Novel Function of Orphan Nuclear Receptor Nur77 in Stabilizing Hypoxia-inducible Factor-1α*

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Hypoxia-inducible factor-1α (HIF-1α) plays a central role in oxygen homeostasis by inducing the expression of a broad range of genes in a hypoxia-dependent manner. Here, we show that the orphan nuclear receptor Nur77 is an important regulator of HIF-1α. Under hypoxic conditions, Nur77 protein and transcripts were induced in a time-dependent manner. When Nur77 was exogenously introduced, it enhanced the transcriptional activity of HIF-1α. The Nur77-induced activation of HIF-1α was repressed at transcription level in the presence of Nur77. Further, we found that the expression of MDM2 was repressed at transcription level in the presence of Nur77 as well as under hypoxic conditions. Finally, PD98059 decreased Nur77-induced HIF-1α stability and recovered MDM2 expression, indicating that the extracellular signal-regulated kinase pathway is critical in the Nur77-induced activation of HIF-1α. Together, our results demonstrate a novel function for Nur77 in the stabilization of HIF-1α and suggest a potential role for Nur77 in tumor progression and metastasis.

Hypoxia-inducible factor-1α (HIF-1α)† plays an essential role in cellular adaptation to changes in oxygen availability (reviewed in Ref. 1). Under hypoxic conditions, HIF-1 stimulates the transcription of diverse genes encoding proteins that function to increase oxygen delivery, to allow metabolic adaptation, and to promote cell survival. HIF-1 consists of α and β subunits, both of which belong to the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) protein family. Whereas HIF-1β, the previously described aryl hydrocarbon receptor nuclear translocator, is quite stable under normoxic conditions, HIF-1α is extremely unstable and is quickly degraded by the ubiquitin-proteasome system (2, 3). The tumor suppressor von Hippel-Lindau (VHL) protein interacts with hydroxylated HIF-1α at proline residues in the presence of oxygen, leading to the proteolysis of HIF-1α (4, 5). Specific prolyl hydroxylases (PHDs) catalyze the hydroxylation of the proline residue in the oxygen-dependent degradation domain of HIF-1α (6–8). Three human PHDs, i.e. PHD1, -2, and -3, are expressed ubiquitously, although each isoform differs in the relative amount of transcripts expressed (7). Further activation of HIF-1α involves its nuclear translocation, dimerization with HIF-1α, DNA binding, and the recruitment of its transcriptional coactivators, such as the cyclic AMP response element-binding protein (CBP)/p300 (9). The cooperative binding of VHL and the factor-inhibiting HIF-1α, which recruits histone deacetylases to HIF-1α under normoxic conditions, represses the transactivation function of HIF-1α (10). Indeed, the factor inhibiting HIF-1α was identified as an asparaginyl hydroxylase enzyme that modifies asparagine 803 of HIF-1α using an oxygen molecule, which results in the dissociation of HIF-1α and its coactivator p300 (11). These processes are regulated by post-translational modifications, in that the phosphorylation of HIF-1α via the Ras/Raf/MEK/p42/p44 signaling pathway leads to an enhanced transcriptional activation of HIF-1α (12, 13). Recently, acetylation of HIF-1α by ARD-1 was shown to enhance its interaction with VHL, resulting in the proteasomal degradation of HIF-1α (14).

Although hypoxia is a strong and universal stimulus that activates HIF-1, a significant number of other hormonal, environmental, and intracellular stimuli have been reported to induce HIF-1 activation, probably in a cell-type-specific manner (15–17). One of these factors, p53, a key regulator of the response to cellular stress, directly interacts with HIF-1α and decreases the level of HIF-1α protein by promoting mouse double minute 2 (MDM2)-mediated ubiquitination and the subsequent proteasomal degradation of HIF-1α (18). In contrast, oncogenic proteins such as v-Src and RasV12 result in the loss of hydroxylated proline 564 and thereby stabilize HIF-1α (17). We have recently shown that hepatitis B virus X protein, a major viral transactivator of hepatitis B virus, increases the transcriptional activity of HIF-1α by stabilizing the protein via the extracellular signal-regulated kinase (ERK) signaling pathway (19). Thus, the stability of HIF-1α is modulated by a variety of intracellular proteins, which may indicate as yet unidentified cross-talk between hypoxia and other physiological signals.

