Stabilization of Mdm2 via Decreased Ubiquitination Is Mediated by Protein Kinase B/Akt-dependent Phosphorylation

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Running title: Regulation of Mdm2 stability by PKB
Summary

The tumor suppressor p53 is commonly inhibited under conditions in which the PI3K/PKB(Akt) pathway is activated. Intracellular levels of p53 are controlled by the E3 ubiquitin ligase Mdm2. Here we show that PKB inhibits Mdm2 self-ubiquitination via phosphorylation of Mdm2 on Ser<sup>166</sup> and Ser<sup>188</sup>. Stimulation of HEK293 cells with IGF-1 increased Mdm2 phosphorylation on Ser<sup>166</sup> and Ser<sup>188</sup> in a PI3K-dependent manner, and the treatment of both HEK293 and COS-1 cells with PI3K inhibitor LY-294002 led to proteasome-mediated Mdm2 degradation. Introduction of a constitutively active form of PKB together with Mdm2 into cells induced phosphorylation of Mdm2 at Ser<sup>166</sup> and Ser<sup>188</sup>, and stabilized Mdm2 protein. Moreover, mouse embryonic fibroblasts lacking PKBα displayed reduced Mdm2 protein levels with a concomitant increase of p53 and p21<sup>Cip1</sup>, resulting in strongly elevated apoptosis after UV-irradiation. In addition, activation of PKB correlated with Mdm2 phosphorylation and stability in a variety of human tumor cells. These findings suggest that PKB plays a critical role in controlling of Mdm2-p53 signalling pathway by regulating Mdm2 stability.
Introduction

The phosphatidylinositide3’-OH kinase (PI3K)\(^1\)-PKB/Akt pathway is a key component of growth factor-induced cell survival. This pathway has been implicated in suppressing apoptosis in a number of cell types in response to a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion, DNA damage and treatment with anti-Fas antibody or TGF\(\beta\) (1-3). A number of PKB substrates that are components of the intrinsic cell death machinery have been identified, including glycogen synthase kinase-3, the Bcl2 family member BAD, the protease caspase-9, and Forkhead transcription factors (1-4). In each case, phosphorylation of these proteins by PKB suppresses their pro-apoptotic function. Stimulation of cells with survival factors causes activation and nuclear translocation of PKB in target cells (5, 6), suggesting that PKB may modulate nuclear targets. Several recent studies have shown that p53-mediated apoptosis is inhibited under conditions in which the PI3K/PKB pathway is activated (7-11). The p53 gene product is a tumor suppressor that mediates growth arrest, senescence, and apoptosis in response to several cellular stresses (12). Protein levels of p53 are the most important determinant of its functions. In normal unstressed cells, p53 is an unstable protein with a half-life of less than 20 min, which is kept at very low cellular levels owing to continuous degradation mediated largely by Mdm2 (13-18). The Mdm2 was originally identified on double-minute chromosomes of spontaneous transformed mouse 3T3 fibroblasts (19). Mdm2 harbors a self- and p53-specific E3 ubiquitin ligase activity within its evolutionarily conserved C-terminal RING-finger domain, which mediates p53 ubiquitination and rapid degradation by the 26S proteasome (20, 21). The current model places p53 and Mdm2 in an autoregulatory feedback loop: Mdm2 transcription is induced by p53, and Mdm2, in turn, binds to the N-terminal transactivation domain of p53, thereby inactivating p53 transcriptional activity (12-14, 17, 18, 20-23). Low levels of Mdm2 activity induce mono-
ubiquitination and nuclear export of p53, whereas high levels promote poly-ubiquitination and nuclear degradation of p53 (24). The levels of Mdm2 expression, the association of Mdm2 with p53, and the E3 ubiquitin ligase activity of Mdm2 that downregulates p53, are regulated by phosphorylation (25), oligomerization (26), and binding to other factors such as p19/p14ARF (27), MdmX (28), and hHR23A (29). More recent data showed that Mdm2 is directly regulated by a deubiquitinase (HAUSP), which reveals a dynamic role of ubiquitination and deubiquitination of Mdm2 in the Mdm2-p53 pathway (30).

Expression of active or kinase-defective PKB has little or no effect on the phosphorylation status of p53 (8), suggesting that p53 does not serve as a direct substrate for PKB, thus raising the possibility that Mdm2 itself might be the principal target of the PI3K/PKB signaling pathway. Indeed, it has been reported that Mdm2 could be phosphorylated by PKB in vitro at Ser^{166} and Ser^{186}, and this phosphorylation promotes the nuclear entry of Mdm2 (31, 32). Here we report that PKB phosphorylates Mdm2 on a previously reported site Ser^{166} (31, 32), and on a novel site Ser^{188} in vitro and in vivo. Phosphorylation of Mdm2 by PKB leads to inhibition of Mdm2 self-ubiquitination and stabilization of Mdm2 by protecting Mdm2 from proteasome-dependent degradation. Thus, our results suggest that PKB-dependent phosphorylation of Mdm2 leads to the increase of Mdm2 stability and a consequent down-regulation of p53.
Experimental Procedures

Materials and chemicals

Chemicals and vendors were as follows: \(\gamma^{32}\text{P}]\text{ATP (Amersham Pharmacia Biotech); ATP bromodeoxyuridine (BrdU) and cycloheximide (Sigma); E1 ubiquitin-activating enzyme, LY-294002, and MG132 (Calbiochem); microcystin-LR (Alexis); IGF-1 (Life Technologies Inc.); protein kinase A inhibitor peptide (PKI) was from Bachem; monoclonal antibody to Mdm2 (SMP-14, Santa Cruz Inc.); monoclonal antibody to p53 (PAb421) and polyclonal anti-p21^{Cip1} (Ab4) (Oncogene Science); monoclonal antibody to HA (12CA5, Roche) and polyclonal anti-phospho-Ser\(^{473}\) PKB antibody (pSer\(^{473}\), Cell Signalling); polyclonal anti-PKB (Ab10) was described previously (5); TRITC-conjugated anti-rabbit IgG antibody (Chemicon); FITC-conjugated anti-BrdU antibody (BD Scicence).

