Akt enhances Mdm2-mediated ubiquitination and degradation of p53

Yoko Ogawara¹, Shohei Kishishita¹, Toshiyuki Obata², Yuko Isazawa¹, Toshiaki Suzuki³, Keiji Tanaka³, Norihisa Masuyama¹ and Yukiko Gotoh¹,⁴,⁵

¹Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
²Institute for Enzyme Research, University of Tokushima, 3-18-15 Kuramoto, Tokushima 770-8503, Japan
³Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan
⁴PRESTO Research Project, Japan Science and Technology Corporation, Osaka, Japan
⁵To whom correspondence should be addressed:
Yukiko Gotoh, Ph.D.
Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan.
TEL +81-3-5841-8473
FAX +81-3-5841-8472
E-mail ygotoh@iam.u-tokyo.ac.jp

Running title: Akt promotes Mdm2 function
Summary

p53 plays a key role in DNA damage-induced apoptosis. Recent studies have reported that the PI3K-Akt pathway inhibits p53-mediated transcription and apoptosis, although the underlying mechanisms have yet to be determined. Mdm2, a ubiquitin ligase for p53, plays a central role in regulation of the stability of p53, and serves as a good substrate for Akt. In this study, we find that expression of Akt reduces the protein levels of p53, at least in part by enhancing the degradation of p53. Both Akt expression and serum treatment induced phosphorylation of Mdm2 at Ser186. Akt-mediated phosphorylation of Mdm2 at Ser186 had little effect on the subcellular localization of Mdm2. However, both Akt expression and serum treatment increased Mdm2 ubiquitination of p53. The serum-induced increase in p53 ubiquitination was blocked by LY294002, a PI3K inhibitor. Moreover, when Ser186 was replaced by Ala, Mdm2 became resistant to Akt enhancement of p53 ubiquitination and degradation. Collectively, these results suggest that Akt enhances the ubiquitination-promoting function of Mdm2 by phosphorylation of Ser186, which results in reduction of p53 protein. This study may shed light on the mechanisms by which Akt promotes survival, proliferation and tumorigenesis.
Introduction

Growth factors, cytokines and certain oncogenes have been shown to be effective inhibitors of apoptosis, and in many cases, their anti-apoptotic effects are mediated by the phosphatidylinositol-3-OH kinase (PI3K)-induced activation of Akt (1, 2). For instance, Ras activation of the PI3K-Akt pathway confers protection from apoptosis in fibroblasts in response to DNA damage or oncogenic Myc (3, 4). In this respect, the PI3K-Akt pathway-mediated survival contributes to the ability of Ras to function as an oncogene (1, 2). Although several Akt targets have been reported, it is not fully understood how Akt promotes survival (5-13).

The tumor suppressor p53 plays a key role in the induction of apoptosis and cell cycle arrest in response to a variety of genotoxic stresses and to the activation of some oncogenes such as Myc, thereby preventing the propagation of damaged cells (14, 15). p53 function is controlled by several mechanisms, including the regulation of p53 protein stability. Central to this process is Mdm2, a ubiquitin ligase that targets p53 for ubiquitination and allows export of p53 from the nucleus to the cytoplasm, where p53 degradation by proteasomes takes place (16-21). Under normal circumstances, p53 is maintained at very low levels by continuous ubiquitination and degradation. Activation of p53 in response to cellular stresses is mediated partly by inhibition of Mdm2 and rapid stabilization of p53 protein (22).

The deregulated activation of mitogenic signals, due to oncogenic activation of Ras or Myc for example, leads to the activation of p53, which provides a mechanism to
prevent the abnormal proliferation associated with tumor development (23, 24). However, this activation of p53 by mitogenic signals must be suppressed during normal cell proliferation, to prevent p53 from inducing cell cycle arrest or apoptosis. Therefore it appears reasonable to assume that mitogenic signals elicit both p53-activating and -inactivating signals.

Recent studies have indeed shown that Ras can inhibit or activate p53, depending on the cellular contexts and the duration of Ras activation (24, 25). The Raf-MEK-MAPK pathway has been shown to mediate Ras activation of p53 (26), most likely through induction of p19\(^{ARF}\), which in turn inactivates Mdm2. The PI3K-Akt pathway has recently been reported to inhibit the transcriptional activity of p53 and reduce the pro-apoptotic functions of p53 (27, 28) (Y.O. and Y.G., unpublished data). Therefore, it is possible that the PI3K-Akt pathway opposes the MAPK pathway in activation of p53. However, it has yet to be determined how Akt suppresses p53.

Here we show that Akt does not affect the mRNA levels of p53, but promotes ubiquitination and degradation of p53 protein. We confirmed very recent studies showing that Mdm2 serves as a good substrate for Akt (29, 30). Although they have shown that Akt promotes nuclear translocation of Mdm2, we could not detect any effect of Akt on Mdm2 subcellular localization. Instead, we found that Akt facilitates the functions of Mdm2 to promote p53 ubiquitination by phosphorylation of Ser186. These findings may explain how mitogenic signal and Ras inhibit p53 during normal cell proliferation, and may also provide a mechanism by which Akt promotes survival.
Experimental Procedures

