MEK-ERK-mediated Phosphorylation of Mdm2 at Ser-166 in Hepatocytes

Mdm2 is activated in response to inhibited Akt signaling*

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Mdm2 inactivates the tumor suppressor p53 and Akt has been shown to be a major activator of Mdm2 in many cell types. We have investigated the regulation of Mdm2 in hepatocytes. We found that growth factor-induced Ser-166 phosphorylation of Mdm2 was inhibited by the MEK inhibitors U0126 and PD98059 in HepG2 cells and in a rat liver cell line, TRL 1215. Also, bile acids and oxidative stress induced phosphorylation of Mdm2 at Ser-166 by an apparently MEK-ERK-dependent mechanism. In contrast, Ser-166 phosphorylation of Mdm2 in lung cells was mediated by Akt. Further studies revealed that phosphatidylinositol 3-kinase inhibitors LY294002 and wortmannin induced phosphorylated ERK Tyr-204 and pMdm2 Ser-166 phosphorylations in hepatocytes in culture and in rat hepatocytes in vivo. In HepG2 cells, this effect was inhibited by U0126 and PD98059. LY294002 also reduced the level of pRaf Ser-259. Furthermore, we have shown that myr-Akt-induced overexpression of pAkt suppressed the levels of pMdm2 Ser-166 in hepatocytes. These data indicate a reversed relationship between Akt and Mdm2 in hepatocytes and suggest that Akt is a negative regulator of Raf-MEK-ERK-Mdm2 in this cell type. Ser-166 phosphorylation of Mdm2 has been shown to increase its ubiquitin ligase activity and increase p53 degradation, and our data indicated an attenuated p53 response induced to DNA damage in hepatocytes exhibiting high levels of pMdm2 Ser-166. Taken together, our data indicate that Mdm2 phosphorylation is regulated via MEK-ERK in hepatocytes. This Mdm2 signaling might be important for the regeneration of hepatocytes after centrilobular cell death.

p53 suppresses tumor development by activating growth arrest, apoptosis, or DNA repair. In unstressed cells, levels of p53 are maintained low by its antagonist murine double minute 2 (Mdm2).2 Mdm2 is an E3 ubiquitin ligase and a major regulator of p53, targeting p53 for proteasomal degradation (1–3). Genotoxicity and other stressful stimuli can induce phosphorylations of both Mdm2 and p53 and thus inhibit the ability of Mdm2 to degrade p53. When accumulated, p53 transactivates target genes, including Mdm2, and the proteins interact in an autoregulatory feedback loop (4–8).

Mdm2 phosphorylations at Ser-395 and Tyr-394 have been shown to decrease the capacity of Mdm2 to target p53 for degradation. In contrast, phosphorylations of Mdm2 at Ser-166 and Ser-186 activate Mdm2. They increase the E3 ligase activity and increase the degradation of p53 (9–12). These phosphorylations have also been associated with an increased nuclear localization of Mdm2 (10). A major regulator of Mdm2, protein kinase B/Akt, phosphorylates Mdm2 at Ser-166 and -186 (10). Akt is an important anti-apoptotic signaling molecule, and the phosphorylation of Mdm2 may serve to protect cells from p53-induced apoptosis (12).

Also, other kinases have been identified to induce Ser-166 phosphorylation of Mdm2 and increase its ability to target p53 for degradation. Thus, Mdm2 has been shown to be phosphorylated by MAPKAP kinase and dampen the duration of p53 response induced by UV light (13). MEK-ERK signaling has also been shown to control levels of Mdm2 by regulating the mRNA export to the cytoplasm in some cell types (14, 15). Recently, we reported that the dioxin model compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (16) and cholesterol-lowering drugs, statins, induced Ser-166 phosphorylation of Mdm2 and an attenuated p53 response in hepatocytes (17). Furthermore, we provided evidence for an involvement of an Akt-independent but mammalian target of rapamycin-ERK-dependent signaling in the statin-induced Ser-166 phosphorylation (17).