Nur77 (also known as NGFI-B, N10, TIS1, and NAK-1) is an orphan member of the steroid/thyroid receptor superfamily of transcriptional factors that positively or negatively regulate gene expression. It is composed of an N-terminal transactivation domain, a DNA-binding domain, and a C-terminal ligand-binding domain (20). Nur77 binds as a monomer to the Nur77-binding
response element (NBRE), which contains the hexanucleotide 5'-AGGTCA-3', a typical recognition motif of the RAR/RXR family, and two A residues preceding this hexanucleotide (21). Nur77 also binds DNA as a homodimer or as a heterodimer with the retinoid X receptor (22, 23). Nur77 is constitutively active when overexpressed, suggesting that the orphan receptor does not require ligand stimulation. Various lines of evidence have suggested a role for Nur77 in cellular proliferation and apoptosis, two major cellular processes. Nur77 expression is rapidly induced by growth factors and mitogens (24–26). Epidermal growth factor and serum induce Nur77 expression, which exerts mitogenic effects (27). Nur77 is also rapidly induced by T-cell receptor signaling in immature thymocytes and T-cell hybridomas, which is followed by apoptotic cell death (28–30). Apoptosis is also induced by many apoptosis-inducing agents, including chemotherapeutic drugs, and the apoptotic process is associated with the translocation of Nur77 into the mitochondria (27, 31). Recently, it was reported that the interaction of Nur77 with the Bcl-2 apoptotic machinery converts Bcl-2 from a protector to a killer (32). Because Nur77 is involved in both cellular proliferation and apoptosis, Nur77 is implicated in the development and progression of tumors, which are considered to result from a disturbance in the balance between these two processes. In the present study, we report a novel function of Nur77 in potentiating the transcriptional activity of HIF-1α and suggest a potential role for Nur77 in tumor progression and metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture and Hypoxic Treatment—Human hepatocellular carcinoma cell lines HepG2 (American Type Culture Collection (ATCC) HB 8065) and Hep3B (ATCC HB 8064), human cervical carcinoma cell line HeLa (ATCC CCL-2), human breast cancer cell line MCF-7 (HTB-22), human embryonic kidney cell line 293 (ATCC CRL-1573), and mouse fibroblast cell line NIH3T3 (CRL-1858) were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in either Dulbecco's modified Eagle's medium or minimal essential medium containing 10% fetal bovine serum at 37 °C in an atmosphere of humidified incubator with 5% CO₂ and 95% air. The cells were exposed to hypoxia (0.1% O₂) by incubating the cells in an anaerobic incubator (Forma Scientific, Marietta, OH) in 5% CO₂, 10% H₂, and 85% N₂ at 37 °C. Hypoxia was also induced chemically by treating the cells with the cell-permeable inhibitor of the mitochondrial electron transport chain, rotenone (Sigma, St. Louis, MO) or 2-deoxyglucose (Calbiochem, San Diego, CA). When the cells were exposed to hypoxia, the medium containing 1% fetal bovine serum was used.

Western Blotting and Immunoprecipitation—Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitors for 30 min on ice, and whole cell lysates were subjected to centrifugation. A fraction of protein from whole cell lysates were subjected to 8–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blocking was performed in 5% (w/v) nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 and then incubated with primary antibody. Blocking was performed in 5% (w/v) nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 and then incubated with primary antibody. The protein concentration was quantified by the Bradford assay (Bio-Rad). The blots were visualized by chemiluminescence (ECL, Amersham, Arlington Heights, IL) or by autoradiography (Kodak, Rochester, NY).