Plasmids and antibodies

Human p53 cDNA, human Mdm2 cDNA and p53-responsive luciferase reporter plasmid (PG13-Luc) are kind gifts from Dr. B. Vogelstein. Flag-tagged and HA-tagged p53 cDNA were cloned into KpnI-BamHI sites of pcDNA3.1(+) (Invitrogen) (Flag-p53 and HA-p53). Mdm2 mutant S186A was generated by QuickChangeKit (STRATAGENE) by utilizing primers 5'-CGCCACAAAGCTGATAGTATTTCCC-3' and 5'-GGGAAATACTATCAGCTTTGTGGCG-3'. Mdm2 mutant S166A/S186A (2SA) was generated by utilizing primers 5'-GGAGAGCAATTGCTGAGACAGAAG-3' and 5'-CTTCTGTCTCAGCAATTGCTCTCC-3' to mutate Ser166 into Ala of S186A Mdm2. Flag-tagged wild type (WT), S186A and 2SA Mdm2 were cloned into KpnI-XhoI sites of pcDNA3.1(+). For bacterial expression, WT and S186A Mdm2 were cloned into EcoRI and XhoI sites of pGEX-6P-1 (Amersham Pharmacia Biotech). Human WT Akt and a constitutively active Akt (CA Akt) were kindly provided by Dr. D. Alessi and Dr. R. Roth (31), respectively. A kinase negative Akt (KN Akt) was made by mutating Lys179 into Ala as described (12). A dominant negative Akt (3A Akt) was made by mutating Lys179, Thr308 and Ser473 into Ala. CA Akt, WT Akt, KN Akt and 3A Akt were cloned into BamHI site of pcDNA3.1(+). Flag-tagged ubiquitin (Flag-Ub) was cloned into KpnI-BamHI sites of pcDNA3.1(+). Antibodies used in this study include polyclonal anti-HA antibody Y-11 (Santa Cruz Biotechnology, Inc.), monoclonal anti-α-tubulin antibody DM 1A (SIGMA), monoclonal anti-topoisomerase
II α antibody 8D2 (MBL), monoclonal anti-MEK1 antibody 25 (Transduction Laboratories), monoclonal anti-Flag antibody M2 (SIGMA), polyclonal anti-Akt antibody (Cell Signaling), monoclonal anti-p53 antibody DO7 (Oncogene) and monoclonal anti-Mdm2 antibody IF2 (CALBIOCHEM). To generate anti-phospho Ser186 Mdm2, a phosphopeptide corresponding to amino acid sequence of human Mdm2 178-193 (CRQRKRHKpSDSISLSF) was synthesized and coupled to keyhole limpet hemocyanin (KLH) (SAWADY Technology). This antigen was injected into Japanese White rabbits, from which serum was collected approximately every two weeks. Serum was affinity purified by passing over a Thiopropyl Sepharose 6B column (Amersham Pharmacia Biotech) coupled with a synthetic peptide of the sequence CRQRKRHKpSDSISLSF, and the bound antibodies were eluted. The elutes containing phosphopeptide-specific antibodies were then passed through a column coupled with the unphosphorylated peptide (i.e. CRQRKRHKpSDSISLSF) to deplete antibodies that react with unphosphorylated Mdm2.

**Cell lines and transfections**

MCF-7, Saos-2 and 293T cells were grown in DMEM containing penicillin-streptomycin and 10% fetal bovine serum. For MCF-7 and Saos-2 cells, transfection was carried out by using LIPOFECTAMINEPLUS Reagent (Invitrogen) in 6 well plates or 10 cm dishes with 5 x 10^5 cells/dish and 3 x 10^6 cells/dish, respectively. For 6 well plates, cells were transfected with 1-3 µg of total DNA together with 6 µl of PLUS Reagent and 4 µl of LIPOFECTAMINE Reagent per well. For 10 cm dishes, cells
were transfected with 4-6 µg of total DNA together with 20 µl of PLUS Reagent and 30 µl of LIPOFECTAMINE Reagent per dish. For 293T cells, transfection was carried out by using FuGENE6 Transfection Reagent (Roche) in 6 cm dishes (2 x 10⁶ cells, 5 µg of total DNA and 12 µl of FuGENE6 Transfection Reagent per dish). For luciferase assay for p53 transcriptional activity, cells were transfected with PG13-Luc together with various constructs and a β-galactosidase-expression plasmid. The β-galactosidase expression was driven by a CMV promoter, and used for a standard to normalize transfection efficiency. Luciferase and β-galactosidase activities were assessed 24 h after transfection.

**RT-PCR**

Total RNA was isolated from MCF-7 cells using the TRIzol Reagent (Invitrogen) and transcribed using ReverTra Ace (TOYOBO) with oligo(dT) primers, according to the manufacturer’s instruction. Aliquots of cDNA corresponding to 100 ng of total RNA were used for PCR amplification in a 50 µl solution containing 1 x KOD Dash buffer (TOYOBO), 0.2 mM dNTPs, 1.25 U KOD Dash (TOYOBO) and 0.4 µM of each primer performed with a Perkin-Elmer DNA thermalcycler. The primers used to amplify p53 were 5’-TCTGGGACAGCCAAGTCTGT-3’ (forward) and 5’-GGAGTCTTCCAGTGTGATGA-3’ (reverse). The primers for GAPDH were 5’-CATTGACCTCAACTACATGG-3’ (forward) and 5’-TTGCCCACAGCCTTGGCAGC-3’ (reverse). The PCR parameters consisted of an initial cycle of 95 °C for 30 sec, followed by 28 cycles of 95 °C for 10 sec, 60 °C for 10
sec and 74 °C for 20 sec, and final extension for 1 min at 74 °C. The amplified PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The products of constitutively expressed GAPDH mRNA served as a control. All the products were assayed in the linear range of the RT-PCR amplification process.

**Degradation analysis of p53 protein**

MCF-7 cells were transfected with Flag-p53 (0.1 µg) and the indicated amounts of either CA Akt or KN Akt for 22 h or treated with LY294002 (10 µM) for 5 h, then treated with 80 µg/ml of cycloheximide for 0, 30, 60, 90 or 120 min as indicated. The cell lysates were subjected to Western blot analysis with anti-Flag antibody or anti-p53 antibody, and the relative intensity of each band was estimated by utilizing a densitometry.