Our findings raised questions about the liver-specific regulation of Mdm2 and p53. Considering the role of the liver in xenobiotic metabolism and the DNA reactivity of many intermediary metabolites formed in the liver (5), it may be speculated that there are safeguarding mechanisms that protects hepatocytes from excess apoptosis. From this perspective, we have characterized the Mdm2 regulation further. Our data indicate that growth factors and toxic stress induce Ser-166 phosphorylation of Mdm2 via MEK-ERK signaling in hepatocytes. Furthermore, an up-regulation of Akt down-regulated Mdm2 in hepatocytes.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Human hepatocellular carcinoma cells, HepG2 cells, and human lung carcinoma cells, A549 cells,
were purchased from the American Type Culture Collection. TRL1215 cells were provided as a generous gift from Dr. Michael P. Waalkes from the National Cancer Institute. This non-tumorigenic cell line was originally derived from the livers of 10-day-old Fischer F344 rats (18). HepG2 cells were grown on collagen-coated dishes in minimal essential medium with Earle’s salts and L-glutamine. Minimal essential medium was also supplemented with sodium pyruvate (1 mM), non-essential amino acids, penicillin/streptomycin, and 10% inactivated fetal bovine serum. The HepG2 cells were serum-starved (0.5% serum) 48 h before exposure. A549 cells were grown in Dulbecco’s modified Eagle’s medium with glucose (4500 mg/liter) and L-glutamine. Dulbecco’s modified Eagle’s medium was also supplemented with sodium pyruvate (1 mM), penicillin/streptomycin, and 10% inactivated fetal bovine serum. The A549 cells were serum-starved (0.1% serum) 24 h before exposure. TRL1215 cells were grown in Williams’ medium E with Glutamax. The medium was also supplemented with sodium pyruvate (1 mM), penicillin/streptomycin, and 10% inactivated fetal bovine serum. The TRL1215 cells were serum-starved (no serum) 24 h before exposure.

LY294002, wortmannin, rapamycin, sodium deoxycholate, chenodeoxycholic acid, anisomycin, 5-fluorouracil, benzo(a)pyrene, and etoposide were purchased from Sigma-Aldrich and dissolved in Me2SO. U0126 and PD98059 were purchased from Cell Signaling Technology (Beverly, MA) and also dissolved in Me2SO. The Me2SO concentration on the dishes was <0.2%. Transforming growth factor α (TGFα) was from Nordic Biosite (Täby, Sweden), insulin and H2O2 from Merck (Darmstadt, Germany), and leptomycin B from Calbiochem (Darmstadt, Germany).

Small Interference RNA Transfection—HepG2 cells were transfected the day after plating by using SignalSilence Akt small interference RNA (Cell Signaling Technology) and TransIT-TKO transfection reagent (Mirus Bio Corporation, Madison, WI). The cells were transfected for 48 h according to the manufacturer’s protocol. The medium was changed to low serum medium (containing 0.5% serum) 24 h before exposure.

Transfection of HepG2 Cells with Myristoylated-Akt (myr-Akt)—For transfection, LNCX plasmid encoding myr-Akt, which is a constitutive active form of Akt, was used. Plasmid encoding myr-Akt was a generous gift from Raphael A. Nemenoff from the University of Colorado Health Science Center (Denver, CO). Myr-Akt plasmid was amplified and purified by using the Qiagen Maxi kit (Qiagen, West Sussex, UK). Twenty-four hours after plating, HepG2 cells were transfected for 48 h with Lipofectin (Invitrogen) using 1 or 2 μg of DNA of the myr-Akt plasmid or an empty vector, according to the manufacturer’s protocol.

