PO$_2$-dependent Differential Regulation of Multidrug Resistance 1 Gene Expression by the c-Jun NH$_2$-terminal Kinase Pathway*§

Received for publication, March 14, 2007 Published, JBC Papers in Press, April 23, 2007, DOI 10.1074/jbc.M702206200

Min Liu‡, Dengwen Li‡, Ritu Aneja‡, Harish C. Joshi‡, Songbo Xie‡, Chao Zhang‡, and Jun Zhou‡1

From the ‡Department of Genetics and Cell Biology and Key Laboratory of Bioactive Materials (Ministry of Education), College of Life Sciences, Nankai University, Tianjin 300071, China and the §Department of Cell Biology, Emory University School of Medicine, Atlanta, Georgia 30322

Hypoxia-induced multidrug resistance 1 (MDR1) gene expression is known to be mediated by c-Jun NH$_2$-terminal kinase (JNK) activation. However, the molecular mechanisms underlying this action of JNK remain elusive. On the contrary, there has been increasing evidence for a negative correlation of JNK activity with MDR1 expression under normoxic conditions. Here, we present evidence that the JNK pathway represses MDR1 expression in normoxia and activates MDR1 expression in hypoxia. Our data show that JNK pathway-induced MDR1 repression in normoxia is mediated by increased c-Jun binding to activator protein 1 site, located in the MDR1 promoter, and requires the activity of histone deacetylase 5. In contrast, JNK pathway-induced MDR1 activation in hypoxia is independent of the activator protein 1 site. Rather, this action is dependent on increased hypoxia-inducible factor 1 (HIF1) binding to the hypoxia response element in the MDR1 promoter, which is promoted by the interaction of HIF1α with c-Jun in the nucleus and requires the activity of the p300/CBP (CREB-binding protein) coactivator.

Tissue hypoxia, a decrease in local oxygen tension (PO$_2$), is a common feature of developing tumors. Tumor cells develop various adaptive programs in response to hypoxia, including up-regulation of specific genes that promote tumor survival and growth (1). The key factor that regulates cellular adaptation to low PO$_2$ is hypoxia-inducible factor 1 (HIF1), a heterodimer comprising α- and β-subunits (2). Although HIF1α protein is stable only in hypoxia and rapidly degraded in normoxia, HIF1β protein level is not altered by PO$_2$. Under hypoxic conditions, HIF1α translocates to the nucleus and dimerizes with HIF1β to form HIF1, which then binds to the hypoxia response elements (HREs) of target genes and activates their transcription (2).

Multidrug resistance 1 (MDR1) gene, encoding the transmembrane drug transporter P-glycoprotein, is induced in response to hypoxia (3, 4), which may contribute to the observed chemotherapeutic resistance of cancer cells under hypoxic conditions (5). Hypoxia-induced MDR1 expression is mediated by the binding of HIF1 to the HRE located in the MDR1 promoter (3). In addition, activation of the c-Jun NH$_2$-terminal kinase (JNK), a member of the mitogen-activated protein kinase family, has been implicated in hypoxia-induced MDR1 expression (6–8). However, it remains to be elucidated how JNK activation contributes to MDR1 expression in hypoxia. On the other hand, there is a growing body of evidence supporting a negative correlation of JNK activity with MDR1 expression under normoxic conditions (9–11). It is unclear at present whether the discrepancy between these studies results from the use of different cell lines or the variation in experimental protocols or whether it simply reflects different roles for JNK in regulating MDR1 gene expression under normoxic and hypoxic conditions.

In this study, our data demonstrate for the first time that the JNK pathway plays PO$_2$-dependent different roles in regulating MDR1 expression, i.e. down-regulation of MDR1 expression in normoxia and up-regulation of MDR1 expression in hypoxia. Moreover, our data provide important mechanistic insights into the above-mentioned PO$_2$-dependent differential regulation of MDR1 expression by the JNK pathway.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Adenoviruses—HOP62 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO$_2$. For hypoxic exposure, cells were placed in an airtight incubator chamber flushed with 1% O$_2$, 5% CO$_2$, and 94% N$_2$. Plasmids and adenoviruses encoding JNK, c-Jun, and other proteins used in this study were prepared as described previously (11, 12).
Western Blot Analysis, Immunocomplex Kinase Assay, and Relevant Antibodies—Proteins were resolved by polyacrylamide gel electrophoresis (10 µg of total protein was loaded per lane), transferred onto polyvinylidene difluoride membranes, and exposed to primary and secondary antibodies, and specific proteins were then visualized with enhanced chemiluminescence reagent as described (13). JNK activity was measured using the immunocomplex JNK kinase assay kit according to the manufacturer’s instructions (Calbiochem). Antibodies against P-glycoprotein (1:500 dilution), JNK (1:2000 dilution), c-Jun (1:1000 dilution), phosphorylated c-Jun (1:1000 dilution), and histone deacetylase 5 (HDAC5) (1:500) were obtained from Calbiochem; HIFα1 antibody (1:500 dilution) was from BD Transduction Laboratories; and β-actin antibody (1:5000 dilution) was from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (both at a 1:2500 dilution) were purchased from Pierce Biotechnology.

