Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase

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The androgen receptor (AR) controls several biological functions including prostate cell growth and apoptosis. However, the mechanism by which AR maintains its stability to function properly remains largely unknown. Here we show that Akt and Mdm2 form a complex with AR and promote phosphorylation-dependent AR ubiquitylation, resulting in AR degradation by the proteasome. The effect of Akt on AR ubiquitylation and degradation is markedly impaired in a Mdm2-null cell line compared with the wild-type cell line, suggesting that Mdm2 is involved in Akt-mediated AR ubiquitylation and degradation. Furthermore, we demonstrate that the E3 ligase activity of Mdm2 and phosphorylation of Mdm2 by Akt are essential for Mdm2 to affect AR ubiquitylation and degradation. These results suggest that phosphorylation-dependent AR ubiquitylation and degradation by Akt require the involvement of Mdm2 E3 ligase activity, a novel mechanism that provides insight into how AR is targeted for degradation.

Keywords: androgen receptor/E3 ligase/phosphorylation/proteasome/ubiquitylation

Introduction

The ubiquitin–proteasome system plays important roles in several biological processes, such as antigen presentation, endocytosis and the cell stress response, and represents one of the most important degradation systems in the cell (Hershko and Ciechanover, 1998; Lee and Goldberg, 1998; Pickart, 2001). Misfolded and unfolded proteins are targets for degradation by the proteasome in order to maintain cell integrity and survival. The loss of the normal control of this system may be associated with human diseases such as cancer (Schwartz and Ciechanover, 1999). Several proteins involved in cell cycle regulation including cyclin E, cyclin B and p27Kip1 are degraded via the ubiquitin–proteasome pathway (Vlach et al., 1997; Zhang et al., 1998; Koepp et al., 2001). Protein ubiquitylation usually requires three processes involving the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) (Pickart, 2001). Protein ubiquitylation provides the recognition signal for 26S proteasome association and leads to protein destruction.

The intrinsic E3 ligase activity serves as the rate-limiting step of ubiquitin modification of proteins and plays a central role in determining the specificity of ubiquitylation. E3s are classified into two groups: the HECT (homology to E6-AP C-terminus) domain-containing E3s and the RING finger-containing E3s. The HECT domain proteins, such as E6-AP, are characterized by the ability to form a thiolester intermediate with the activated ubiquitin before transfer of ubiquitin to substrates (Huibregtse et al., 1995; Pickart, 2001). In contrast, the RING finger-containing E3s do not form a covalent bond with the activated ubiquitin and instead catalyze ubiquitylation by association with substrates (Pickart, 2001). Mdm2, a RING finger-containing protein, has been found to play an important role in the control of tumor suppressor p53 activity via promotion of p53 ubiquitylation and degradation (Honda et al., 1997; Fang et al., 2000; Weissman, 2001). Many RING finger-containing proteins are part of a large protein complex, such as SCF (Skp1–cullin–F-box protein) and anaphase-promoting complex (APC), which are involved in cell cycle regulation (Gmachl et al., 2000; Tyers and Jorgensen, 2000).

It is thought that protein phosphorylation may play a critical role in the initiation of protein ubiquitylation. The loss or inhibition of protein phosphorylation usually prevents protein ubiquitylation and causes aberrant protein accumulation. One example is IκBα, an inhibitor of nuclear factor-κB (NF-κB), which associates with NF-κB and prevents its activation by sequestering it in the cytoplasm. Under cytokine treatment, such as tumor necrosis factor-α (TNF-α), IκBα is phosphorylated at two specific sites, Ser32 and Ser36, leading to NF-κB activation via destruction of IκBα (Brockman et al., 1995; Brown et al., 1995). When either Ser32 or Ser36 is mutated, IκB cannot be phosphorylated, preventing its ubiquitylation and degradation (Brockman et al., 1995; Brown et al., 1995). Protein phosphorylation may provide the recognition motif for an E3, a concept supported by the demonstration that Skp2, a component of the SCF complex, binds to phosphorylated p27Kip1, but not to the p27Kip1 mutant defective in the phosphorylation, promoting p27Kip1 ubiquitylation and degradation (Carrano et al., 1999; Ganoth et al., 2001).