Luciferase—Luciferase reporter plasmids were cotransfected with 1 μg of either pCMV-HIF-1α or pCMV-HIF-1α-MDM2 expression vector into HepG2 cells (2 × 10⁵ cells/well) seeded in 12-well culture plates and incubated overnight. The cells were transfected with reporter plasmid (0.2–0.3 μg), β-galactosidase (β-gal) expression vector (0.2 μg) in the presence or absence of Nur77 expression vector using LipofectaminePlus® (Invitrogen) or Polyfect® (Qiagen Inc., Chatsworth, CA), as described previously (19). For reporter gene analysis, HepG2 cells (2 × 10⁵ cells/well) were seeded in 12-well culture plates and incubated overnight. The cells were transfected with reporter plasmid (0.2–0.3 μg), β-galactosidase (β-gal) expression vector (0.2 μg) and Nur77 expression vector using LipofectaminePlus® (Invitrogen). At the end of treatment, luciferase activity was determined using an analytical luminescence luminometer. Luciferase activity was normalized for transfection efficiency using the corresponding β-gal activity. For statistical analysis, one-way analysis of variance was performed using GraphPad Instat® (GraphPad Software, San Diego, CA). A value of p < 0.05 was considered statistically significant.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time PCR—Total RNA was prepared using RNaseasy kit (Qiagen Inc.). PCR reaction was performed as described previously (19, 33) with specific primers for Nur77 (forward, 5'-GGCGCTGCGTGGACAGGTC-3'; reverse, 5'-CCCTTCCACATTTTAACTGA-3'). The transcripts of HepG2, Hep3B, NIH3T3, and HeLa were amplified in a real-time PCR (Bio-Rad) according to the manufacturer's instructions. The thermal profile was 95 °C for 5 min, 30 cycles of 95 °C for 30 s, and 60 °C for 30 s. At the end of each phase, fluorescence was measured and used for quantitative purposes. Nur77 expression data were normalized to GAPDH content, and the relative transcript level was calculated using the formula as described previously (35).

RESULTS

Induction of Nur77 Gene Expression under Hypoxia—To examine the possibility that Nur77 is involved in the hypoxic signaling pathway, we investigated the regulation of Nur77 expression under hypoxia. As shown in Fig. 1A, when HepG2 cells were incubated under hypoxic conditions, the expression of Nur77 protein was dramatically induced. The induction of Nur77 was observed as early as 30 min after exposure and continued for 24 h. When the cells were treated with a hypoxia-mimicking agent, CoCl₂, a similar pattern of induction was observed. The level of Nur77 induction under hypoxia was as close as that induced by phorbol 12-myristate 13-acetate (PMA) and ionomycin, well known activators of Nur77 (36). The induction of Nur77 was accompanied by an increase in HIF-1α. PMA and ionomycin treatment also induced the expression of HIF-1α protein, which confirms recently published data that indicate PMA induces stabilization of HIF-1α (37). To examine whether the increased Nur77 protein was transcriptionally active, we performed reporter gene analysis using a reporter construct encoding NRE, a Nur77-binding response element (33). Consistent with the induction at the protein level, the
The reporter was activated in the presence of CoCl₂ at a level even higher than that observed in the presence of PMA and ionomycin. The reporter activity was further enhanced when HIF-1α/H9251 was cotransfected, indicating that HIF-1α/H9251 may have a role in the induction of Nur77 under hypoxia (Fig. 1B). Next, we evaluated whether the increase in Nur77 was mediated at the level of transcription. The Nur77 transcripts increased after 30 min and were maximal at 9 h after CoCl₂ treatment (Fig. 1C). These results were reproduced when Nur77 mRNA was measured by real-time PCR. Consistently, the activity of the reporter encoding the Nur77 promoter was enhanced in the presence of CoCl₂, and it was further increased by cotransfection with HIF-1α (Fig. 1D). These results demonstrate that the expression of Nur77 is induced under hypoxia at the level of transcription.