**Recombinant Mdm2 production**

For bacterial expression of Mdm2, BL21-Gold (DE3) cells (STRATAGENE) were transformed with Mdm2 pGEX-6P-1, cultured in LB medium containing 50 µg/ml of ampicillin, and induced with isopropyl-β-D-thiogalactopyranoside (1 mM) for 5 h. Cells were harvested, resuspended in a sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM PMSF) and lysed by sonication. Triton X-100 was added to a final concentration of 1%, and the lysates were centrifuged at 20,000 x g for 20 min at 4 °C. The crude lysate was filtered (0.45 µm pore size; Millipore, Bedford, Mass.), and loaded onto a 2 ml Glutathione Sepharose™ 4B
(Amersham Pharmacia Biotech) column equilibrated with 5 volumes of PBS. The column was washed with 10 volumes of Cleavage Buffer (Amersham Pharmacia Biotech). The Glutathione Sepharose was mixed with PreScission Protease (Amersham Pharmacia Biotech) and incubated at 4 °C for 12 h, and Mdm2 was eluted. Mdm2 was dialyzed against a dialysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF and 0.1% (v/v) aprotinin.

**In vitro kinase assays**

Recombinant active Akt and kinase negative Akt were prepared as described previously (32, 33). Recombinant Akt was incubated with substrates (1 µg of recombinant Mdm2 protein) in 15 mM MgCl₂, 1 mM dithiothreitol, 100 µM ATP and 20 mM Tris-HCl (pH 7.5) for 30 min at 37 °C in the presence of [γ-³²P]ATP (2 µCi) (Amersham Pharmacia Biotech). The reaction was stopped by the addition of Laemmli’s sample buffer. The samples were subsequently resolved by SDS-PAGE, and analyzed by autoradiography. The phosphorylation reaction was also carried out without radiolabeled ATP, and the samples were resolved by SDS-PAGE and subjected to Western blot analysis with anti-phospho Ser186 Mdm2 antibody.

**Immunostaining**

Cells grown on coverslips were fixed for 10 min in PBS containing 3.7% formaldehyde. Fixed coverslips were permeabilized in PBS containing 0.1% Triton X-100 for 10 min, washed twice in PBS (5 min) and incubated in a blocking buffer (PBS containing 0.2%
bovine serum albumin) for 30 min. Cells were then incubated in the blocking buffer containing the primary antibody for 1 h and washed 3 times in PBS (5 min) before incubation with the appropriate fluorescein-conjugated secondary antibody plus Hoechst33258 (Molecular Probes, Inc.) for a further 30 min. Cells were washed 3 times in PBS (5 min) and washed in water. Stained cells were mounted on glass slides and examined under a fluorescent microscope (Nikon).

**Subcellular fractionation**

Cells were trypsinized, rinsed with PBS and collected by centrifugation. Cells were then suspended in 300 µl of a hypotonic buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 10 mM NaCl and 0.005% Nonidet P-40) and placed on ice for 15 min. Cells were then homogenized and spun at 500 x g for 5 min before the supernatant (cytoplasmic fraction) was collected. The remaining pellet was washed with 300 µl of the hypotonic buffer, resuspended in 100 µl of RIPA buffer (2 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1.0% Nonidet P-40, 1.0% deoxycholate and 0.025% SDS), sonicated and spun at 15,000 x g for 15 min to remove debris and collect the supernatant (nuclear fraction). We confirmed the separation of the cytoplasmic and nuclear fractions by Western blotting of MEK1 (a cytoplasmic marker (34)) and topoisomerase II α (a nuclear marker (21)), respectively.

**Immunoprecipitation**

Cells were rinsed with PBS and scraped into 400 µl of RIPA buffer. Cells were then
sonicated and spun at 15,000 x g for 15 min to remove cellular debris. The supernatants were used as cell lysates. For immunoprecipitation, the cell lysates were incubated with antibody for 1 h on ice and then with Protein A Sepharose (Amersham Pharmacia Biotech) beads for 1 h at 4 °C. The beads were washed 4 times with RIPA buffer, then eluted in Laemmli’s SDS sample buffer. The elutes were subjected to SDS-PAGE and Western blot analysis.

**Ubiquitination assays**

Cells were transfected with HA-p53 and Flag-Ub together with various constructs. Cells were exposed to β-lactone (5 μM) (CALBIOCHEM) for 2 h before the preparation of cell lysates to inhibit proteasome-mediated degradation of ubiquitinated proteins. Cell lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane. For detection of p53, the blot was probed with anti-p53 antibody. For detection of ubiquitinated p53, the blot was probed with anti-Flag antibody or anti-p53 antibody.
Results

Akt reduces p53 protein by enhanced degradation

Akt has been shown to suppress p53-dependent apoptosis triggered by hypoxia (28), etoposide, γ-irradiation (data not shown) or ectopic expression of p53 ((28) and data not shown). Previous reports have shown that Akt is capable of inhibiting the transcriptional activity of p53 (28, 29). We confirmed that expression of active Akt reduced the transcriptional activity of a p53 reporter plasmid in MCF-7 cells (Fig. 1A). However, the underlying mechanisms of Akt inhibition of p53 remain unclear.

To dissect the mechanisms by which Akt inhibits the transcriptional activity of p53, we first investigated whether Akt expression has any effect on the protein and mRNA levels of p53. To examine this, we transfected MCF-7 cells with Akt constructs (the transfection efficiency was about 70%). The amounts of endogenous p53 protein were markedly reduced by expression of active Akt (Fig. 1B). In contrast, the mRNA levels of p53 detected by RT-PCR were unchanged by expression of active Akt (Fig. 1C). These results indicate that Akt reduces the levels of p53 protein, but not p53 mRNA in MCF-7 cells.

Since it is well established that the level of p53 protein is regulated largely by stability, we then asked whether the stability of p53 was affected by Akt. Flag-tagged p53 was ectopically expressed in MCF-7 cells along with an Akt plasmid. Titration of the amount of co-transfected Akt plasmid showed that increasing amounts of active Akt correlated with decreased levels of p53 protein (Fig. 1D).
was then assessed by the addition of cycloheximide, a translational inhibitor. Two hours of cycloheximide treatment decreased p53 protein by 40% in control cells, whereas the same treatment decreased p53 protein by 80% in active Akt-expressing cells (Fig. 2A), indicating that degradation rate of p53 protein was greater in active Akt-expressing cells. The amounts of p53 protein were then estimated by the use of a densitometry. As shown in Fig. 2B, p53 decayed faster when active Akt was expressed. The degradation enhanced by Akt was blocked by treatment with MG132, a proteasome inhibitor (data not shown). When MCF-7 cells were treated with LY294002, a PI3K inhibitor, the stability of endogenous p53 protein increased (Fig. 2C). These results suggest that the PI3K-Akt pathway accelerates p53 degradation.

