₂O₂ enhanced the expression. ROS from NADPH oxidase are also mediators of HIF-1α mRNA induction in lipopolysaccharide-stimulated microglial cells and thrombin-stimulated pulmonary artery smooth muscle cells (15, 19). Furthermore, we showed that PI3K-Akt and PKC, but not ERK or JNK, regulate HIF-1α mRNA expression. Because both LY29004 and Ro31-8220 suppressed HIF-1α mRNA expression more effectively in A11 cells than in P29 cells, PI3K-Akt and PKC may engage in the ROS-mediated expression of HIF-1α mRNA. Consistent with these results, either PI3K or PKC or both are shown to regulate HIF-1α transcription in lipopolysaccharide-stimulated glial cells, BCR/ABL-expressing Ba/F3 hematopoietic cells, and angiotensin II-treated vascular smooth muscle cells (12, 15, 20). In contrast, ERK and JNK are reported to mediate lipopolysaccharide-stimulated HIF-1α mRNA induction in human monocytes/macrophages and hepatoma cells, respectively (21, 22). Further study is required to determine which PKC isoform is responsible for the ROS-mediated expression of HIF-1α mRNA using a molecular approach.

The HIF-1α gene promoter contains putative binding sites for several transcription factors, including Sp1, AP-1, and NF-κB (23–25). Treatment of A11 cells with mithramycin A resulted in a marked suppression of HIF-1α mRNA expression in A11 cells, whereas sulfasarsazine and curcumin showed no effect, suggesting the importance of Sp1 for the promoter activity. Luciferase reporter assays also indicated that the Sp1 bind-
ing site is indispensable for the promoter activity. Unexpectedly, however, we could not find any difference in the level of Sp1 binding to and histone acetylation around the binding site between A11 and P29 cells. In contrast, Oh et al. (19) reported that lipopolysaccharide induces HIF-1α/H9251 mRNA in an Sp1-dependent pathway. It is necessary to determine whether other regions of the promoter and transcription factors are involved in the overexpression of HIF-1α mRNA in A11 cells.

In the present study, we showed that TSA markedly repressed the expression of HIF-1α mRNA in A11 cells. Based on this observation, we found a correlation between HDAC activity and HIF-1α transcription; that is, HDAC activity was higher in A11 than in P29 cells. It was also higher in H2O2-treated P29 cells and antimycin A-treated A11 cells than in the respective control cells. Furthermore, HDAC activity in A11 cells was repressed by ebselene, LY294002, and Ro31-8220. Together, these data indicate that ROS lead to HDAC activation through PI3K and PKC pathways, thereby activating HIF-1α transcription. In general, histone acetylation enhances gene expression through the chromatin remodeling caused by histone modification (26). Therefore, it is not clear how HDAC inhibition can lead to the transcriptional repression of the HIF-1α gene. However, many genes such as proinflammatory genes, including tumor necrosis factor-α, interleukin-1β, interferon-γ, and inducible nitric-oxide synthase, are reported to be repressed by HDAC inhibitors (27–30). The repression of these proinflammatory genes has been suggested to be a result of inhibition of NF-κB activation and the acetylation of non-histone proteins (30). Because our data indicate little contribution of NF-κB in the ROS-mediated HIF-1α mRNA overexpression in A11 cells, acetylation of other non-histone proteins may be important. It should be noted that Noh et al. (31) have recently shown that TSA decreases mRNA of extracellular matrix components. They also show that HDAC2 plays an important role in the development of extracellular matrix accumulation and that ROS mediate transforming growth factor-β1-induced activation of HDAC2 (31). HDACs constitute a family of 18 enzymes (32). Therefore, it will be interesting to determine which HDAC is responsible for the ROS-mediated HIF-1α transcription in the cells carrying mtDNA with the ND6 mutation.

In conclusion, our findings show that the ROS-generating ND6 mutation causes HIF-1α transcription via PI3K-Akt/PKC/
mtDNA Mutations Control HIF-1α Transcription

**A**

|        | P29   | A11   | TSA (ng/ml) |
|--------|-------|-------|-------------|
|        | 0     | 0     | HIF-1α      |
|        | 10    | 10    | β-Actin     |
|        | 25    | 25    | 28S         |
|        | 50    | 50    | 18S         |
|        | 100   | 100   |             |

**B**

HDAC pathway. Because mtDNA mutations have been implicated to be a factor in cancer etiology and shown to be gradually accumulated in tumor cells, some of them, especially pathogenic somatic mutations, may contribute to malignant progression by causing the up-regulation of HIF-1α protein in tumors.

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FIGURE 7. HDAC activity is involved in the ROS-mediated HIF-1α transcription. A, P29 and A11 cells were treated with TSA at the indicated concentrations for 18 h. Total RNA was extracted and subjected to Northern blot analysis. The blots were hybridized with a 32P-labeled HIF-1α cDNA. Ethidium bromide staining of the gel is also shown. B, HDAC activity is shown in untreated P29 and P29 cells treated with 25 μM H2O2 for 16 h, and untreated A11 and A11 cells treated with ebelsene (20 μM), antimycin A (20 μM), LY294002 (20 μM), Ro31-8220 (5 μM), and TSA (100 ng/ml) for 18 h.