**Nur77 Increases the Transcriptional Activity as Well as the Nuclear Accumulation of HIF-1α**—Next, we asked the biochemical and pathophysiological meaning of Nur77 induction in response to hypoxic stress. We predicted cross-talk between Nur77 and HIF-1α, because HIF-1α is a major regulator of gene expression in response to hypoxia. Therefore, we examined whether Nur77 increases HIF-1 activity using reporter gene analysis with transient cotransfection of the Nur77 expression vector and a reporter gene containing hypoxia response element (HRE) sequences that locates in the erythropoietin gene promoter (9). As shown in Fig. 2A, cotransfection of the Nur77 expression vector into HepG2 cells activated HRE reporter gene activity in a dose-dependent manner. Importantly, DN-Nur77 strongly suppressed CoCl₂-induced HRE reporter activity. These results indicate that Nur77 enhances the transcriptional activity of HIF-1 under hypoxic conditions and even in the absence of hypoxic stress.
HIF-1α was translocated within 5 min under normoxia, but the integrity of pcDNA3.0-HIF-1α was preserved. Nur77 luciferase activity was measured and normalized by CoCl2 treatment. The majority of HIF-1α expression of Nur77 and was further increased in the presence of endothelial growth factor (VEGF), was increased with the expression of HIF-1α. The subcellular localization of HIF-1α was localized diffusely in both the nucleus and cytoplasm in the presence of CoCl2. Similarly, the level of GFP-HIF-1α was enhanced and accumulated in the nucleus when Nur77 was cotransfected. These results demonstrate that Nur77 increases protein level as well as the nuclear translocation of HIF-1α.

Nur77 Increases the Stability of HIF-1α—To evaluate whether Nur77 increases the stability of HIF-1α, we introduced Nur77 into 293 cells and measured the level of HIF-1α protein. Expression of HIF-1α, as well as that of vascular endothelial growth factor (VEGF), was increased with the expression of Nur77 and was further increased in the presence of CoCl2 (Fig. 3A). Although VEGF mRNA was greatly induced, HIF-1α mRNA levels remained unchanged in the presence of Nur77 (Fig. 3B). Because these results indicate that Nur77 increases the level of HIF-1α protein (perhaps through the stabilization of HIF-1α), we measured HIF-1α protein in the presence of cycloheximide, which blocks de novo protein synthesis. As shown in Fig. 3C, Nur77 induced strong expression of HIF-1α and blocked the degradation of HIF-1α as efficiently as CoCl2 treatment did. The majority of HIF-1α protein was degraded within 5 min under normoxia, but the integrity of HIF-1α was maintained in the presence of either CoCl2 or Nur77 up to 60 min.

N-terminal Transactivation Domain of Nur77 Is Sufficient to Stabilize HIF-1α—Nur77 is composed of an N-terminal transactivation domain, a DNA-binding domain, and a C-terminal ligand-binding domain (Fig. 4A). We dissected Nur77 to identify the functional domain that enhances the stability of HIF-1α. Interestingly, the N-terminal transactivation domain alone showed almost full activity in stabilizing HIF-1α, whereas the Nur77Hga construct showed little stabilizing activity (Fig. 4B). Because the Nur77Hga construct has dominant negative function (20, 29), we tested the effect of the construct on the stabilization and transactivation of HIF-1α. As shown in Fig. 4C, Nur77Hga repressed HIF-1α stabilization as well as the expression of VEGF that is induced by CoCl2. These results are consistent with the results of the reporter gene analysis shown in Fig. 2A. The function of the N-terminal transactivation domain that induced the activation of HIF-1α was further confirmed by the reporter gene assays using the HRE reporter (Fig. 4D).

Nur77 Represses the Expression of MDM2, thereby Decreasing the Association between HIF-1α and MDM2—Finally, we investigated the molecular mechanism by which Nur77 enhances the stabilization of HIF-1α protein. We examined whether Nur77 influences the association between HIF-1α and VHL, which subsequently drives the ubiquitin proteasomal degradation of HIF-1α. As shown in Fig. 5A, when cells were treated with MG132 (which blocks proteasome function), the binding of VHL to HIF-1α preceded degradation of HIF-1α under normoxia, as described previously (4, 5). However, this binding was largely diminished in the presence of CoCl2, although this binding was not affected when Nur77 was expressed. This result indicates that VHL is involved in the hypoxia-induced stabilization of HIF-1α, but it may not be associated with the Nur77-induced stability of HIF-1α. Because the ubiquitination/proteasomal degradation of HIF-1α is also regulated by MDM2, we investigated whether Nur77 reduces the binding of HIF-1α to MDM2. MDM2 bound strongly to HIF-1α in the presence of MG132. However, this binding was completely abolished in the presence of either CoCl2 or Nur77 (Fig. 5B). In the presence of CoCl2 or Nur77, the expression of MDM2 protein was largely decreased, suggesting that the loss
Fig. 3. Expression of Nur77 increases stability of HIF-1α protein. A, 293 cells were transfected with 2 μg of p3XFLAG™7.1-Nur77 or empty vector (EV). After 1 h of transfection, the cells were incubated in the presence (+) or absence (−) of 100 μM CoCl₂ for 24 h. At the end of incubation, the cells were lysed, and 50-μg cell lysates were analyzed for expression of the indicated proteins by Western blot analysis. B, 293 cells were transfected and treated with CoCl₂ as shown in A, and total RNA was extracted at the end of incubation. The expression of HIF-1α and VEGF was analyzed by RT-PCR. The expression of β-actin was monitored as a control. C, 293 cells were transfected with 2 μg of p3XFLAG™7.1-Nur77 (●) or empty vector (EV). Cells transfected with empty vector were treated with (●) or without (●) 100 μM CoCl₂ for 24 h. At the end of incubation, the cells were treated with 10 μM cycloheximide (CHX) for the indicated time period. The expression of HIF-1α, FLAG-Nur77, and α-tubulin was analyzed by Western blot analysis. The density of the HIF-1α protein band was determined using an image analysis system. The values were normalized to that of α-tubulin and expressed as percent of the cycloheximide-untreated control value.