Western Blot Analysis—The cells were washed with phosphate-buffered saline and lysed in IPB7 (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM NaF, 1 mM NaVO3, 0.1 mg/ml trypsin inhibitor, and 1 mg/ml aprotinin). Rats were injected intraperitoneally with diethyl- nitrosamine (DEN), 1.32 mmol/kg body weight (Sigma-Aldrich), 4 or 24 h before sacrifice. The livers were homogenized and subfractionated. The protein was quantified by using Coomassie Plus, the better Bradford assay kit (Pierce). The samples were subjected to SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad), and the protein bands were subsequently probed with antibodies. Primary antibodies used in the analyses were phospho-Mdm2 (Ser-166), Mdm2, phospho-p53 (Ser-46), phospho-Akt (Ser-473), phospho-Raf (Ser-259) (Cell Signaling Technology), Mdm2 (4B11), Mdm2 (4B2) (gifts from Dr. A. Levine), pAkt1/2/3 (Ser-473)-R catalog number sc 7985-R, Akt1 (B-1) sc-5298, p-ERK (E-4) sc-7383, ERK1 (K-23) sc-94, actin (C-11)-R sc1615, Cdk2 (M2) sc-163, p21 (F-5) sc-6246 (Santa Cruz Biotechnology, Santa Cruz, CA), and p53 CM-1 (Novoceastra, Newcastle, UK). Secondary antibodies used in the analyses were goat anti-rabbit IgG-horseradish peroxidase sc-2004, goat antimouse IgG-horseradish peroxidase sc-2005 (Santa Cruz Biotechnology), and anti-rabbit IgG horseradish peroxidase P0217 (DAKO, Glostrup, Denmark). The results were visualized by the ECL detection kit (Amersham Biosciences AB, Uppsala, Sweden). Cdk2, used as a loading control, did not vary between different treatments and correlated with actin, total ERK, and total Mdm2. The results were analyzed with NIH Image version 1.62 software and ImageJ version 1.34s software.

Statistical Analysis—Statistical analysis was performed using Student’s t test. The statistical analysis was based on at least three different experiments, and the results were considered to be statistically significant when p < 0.05.

Immunohistochemical Staining—Female Sprague-Dawley rats were injected intraperitoneally with LY294002 (5 mg/kg body weight) or wortmannin (15 μg/kg body weight) 90 min before sacrifice or with DEN (0.99 mmol/kg body weight) 3 or 48 h before sacrifice. Livers were fixed and slices were stained as described previously (5). In brief, to achieve a rapid fixation, the livers were perfused in 3.7% buffered formaldehyde for 1.5 h and subsequently fixed for 24 h. The sections were incubated overnight with primary antibodies, phospho-Mdm2 (Ser-166) (Cell Signaling Technology), p-ERK (E-4) catalog number sc-7383 (Santa Cruz Biotechnology), and p53 (Ab-3) (Calbiochem). Primary antibodies were visualized using the EnVision+™ peroxidase kit (DAKO). No staining was detected when the primary antibodies were omitted. All animals received humane care, and the experimental protocol was approved by the Swedish Board for Laboratory Animals and was in accordance with National Institutes of Health guidelines.

RESULTS

Akt has been shown to activate Mdm2 in many cell types by inducing phosphorylation at Ser-166 and -186 (9–12). In the present study, we have investigated the effect of Akt activation on Mdm2 phosphorylation in HepG2 cells. We found that insulin phosphorylated Akt at Ser-473 (pAkt Ser-473) and Mdm2 at Ser-166 (pMdm2 Ser-166) in serum-starved HepG2 cells (Fig. 1A). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 reduced the insulin-induced pAkt Ser-473 without affecting pMdm2 Ser-166 (Fig. 1A). The same result was obtained by using another PI3K inhibitor, wortmannin (not shown), or by silencing Akt with small interference RNA (Fig. 1B). These results suggested that the phosphorylation of Mdm2...
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A

HepG2 cells

B

HepG2 cells

C

HepG2 cells

D

HepG2 cells

E

A549 cells

(123 ± 15%, ratio pERK Tyr-204:Cdk2 after 1 h with insulin). Total Mdm2, Akt, or ERK were not affected (Fig. 1A).

Next, we tested the effect of TGFα. We found that TGFα induced the same response as insulin in HepG2 cells. Thus, PI3K inhibitors LY294002, wortmannin, and a mammalian target of rapamycin inhibitor, rapamycin, did not inhibit the TGFα-induced pMdm2 Ser-166, although the MEK inhibitors U0126 and PD98059 did so (Fig. 1D). The phosphorylation of ERK, the downstream target of MEK, was completely inhibited by these inhibitors (Fig. 1D). Together, these findings suggest that growth factor-induced phosphorylation of Mdm2 at Ser-166 in HepG2 cells was mediated by the MEK-ERK pathway. We also analyzed levels of total Mdm2, as it has been indicated that MEK-ERK can control nuclear export of Mdm2 mRNA, at least in some cell types (14, 15). However, Mdm2 levels were not altered by the MEK inhibitors (Fig. 1A) under our experimental conditions.