Quantitative Real Time RT-PCR—Total cellular RNA was prepared using the TRIzol reagent (Invitrogen). Quantitative real time RT-PCR was performed in triplicate as described (14). The Ct values in log linear range representing the detection threshold values were used for quantification by the ΔΔCt method, and glyceraldehyde-3-phosphate dehydrogenase was used as a control to normalize the reading for MDR1 in each sample.

 Luciferase Reporter Assay—MDR1 promoter activity was measured by using the dual luciferase reporter assay system following the manufacturer’s protocol (Promega), with the MDR1 promoter-driven firefly luciferase reporter plasmid pMDR1-Luc and the thymidine kinase-driven Renilla luciferase reporter plasmid pRL-TK. In the pMDR1-Luc plasmid, the MDR1 promoter (from −189 to +133 relative to the major site of transcriptional initiation) was used to drive the firefly luciferase gene as described (15). To minimize experimental variability caused by differences in cell viability and transfection efficiency, the measured firefly luciferase activity was normalized to the thymidine kinase-driven Renilla luciferase activity.

 Transcription Factor Binding Assay—Nuclear extracts, prepared as described (12), were incubated in 96-well plates with immobilized oligonucleotide sequences that contain the activator protein 1 (AP1) site or HRE in the MDR1 promoter. The plates were incubated in succession with c-Jun or HIFα1 antibody, horseradish peroxidase-conjugated secondary antibody, and SigmaFast peroxidase substrate (Sigma-Aldrich). JNK activity was measured by using the dual luciferase reporter assay system following the manufacturer’s protocol (Promega), with the pMDR1-Luc and the thymidine kinase-driven firefly luciferase reporter plasmid pRL-TK, together with the Renilla luciferase reporter plasmid pRL-TK, with the thymidine kinase promoter-driven Renilla luciferase reporter plasmid pRL-TK, and the control plasmid pCDNA3. MDR1 promoter activity was calculated by dividing the measured firefly luciferase activity with the measured Renilla luciferase activity. The unit shown in the y axis is relative, with MDR1 promoter activity in cells transfected with control adenovirus in normoxia set arbitrarily at 1. D, quantitative real time RT-PCR analysis of MDR1 mRNA expression in HOP62 cells treated with JNK adenovirus (m.o.i. = 10) or control (Con; β-galactosidase) adenovirus under normoxic or hypoxic conditions. AD, adenovirus. B, experiments were performed as in A, and the P-glycoprotein level was quantified by densitometric analysis of the Western blot bands. The unit shown in the y axis is relative, with the P-glycoprotein level in cells treated with control adenovirus in normoxia set at 1. E, western blot analysis of the expression of P-glycoprotein (Pgp), JNK, and actin in HOP62 cells treated for 24 h with specific JNK siRNA or control (luciferase) siRNA under normoxic or hypoxic conditions. The unit shown in the y axis is relative, with the P-glycoprotein level in cells treated with control siRNA in normoxia set at 1. F, experiments were performed as in E, and the P-glycoprotein level was quantified by densitometry. The unit shown in the y axis is relative, with the P-glycoprotein level in cells treated with the control siRNA in normoxia set at 1.

Hypoxia alone significantly increased MDR1 mRNA expression (Fig. 1C) despite its modest effect on the P-glycoprotein level. Luciferase reporter assay using the MDR1 promoter further showed that transfection of cells with JNK reduced MDR1 promoter activity in normoxia and enhanced MDR1 promoter activity in hypoxia (Fig. 1D). There was also a dramatic increase in MDR1 promoter activity in response to hypoxia alone (Fig. 1D). Together these data demonstrate that JNK regulates MDR1 gene expression negatively in normoxia and positively in hypoxia, in addition to documenting the previous finding that hypoxia is able to activate MDR1 expression (3, 4, 6).