Fig. 4. N-terminal transactivation domain of Nur77 is enough to enhance HIF-1α activity. A, schematic representation of Nur77 deletion mutants. B, 293 cells were transfected with 2 μg of p3XFLAG™7.1-Nur77, p3XFLAG™7.1-Nur77NT, or p3XFLAG™7.1-Nur77Hga. After 24 h of transfection, whole cell lysates were obtained, and the indicated proteins were analyzed by Western blot analysis. The expression of α-tubulin was monitored as a control. C, 293 cells were transfected with 2 μg of p3XFLAG™7.1-Nur77Hga or empty vector (EV), and the transfected cells were treated with (+) or without (−) CoCl₂ for 24 h. At the end of incubation, whole cell lysates were obtained and the indicated proteins were analyzed by Western blot analysis. The expression of α-tubulin was monitored as a control. D, the HRE-tk-Luc reporter (300 ng) was transfected into HepG2 cells. After 24 h of transfection, luciferase activity was measured and normalized by β-gal activity. Data shown are the mean ± S.D. of three independent determinations. TAD, transactivation domain; DBD, DNA binding domain; LBD, ligand binding domain.
of MDM2 protein caused the decrease in association of HIF-1α and MDM2.

We further studied the down-regulation of MDM2 expression by Nur77. When Nur77 was expressed in 293 cells, MDM2 expression was completely inhibited. Similar results were obtained with other cell lines such as MCF-7 and NIH3T3 (Fig. 6A). The increase in HIF-1α stability in the presence of Nur77 was blocked when MDM2 was exogenously introduced. Not only was the N-terminal transactivation domain of Nur77 effective in stabilizing HIF-1α (Fig. 5), the expression of MDM2 was strongly repressed by the N-terminal transactivation domain. The result was contrasted to that obtained with the Nur77Hga mutant (Fig. 6B). When we examined the level of MDM2 mRNA, it was largely diminished in the presence of Nur77, indicating that the down-regulation of MDM2 is obtained at transcription level (Fig. 6C). Together, these results clearly show that MDM2 is down-regulated by Nur77, which may subsequently lead to stabilization of HIF-1α.

Expression of MDM2 Is Down-regulated under Hypoxia—We expected that MDM2 would be down-regulated under hypoxia, because Nur77 is induced under these conditions. This possibility was investigated in HepG2 and 293 cells, and we found that MDM2 expression was reduced as expected. The decrease in MDM2 was accompanied by increases in the levels of Nur77 and HIF-1α proteins (Fig. 7A). When the time course of the decrease in MDM2 was examined, it exactly matched the increases in
Nur77 and HIF-1α (Fig. 7B). Furthermore, when the dominant negative construct, Nur77_{Hga}, was expressed, levels of MDM2 were restored (Fig. 7C). Down-regulation of MDM2 under hypoxia was achieved at transcription level, because the mRNA level of MDM2 was largely decreased under hypoxic conditions (Fig. 7D). Together, our results demonstrate that the Nur77-induced down-regulation of MDM2 is an important mechanism by which Nur77 enhances the stabilization of HIF-1α.