The non-small cell lung cancer cell line A549 was used as a control. In this cell line, we found that insulin induced pAkt Ser-473 and pMdm2 Ser-166 and that the PI3K inhibitor LY294002 inhibited these effects (Fig. 1E). In this case, inhibition of MEK by PD98059 or U0126 did not affect insulin-induced Ser-166 phosphorylation. These results thus deviated from those obtained with HepG2 cells and indicated an involvement of Akt in pMdm2 Ser-166 formation in lung cells. These data are in line with previous publications (9–11, 19) showing an Akt-mediated phosphorylation of Mdm2 at Ser-166 in many cell types.

As indicated in Fig. 1, increases in pMdm2 Ser-166 levels were paralleled by increases in pERK levels. To further investigate the role of ERK in the phosphorylation of Mdm2, known ERK-activating stimuli were tested. Rosseland et al. (20) have recently shown that oxidative stress phosphorylates ERK in hepatocytes. To investigate whether oxidative stress also phosphorylates Mdm2, we treated HepG2 cells with H2O2. It was found that H2O2 induced pERK Tyr-204, pAkt Ser-473, as well as pMdm2 Ser-166 in a concentration-dependent manner in HepG2 cells (Fig. 2A). Elevated levels of pERK and pMdm2 were registered for at least 2 h (Fig. 2B); whereas the Akt phosphorylation was more transient (data not shown). Fig. 2C shows the densitometric analysis of H2O2-induced pMdm2 and pERK in four experiments. Importantly, pretreatment with the MEK inhibitor PD98059 abolished the H2O2-induced phosphorylation of Mdm2 at Ser-166 (Fig. 2D). The MEK inhibitor U0126 also inhibited the H2O2-induced pMdm2 Ser-166 (data not shown). This indicates that oxidative stress also induced pMdm2 via MEK-ERK in HepG2 cells. In A549 cells, H2O2 induced pERK and pAkt but not pMdm2 (Fig. 2E). Bile acids have been shown to activate ERK (21). As shown in Fig. 3A, both deoxycholic acid and chenodeoxycholic acid induced pERK levels, which correlated with increased pMdm2 levels. Also, this effect was inhibited by U0126, whereas levels of total ERK and Mdm2 were unaffected (Fig. 3B).

Anisomycin has been shown to induce phosphorylation of Mdm2 at Ser-166 in a MAPKAP-dependent manner (13, 22). We found that anisomycin induced pMdm2 and pERK (but not pAkt) in HepG2 cells (Fig. 3C). In A549 cells, anisomycin induced pAkt and pMdm2 but not pERK (data not shown).
PI3K phosphorylates Akt and its inhibitors decreased pAkt levels in growth factor-stimulated cells without inhibiting pMdm2 Ser-166 (Fig. 1, A and D). Next, we investigated whether constitutive levels of pMdm2 Ser-166 were affected by PI3K inhibitors. To our surprise, we found that LY294002 and wortmannin increased pMdm2 levels (Fig. 4, A and E). The increase in pMdm2 levels in HepG2 cells was preceded by decreased levels of pAkt and increased levels of pERK, whereas total ERK, Mdm2, and Akt were unaffected (Fig. 4A). Densitometric analysis of results from three experiments shows that pMdm2 Ser-166 levels were significantly increased after 15 min of exposure (Fig. 4B). The peak pERK level was seen within 2 h (data not shown). The LY294002-induced pMdm2 Ser-166 was inhibited by the MEK inhibitor PD98059 (Fig. 4C). Raf can regulate ERK activity (23–26), and we found that LY294002 decreased the inactivating Ser-259 phosphorylation of Raf (Fig. 4D). These data are in line with a previously discussed cross-talk between Akt and Raf-ERK (23–26). The data presented here extend previously published data and suggest a cross-talk regulating Mdm2 activation in hepatocytes. In A549 cells, the levels of pMdm2 were unaffected or decreased by LY294002 and wortmannin, whereas pERK levels increased by LY294002 (Fig. 4F). Insulin was used as a positive control (Fig. 4, E and F).