Preparation of plasmids and proteins

pCMV5-HA-PKB expression vectors have been described (5). pGEX2T-Mdm2 was made by inserting the BamHI-EcoRI fragment of human Mdm2 cDNA released from pcDNA3-Mdm2 (a kind gift of Dr. C. G. Maki, University of Chicago) into pGEX2T vector (Amersham Pharmacia Biotech). The same fragment was also ligated into the BglII-EcoRI sites of pEGFP-C1 (Invitrogen) vector to generate pEGFP-Mdm2. The Mdm2 mutants were generated using the Quick Change kit (Stratagene). pGEX2T-UbcH5 and pGEX2KT-ubiquitin were obtained from Dr. W. Krek (Eidgenössische Technische Hochschule, Zürich, Switzerland). All constructs were verified by DNA sequencing. GST-fusion proteins were expressed in Escherichia coli BL21 strain and purified on Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s
instructions. Human ΔPH-PKBα cDNA (118-480) was released from pMV1 vector as a EcoRI-EcoRI fragment and was inserted into pFastBacHTc vector (Life Technologies Inc.) to generate pFastBacHTc.ΔPH-PKBα B. After transposition into the DH10Bac competent cells, the recombinant Bacmid DNA was isolated, and then transfected into Sf9 cells. The resulting recombinant baculovirus particles were used to infect Sf9 cells and the expressed His-tagged ΔPH-PKBα protein was purified on Ni-NTA resin according to the manufacturer’s instructions (Qiagen).

Production of antibodies

The peptides: (1) Ser160-Arg-Arg-Arg-Ala-Ile-Ser166-Glu-Thr-Glu-Glu-Asn-Ser-Asp173, (3) Ser160-Arg-Arg-Arg-Ala-Ile-phospho-Ser166-Glu-Thr-Glu-Glu-Asn-Ser-Asp173, (3) Lys182-Arg-His-Lys-Ser-Asp-Ser188-Ile-Ser-Leu-Ser-Phe-Asp-Glu195, (4) Lys182-Arg-His-Lys-Ser-Asp-phospho-Ser188-Ile-Ser-Leu-Ser-Phe-Asp-Glu195 were synthesized by Neosystem (Strasbourg, France). The rabbit polyclonal anti-phosphopeptide antibodies were prepared and affinity-purified using a nonphosphopeptide affinity column followed by a phosphopeptide affinity column. These antibodies are referred to as pSer166, and pSer188. The polyclonal antibody against Mdm2 was raised by immunizing rabbits with GST-Mdm2 (full-length of human Mdm2), and affinity purified using a GST-coupled Sepharose column followed by GST-Mdm2-coupled Sepharose column. This antibody is referred to as anti-Mdm2.

Cell culture, transfections, immunoprecipitation, and immunofluorescence

Generation of PKBα knockout mouse has been described in (33). MEFs were prepared from E13.5 embryos generated by the Pkbaα+/− wild type (Pkbaα+/−) or knockout (Pkbaα−) intercrosses as described previously (33, 34). The genotype of each embryo was verified by PCR (data not shown).
Cells were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Transfections were performed by calcium phosphate precipitation (5). Cells were lysed in NP-40 lysis buffer and immunoprecipitated exactly as described (5). For cell immunofluorescence, the cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton-X 100. After blocking, cells were incubated with monoclonal anti-Mdm2 antibody (SMP14, Santa Cruz Inc., 2 µg/ml diluted in PBS) for 2 hr, followed with polyclonal Mdm2 phospho-antibodies (2 µg/ml diluted in PBS) for additional 2 hr. Cells were then extensively washed, and incubated with FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG (both 1:100 dilution). After washing, the cells were visualized by Confocal microscopy (Olympus BX61).

**Cell cycle and cell death analysis**

Exponentially growing MEFs were labeled for 30 min with 10 µM 5-bromo-2'-deoxyuridine (BrdU; Sigma). Subsequently, the cells were exposed to 120 J/m² UV-C irradiation (UV Stratalinker 1800; Stratagene) or left untreated, harvested at indicated intervals by trypsinization, and fixed with 70% ethanol at -20°C. Cells were then treated as follows: (i) incubated in 2 N HCl containing 0,2 mg/ml porcine pepsin (Sigma) for 20 min at room temperature; (ii) neutralized with 0,1 M sodium tetraborate (Sigma) and washed twice in PBS; (iii) incubated for 1 hr at room temperature in PBS containing 0.5% Tween-20, 1% BSA, and 1:50 (v/v) FITC-labeled anti-BrdU mouse monoclonal antibody (Becton Dickinson); (iv) washed in PBS and resuspended in PBS containing 20 µg/ml propidium iodide (Sigma). Bivariatic flow cytometry analysis was performed on fluorescence-activated cell analyzer (FACS Calibur; Becton Dickinson) equipped with an air-
cooled 15 mW 488 nm argon ion laser. Collected data were analyzed for cell cycle distribution and cell death rate using the Cell Quest software (Becton Dickinson).

*In vitro PKB kinase assay*

Phosphorylation of Mdm2 and mutants were carried out at 30°C for different times in a final volume of 50 µl of kinase assay buffer containing 50 µg/ml of GST-Mdm2 or mutants and PKB as indicated in each experiment. The reactions were started by addition of 100 µM of [γ-32P]ATP and terminated by addition of SDS-sample buffer and immediately boiled for 3 min. Samples were then subjected to 8% SDS-PAGE, followed by autoradiography or 32P determination in excised gel slices, at positions corresponding to Mdm2. To determine the effect of phosphorylation on Mdm2 self-ubiquitination, phosphorylation of Mdm2 was carried out under the similar conditions using nonradioactive ATP instead of [γ-32P]ATP. At each time point, the reaction was terminated by addition of 10 mM EDTA (final) and 20 µl of 50% Glutathione Sepharose 4B. After extensively washed, the beads bound GST-Mdm2 was used for ubiquitination assay.