Akt phosphorylates Mdm2 at Ser186

We asked whether Akt might regulate p53 stability by a direct phosphorylation of p53. We found that immunoprecipitated active Akt was not able to phosphorylate p53 in vitro (data not shown), suggesting an indirect regulation of p53 by Akt. The major way in which p53 is degraded is by Mdm2-mediated ubiquitination. Mdm2 is phosphorylated at multiple sites in vivo (35). Interestingly, analysis of human Mdm2 sequence revealed two sites (Ser166 and Ser186) that conform to the consensus site phosphorylated by Akt (R x R x x S/T), and recent studies have shown that Mdm2 can be phosphorylated by Akt at these sites in vitro and in IGF-1-treated cells (29, 30). The first site (Ser166) is not conserved across species, however the second site (Ser186) is conserved among species as far as we know, suggesting its possible functional
importance. We confirmed that active Akt, but not kinase negative Akt, was capable of inducing Mdm2 phosphorylation in vitro (Fig. 3A). To further examine if Akt phosphorylates Mdm2 at Ser186, we generated a polyclonal antibody that specifically recognizes phosphorylated Ser186 of Mdm2. Upon Western blot analysis, this antibody detected Mdm2 that had been phosphorylated by Akt in vitro (Fig. 3B). The specificity of this antibody was confirmed by its failure to recognize the Mdm2 mutant in which Ser186 was mutated into Ala (S186A Mdm2) (Fig. 3B).

By the use of anti-phospho Ser186 Mdm2 antibody, we found that serum stimulation increased Ser186 phosphorylation (Fig. 3C). The increase in Ser186 phosphorylation was blocked by LY294002, suggesting that PI3K is required for serum induction of Ser186 phosphorylation (Fig. 3C). We also found that active Akt expression was sufficient for inducing Ser186 phosphorylation of Mdm2 in vivo. In addition, expression of kinase negative Akt blocked the serum induction of Ser186 phosphorylation (Fig. 3D). These results strongly support that the PI3K-Akt pathway mediates Mdm2 phosphorylation at Ser186 in vivo.

Akt does not affect the subcellular localization of Mdm2

We next asked whether Akt phosphorylation of Mdm2 at Ser186 has any impact on Mdm2. We first examined if Akt regulates the stability of Mdm2 protein. Western blot analysis indicated that expression of active Akt did not affect the levels of ectopically-expressed Mdm2 (Fig. 4A). In addition, the levels of wild type and S186A mutant of Mdm2 were almost the same when expressed in MCF-7 cells (Fig. 4B).
Therefore, Ser186 phosphorylation does not appear to alter the stability of Mdm2 protein.

Since Ser186 is located close to the nuclear localization sequence and nuclear export signal of Mdm2 (residues 178-185 and 191-199, respectively), we asked whether Akt phosphorylation of Mdm2 alters its subcellular localization. In MCF-7 cells, endogenous Mdm2 was localized mainly in the nucleus and slightly in the cytoplasm (Fig. 5A). In subcellular fractionation experiments, more than 90% of Mdm2 was found in the nuclear fraction (Fig. 5B). Serum and LY294002 treatment did not change the amounts of Mdm2 protein in the nuclear and cytoplasmic fractions (Fig. 5B). The localization of Mdm2 did not change upon serum or LY294002 treatment in immunostaining experiments, either (Fig. 5A). Expression of active Akt did not induce nuclear translocation of Mdm2 as shown in Figs. 5B, 5C and 5D. Furthermore, we found that expression of a dominant negative Akt (3A Akt) did not change the localization of endogenous Mdm2 in MCF-7 cells (Fig. 5C). Importantly, the localization of S186A Mdm2 as well as S166A/S186A Mdm2 (2SA Mdm2) was mainly in the nucleus, indistinguishable from that of wild type Mdm2 when expressed in Saos-2 cells (Fig. 5D). Therefore we conclude that Akt does not induce nuclear translocation of Mdm2 in our system, in apparent contradiction to the previous reports (29, 30) (see Discussion).

Serum facilitates Mdm2-mediated p53 ubiquitination in a PI3K-dependent manner

We then tested the possibility that Ser186 phosphorylation regulates the function of
Mdm2. Mdm2 is known to promote p53 degradation by facilitating ubiquitination (19). Since serum treatment increased Ser186 phosphorylation (Fig. 3C), we examined the ability of Mdm2 to promote ubiquitination of p53 in the presence or absence of serum. To detect ubiquitination of p53, MCF-7 cells were transfected with Flag-tagged ubiquitin and HA-tagged p53, and treated with a proteasome inhibitor for 2 h. p53 was immunoprecipitated and subjected to Western blot analysis with both anti-Flag antibody and anti-p53 antibody, to visualize the ubiquitination of p53. As shown in Figs. 6A and 6B, serum treatment markedly enhanced the ubiquitination-inducing effect of Mdm2. This enhancement of p53 ubiquitination was reduced by LY294002 treatment (Fig. 6B), suggesting that serum enhancement of p53 ubiquitination is PI3K-dependent.

**Akt facilitates p53 ubiquitination**

We examined whether Akt is sufficient to enhance p53 ubiquitination. MCF-7 cells were transfected with Akt constructs together with p53. Expression of active Akt enhanced the ubiquitination of p53 (Fig. 6C). These results, taken together, suggest that growth factor-stimulation activates Mdm2-mediated p53 ubiquitination by way of the PI3K-Akt pathway.