To study these effects in another liver cell line, TRL 1215 cells were employed. As shown in Fig. 5A, LY294002 increased pMdm2 and pERK levels in these cells as well. This effect was inhibited by the MEK inhibitor PD98059 (Fig. 5B). Also, growth factor-induced pMdm2 Ser-166 was inhibited by MEK inhibitors in TRL 1215 cells (data not shown). Next, the effect of Akt overexpression was studied. In non-starved myr-Akt-overexpressing cells, the levels of pMdm2 Ser-166 and pERK Tyr-204 were suppressed (Fig. 6). Interestingly, a dose-effect relationship was indicated, so that increasing doses of myr-Akt gave decreasing levels of pMdm2.

The effect of PI3K inhibitors on phosphorylated Mdm2 and ERK was studied in vivo in rat liver. In control livers, no or very weak nuclear staining for pMdm2 Ser-166 or pERK Tyr-204 was detected (Fig. 7, A and D). Ninety minutes after an intraperitoneal injection of LY294002 (5 mg/kg) or wortmannin (15 μg/kg), a nuclear staining for pERK in hepatocytes was markedly increased (Fig. 7, E and F). There was also an increased staining in other cell types than just hepatocytes. No clear zonal distribution was observed. Both treatments also induced...
nuclear staining for pMdm2 Ser-166, affecting a major part of the lobule including centrilobular and midzonal areas (Fig. 7, B and C). Of interest is that this increase only affected hepatocytes. For example, non-hepatocytes stained for pERK Tyr-204 were not stained for pMdm2 Ser-166 (Fig. 7, B, C, E and F). These results corroborate the data obtained with cell lines and indicate that PI3K inhibitors induced phosphorylation of Mdm2 at Ser-166 in hepatocytes in vivo. Other cell types in the liver were not affected.

We have previously described the p53/Mdm2 autoregulatory loop and the induction of apoptosis in rat liver in response to DNA-damaging agents (5). We subsequently tested the possible involvement of pMdm2 Ser-166 in this response. As shown in Fig. 8, treatment with DEN induced nuclear staining for pMdm2 Ser-166 in hepatocytes within 3 h (Fig. 8A). The zonal distribution of pMdm2 Ser-166-positive hepatocytes seen at 3 h was the same as the previously described zonal distribution of cytoplasmic and nuclear staining.
PI3K inhibitors induce phosphorylation of Mdm2 at Ser-166 and phosphorylation of ERK at Tyr-204 in rat liver in vivo. Liver section stained for pMdm2 Ser-166 in untreated rat (A), LY294002-treated rat (5 mg/kg, 90 min) (B), or wortmannin-treated rat (15 μg/kg, 90 min) (C). Liver section stained for pERK Tyr-204 in untreated rat (D), LY294002-treated rat (5 mg/kg, 90 min) (E), or wortmannin-treated rat (15 μg/kg, 90 min) (F). In B and C, hepatocytes with stained nuclei are shown in high magnification (inset, thin arrow points to the stained nucleus). In E and F, hepatocytes and non-parenchymal cells with stained nuclei are shown in high magnification (inset, thin arrow points to stained nucleus in hepatocytes and open arrow to stained nucleus in non-parenchymal cells).

PI3K inhibitors induce phosphorylation of Mdm2 at Ser-166 and phosphorylation of ERK at Tyr-204 in rat liver in vivo. Liver section stained for pMdm2 Ser-166 in untreated rat (A), LY294002-treated rat (5 mg/kg, 90 min) (B), or wortmannin-treated rat (15 μg/kg, 90 min) (C). Liver section stained for pERK Tyr-204 in untreated rat (D), LY294002-treated rat (5 mg/kg, 90 min) (E), or wortmannin-treated rat (15 μg/kg, 90 min) (F). In B and C, hepatocytes with stained nuclei are shown in high magnification (inset, thin arrow points to the stained nucleus). In E and F, hepatocytes and non-parenchymal cells with stained nuclei are shown in high magnification (inset, thin arrow points to stained nucleus in hepatocytes and open arrow to stained nucleus in non-parenchymal cells).