*In vitro ubiquitination assay*

32P-labeling of GST-ubiquitin and ubiquitination assay were carried out as described previously (35). Assay conditions were 30 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM dithiothreitol, 2 mM ATP, 15 ng of E1 ubiquitin-activating enzyme, 0.5 µg of GST-UbcH5, and 1.5 µg GST-Mdm2 or mutants in a final volume of 50 µl. The reactions were started by the addition of 2 µg 32P-labeled GST-ubiquitin and incubated at 37°C for 1 hr. GST-Mdm2 was then precipitated by Glutathione Sepharose 4B and eluted in SDS sample buffer. The samples were separated by 8% SDS-PAGE, and then analyzed by autoradiography.
Results

Effect of phosphorylation on Mdm2 self-ubiquitination

We identified two major phosphorylation sites of Mdm2 on Ser\textsuperscript{166} and Ser\textsuperscript{188} by PKB \textit{in vitro} (data not shown). Residue Ser\textsuperscript{166} has been previously described as a PKB site (31, 32), whereas Ser\textsuperscript{188} was identified as a novel PKB site here for the first time. We also confirmed the sites of phosphorylation by in vitro kinase assay using peptides corresponding to the two sites as substrates (data not shown). We failed to detect phosphorylation on Ser\textsuperscript{186}, which was previously described by other groups (31, 32). The region surrounding Ser\textsuperscript{166} and Ser\textsuperscript{188} in human Mdm2 conforms to a consensus sequence motif for PKB phosphorylation, i.e. RxRxxS/T (36). It was also found that Ser\textsuperscript{166} and Ser\textsuperscript{188} are the best predicted sites for PKB using the Scansite motif-profile scoring algorithm program generated by Yaffe and Cantley (37, http://scansite.mit.edu/).

Since Mdm2 is capable of self-ubiquitination, and ubiquitin proteasome pathway-dependent degradation is an important mechanism for regulating Mdm2 levels in cells (20, 21), we asked whether PKB-induced phosphorylation of Mdm2 might have an effect on its ubiquitination. We examined the effect of phosphorylation on reconstituted Mdm2 self-ubiquitination \textit{in vitro}. As shown in Fig. 1A, Mdm2 self-ubiquitination was observed only in presence of both ubiquitin-activating enzyme E1 and the ubiquitin-conjugating enzyme E2, UbcH5. The degree of Mdm2 self-ubiquitination was reduced over time by PKB mediated phosphorylation. To determine which phosphorylation site was responsible for this reduction of Mdm2 self-ubiquitination, two mutants i.e. GST-Mdm2\textsuperscript{S166A} and GST-Mdm2\textsuperscript{S188V} (in which the serine residue was individually changed to alanine or valine) were first phosphorylated by recombinant \(^\Delta\)PH-PKB\textsubscript{\alpha}, and then subjected to the ubiquitination assay. As shown in Fig. 1B, the non-phosphorylated forms of Mdm2 and the mutants...
were all ubiquitinated in the presence of both E1 and UbcH5. Phosphorylation of wild-type GST-Mdm2 completely abolished Mdm2 self-ubiquitination. However, phosphorylation of either GST-Mdm2S166A or GST-Mdm2S188V only partially inhibited Mdm2 self-ubiquitination. The Mdm2S188A and Mdm2S166A/S188V double mutant were highly degraded in E. coli. and could not be tested. Three Asp mutants (GST-Mdm2 S166D, GST-Mdm2S188D, and GST-Mdm2S166D/S188D) were also generated and tested in this in vitro ubiquitination assay. As shown in Fig. 1C, all three Asp mutants were poorly ubiquitinated, further supporting the idea that the protective effect of PKB on Mdm2 self-ubiquitination may be due primarily to the phosphorylation of Mdm2 at Ser166 and Ser188.

**Production and characterization of Mdm2 phospho-specific antibodies**

To monitor the phosphorylation of Mdm2 in vivo, two rabbit polyclonal antibodies that recognized phosphorylated Mdm2 at Ser166 and Ser188 were prepared. These antibodies are referred to as pSer166 and pSer188, respectively. The specificity of the purified antibodies was evaluated by immunoblotting. As shown in Fig. 2A, GST-Mdm2 phosphorylated by either pervanadate-activated HA-PKBα (WT, van) or recombinant ∆PH-PKBα was recognized by each antibody. Non-phosphorylated GST-Mdm2 or GST-Mdm2 phosphorylated by either untreated HA-PKBα (WT) or kinase-defective HA-PKBα (KD) was not detected. The pSer166 antibody detected both GST-Mdm2 and GST-Mdm2S188V phosphorylated by ∆PH-PKBα, but did not detect GST-Mdm2S166A (Fig. 2A). Similarly, pSer188 could also detect GST-Mdm2 and GST-Mdm2S166A but not GST-Mdm2S188V when phosphorylated by ∆PH-PKBα. These results suggest that each phospho-antibody specifically detected phosphorylation of Mdm2 by PKB at Ser166 and Ser188, respectively.