As a transcription factor, the androgen receptor (AR) associates with androgen and translocates into the nucleus where it binds to an AR response element (ARE) and turns on its target genes, which are thought to mediate androgen–AR signals in the regulation of diverse cell functions such as cell growth and apoptosis (Heisler et al., 1997; Grossmann et al., 2001). AR consists of an N-terminal transactivation domain, a DNA-binding domain (DBD), a hinge region that contains the bipartite nuclear translocation signal involved in AR nuclear translocation, and a ligand-binding domain (LBD),
which is responsible for ligand binding and transactivation (Chang et al., 1995). Recent studies suggest that AR may require co-regulators such as ARA70, ARA55, ARA54, and the p160 family of co-activators, such as SRC-1 and TIF2, for optimal transcriptional activation (Yeh and Chang, 1996; Alen et al., 1999; Fujimoto et al., 1999; Kang et al., 1999; Heinlein and Chang, 2002). This idea is supported by the fact that overexpression of dominant-negative ARA54 suppresses androgen-induced AR transactivation in prostate cancer cells (Miyamoto et al., 2002).

Since androgen–AR signaling plays important roles in cell functions, it is conceivable that this signal needs to be tightly controlled in order to maintain normal cell function. Deregulation of this signal may lead to pathological problems such as cancer or androgen insensitivity syndromes (Quigley et al., 1995). One way to control this signal is via regulation of AR protein synthesis or turnover. Although control of AR synthesis has been studied extensively, little is known about how AR is targeted for degradation. Accumulating evidence indicates that AR may be targeted for degradation via ubiquitin–proteasome pathways. First, there is a highly conserved PEST sequence, which is thought to target proteins for ubiquitylation and degradation, located in the hinge region of AR throughout many species (Sheflin et al., 2000). Secondly, AR protein levels were elevated under proteasome pathways. First, there is a highly conserved PEST sequence, which is thought to target proteins for ubiquitylation and degradation, located in the hinge region of AR throughout many species (Sheflin et al., 2000). Moreover, several RING finger-containing E3s, such as BRCA-1, ARA54 and Snurf-1, have been reported to associate with AR and modulate AR activity (Kang et al., 1999; Poukka et al., 2000; Yeh et al., 2000).

AR is a phosphoprotein, and its activity can be regulated via phosphorylation. Recently, we reported that Akt phosphorylates AR at Ser210 and Ser790 and suppresses AR activity (Lin et al., 2001). It remains to be determined whether AR phosphorylation by Akt leads to AR ubiquitylation and degradation. Here we show that activation of the phosphatidylinositol 3-kinase (PI3K)–Akt pathway promotes AR ubiquitylation and leads to AR degradation via a proteasome-dependent pathway. We also demonstrate that Mdm2 and Akt form a complex with AR and induce AR ubiquitylation and degradation in a phosphorylation-dependent manner. The effect of Akt on AR ubiquitylation and degradation is markedly lower in a Mdm2-null cell line compared with the wild-type cell line. Thus, our results represent novel findings that phosphorylation-dependent AR ubiquitylation and degradation by Akt require the involvement of Mdm2 E3 ligase activity and provide further insight into how AR is targeted for degradation.

**Results**

**Akt promotes AR degradation via a proteasome-dependent pathway**

Phosphorylation plays a critical role in proteasome-mediated degradation of proteins such as the p27Kip1, cyclin E and IκBα proteins (Brown et al., 1995; Carrano et al., 1999; Koeppe et al., 2001). We have previously reported that Akt could suppress AR transactivation via phosphorylation of AR at two Akt consensus sites (Ser210 and Ser790) (Lin et al., 2001). To explore the possibility that phosphorylation may mediate AR degradation, COS-1 cells were transfected with AR in combination with vector (pCDNA3), a constitutively active form of Akt (cAkt) or a kinase-inactive Akt mutant (dAkt) for a western blot assay. The western blot revealed that cAkt, but not dAkt, markedly reduced AR protein levels in the presence or absence of the androgen dihydrotestosterone (DHT) (Figure 1A). To confirm further the effect of the Akt on AR protein levels, we also used immunocytochemistry. As shown in Figure 2B, cAkt, but not dAkt, could reduce AR protein levels, as determined by the lower fluorescence intensity compared with vector. These results indicate that Akt activity is important for the down-regulation of AR protein levels. We further demonstrated that cAkt could reduce AR protein levels in a dose-dependent manner (Figure 1B). cAkt could also reduce endogenous AR protein levels in prostate cancer LNCaP cells (Figure 1C). To avoid the possibility that these results are due to overexpression, which may not be physiologically relevant, we used insulin-like growth factor-1 (IGF-1), which induces AR phosphorylation via activation of the PI3K–Akt pathway (Lin et al., 2001), to mimic physiological conditions. As shown in Figure 1D, IGF-1 could activate Akt and reduce AR protein levels in the LNCaP cells. LY294002, a selective PI3K inhibitor, blocked IGF-1-mediated Akt activation and reversed the suppressive effect of IGF-1 on AR protein levels, suggesting that IGF-1 down-regulates AR protein levels via the PI3K–Akt pathway.