FIGURE 8. DEN-induced phosphorylation of Mdm2 at Ser-166 in rat liver in vivo. Liver section stained for pMdm2 Ser-166 following DEN treatment (0.99 mmol/kg) for 3 h (A) or for 48 h (B). Liver section stained for p53 following DEN treatment (0.99 mmol/kg) for 48 h (C). D, Western blot showing the level of pMdm2 Ser-166 in rat liver samples following DEN treatment (1.32 mmol/kg, times indicated). The nuclear fractions of the liver samples were used for Western blot analysis. Actin was used as a loading control.

DISCUSSION

In this study, we have shown that bile acids, H2O2, genotoxic compounds such as the liver carcinogen DEN, and PI3K inhibitors all induce Ser-166 phosphorylation of Mdm2 in hepatocytes. We also have provided evidence that this phosphoryla-
Mdm2 phosphorylation in hepatocytes, even though Akt was down-regulated. Furthermore, transfection of cells with constitutively active myr-Akt decreased Mdm2 phosphorylation. We conclude that, in many cell types, pAkt has been shown to phosphorylate and activate Mdm2 (9–12), whereas data presented here indicate that Akt rather inactivates Mdm2 in hepatocytes. It is perhaps important to add that we used cell lines with wild-type p53 function in this study; it is not known how non-functional p53 influences this regulation.

The activation of the MEK-ERK pathway by PI3K inhibitors can be explained by a cross-talk between Akt and Raf. Thus, high levels of pAkt can inhibit Raf-1-MEK-ERK signaling by inactivating Raf-1 (30), and our data show that LY294002 might activate Raf. This cross-talk has been documented in vitro in several cell types (23–26) including the Hep3B cell line (31). It has also been shown that an inhibition of Akt by LY294002 up-regulates B-Raf in a human kidney epithelial cell line (32). Interestingly, our data show that both LY294002 and wortmannin induced increased levels of pERK in rat hepatocytes and in non-parenchymal liver cells in situ, indicating an in vivo relevance of our findings. These results and the dose-effect relationship obtained with transfected myr-Akt suggest that Akt maintains a negative control and modulates Raf-MEK-ERK in quiescent rat liver cells in situ.

Many cell types have been employed in studies showing Mdm2 activation by Akt, and hepatocytes seem to be the only cell type, so far, in which ERK-dependent Mdm2 activation is documented. Furthermore, we have shown that pMdm2 Ser-166 accumulated in hepatocytes but not in lung cells or in non-parenchymal liver cells. Both toxicological stress and growth signals were mediated to Mdm2 via MEK-ERK (Fig. 10). In a previous publication (17), we showed that a statin-induced pMdm2 Ser-166 accumulation was prevented by MEK-inhibitors as well as by rapamycin, suggesting an involvement of MEK and mammalian target of rapamycin in this response in hepatocytes. It is unknown why pAkt in hepatocytes, even in high levels, was unable to phosphorylate Mdm2 or why pERK was unable to induce pMdm2 Ser-166 in lung cells or non-parenchymal liver cells.

It is well established that Mdm2 ablation is embryonically lethal and that the apoptotic phenotype is rescued by p53 knock-out (33). Also Raf-1 knock-out is lethal, and embryonic liver cell apoptosis seems to be a primary event in this early death, at least in some genetic backgrounds (34). Further studi-
loberal cell death, and it can be speculated that an ERK-Mdm2 signaling is needed for coordinating this replication under genotoxic stress. Another reasoning suggests that an ERK-Mdm2 signaling is important for hepatocytes to endure chronic stress; it has been reported that chronic ethanol intake is associated with a down-regulated Akt signaling (37). Our data raise the question whether this chronic down-regulation of pAkt levels may serve to up-regulate Raf-MEK-ERK signaling.

In summary, we suggest that hepatocytes respond to low pAkt levels with an up-regulation of Mdm2 phosphorylation at Ser-166 via the MEK-ERK pathway. This ERK-mediated effect on Mdm2 may attenuate the duration and intensity of p53 responses in liver.

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