To examine whether endogenous JNK also plays distinct roles in regulating MDR1 expression in normoxia and hypoxia,
we antagonized the expression of endogenous JNK using specific small interfering RNA (siRNA) and then examined the P-glycoprotein level. Western blot analysis revealed that knockdown of JNK was able to induce P-glycoprotein expression in normoxia and decrease P-glycoprotein expression in hypoxia (Fig. 1, E and F). Similarly, knockdown of JNK by siRNA could increase MDR1 mRNA expression and promoter activity in normoxia and decrease MDR1 mRNA expression and promoter activity in hypoxia (data not shown). Thus, endogenous JNK also possesses the ability to differentially regulate MDR1 expression under normoxic and hypoxic conditions.

Regulation of MDR1 Expression by JNK Is Dependent on JNK Activity and c-Jun in Both Normoxia and Hypoxia—JNK activation is known to contribute to hypoxia-induced MDR1 expression (6–8). In agreement with these studies, we found a time-dependent increase of JNK activity in response to hypoxia (Fig. 2, A and B). Suppression of JNK activity by a specific inhibitor, SP600125, completely abrogated hypoxia-induced MDR1 expression and promoter activity (Fig. 2C and data not shown). Moreover, SP600125 completely inhibited the ability of JNK to repress MDR1 in normoxia and activated MDR1 in hypoxia (Fig. 2C and data not shown), indicating a critical requirement of JNK activity for its regulation of MDR1 expression under both normoxic and hypoxic conditions.

We next investigated whether JNK exerts its PO2-dependent regulatory effect on MDR1 expression through its substrate, the c-Jun transcription factor. As shown in Fig. 2D, knockdown of c-Jun expression using specific siRNA blocked hypoxia-induced MDR1 promoter activity. In addition, JNK-induced repression of MDR1 in normoxia and activation of MDR1 in hypoxia were both abolished by c-Jun siRNA (Fig. 2D). Transfection of cells with c-Jun also exhibited a PO2-dependent regulatory effect on MDR1 promoter activity (Fig. 2E) similar to that observed for transfection with JNK. Thus, c-Jun acts as a pivotal mediator for JNK to regulate MDR1 expression in both normoxia and hypoxia.

We also checked the effects of other components of the JNK pathway, including the upstream kinase MKK4 and upstream kinase kinase MEKK1 (16), on MDR1 gene expression. As shown in Fig. 2E, both MKK4 and MEKK1 could decrease MDR1 promoter activity in normoxia and increase MDR1 promoter activity in hypoxia. In contrast, MKK3 and MEKK2, two molecules not critically involved in the JNK pathway (16), did not have obvious effects on MDR1 promoter activity (Fig. 2E). We also found that MKK4 and MEKK1, but not MKK3 and MEKK2, were able to decrease MDR1 mRNA expression in normoxia and increase MDR1 mRNA expression in hypoxia (data not shown). These results indicate that the PO2-dependent differential regulation of MDR1 expression represents a specific property of the JNK pathway.

Distinct Roles for the AP1 Site and HRE in the MDR1 Promoter in Mediating PO2-dependent Differential Regulation of MDR1 Expression by the JNK Pathway—The MDR1 promoter is known to harbor multiple binding elements, including a negative binding site for the heterodimeric transcription factor AP1 (notably c-Jun/c-Fos), located from −119 to −113 relative to the major transcription start site, and a functional HRE (3, 17, 18). We investigated whether the regulatory effect of the JNK pathway on MDR1 expression is mediated by the alteration of c-Jun binding to the AP1 site. As shown in Fig. 3A, in normoxia both JNK and c-Jun adenoviruses significantly increased c-Jun binding to the AP1 site, whereas in hypoxia neither of them changed the binding of c-Jun to the AP1 site. To further investigate the role for the AP1 site, we performed luciferase reporter assays using an MDR1 promoter harboring mutations in the AP1 site. We found that JNK/c-Jun-induced reduction of
MDR1 promoter activity in normoxia was completely abrogated by AP1 site mutations; however, JNK/c-Jun-induced enhancement of MDR1 promoter activity in hypoxia was not obviously affected (Fig. 3B). These data indicate that JNK pathway-induced MDR1 down-regulation in normoxia is mediated by increased c-Jun binding to the AP1 site, whereas JNK pathway-induced MDR1 up-regulation in hypoxia is independent of the AP1 site.