**Ser186 of Mdm2 is essential for the Akt enhancement of its functions**

To further examine if Akt facilitates the ability of Mdm2 to induce p53 ubiquitination, we examined the synergy between Akt and Mdm2, and the possible requirement of Ser186 for this synergy. This experiment was performed under low serum conditions...
(0.1% serum) to reduce the possible contribution of endogenous Akt activity. Expression of either active Akt alone or wild type Mdm2 alone induced p53 ubiquitination to some extent, but expression of both active Akt and wild type Mdm2 synergistically increased p53 ubiquitination (Fig. 7). If this synergy is due to direct activation of Mdm2 by Akt, S186A mutation should hamper it. The S186A mutation of Mdm2 almost completely abrogated the ubiquitination promoting activity of Mdm2 enhanced by active Akt (Fig. 7), suggesting that Akt activates Mdm2 by direct phosphorylation. The loss of p53 ubiquitination by S186A mutation was not due to reduction of Mdm2 protein, since the protein levels were almost the same between WT and S186A Mdm2 (Fig. 4B).

We then examined the effects of S186A mutation of Mdm2 on the levels of p53 protein, by expression of Mdm2 and p53 in the absence of proteasome inhibitors. Expression of active Akt as well as wild type Mdm2 reduced the levels of p53 protein, presumably due to the enhanced degradation of p53 (Fig. 8A, also see Fig. 2). However, the S186A mutation of Mdm2 abrogated its activity to reduce p53 protein (Fig. 8A). Consistent with this, the inhibitory effect of Mdm2 on the transcriptional activity of p53 was also reduced when Ser186 was mutated to Ala (Fig. 8B). These results strongly suggest that Akt facilitates the functions of Mdm2 to induce ubiquitination and degradation of p53 in a Ser186-dependent manner.
Discussion

In this study, we investigated the mechanism by which Akt antagonizes p53. Expression of active Akt reduced the levels of p53 protein, but not p53 mRNA. The reduction of p53 protein by Akt appeared to be due at least in part to the reduced stability of p53 protein, because active Akt was capable of reducing the levels of ectopically-induced p53, and because active Akt increased the degradation rate of p53. This finding is consistent with the report by Mayo and Donner (29), but is apparently contradictory to the report by Yamaguchi et al., in which adenoviral expression of Akt did not reduce the amount of p53 protein in hippocampal neurons (28). It is possible that the difference is due to the types of cells used, although we have observed Akt reduction of p53 protein in a number of cell types.

We propose that Akt promotes degradation of p53 via direct phosphorylation of Mdm2, based on several lines of evidence. First, Akt is capable of phosphorylating Mdm2 \textit{in vitro} at Ser186, which is conserved among species. This site of Mdm2 can also be phosphorylated \textit{in vivo} in response to Akt expression or serum treatment in a PI3K-dependent manner. Second, the ability of Mdm2 to facilitate p53 ubiquitination and degradation was enhanced by co-expression of active Akt. Third, Akt enhancement of Mdm2 mediated p53 ubiquitination and degradation was abolished by S186A mutation of Mdm2.

Akt activation of Mdm2 may well account for the enhanced degradation of p53 by Akt, but these results do not rule out the possibility that Akt promotes p53
degradation through regulation of other targets, such as p19ARF, p300 and the proteasome. However, the contribution of these other possible targets may be small, as the S186A mutant Mdm2 appeared to behave as a dominant negative preventing the phosphorylation of Mdm2, and its expression reversed the ability of Akt to promote p53 degradation (see Fig. 8A).

Mdm2 has been shown to shuttle between the nucleus and the cytoplasm by utilizing nuclear export signals (NESs) and nuclear localization sequences (NLSs) (36, 37), and the nuclear localization of Mdm2 is prerequisite for the degradation of p53 (37). After the completion of our study, two papers reported \textit{in vitro} and \textit{in vivo} phosphorylation of Mdm2 by Akt and nuclear translocation of Mdm2 by Akt-mediated phosphorylation (29, 30). We confirmed Akt-mediated phosphorylation of Mdm2 at Ser186 by utilizing the phospho Ser186-specific antibody. However, we could not detect any effect of Akt on Mdm2 localization. The nuclear localization of Mdm2 was not affected by the expression of Akt constructs, serum treatment, PI3K inhibitor treatment or the mutation of Mdm2 at the phosphorylation site(s). We thus concluded that Akt modulates the activity of Mdm2 independently of its subcellular localization.

How Ser186 phosphorylation enhances Mdm2 function is still an open question. Given that the protein stability and the subcellular localization of Mdm2 are not regulated by Akt-mediated phosphorylation, it is possible that Ser186 phosphorylation regulates the affinity of Mdm2 toward p53, although Ser186 does not reside in the p53-binding domain of Mdm2 determined in the previous study (38). Alternatively, Ser186 phosphorylation may affect the ubiquitin ligase activity of Mdm2,
or the affinity of Mdm2 toward other proteins including p19ARF, which has been reported to sequester Mdm2 from p53 (30, 39-41). The exact roles of Ser186 phosphorylation of Mdm2 await future investigation.

In our experiments, the S186A mutation abolished Mdm2 induction of p53 ubiquitination, but only partially impaired the Mdm2 reduction of the transcriptional activity of p53. This result suggests that Mdm2 suppresses the transcriptional activity of p53 not just by induction of ubiquitination, but also by other mechanisms, as previously described (42-44), and that Ser186 phosphorylation regulates the former, but not the latter, functions of Mdm2.