To determine whether cAkt reduced AR protein levels via proteasome-dependent degradation, the 26S proteasome inhibitor MG132 was used (Lee and Goldberg, 1998). MG132 was able to block the cAkt effect on AR protein levels (Figure 1B), suggesting that cAkt promoted AR degradation via the proteasome-dependent pathway. These results, together with the previous reports showing that cAkt, but not dAkt, phosphorylated AR (Lin et al., 2001), suggest that phosphorylation of AR by Akt induces AR degradation via a proteasome-dependent pathway.

**Phosphorylation of AR is critical for Akt-mediated AR degradation**

To confirm further that promotion of AR degradation by Akt results from AR phosphorylation, we compared the effect of Akt on protein levels of the wild-type AR (wtAR) and a mutant AR (mtAR) with two serine residues, Ser210 and Ser790, replaced with alanine (A), preventing Akt-mediated phosphorylation (Wen et al., 2000; Lin et al., 2001). Western blotting revealed that cAkt reduced wtAR protein levels, but not mtAR protein levels (Figure 2A). Immunocytochemistry further confirmed this result (Figure 2B). Taken together, these results indicate that AR phosphorylation is essential for Akt-mediated AR degradation.

If the phosphorylation of AR by Akt induces AR degradation, then the mtAR would be more stable than wtAR. To test this hypothesis, we compared the stabilities of wtAR and mtAR. Figure 2C shows that wtAR was readily degraded with a 90 min half-life ($t_{1/2}$), but the mtAR was very stable even after 4 h of cycloheximide treatment. These results support the notion that phosphorylation of AR by Akt reduces AR stability.
Akt promotes AR ubiquitylation dependent on AR phosphorylation

The above experiments demonstrated that Akt promotes AR degradation via a proteasome-dependent pathway, implying that Akt may promote AR ubiquitylation, thus resulting in AR degradation by 26S proteasome. To test the hypothesis that Akt promotes AR ubiquitylation, COS-1 cells were transfected with AR along with cAkt in the presence or absence of hemagglutinin-tagged ubiquitin (HA-Ub). AR proteins were immunoprecipitated with an AR antibody, followed by western blotting with an HA antibody. AR ubiquitylation occurred in the presence of HA-Ub and, as expected, cAkt notably enhanced AR ubiquitylation (Figure 3). However, mtAR was significantly less ubiquitylated in the presence of HA-Ub, and cAkt showed no effect on mtAR ubiquitylation (Figure 3), which correlates with Figure 2A and B, showing that cAkt reduces wtAR, but not mtAR protein levels. These results indicate that AR phosphorylation by Akt promotes AR ubiquitylation and subsequent degradation by the 26S proteasome.

Mdm2 interacts with AR in vivo and in vitro

We have demonstrated that AR undergoes ubiquitin-dependent degradation, which is promoted by Akt. However, it is unknown currently which E3 ligase is involved in this ubiquitylation. Recently, two groups have individually reported that Akt interacts with and phosphorylates Mdm2, a RING finger E3 ligase responsible for p53 ubiquitylation and degradation (Mayo and Donner, 2001; Zhou et al., 2001), at Ser166 and Ser186. This finding led us to hypothesize that Mdm2 may be able to associate with Akt and form a complex with AR, leading to AR ubiquitylation. To test this hypothesis, we used co-immunoprecipitation to determine whether Mdm2 forms a complex with AR and Akt. Mdm2 was immunoprecipitated with an anti-Mdm2 antibody from LNCaP cells, which express endogenous Mdm2, Akt and AR, and the immunocomplexes were subjected to SDS–PAGE, and followed by western blot analysis. Figure 4A reveals that both Akt and AR were detected in the Mdm2 immunocomplexes. A similar result was obtained in MCF-7 breast cancer cells (data not shown). These results indicate that endogenous Mdm2 forms a complex with endogenous AR and Akt in vivo. We further determined whether phosphorylation of AR by Akt increases the interaction between AR and Mdm2. COS-1 cells were transfected with AR and Mdm2 in the presence of cAkt. The co-immunoprecipitation experiment revealed that Mdm2 interacts with AR, and cAkt enhanced the interaction between Mdm2 and AR under the overexpression conditions (Figure 4B). This result suggests that phosphorylation of AR by Akt may increase the interaction between Mdm2 and AR. To confirm this result further, we compared the interaction of Mdm2 with wtAR or mtAR in the presence of cAkt. Figure 4B shows that wtAR binds to Mdm2, whereas mtAR only weakly interacts with Mdm2.