*IGF-1 induces in vivo phosphorylation of Mdm2 at Ser166 and Ser188*
To determine whether our phospho-antibodies could detect Mdm2 phosphorylation \textit{in vivo}, the effect of insulin-like growth factor-1 (IGF-1) on HEK293 cells was examined. Serum-starved cells were incubated with 100 ng/ml IGF-1 for different times, and cell lysates were analyzed by immunoblotting using pSer$^{166}$ and pSer$^{188}$. Following IGF-1 stimulation a dramatic increase in phosphorylation on Mdm2 residues Ser$^{166}$ and Ser$^{188}$ was observed, and a relatively high level of phosphorylation was maintained for the 30 min duration of the experiment (Fig. 2B). The IGF-1-induced phosphorylation of Mdm2 was suppressed by pretreatment with 50\(\mu\)M LY-294002, an inhibitor of PI3Kinase (Fig. 2B). These results suggest that the activation of PI3K/PKB pathway is required for Mdm2 phosphorylation at Ser$^{166}$ and Ser$^{188}$ \textit{in vivo}.

\textbf{PKB promotes Mdm2 phosphorylation \textit{in vivo} and prevents its degradation}

To test whether Mdm2 is phosphorylated in transfected cells, a pEGFP-Mdm2 was co-transfected with pCMV5-HA-m/p-PKB\(\alpha\) (m/p-PKB\(\alpha\), a constitutively active membrane targeted myristoylated/palmitoylated PKB\(\alpha\)), pCMV5-HA-KD-PKB\(\alpha\) (KD-PKB\(\alpha\), a kinase deficient PKB\(\alpha\)), or pCMV5 into COS-1 cells as described in “Experimental Procedures”. The expression of m/p-PKB\(\alpha\) but not KD-PKB\(\alpha\) caused an increase in the phosphorylation level of GFP-Mdm2 at both Ser$^{166}$ and Ser$^{188}$ (Fig. 3A), indicating that PKB kinase activity is required for the phosphorylation of Mdm2 at Ser$^{166}$ and Ser$^{188}$ \textit{in vivo}. Significantly, the level of GFP-Mdm2 expression was lower in the mock or KD-PKB\(\alpha\) transfected cells but remained high in the m/p-PKB\(\alpha\) transfected cells (Fig. 3A, left panel). To explore whether the lower levels of Mdm2 were due to degradation of protein, transfected cells were exposed to the proteasome inhibitor MG132. As shown in the right panel of Fig. 3A, the reduction of Mdm2 protein levels in both mock and KD-PKB\(\alpha\) transfected cells was reversed upon MG132 treatment, and reached a similar level to
that of the m/p-PKBα transfected cells. The efficiency of transfection was monitored by co-expression of GFP as indicated. Lysates were probed with anti-phospho-Ser\textsuperscript{473} antibody (pSer\textsuperscript{473}) to confirm the activation status of PKB. These data suggested that transfected GFP-Mdm2 is a labile protein that is a target for degradation (20, 21), and furthermore that PKB phosphorylation is required for protecting Mdm2 from proteasome-dependent destruction, thus increasing Mdm2 stability.

To further confirm the hypothesis that PKB activity is sufficient to stabilize Mdm2, GFP-Mdm2 was co-transfected with m/p-PKB\textalpha, KD-PKB\textalpha, or pCMV5 empty vector in COS-1 cells. The transfected cells were treated with a protein synthesis inhibitor cycloheximide (CHX) for the indicated period of time before harvesting. Levels of GFP-Mdm2 were markedly decreased in mock or KD-PKB\textalpha cotransfected cells, but remained relatively high in m/p-PKB\textalpha co-transfected cells over time (Fig. 3B). This was not a result of variations in transfection efficiency as shown by the levels of GFP control. These data suggest that maintenance of a higher level of GFP-Mdm2 in the m/p-PKB\textalpha cotransfected cells may be primarily due to prevention of Mdm2 protein degradation by PKB, rather than activation of transcriptional machinery.

To investigate whether phosphorylation of Mdm2 by PKB was sufficient to increase its stability, three Ser to Asp phosphorylation site mutants of Mdm2 were constructed and transfected into COS-1 cells. As shown in Fig. 3C, wild-type GFP-Mdm2 (WT) was rapidly degraded in the absence of MG132, but markedly accumulated in the presence of this inhibitor. Substitution of Ser\textsuperscript{166} to Asp (GFP-Mdm2\textsuperscript{S166D}) and Ser\textsuperscript{188} to Asp (GFP-Mdm2\textsuperscript{S188D}) resulted in increased Mdm2 stability even in the absence of MG132. A double mutant in which both Ser\textsuperscript{166} and Ser\textsuperscript{188} were changed to Asp (GFP-Mdm2\textsuperscript{S166D/S188D}, DD) was tested, and was as stable as each single mutant. These results
indicate that phosphorylation of Mdm2 at Ser\textsuperscript{166} and Ser\textsuperscript{188} by PKB regulates Mdm2 protein stability, probably via inhibition of proteasome-mediated degradation.

To examine if Mdm2 ubiquitination is regulated by PKB in vivo, a HA-ubiquitin construct was co-transfected with wild-type GFP-Mdm2 and its mutant in HEK293 cells. As shown in Fig. 3D, ubiquitination of Mdm2 accumulated in wild-type GFP-Mdm2 and HA-ubiquitin co-transfected HEK293 cells, but was significantly reduced in GFP-Mdm2\textsuperscript{S166D/S188D} and HA-ubiquitin transfected cells. These data further support that PKB-mediated Mdm2 phosphorylation prevents Mdm2 from self-ubiquitination.

**Inhibition of the PI3Kinase pathway induces proteasome-dependent degradation of Mdm2**

The above results suggest that PKB-mediated phosphorylation might be involved in stabilizing Mdm2. This prompted us to investigate whether the PI3K/PKB pathway could regulate the stability of endogenous Mdm2. The protein levels of endogenous Mdm2 in HEK293 (Fig. 4A) and COS-1 (Fig. 4B) cells were measured in the presence of LY-294002. The level of Mdm2 protein declined rapidly in LY-294002 treated cells, and this effect was significantly delayed by treating cells with the proteasome inhibitor MG132. These data suggest that the PI3K/PKB signaling pathway plays a critical role in regulating the stability of endogenous Mdm2 protein, and implies that this process is of physiological significance in cells.