**DISCUSSION**

Hypoxia, a reduction in tissue oxygen below physiological levels, commonly develops within solid tumors and often results in the formation of aberrant vasculature by inducing the expression of VEGF through the activation of HIF-1α, a key transcriptional regulator of hypoxic events (reviewed in Refs. 1 and 40). Therefore, great attention has been paid to the factors that regulate the activity of HIF-1α to extend understanding of tumor progression and to facilitate the development of new therapeutic strategies directed against cancer. In this study, we have shown that an orphan nuclear receptor, Nur77, is induced under hypoxia and that it has a role in stabilizing HIF-1α. We have also identified the link between Nur77 and tumor suppressor MDM2 by showing that Nur77 lowers the expression of MDM2, which subsequently decreases degradation of HIF-1α.

Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin proteasomal pathway, and the ubiquitination of HIF-1α is mediated by its interaction with VHL (4, 5). In contrast, when Nur77 stabilizes HIF-1α under normoxic conditions, the binding of HIF-1α to VHL is unchanged (Fig. 6A). Instead, the expression of MDM2 and its subsequent binding to HIF-1α is significantly reduced, indicating that the modulation of MDM2 expression is the main mechanism underlying the Nur77-induced stabilization of HIF-1α (Fig. 4). Consistent with these results, when the expression of Nur77 is induced under hypoxia, the expression of MDM2 is largely diminished (Fig. 8). A decrease in MDM2 protein under hypoxia has also been observed by others (41, 42). Furthermore, exogenously introduced DN-Nur77 restored the expression of MDM2 but decreased the expression of HIF-1α (Fig. 8). This result indicates that MDM2 is an important regulator of HIF-1α stability and may be a major target of Nur77 function. Although the mechanism by which Nur77 down-regulates the expression of MDM2 is largely unknown, direct transcriptional regulation of the MDM2 promoter by Nur77 is unlikely, because DNA binding of Nur77 is not required in this process (Fig. 7B). Recently, Bardos et al. (43) reported a conflicting observation that the transient expression of MDM2 led to increased expression of MDM2 is decreased under hypoxia. A, HepG2 cells (5 × 10⁶ cells/dish) and 293 cells (3 × 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated overnight. The cells were incubated in hypoxic conditions (0.1% O₂) for 24 h. The expression of indicated proteins was analyzed by Western blot or immunoprecipitation/Western blot analysis. The expression of α-tubulin was monitored as a control. B, HepG2 cells (5 × 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated overnight. The cells were incubated and treated with 100 µM CoCl₂ for the indicated time periods. The expression of indicated proteins was analyzed by Western blot or immunoprecipitation/Western blot analysis. The expression of α-tubulin was monitored as a control. C, 293 cells were transfected with 2 µg of DN-Nur77 or empty vector (EV). After 1 h of transfection, the cells were incubated in the presence (+) or absence (−) of 100 µM desferrioxamine (DFO) for 24 h. At the end of incubation, the cells were lysed, and 50-µg cell lysates were analyzed for expression of the indicated proteins by Western blot analysis. D, HepG2 cells (5 × 10⁶ cells/dish) were seeded in 100-cm² dishes and incubated overnight. The cells were incubated in hypoxic conditions (0.1% O₂) for the indicated time periods. Total RNA was prepared and the expression of MDM2 transcripts was analyzed using RT-PCR. Representatives of at least three independent experiments with similar results are shown.
HIF-1α and did not affect the half-life of HIF-1α. However, when MDM2 was overexpressed in our experimental system, levels of endogenous HIF-1α protein decreased (data not shown). The reasons for this discrepancy are not fully understood at this time. The tumor suppressor, p53, is a well-known transcriptional activator of MDM2, and MDM2 itself inactivates p53 transcriptional activity by inducing the ubiquitin-mediated degradation of p53 (44). Under hypoxic conditions, p53 regulates the stability of HIF-1α by promoting the MDM2-mediated ubiquitination and proteasomal degradation of HIF-1α (18). However, reports of the expression of p53 under hypoxic conditions in literature are inconsistent, which may be the result of different experimental conditions, such as the degree of hypoxic stress, the status of the cell cycle, and the cell density used in experiments (45, 46). Whether or not p53 is associated with the function of Nur77 is an interesting question to be addressed in the future.