Taken together, these results suggest that Mdm2 interacts with AR in vivo, and AR phosphorylation by Akt increases the binding between Mdm2 and AR.

To test whether Mdm2 interacts with AR in vitro, we used the glutathione S-transferase (GST) pull-down assay. GST–Mdm2 was expressed in bacteria and purified by
glutathione-coupled beads. GST–Mdm2 was then incubated with in vitro expressed [35S]methionine-labeled AR, subjected to SDS–PAGE and followed by autoradiography. Figure 4C shows that Mdm2 interacts with AR in vitro. Mdm2 C464A (C464A), a RING finger mutant with cysteine changed to alanine that has lost its E3 ligase activity (Honda et al., 1997; Fang et al., 2000), also interacts with AR, but to a lesser extent compared with Mdm2 (Figure 4C). The lower binding of C464A to AR was not due to the decreased protein amounts, since equal amounts of GST–Mdm2 and GST–C464A were used in this experiment, as determined by Coomassie Blue staining (data not shown). We next compared the interaction of GST–Mdm2 with wtAR or mtAR in vitro. As shown in Figure 4D (left panel), GST–Mdm2 was able to interact with wtAR, whereas mtAR only weakly interacted with Mdm2, which was consistent with the weaker in vivo interaction of Mdm2 with mtAR (Figure 4B). The decreased interaction of GST–Mdm2 with mtAR compared with wtAR suggests that AR may be phosphorylated during the in vitro translation process. To prove this hypothesis, we demonstrated that Akt activity was detected in the in vitro translation system, which probably could induce AR phosphorylation (data not shown). Furthermore, treatment with alkaline phosphatase, which dephosphorylates proteins, obviously reduced the interaction of AR with GST–Mdm2 (Figure 4D, right panel). These results indicate that AR phosphorylation by Akt may play an important role in the interaction between AR and Mdm2.

To map the Mdm2 interaction domain on AR, several AR deletion mutants were constructed and expressed in vitro. Figure 4E shows that Mdm2 can interact with full-length AR, AR1 (amino acids 34–560), AR2 (amino acids 552–918) and AR3 (amino acids 486–651), but not AR4 (amino acids 66–918), indicating that Mdm2 can interact with the AR N-terminus and DBD. To map the AR interaction domain on Mdm2 further, several Mdm2 deletion mutants were constructed and expressed in bacteria. AR was able to interact with full-length Mdm2, M2 (amino acids 191–491) and M3 (amino acids 341–491), but not M1 (amino acids 1–155) (Figure 4F), suggesting that AR interacts with Mdm2 at amino acids 341–491, which covers the RING finger domain.

Since we have mapped the interaction domains between AR and Mdm2, we next determined whether these interaction domains could interrupt the interaction between endogenous AR and Mdm2 by co-immunoprecipitation assay in LNCaP cells. Figure 4G shows that AR interacts with Mdm2, and this interaction is abrogated by M3 (amino acids 341–491), which covers the RING finger domain of Mdm2. AR-D (amino acids 483–651) can also dramatically reduce the interaction between endogenous AR and Mdm2 (Figure 4G). These results suggest that the appropriate interaction domain within AR or Mdm2 can interrupt the interaction of endogenous AR and Mdm2.

Fig. 2. Phosphorylation-dependent AR degradation by Akt. (A) COS-1 cells were transfected with wtAR or mtAR in combination with vector or cAkt for 16 h in 10% CDS medium, treated with 10 nM DHT for 16 h, and then harvested for a western blot assay. (B) COS-1 cells were transfected with wtAR or mtAR in combination with pCDNA3, cAkt or dAkt in 10% CDS medium for 16 h and treated with ethanol or 10 nM DHT for another 16 h. Cells were fixed and stained with AR antibodies, followed by examination with a confocal microscope. The red and blue colors represent AR and nuclei staining, respectively. (C) COS-1 cells were transfected with wtAR or mtAR for 16 h in 10% CDS medium, treated with 20 μg/ml cycloheximide (CYH) in the presence of 10 nM DHT, and then harvested for a western blot assay at different times as indicated.
Mdm2 is involved in Akt-mediated AR ubiquitylation and degradation