We then investigated the effects of JNK/c-Jun overexpression on HIF1 binding to the HRE in the MDR1 promoter. We found that JNK and c-Jun adenoviruses did not alter HIF1/HRE binding in normoxia (Fig. 3C). Hypoxia alone significantly increased HIF1/HRE binding, which was further enhanced by overexpression of JNK or c-Jun (Fig. 3C). Luciferase reporter assay using an MDR1 promoter harboring mutations in the HRE revealed that JNK/c-Jun overexpression enhanced of MDR1 promoter activity in hypoxia was entirely blocked by HRE mutations (Fig. 3D). In contrast, JNK/c-Jun-induced reduction of MDR1 promoter activity in normoxia was not affected by HRE mutations (Fig. 3D). These data thus indicate that the JNK pathway-induced MDR1 up-regulation in hypoxia is mediated by increased binding of HIF1 to the HRE in MDR1 promoter, but the JNK pathway-induced MDR1 down-regulation in normoxia is independent of the HRE.

The JNK Pathway-induced Increase of HIF1 Binding to the HRE Is Mediated by the Interaction of HIF1α with c-Jun in the Nucleus—One possibility for the JNK pathway-induced increase of HIF1/HRE binding in hypoxia is that the JNK pathway may increase the HIF1α protein level. It is also possible that the JNK pathway increases HIF1/HRE binding through a direct or indirect association between nuclear c-Jun and HIF1α. Western blot analysis of the HIF1α protein level revealed that JNK/c-Jun overexpression did not affect HIF1α level in hypoxia (data not shown), thus ruling out the first possibility. We also tested the potential interaction between nuclear c-Jun and HIF1α in hypoxia. Immunoprecipitation of the nuclear lysate with a c-Jun antibody showed a clear association of endogenous HIF1α and c-Jun in the nucleus (Fig. 3E). To investigate whether the JNK pathway-induced increase of HIF1/HRE binding in hypoxia is mediated by HIF1α/c-Jun interaction, we depleted c-Jun from the nuclear lysate using a c-Jun antibody and then examined HIF1/HRE binding. As shown in Fig. 3F, c-Jun depletion not only largely abolished hypoxia-induced HIF1/HRE binding, but it also abolished the ability of JNK and c-Jun to increase HIF1/HRE binding. Furthermore, add-back of purified c-Jun significantly restored HIF1/HRE binding. These results thus indicate that in hypoxia, nuclear HIF1α/c-Jun interaction plays an important role in mediating the JNK pathway-induced increase of HIF1 binding to the HRE in the MDR1 promoter.

MDR1 Repression in Normoxia and Activation in Hypoxia by the JNK Pathway Are Mediated by HDAC5 and p300/CREB, Respectively—To gain more mechanistic insights into the PO2-dependent differential regulation of MDR1 expression by the
JNK pathway, we sought to look for additional protein partners of the c-Jun transcription factor. In yeast two-hybrid experiments using c-Jun as bait, we identified several clones of different sizes that encode for HDAC5, a transcriptional repressor belonging to the class II HDAC family (19). Immunoprecipitation of the nuclear lysate with a c-Jun antibody revealed that in normoxia c-Jun interacted with HDAC5 in the nucleus (Fig. 4A). However, the interaction between nuclear HDAC5 and c-Jun was not detected under the hypoxic condition (Fig. 4A). A parallel immunoprecipitation experiment with a HIF1α antibody revealed an association of nuclear HIF1α with c-Jun, but not with HDAC5, in hypoxia (Fig. 4B).

HDAC5 is known to repress gene transcription by promoting histone deacetylation and generating a stabilized, transcriptionally repressive chromatin structure (20). The interaction between HDAC5 and c-Jun in normoxia suggests that the activity of HDAC5 might underlie the JNK pathway-induced repression of MDR1 under the normoxic condition. To test this possibility, we examined the effects of JNK and c-Jun on MDR1 promoter activity in the presence of a dominant-negative, catalytic mutant of HDAC5. As shown in Fig. 4C, the repression of MDR1 promoter activity by JNK and c-Jun in normoxia was largely blocked by dominant-negative HDAC5. In contrast, JNK or c-Jun-induced up-regulation of MDR1 in hypoxia was not obviously affected. These results thus reveal a role for HDAC5 in mediating the down-regulatory effect of the JNK pathway on MDR1 expression in normoxia.