Multiple signaling pathways, including the calcium, protein kinase A, protein kinase C, and MAPK pathways, are involved in the gene expression as well as transactivation function of Nur77 (47, 48). Phosphorylation regulates the biological function and cellular localization of Nur77 family members. Nerve growth factor and fibroblast growth factor, which promote the differentiation of PC12 cells, strongly phosphorylate Nur77, which is then found diffusely located in both the nucleus and cytoplasm, whereas underphosphorylated Nur77 is found only in the nucleus (49). Recently, Kolluri et al. (27) reported that MAP/ERK kinase kinase 1 (MEKK1) inhibits Nur77 transcriptional activity and Nur77-induced cellular proliferation through the activation of the Jun N-terminal kinase, which efficiently phosphorylates Nur77 and thereby blocks the binding of Nur77 to DNA. In contrast, Slagsvold et al. (50) showed that ERK2 mediates the phosphorylation of Nur77 in vitro when a survival signal is received from growth factors, such as epidermal growth factor (50). In this study, we showed that Nur77 induced activation of the ERK pathway, which may result in stabilization of HIF-1α (Fig. 8), suggesting that Nur77 and ERK pathway may positively cooperate to achieve an intensive intracellular signaling that potentiates HIF-1α function. However, the molecular details of how Nur77 affects the ERK activity remain unknown at present.

Interestingly, the fact that the N-terminal transactivation domain of Nur77 is sufficient to activate HIF-1α suggests that neither the DNA binding nor the transcriptional function of Nur77 is required for the activation of HIF-1α. A previous study showed that Nur77 transactivation in the nucleus is associated with cell proliferation, whereas mitochondrial translocation induces apoptosis (3, 27, 32). Both full-length Nur77 and the N terminus of Nur77 localize mainly in the nucleus (Fig. 3 and data not shown), indicating that the nuclear location of Nur77 without DNA-binding activity could activate HIF-1α. Therefore, the function of Nur77 in stabilizing HIF-1α is a unique property of Nur77 that is independent of other previously described functions of Nur77, such as the transcriptional regulation of mitogenic effects in the nucleus and the induction of apoptosis in the cytoplasm.

An increasing corpus of data supports the role of Nur77 as a survival factor that promotes cellular proliferation. Nur77 is often overexpressed in cancer cells due to the uncontrolled expression of the growth factors that induce its expression (51, 52). Overexpression of Nur77 has been reported to prevent ceramide-induced cell death in neuronal cells and to be associated with retinoic acid-induced apoptosis of lung cancer cells (51, 53). Moreover, the expression of Nur77 is critical for the protection of cells from tumor necrosis factor α-induced apoptosis in mouse embryonic fibroblasts (54). Nur77 is induced by epidermal growth factor and serum in lung cancer cells, and the ectopic expression of Nur77 stimulates cell cycle progression and proliferation (27). Similarly, HIF-1α is present at higher levels in human tumors than in normal tissues, and the expression of HIF-1α in various solid tumors has been associated with tumor aggressiveness, vascularity, treatment failure, and mortality (reviewed in Refs. 1 and 40). In the present study, we have shown that the expression of Nur77 is induced under hypoxic conditions, and stabilizes and transactivates HIF-1α. Recently, it was reported that HIF-1α binds and transactivates Nur77 in renal cell carcinoma (55). In an independent study, VEGF has been shown to induce the expression of the Nur77 family genes (56). These results, together with our observations, suggest a complicated positive feedback regulation of HIF-1α and Nur77. Our results also suggest that Nur77
confers a proliferative advantage on tumor cells, even in the absence of hypoxic stress, through the activation of HIF-1α. An understanding of the underlying mechanisms involved in the stabilization of HIF-1α has important implications, because malignant cells with high level expression of HIF-1α are aggressive and metastatic. Therefore, Nur77 may be a novel target for the development of new anticancer agents that restrict HIF-1α-induced tumor progression and metastasis.

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