**Cells lacking PKB\(\alpha\) display decreased Mdm2 protein levels and increased susceptibility to UV-induced apoptosis**

To confirm the role of PKB in stabilizing Mdm2 in vivo, we examined the level of Mdm2 protein in \(Pkb\alpha^-\) (\(Pkb\alpha\) knockout) and \(Pkb\alpha^{+/+}\) (\(Pkb\alpha\) wild type) MEFs by immunostaining and
immunoblotting. As shown in Fig. 5A, Mdm2 was mainly localized in the nucleus in both $Pkb\alpha^{-/-}$ and $Pkb\alpha^{+/+}$ cells. The intensity of Mdm2 immunoreactivity in nucleus was significantly reduced in $Pkb\alpha^{-/-}$ cells compared to that of $Pkb\alpha^{+/+}$ cells. Immunoblotting analysis revealed that the levels of Mdm2 protein in $Pkb\alpha^{-/-}$ cells was also much lower than that in $Pkb\alpha^{+/+}$ cells (Fig. 5B).

Phosphorylation of Mdm2 at Ser$^{166}$ was detected in $Pkb\alpha^{+/+}$ cells, but was dramatically reduced in $Pkb\alpha^{-/-}$ cells (Fig. 5B). Phosphorylation of Mdm2 at Ser$^{188}$ was not analyzed because it is likely that the pSer$^{188}$ antibody is unable to detect phosphorylation of Ser$^{188}$ due to the divergence in the sequences of human and mouse in this region. Since PKB$\alpha$ is the main PKB isoform in MEFs$^2$ (38), and the lack of PKB$\alpha$ in MEFs caused a decrease in Mdm2 protein levels, this suggest that PKB activity was required to stabilize Mdm2 in MEFs. As Mdm2 is a key determinant of p53 stability, we next examined the p53 protein level in these cells. As shown in Fig. 5B, levels of p53 were significantly elevated in $Pkb\alpha^{-/-}$ MEFs. Concomitant with the increase in p53, the expression level of p21$^{\text{Cip1}}$ in $Pkb\alpha^{-/-}$ MEFs was also increased as compared to control wild-type cells (Fig. 5B).

Since p53 and its transcriptional target p21$^{\text{Cip1}}$ play an important role in controlling the G1 and G2/M cell cycle checkpoints that mediate growth arrest and apoptosis (39-41), we next analyzed the cell cycle of MEFs in response to UV-irradiation. As shown in Fig. 5C, the exponentially growing $Pkb\alpha^{-/-}$ MEFs displayed a small but significant increase in the G2/M cell population as compared to their wild-type counterparts. These results correspond well with recent findings by Kandel $et$ $al.$ (42), who reported on attenuated transition from G2/M to G1 in $Pkb\alpha^{-/-}$ MEFs. Upon treatment with UV-C radiation, a large proportion (up to 60%) of the wild-type MEFs were arrested in G1 phase of cell cycle for the whole period of examination. In contrast, the $Pkb\alpha^{-/-}$ cells displayed only a compromised (50%) and temporary G1 arrest accompanied by a partial G2/M
block. Importantly, the majority of Pkbα−/− MEFs underwent apoptotic cell death in the course of experiment, which was manifested by a continuous increase of cell population with sub-G1 DNA content (Fig. 5C). Since most of the wild-type cells resisted the treatment with UV radiation, these results imply that PKBα protects cells against the UV-induced apoptotic cell death. This is consistent with observations that the knockout of PKBα/Akt1) sensitizes cells to apoptosis after a variety of stimuli (38). Although the molecular basis for these phenomena is not yet entirely elucidated, it is conceivable that the increased p53 level in the Pkbα−/− knockout MEFs can, at least in part, account for the G2/M delay, compromised G1 arrest, and the elevated apoptosis rate after UV treatment in these cells.

*Activation of PKB correlates with Mdm2 phosphorylation in human tumor cell lines*

Elevated PKB activity via amplification of PI3K and PKB genes, mutational loss of PTEN or activation of oncogenic receptor tyrosine kinase is a common feature of many human cancers (1, 4, 43). This prompted us to investigate the phosphorylation status of Mdm2 in various human tumor cell lines. Levels of PKB and PKB phosphorylation varied greatly between different cell lines3 (Fig. 6A). Fig. 6A shows that the PTEN-deficient cell lines LNCaP and U87MG exhibited high levels of PKB phosphorylation on Ser473, with a concomitant increase in Mdm2 protein level and its phosphorylation on Ser166 and Ser188. In contrast, in cell lines with lower PKB activity, (e. g. LN229, LN215, and BS149) the phosphorylation levels of Mdm2 were reduced. These data suggest that Mdm2 is a target for PKB in tumor cell lines with elevated PKB activity, and that constitutive activation of the PKB-Mdm2 signaling pathway is an important characteristic of certain tumor cell lines. Immunostaining of LNCaP and LN215 cells showed that phosphorylation of Mdm2 residues Ser166 and Ser188 was elevated in LNCaP cells in which PKB activity is high, compared to Mdm2
levels in LN215 cells in which PKB activity is relatively low (Fig. 6B and 6C). In addition, the
subcellular localization of endogenous Mdm2 was observed mainly in the nucleus in both cell lines
(Fig. 6B and 6C), arguing that the nuclear localization of Mdm2 might be independent on the
Mdm2 phosphorylation status. These data suggest that activation of PKB leads to the
phosphorylation of Mdm2 and plays an essential role in regulating Mdm2 stability in human tumor
cells.
Discussion