It has been shown that mitogenic signals including Ras activation regulate Mdm2 at different levels (24). For instance, Ras activation of the Raf-MEK-MAPK pathway upregulates the transcription of Mdm2 by direct activation of the Ets binding elements upstream of mdm2 gene (25). On the other hand, the Ras-Raf pathway induces p19ARF resulting in the sequestration/inhibition of Mdm2 (25, 45). Mdm2 has been reported to serve as a good substrate for several kinases including ATM, DNA-dependent protein kinase and Cdk2/cyclinA complex, which negatively regulate Mdm2 functions (46-48). Our study has shown that Mdm2 can be positively regulated by Akt-mediated phosphorylation. Mdm2 is thus likely to be extensively regulated by phosphorylation and by other means in various ways.

It is now widely appreciated that the PI3K-Akt pathway plays a central role in promoting survival by cytokines, growth factors, neurotrophic factors and cell attachment (1, 2). Previous studies on Akt-mediated survival have revealed several
substrates of Akt such as BAD, caspase-9, members of the Forkhead family, Nur77 and IKKα (6-13, 49), although it is still largely unknown how Akt promotes survival. It has been reported that Akt can inhibit apoptosis at both pre- and post-mitochondrial steps (50, 51). Mdm2-mediated p53 inhibition might contribute to the Akt inhibition of apoptosis at the pre-mitochondrial steps. As p53 mediates a wide variety of apoptosis signals, inhibition of p53 might account for a part of the potent anti-apoptotic effects of Akt.

Akt was originally discovered as a cellular counterpart of the viral oncogene, v-akt (52) (and as a kinase related to protein kinase A/C (53, 54)). Based on mutational analysis and other experiments, it has been shown that the PI3K-Akt pathway is necessary for Ras transformation of fibroblasts (1, 2, 55). Akt activation and requirement have also been shown in tumors associated with pten deficiencies (56, 57). Akt activation of Mdm2 might contribute to oncogenic activity of Akt under certain circumstances, considering the tumor-promoting effects of Mdm2, which is amplified in about 30% of human sarcomas, and promotes tumorigenesis in fibroblasts when overexpressed (58).

Our results show growth factor-dependent ubiquitination of p53 for the first time. Why is negative regulation of p53 necessary in response to the mitogenic signals? The simplest idea is that p53, a cell proliferation inhibitor, must be downregulated during normal cell proliferation. Mitogenic signals need to suppress p53, especially to counteract the induction of p53 by the mitogenic signal-activated Ras-MAPK pathway. High levels of mitogenic signals (or activation of the Ras-MAPK
pathway) activate p53 and induce senescence or apoptosis presumably to prevent tumorigenesis. Therefore, the levels, duration, or other parameters of the mitogenic signals might determine whether cells undergo proliferation or senescence/apoptosis, in response to mitogenic signals. In this respect, it is intriguing that transient activation of Ras (which promotes proliferation), but not sustained activation of Ras (which promotes senescence), induces activation of the PI3K-Akt pathway in epithelial cells (59). Therefore, the PI3K-Akt pathway might contribute to the cell fate determination by inhibition of p53 accumulation.
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Footnotes

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†The abbreviations used are: CA Akt, constitutively active Akt; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IB, immunoblotting; IP, immunoprecipitation; KN Akt, kinase negative Akt; MAPK, mitogen-activated protein kinase; Mdm2, murine double minute; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered-saline; PI3K, phosphatidylinositol-3-OH kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium lauryl sulfate; Ub, ubiquitin.
Figure legends

Fig. 1  Akt reduces the amounts of p53 protein, but not p53 mRNA
A. MCF-7 cells plated in 6 well plates were transfected with PG13-Luc, a reporter plasmid to monitor the transcriptional activity of p53 (60), and the indicated amounts of active Akt (CA, myristylated Akt without PH domain) for 24 h. The luciferase activity in the cell extracts was measured and normalized by β-galactosidase activity. Experiments were performed in triplicate. Error bars, s.d.  B, C. MCF-7 cells were transfected with the indicated amounts of CA Akt. The transfection efficiency was about 70% as judged by co-transfected green fluorescence protein. The cell lysates were subjected to Western blot analysis with anti-p53 antibody. Western blot analysis with α-tubulin was performed for a loading control (B). The isolated total RNAs were subjected to RT-PCR to detect p53 and GAPDH mRNA levels, as described in Experimental Procedures (C).  D. MCF-7 cells were transfected with Flag-p53 (0.1 µg) and the indicated amounts of CA Akt for 22 h. The cell lysates were subjected to Western blot analysis with anti-Flag antibody and with anti-α-tubulin antibody.

Fig. 2  Akt promotes degradation of p53 protein
A, B. MCF-7 cells plated in 6 well plates were transfected with Flag-p53 (0.1 µg) either alone or together with CA Akt (0.05 or 0.1 µg) or kinase negative Akt (KN, K179A) (0.1 µg). Twenty-two hours after transfection, cells were treated with cycloheximide (CHX) (80 µg/ml) for the indicated times and lysed. The cell extracts
were subjected to Western blot analysis with anti-Flag antibody (A). The amount of p53 was quantified by utilizing a densitometry and shown as a relative value to the p53 amount without CHX treatment under each condition (B). Essentially the same results were obtained in seven independent experiments. C. MCF-7 cells plated in 6 well plates were treated with or without LY294002 (LY) (10 µM). Five hours after LY treatment, cells were treated with cycloheximide (CHX) (80 µg/ml) for the indicated times and lysed. The cell extracts were subjected to Western blot analysis with anti-p53 antibody. Essentially the same results were obtained in three independent experiments.