We next investigated whether in hypoxia the HIF1α-dependent MDR1 activation by the JNK pathway requires the p300/CBP transcriptional coactivator, which is known to contain histone acetyltransferase activity and promote the transcriptional activity of HIF1α (21, 22). Consistent with previous findings, our data also revealed that p300/CBP interacted with HIF1α in the nucleus (data not shown). In addition, we found that the JNK pathway-induced MDR1 activation in hypoxia was significantly abrogated by co-transfection with the adenosinon onc protein E1A, a well known suppressor of p300/CBP activity (23); in contrast, a mutant E1A, which is unable to bind p300/CBP (23), did not inhibit JNK pathway-induced MDR1 activation (Fig. 4D). These results indicate that in hypoxia the HIF1α-dependent up-regulatory effect of the JNK pathway on MDR1 expression requires the activity of the p300/CBP coactivator.

**DISCUSSION**

Drug resistance, particularly multidrug resistance, is a primary cause of cancer chemotherapy failure. A major mechanism underlying MDR is excessive induction of the MDR1 gene, ultimately leading to enhanced expression of P-glycoprotein in the plasma membrane. P-glycoprotein extrudes a wide spectrum of chemotherapeutic agents out of cells, causing decreased intracellular drug accumulation and therefore decreased drug effectiveness. MDR1/P-glycoprotein overexpression has been reported to confer MDR in cultured cancer cells and has also been correlated with drug resistance and poor prognosis in cancer patients (24, 25). However, it remains largely unknown how MDR1 expression is up-regulated in various types of cancer. Elucidating the molecular events that regulate MDR1 expression may contribute to the development of appropriate agents combating MDR and improving chemotherapeutic efficacy.

Recent studies have demonstrated that oxygen deprivation within the microenvironment of developing tumors can induce MDR1 gene expression in a HIF1α-dependent manner (3, 4), and JNK activation has been suggested to mediate the above effect (6–8). Intriguingly, in normoxia JNK activity appears to nega-
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...tively correlate with MDR1 expression (9–11). Although it is still ambiguous today as to how JNK functions in MDR1 expression, our results demonstrate clearly that the JNK pathway down-regulates MDR1 expression in normoxia and up-regulates MDR1 expression in hypoxia. These data provide the first direct evidence that the JNK pathway plays different PO$_2$-dependent roles in regulating MDR1 expression. In addition, these data suggest that strategies targeting the JNK pathway for the manipulation of MDR should include PO$_2$ as a critical factor of consideration.

MDR1 expression is known to undergo transcriptional regulation (24, 26). The MDR1 promoter bears a number of binding sites for transcription factors such as Y-box-binding protein 1, nuclear transcription factor Y, and the Sp1 transcription factor (27–29), as well as AP1 (c-Jun/c-Fos, etc.) and HIF1 (3, 18). In addition, cross-coupled nuclear factor-κB/p65 and c-Fos transcription factors are shown to bind to the CAAT box in the MDR1 promoter, to negatively regulate MDR1 expression (30), and p53 is shown to regulate MDR1 either positively or negatively (31, 32). The data in the present study show that the JNK pathway-induced down-regulation of MDR1 in normoxia is mediated by increased c-Jun binding to the AP1 site in the MDR1 promoter, whereas the JNK pathway-induced up-regulation of MDR1 in hypoxia is mediated by increased HIF1 binding to the HRE in the MDR1 promoter. A natural question to be addressed in the future then is how c-Jun and HIF1 coordinate with other transcription factors in regulating MDR1 expression under both normoxic and hypoxic conditions. Our data reveal that in hypoxia, the JNK pathway-induced increase in HIF1 binding to the HRE in the MDR1 promoter is mediated by the interaction between nuclear HIF1α and c-Jun. It will be interesting to investigate, in the future, whether the HIF1α/c-Jun interaction is direct or is mediated by other protein(s) present in the nucleus. In this study, we have also presented evidence that MDR1 repression in normoxia and activation in hypoxia by the JNK pathway are mediated by the HDAC5 transcriptional repressor and the p300/ CBP transcriptional coactivator, respectively. These data provide important mechanistic insights into the PO$_2$-dependent differential regulation of MDR1 expression by the JNK pathway.

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