Growth factor-mediated activation of PI3K/PKB is a key element of cellular survival and proliferation. Activation of PI3K/PKB signaling is thought to be required for the inhibition of p53-dependent apoptosis (7-11), but the exact mechanism for this effect has remained elusive. The stoichiometric balance between the tumor suppressor p53 and its negative regulator Mdm2 may determine the extent of cellular p53 activity and its functions. Levels and activity of both Mdm2 and p53 are regulated by posttranslational modifications and protein-protein interaction in cells. The stability of p53 can be regulated by phosphorylation at multiple sites (13, 14). Similarly, Mdm2 is also a phosphoprotein, and phosphorylation of Mdm2 affects Mdm2-p53 complex formation (12-14, 17, 18, 20-23). Several reports have demonstrated that Mdm2 can be phosphorylated by DNA-dependent protein kinase at Ser^{17} (44), by cyclin A-dependent kinase at Thr^{216} (45), by casein kinase II at Ser^{269} (human)/Ser^{267} (murine) (46, 47), and by ataxia telangiectasia mutated protein kinase (ATM) at Ser^{395} (48). Phosphorylation at these sites results in the inhibition of Mdm2-p53 complex formation, thereby increasing p53 stability. Mdm2 could be phosphorylated at Tyr^{394} by a non-receptor tyrosine kinase c-Abl (49). Phosphorylation of Mdm2 by c-Abl impairs its ability to inhibit the transcriptional and apoptotic activities of p53, as well as its efficiency to promote p53 degradation (49). C-terminal Ser^{407} phosphorylation of Mdm2 by ATM- and RAD-3-related kinase (ATR) reduces Mdm2-dependent export of p53 from nuclei to cytoplasm (50). Mdm2 is also hypophosphorylated on several residues in its conserved region II (Ser^{240}, Ser^{240}, Ser^{260}, and Ser^{262}) in response to ionizing irradiation, which contributes to p53 stabilization (51). More recent data showed that Mdm2 destabilization requires the activity of DNA-damage-dependent PI 3-kinase family members, most likely through Mdm2 phosphorylation on multiple sites (52). It has also been demonstrated that the stability of Mdm2 can be modulated
via association of Mdm2 with p53 or p19/p14\textsuperscript{ARF} or MdmX (27, 28). However, the stability of Mdm2 is not solely controlled by association with p53 or by p19/p14\textsuperscript{ARF} expression, for example the acetylase activity of p300 is dispensable for Mdm2 stabilization (53). Other unknown growth control molecules that are important in the regulation of Mdm2 stability may exist (54-57).

In this paper, two PKB phosphorylation sites on Mdm2 were identified (Ser\textsuperscript{166} and Ser\textsuperscript{188}), and Ser\textsuperscript{188} is novel. The sequences around Ser\textsuperscript{188} in Mdm2 are among the most conserved regions in homologues of this protein in mouse (Ser\textsuperscript{185}), dog (Ser\textsuperscript{188}), zebrafish (Ser\textsuperscript{169}), and Xenopus (Ser\textsuperscript{182}). The flanking sequences of Ser\textsuperscript{166} are also conserved in mouse (Ser\textsuperscript{163}), dog (Ser\textsuperscript{166}), and zebrafish (Ser\textsuperscript{146}), but not in Xenopus (13, 14). The presence of conserved sequences argues in favor of a functional role for this region, presumably via phosphorylation, and this has been demonstrated in human and mouse species. Using the Scansite motif program (37), two Mdm2 sites that fit the theoretically optimal PKB phosphorylation consensus motif were identified as Ser\textsuperscript{166} and Ser\textsuperscript{188}.

Other groups have identified a third putative phosphorylation site (Ser\textsuperscript{186}) by site-directed mutagenesis (31, 32, 58), but this site possesses a relatively poor PKB-phosphorylation consensus motif (59). Consistent with the results of Ashcroft et al. (60), we also could not find any phosphorylation of Mdm2 on Ser\textsuperscript{186} by m/z –79 precursor ion scanning and by phosphate release experiments when GST-Mdm2 was maximally phosphorylated either by transfected HA-PKB\textalpha or by recombinant ΔPH-PKB\textalpha (data not shown). The physiological relevance of phosphorylation on this amino acid in vivo will require further investigation.

Previous reports have indicated that survival signals positively regulate Mdm2 and lead to stabilization of the protein (54-57). However, the mechanisms controlling Mdm2 protein stability remain to be elucidated. Our results demonstrate that phosphorylation of Mdm2 by PKB prevents its self-ubiquitination in vitro and thereby stabilizes the protein in vivo. Similar data from Ashcroft
et al. (60) indicated that phosphorylation of Mdm2 by PKB increased the ability of Mdm2 to target p53 for ubiquitin-dependent degradation. Indeed, knockout of PKBα resulted in decreased Mdm2, and accumulated p53 and p21Cip1 in MEFs in vivo (Fig. 5). The results are supportive of a physiological role for PKB in counter-balancing the effect of p53 on cell proliferation and apoptosis. This positive regulatory effect is regulated via stabilization of Mdm2 by phosphorylation and reduced Mdm2 self-ubiquitination. Data from human tumor cells where elevated levels of PKB activation correlate with elevated levels of Mdm2 phosphorylation further support this hypothesis.

A tentative model for regulation of Mdm2-p53 pathway by PKB is shown in Fig. 7. Our model proposes that PKB-mediated phosphorylation of Mdm2 may be a central event in negatively regulating p53 function under conditions where the PI3K/PKB signaling is activated. However, how phosphorylation by PKB affects the Mdm2 self-ubiquitination still remains to be elucidated. Since the ubiquitin-conjugating site on Mdm2 is located at the C-terminal RING finger domain (61), it is difficult to envision a reduction of Mdm2 self-ubiquitination via direct steric hindrance resulting from phosphorylation in the N-terminal half of the molecule. Nevertheless, it is possible that phosphorylation could induce a conformation change in Mdm2 that may efficiently block the ubiquitin conjugating site(s) at the C-terminal RING finger domain, which would, in turn, protect Mdm2 from degradation.