Fig. 3  Akt phosphorylates Mdm2 at Ser186

A. Recombinant Mdm2 protein (1 µg each) was phosphorylated with active (CA) or kinase negative (KN) Akt prepared as described (32, 33) in the presence of [γ-32P]ATP. Phosphorylation was detected by autoradiography. Asterisk represents a degraded band of recombinant Mdm2. B. Recombinant wild type (WT) or S186A Mdm2 was phosphorylated in vitro in the presence or absence of active Akt, and subjected to Western blot analysis with anti-phospho Ser186 Mdm2 antibody (anti-pS186). C. 293T cells were transfected with a plasmid encoding wild type Mdm2 for 18 h, and were serum-starved in the presence or absence of 1 µM of LY294002 for 6 h. Before harvesting, cells were treated with or without 10% serum for 45 min in the presence or absence of 10 µM of LY294002. The cell extracts were immunoprecipitated with anti-Mdm2 antibody (IF2), and the immunoprecipitates were subjected to Western blot
analysis with anti-phospho Ser186 Mdm2 antibody (anti-pS186) or anti-Mdm2 antibody (anti-Mdm2). D. 293T cells were transfected with a plasmid encoding wild type Mdm2 either alone or together with active Akt (CA) or kinase negative Akt (KN) for 18 h, and were serum-starved for 6 h. Before harvesting, cells were treated with or without 10% serum for 45 min. The cells were immunoprecipitated and subjected to Western blot analysis as in Fig. 3C.

Fig. 4  Akt does not change the stability of ectopically-expressed Mdm2 protein
A. MCF-7 cells plated in 6 well plates were transfected with Mdm2 (1 µg) either alone or together with active Akt (1 µg). Twenty-two hours after transfection, cells were lysed and subjected to Western blot analysis with anti-Mdm2 antibody or anti-Akt antibody. B. MCF-7 cells transfected with vector alone or plasmids encoding either wild type or S186A Mdm2 were lysed, and subjected to Western blot analysis with anti-Mdm2 antibody.

Fig. 5  Akt does not change the subcellular localization of Mdm2 protein
A. MCF-7 cells plated on coverslips in 6 well plates were treated with or without serum (10%) and LY294002 (10 µM) for 8 h. Cells were fixed and stained with anti-Mdm2 antibody (green). The cells were counterstained with Hoechst33258 (Hoechst) to visualize the nucleus (blue). Mdm2 was found mainly in the nucleus in 100% of the cells, when more than 100 cells were observed under each condition. B. MCF-7 cells plated in 6 well plates were transfected with Mdm2 (2 µg) either alone or together
with active Akt (0.1 µg). Twenty-four hours after transfection, cells were treated with 10% (+) or 0.1% (-) serum in the presence (+) or absence (-) of LY294002 (10 µM) for 10 h. The nuclear (N) and cytoplasmic (C) fractions were prepared as described in Experimental Procedures, and subjected to Western blot analysis with anti-Mdm2 antibody. Each lane corresponds to the cytoplasmic or nuclear fraction from 5 x 10^5 cells. MEK1 and topoisomerase II α were also assessed as cytoplasmic and nuclear markers, respectively. C. MCF-7 cells were transfected with a plasmid encoding CA Akt or 3A Akt for 24 h. The cells were fixed and stained with both anti-Mdm2 antibody (IF2) and anti-Akt antibody. Mdm2 staining was visualized with Alexa 488-conjugated anti-mouse antibody (green), and Akt staining was visualized with Alexa 594-conjugated anti-rabbit antibody (red). The cells were counterstained with Hoechst33258 to visualize the nuclei (blue). The cells expressing Akt were indicated by arrows. Mdm2 was found mainly in the nucleus in 100 % of the cells, when more than 100 cells were observed under each condition. D. Saos-2 cells were transfected with plasmids encoding Mdm2 (either wild type, S186A or S166A/S186A (2SA)) and Akt (either CA or 3A) for 24 h. Saos-2 cells were used because the level of endogenous Mdm2 is negligible. The cells were fixed and stained with anti-Mdm2 antibody (green) and with Hoechst33258 (blue). Essentially the same results were obtained in at least three independent experiments in A-D.

Fig. 6 The PI3K-Akt pathway promotes p53 ubiquitination

A. MCF-7 cells plated in 10 cm dishes were transfected with plasmids encoding HA-
p53 (2 µg), Mdm2 (2 µg) and Flag-tagged ubiquitin (2 µg) for 24 h as indicated. The cells were then exposed to β-lactone (5 µM), a proteasome inhibitor, for 2 h and lysed. p53 was immunoprecipitated with anti-HA antibody, and subjected to Western blot analysis with anti-p53 antibody. The ladder of bands indicated by a bracket (Ub₆-p53) represents ubiquitinated p53. B. MCF-7 cells plated in 10 cm dishes were transfected with plasmids encoding HA-p53 (2 µg) and Flag-tagged ubiquitin (2 µg) as indicated. The cells were treated with (+) or without (-) 10% serum and 10 µM of LY294002 (LY) for 12 h. The cells were exposed to β-lactone (5 µM) for 2 h and lysed. p53 was immunoprecipitated from the cell extracts with anti-HA antibody, and subjected to Western blot analysis with anti-Flag antibody (upper panel) or anti-p53 antibody (lower panel). The ladder of bands represents ubiquitinated p53. C. MCF-7 cells plated in 10 cm dishes were transfected with plasmids encoding HA-p53 (2 µg), Akt (either active or wild type; 0.1 µg) and Flag-tagged ubiquitin (2 µg) as indicated. One day after transfection, the cells were serum-starved for 12 h, exposed to β-lactone (5 µM) for 2 h and lysed. p53 was immunoprecipitated with anti-HA antibody, and subjected to Western blot analysis with anti-p53 antibody.

**Fig. 7  Akt promotes Mdm2 ubiquitination of p53 in a Mdm2 Ser186-dependent manner**

MCF-7 cells plated in 10 cm dishes were transfected with plasmids encoding HA-p53 (2 µg), Mdm2 (either wild type or S186A; 2 µg), Akt (either active, wild type or kinase negative; 0.1 µg) and Flag-tagged ubiquitin (2 µg) as indicated. One day after
transfection, cells were incubated in the presence of 0.1% serum for 12 h, and then 5 
µM β-lactone for 2 h. p53 was immunoprecipitated from the cell lysates with anti-HA 
antibody, and subjected to Western blot analysis with anti-Flag antibody. The ladder 
of bands represents ubiquitinated p53.