The PKB phosphorylation sites on Mdm2 identified in our study lie in the vicinity of the nuclear localization sequence (NLS, residues 181-185) and nuclear export sequence (NES, residues 191-202) domain, which regulates Mdm2 nuclear import and export, raising the possibility of a functional connection between Mdm2 phosphorylation and nuclear translocation. Indeed, phosphorylation of Mdm2 by PKB was reported to result in Mdm2 nuclear translocation (31, 32) leading to a reduction in p53 transactivation (31) and enhancement of Mdm2-mediated
ubiquitination of p53 (32). It is possible that the subcellular relocalization of Mdm2 might also impair Mdm2 self-ubiquitination and increase the stability of Mdm2 protein, but this model has yet to be tested experimentally. However, it should be pointed out that both endogenous Mdm2 (Fig. 5A and Fig. 6B, C) and transfected Mdm2 (31, 51, 60) were exclusively located in the nucleus independent of phosphorylation status of Mdm2. We and other groups (58) did not observe significant changes in the subcellular localization of Mdm2 when PKB was activated by a variety of stimuli (data not shown). The reason for this disparity is not clear. Our data suggests that activation of PKB promotes translocation of PKB to the nucleus (5, 6) and phosphorylates Mdm2 in the nuclear compartment.

In summary, these results demonstrate that Mdm2 can be phosphorylated by activated PKB at Ser\textsuperscript{166} and Ser\textsuperscript{188}. Phosphorylation by PKB inhibits Mdm2 self-ubiquitination and this effect is mediated by phosphorylation on amino acids Ser\textsuperscript{166} and Ser\textsuperscript{188}. We also demonstrate that phosphorylation of Mdm2 at Ser\textsuperscript{166} and Ser\textsuperscript{188} resulting in the stabilization of Mdm2 protein by protecting Mdm2 from proteasome-mediated degradation. The above data support a physiological role for PKB in the regulation of Mdm2-p53 signaling, and reveal a novel mechanism of PKB-induced cellular transformation that may contribute to tumorigenesis.
References


Footnotes

∗The Friedrich Miescher Institute is part of the Novartis Research Foundation. R. T. and D. P. B were supported by grants from the Swiss Cancer League (KFS01342-02-2003 and OCS1167-09-2001).

†Present address: Conway Institute of Biomolecular and Biomedical Research, Department of Medicine and Therapeutics, University College Dublin and Dublin Molecular Medicine Centre, Dublin 4, Ireland.

The abbreviations used are: PI3K, phosphatidylinositide-3’-OH kinase; PKB, protein kinase B; m/p-PKB, myristoylated/palmitoylated PKB; Mdm2, murine double minute 2; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; IGF-1, insulin-like growth factor-1; GST, gluathione S-transferase; MEF, mouse embryonic fibroblast; UV, ultraviolet.


D. P. Brazil and B. A. Hemmings, unpublished data.

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Figure Legends

Fig. 1. Effect of Mdm2 phosphorylation by PKB on Mdm2 self-ubiquitination.

(A) GST-Mdm2 (50 µg/ml) was phosphorylated by recombinant ΔPH-PKBα for the indicated times. Both phosphorylated and unphosphorylated GST-Mdm2 was subjected to a self-ubiquitination assay using 32P-GST-ubiquitin as described in “Experimental Procedures”. The gel was then stained with Coomassie blue followed by autoradiography.

(B and C) GST-Mdm2 wild-type (WT) and various mutants (GST-Mdm2S166A, S166A; GST-Mdm2S188V, S188V; GST-Mdm2S166D, S166D; GST-Mdm2S188D, S188D; GST-Mdm2S166D/188D, D D) were phosphorylated in the presence (+) or absence (-) of ΔPH-PKBα for 2 hr and subjected to the Mdm2 self-ubiquitination assay as described above.

Fig. 2. IGF-1 induces phosphorylation of Mdm2 in HEK 293 cells.

(A) Characterization of phosphorylation site-specific antibodies for Mdm2. Wild-type HA-PKBα (WT), kinase deficient HA-PKBα (KD) or pCMV5 empty vector (mock) were transfected into HEK293 cells and immunoprecipitated with anti-HA antibody. Cells treated with 0.1 mM pervanadate (van) for 15 min are also indicated. The immunoprecipitates or recombinant ΔPH-PKBα was used to phosphorylate GST-Mdm2 and various mutants as described in “Experimental Procedures”. Samples were analyzed by immunoblotting with pSer166 or pSer188. The blots were stained with Coomassie Blue (CBB) as indicated.

(B) Serum-starved HEK293 cells were untreated (-) or pretreated with 50 µM LY-294002 (+) for 30 min and then stimulated with IGF-1 for the indicated times before lysis. Whole cell lysates were analyzed by immunoblotting using pSer166 or pSer188, or anti-Mdm2 antibody.
Fig. 3. Effect of phosphorylation on Mdm2 stability.

(A) pEGFP-Mdm2 was cotransfected with either pCMV5-HA-m/p-PKB (m/p), pCMV5-HA-KD-PKB (KD), or pCMV5 empty vector (mock), together with a GFP encoding plasmid (to control for expression) in COS-1 cells for 24 hr. After serum-starvation for 24 hr, cells were treated with (+) or without (-) 40 \( \mu \)M of MG132 for 4 hr before harvesting.

(B) COS-1 cells were cotransfected with pEGFP-Mdm2 and various PKB constructs, together with a GFP encoding plasmid for 24 hr as in (A). Cells were treated with 50 \( \mu \)g/ml of cycloheximide for different times before harvesting.