Fig. 8 Akt promotes Mdm2 degradation of p53 in a Mdm2 Ser-186-dependent 
manner

A. MCF-7 cells plated in 6 well plates were transfected with plasmids encoding HA-
p53 (0.05 µg), Mdm2 (either wild type or S186A; 1 µg) and active Akt (1 µg) as 
indicated. One day after transfection, the cells were lysed, and p53 was 
immunoprecipitated with anti-HA antibody and subjected to Western blot analysis with 
anti-p53 antibody. The positions of p53 and Immunoglobulin (IgG) heavy chain are 
indicated by arrowheads. B. MCF-7 cells were transfected with PG13-Luc and 
Mdm2 (either wild type or S186A; 0.1 µg) for 24 h, and then the luciferase activity in 
the cell lysates was measured. Experiments were performed in triplicate. Error bars, 
s.d.
Fig. 1

A

Relative Luciferase Activity (Arbitrary unit)

CA Akt (µg)

0 0.1 1.0

B

CA Akt (µg) 0 1.0

anti-p53

anti-α-tubulin

C

CA Akt (µg) 0 0.1 1.0

p53

GAPDH

D

CA Akt (µg) 0 0.02 0.05 0.10 0.15

anti-Flag

anti-α-tubulin
Fig. 2

A

CHX treatment (min)

0 30 60 90 120

- CHX

+CHX

+CHX CA Akt 0.05 µg

+CHX CA Akt 0.1 µg

B

p53 protein (%)

0 20 40 60 80 100 120

CHX treatment (min)

- CHX

+CHX

+CHX CA Akt 0.1 µg

+CHX KN Akt 0.1 µg

C

CHX treatment (min)

0 30 60 90 120

+CHX

+CHX +LY

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Fig. 3

A

| Mdm2 | Akt  | Serum | LY  |
|------|------|-------|-----|
| +    | +    | +     |     |

* Mdm2

B

| CA  | Akt  | Mdm2 | anti-pS186 |
|-----|------|------|------------|
|     | -    | WT   |            |
|     | -    | S186A|            |
|     | +    | WT   |            |
|     | +    | S186A|            |

C

| LY   | serum | Mdm2 |
|------|-------|------|
| -    | -     | -    |
| -    | +     | +    |
| -    | +     | +    |

* pS186 Mdm2

D

| Serum | Mdm2 | Akt  |
|-------|------|------|
| -     | -    | -    |
| -     | +    | +    |
| -     | +    | CA   |
| -     | +    | KN   |

* anti-Mdm2
Fig. 4
Fig. 5

A

| LY | serum | Mdm2 |
|----|-------|------|
| -  | -     | +    |
| -  | +     | +    |

B

| LY | CA Akt | serum | Mdm2 |
|----|--------|-------|------|
| -  | -      | -     | -    |
| -  | +      | -     | +    |
| -  | -      | +     | +    |

|        | C   | N   | C   | N   | C   | N   | C   | N   |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|
| anti-Mdm2 |
| anti-MEK1 |
| anti-topo II α |
D

|                  | Akt     | Mdm2    | Hoechst |
|------------------|---------|---------|---------|
| WT Mdm2          | ![Image](red) | ![Image](green) | ![Image](blue) |
| WT Mdm2 +CA Akt  | ![Image](red) | ![Image](green) | ![Image](blue) |
| WT Mdm2 +3A Akt  | ![Image](red) | ![Image](green) | ![Image](blue) |
| S186A Mdm2       | ![Image](red) | ![Image](green) | ![Image](blue) |
| 2SA Mdm2         | ![Image](red) | ![Image](green) | ![Image](blue) |
Fig. 6

A

|        | FLAG-Ub | Serum | Mdm2 | HA p53 |
|--------|---------|-------|------|--------|
| +      | +       | +     | +    | +      |
| +      | +       | -     | -    | +      |
| +      | +       | +     | +    | +      |

IP anti-HA
IB anti-p53

B

|        | FLAG-Ub | LY   | Serum | HA p53 |
|--------|---------|------|-------|--------|
| +      | +       | -    | +     | +      |
| +      | +       | +    | -     | +      |
| +      | +       | +    | +     | +      |

IP anti-HA
IB anti-Flag

C

|        | FLAG-Ub | Serum | Akt   | HA p53 |
|--------|---------|-------|-------|--------|
| +      | +       | -     | CA    | +      |
| +      | +       | -     | WT    | +      |
| +      | +       | +     | +     | +      |

IP anti-HA
IB anti-p53

Ub<sub>n</sub>-p53
p53
|        | Flag-Ub | Akt   | Mdm2 | HA p53 |
|--------|---------|-------|------|--------|
|        | +       | --    | -    | +      |
| +      | +       | CA    | -    | +      |
| +      | +       | WT    | -    | +      |
| +      | +       | KN    | -    | +      |
| +      | +       | CA    | WT   | +      |
| +      | +       | -     | WT   | +      |
| +      | +       | -     | S186A| +      |
| +      | +       | -     | -    | +      |
| +      | +       | -     | -    | +      |

Fig. 7
### A

| Experiment       | CA Akt | Mdm2  | HA p53 |
|------------------|--------|-------|--------|
| -                | -      | -     | -      |
| Mdm2 WT          | +      | WT    | S186AS186A |
| HA p53 -         | +      | +     | +      |

**IP anti-HA**

**IB anti-p53**

Arrow indicates p53, IgG

### B

| Mdm2     | Relative Luciferase Activity (Arbitrary unit) |
|----------|---------------------------------------------|
| -        | 80 ± 10                                     |
| WT       | 20 ± 2                                      |
| S186A    | 30 ± 3                                      |

**Fig. 8**
Akt enhances Mdm2-mediated ubiquitination and degradation of p53
Yoko Ogawara, Shohei Kishishita, Toshiyuki Obata, Yuko Isazawa, Toshiaki Suzuki, Keiji Tanaka, Norihisa Masuyama and Yukiko Gotoh

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