(C) COS-1 cells were transfected with wild-type pEGFP-Mdm2 (WT), or with its mutants (S166D, pEGFP-Mdm2\(^{S166D}\); S188D, pEGFP-Mdm2\(^{S188D}\); DD, pEGFP-Mdm2\(^{S166D/S188D}\)) together with a GFP encoding plasmid. After serum-starvation for 24 hr, cells were treated with (+) or without (-) 40 \( \mu \)M of MG132 for 4 hr before harvesting. Total cell lysates were analyzed by immunoblotting with anti-GFP, pSer\(^{166}\), pSer\(^{188}\), anti-HA, or pSer473 antibodies as indicated.

(D) HEK293 cells were transfected either with wild-type pEGFP-Mdm2 (WT) or with pEGFP-Mdm2\(^{S166D/S188D}\) (DD) together with a pCMV5-HA-ubiquitin plasmid. After 24 hr transfection, cells were treated with 40 \( \mu \)M of MG132 for 4 hr before harvesting. 500 \( \mu \)g cell lysates were immunoprecipitated with anti-GFP, and followed by immunoblotting with anti-HA and anti-GFP as indicated.

Fig. 4. Inhibition of PI3K induces proteasome-dependent degradation of endogenous Mdm2.

HEK293 (A) and COS-1 (B) cells were treated with 50 \( \mu \)M LY-294002 for the indicated time in the presence (+) or absence (-) of 40 \( \mu \)M of MG132 before harvesting. Cell lysates were analyzed by
immunoblotting with monoclonal antibody to Mdm2, and anti-β-tubulin antibody as indicated.

Fig. 5. Cells lacking PKBα have reduced levels of Mdm2 and increased susceptibility to UV-induced apoptosis.

(A) Immunofluorescence photomicrographs showing Mdm2 immunoreactivity in Pkbα−/− and Pkbα+/+ MEFs.

(B) For detection of Mdm2, PKB, pSer473 and actin in Pkbα−/− and Pkbα+/+ MEFs, 50 µg of total cell lysate were analyzed by immunoblotting with a monoclonal antibody to Mdm2, pSer166, pSer188, pSer473, polyclonal anti-PKB (Ab10), anti-actin antibodies as indicated. For detection of p53, p21Cip1, 500 µg of total cell lysates were first immunoprecipitated followed by immunoblotting with monoclonal anti-p53 and anti-p21Cip1 antibodies as indicated.

(C) Induction of apoptosis in MEFs by UV-irradiation. Cells were labelled with BrdU for 30 min before UV-irradiation. At each time point, the UV treated cells were fixed in 70% ethanol and then stained with anti-BrdU-FITC and 20 µg/ml propidium iodide as described in “Experimental Procedures”.

Fig. 6. Activation of PKB and phosphorylation of Mdm2 in tumor cell lines.

All cells were cultured in normal FCS containing medium. Each 40 µg of total cell lysates were analyzed by immunoblotting with with pSer166, pSer188, pSer473, anti-Mdm2, anti-PKB, and anti-Actin antibodies as indicated (A). Cells were double immunostained with a monoclonal anti-Mdm2 and a polyclonal phospho-antibody against pSer166 antibody (B) and pSer188 antibody (C) as indicated.
Fig. 7. Model for regulation of Mdm2-p53 via the PI3K/PKB pathway.

Mdm2 stability is regulated by ubiquitination and proteasome-dependent degradation. When the PI3K/PKB pathway is activated by growth factors, Mdm2 is phosphorylated at two sites, i.e. Ser$^{166}$ and Ser$^{188}$. Phosphorylation of Mdm2 by PKB protects Mdm2 from self-ubiquitination, thereby stabilizing the protein. This is reflected in an increased ability of Mdm2 to promote the ubiquitination and degradation of p53.
Figure 1, Feng et al.

A

\[ \Delta PH-PKB_\alpha \quad - \quad - \quad 0 \quad 5 \quad 30 \quad 120 \text{ min} \]

GST-Mdm2 \quad - \quad + \quad + \quad + \quad + \quad + \quad +

UbcH5 \quad + \quad - \quad + \quad + \quad + \quad + \quad +

E1 \quad + \quad + \quad - \quad + \quad + \quad + \quad +

B

GST-Mdm2 \quad WT \quad S166A \quad S188V

\[ \Delta PH-PKB_\alpha \quad - \quad - \quad + \quad - \quad + \quad - \quad + \]

E1 \quad - \quad + \quad + \quad + \quad + \quad + \quad +

UbcH5 \quad + \quad + \quad + \quad + \quad + \quad + \quad +

C

GST-Mdm2 \quad S166D \quad S188D \quad DD

\[ \Delta PH-PKB_\alpha \quad - \quad + \quad - \quad - \quad + \quad - \quad + \]

E1 \quad + \quad + \quad + \quad + \quad + \quad + \quad +

UbcH5 \quad + \quad + \quad + \quad + \quad + \quad + \quad +
Figure 2, Feng et al.

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Figure 3, Feng et al.

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Figure 3, Feng et al.

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Figure 4, Feng et al.

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Figure 5, Feng et al.

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Pkbα^+/+

Pkbα^−/−

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Pkbα^+/+, control

Pkbα^+/+, UV

Pkbα^−/−, control

Pkbα^−/−, UV

% cell-cycle phase

Time [hours]

G2/M

S

G1

subG1

0 6 12 18 24 30 36

0 6 12 18 24 30 36

0 6 12 18 24 30 36

0 6 12 18 24 30 36

100

80

60

40

20

0

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40

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Figure 6, Feng et al.

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Mdm2 | pSer^{188}

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Figure 7, Feng et al.
Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation
Jianhua Feng, Rastislav Tamaskovic, Zhongzhou Yang, Derek P. Brazil, Adrian Merlo, Daniel Hess and Brian A. Hemmings

*J. Biol. Chem.* published online May 28, 2004

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