As Mdm2 forms a complex with Akt and AR, we determined whether Mdm2 is involved in Akt-mediated AR ubiquitylation and degradation. We compared the effect of Akt on AR degradation and ubiquitylation in Mdm2-intact mouse embryonic fibroblasts (MEFs) with that in Mdm2-deficient MEFs. Figure 5A shows that cAkt can reduce AR protein levels in the Mdm2-intact MEFs. However, AR protein levels remained unchanged when cAkt was transfected into the Mdm2-deficient MEFs. Restoration of Mdm2 expression in Mdm2-deficient MEFs rescued the effect of cAkt on AR protein levels (Figure 5A). It should be noted that AR protein levels in the Mdm2-deficient MEFs were much higher than in the Mdm2-intact MEFs (Figure 5A). Moreover, cAkt promoted AR ubiquitylation in the Mdm2-intact MEFs, but failed to enhance AR ubiquitylation in the Mdm2-deficient MEFs, and restoration of Mdm2 expression rescued the effect of cAkt on AR ubiquitylation in the Mdm2-deficient MEFs (Figure 5B). In contrast, restoration of C464A or Mdm2 S166A/S186A (S166A/S186A) expression, defective in Akt-mediated phosphorylation, in the Mdm2-deficient MEFs failed to rescue AR ubiquitylation and degradation by cAkt (Figure 5B, right panel). To confirm further that Mdm2 is involved in the Akt-mediated AR ubiquitylation and degradation, we used C464A or S166A/S186A, each of which acts as a dominant-negative mutant, to block endogenous Mdm2 function (Mayo and Donner, 2001). Figure 5B (left panel) and C reveals that both C464A and S166A/S186A attenuated the effect of Akt on AR ubiquitylation and degradation. These results suggest that Mdm2 is involved in Akt-induced AR ubiquitylation and degradation.

Mdm2 is an E3 ligase for AR and promotes AR ubiquitylation and degradation

Since Mdm2 is involved in Akt-mediated AR ubiquitylation, we next evaluated whether Mdm2 induces AR ubiquitylation. AR proteins were immunoprecipitated from Mdm2-deficient MEF extracts by an AR antibody, and the AR ubiquitylation was detected by an HA antibody. As shown in Figure 6A, Mdm2 markedly induced AR ubiquitylation. In contrast, C464A or S166A/S186A did not induce AR ubiquitylation. A similar result was also obtained in COS-1 cells (data not shown). We further used a pulse-chase experiment to determine whether Mdm2 promoted AR degradation. Figure 6B shows that Mdm2 accelerates AR degradation with a t1/2 from 180 to 90 min. Western blot analysis revealed that Mdm2 reduced AR protein levels in a dose-dependent manner, and this effect could be blocked by MG132 (Figure 6C), suggesting that Mdm2 promotes AR degradation via a proteasome-dependent pathway. In contrast, C464A or S166A/S186A only marginally influenced AR protein levels (Figure 6D). These results suggest that Mdm2 is indeed an E3 ligase for AR, and its E3 ligase activity and phosphorylation of Mdm2 by Akt are important for AR ubiquitylation and degradation by the proteasome.

Phosphorylation of AR by Akt is critical for Mdm2-induced AR ubiquitylation and degradation

The above experiments have demonstrated that promotion of AR ubiquitylation and degradation by Akt required AR phosphorylation. We reason that Akt may first phosphorylate AR, then recruit Mdm2 to the AR–Akt complex, leading to AR ubiquitylation, since Mdm2 binds more weakly to mtAR than to wtAR (Figure 4B and D). To test this hypothesis, we compared the effect of Mdm2 on wtAR and mtAR ubiquitylation and degradation. Figure 3 showed that Mdm2 induced wtAR ubiquitylation, but not mtAR ubiquitylation. Mdm2 reduced wtAR protein levels, but not mtAR protein levels (Figure 6E). These results therefore suggest that phosphorylation of AR by Akt is critical for Mdm2-induced AR ubiquitylation and degradation, and further support the hypothesis that Akt first phosphorylates AR and then facilitates Mdm2 binding to AR, leading to AR ubiquitylation and degradation.

Mdm2-mediated AR degradation reduces AR transcriptional activity

AR activity is regulated by several mechanisms including changes in protein stability, subcellular localization and post-translational modifications such as phosphorylation and acetylation (Fu et al., 2000; Poukka et al., 2000; Lin et al., 2001). It remains unclear whether ubiquitylation is also involved in the regulation of AR activity. We have observed that Mdm2 induced AR ubiquitylation and accelerated AR degradation. To determine whether Mdm2-mediated AR degradation affects AR transcriptional activity, we used two AR reporters including mouse mammary tumor virus luciferase (MMTV-luc) and four copies of a synthetic ARE (ARE4-luc) in a transient transfection assay. Mdm2 or cAkt suppressed AR transcriptional activity from two AR reporters, and Mdm2 and cAkt could enhance further the suppressive effect of cAkt on AR transactivation (Figure 7A). Northern blotting further confirmed that cAkt or Mdm2 could inhibit androgen-induced prostate-specific antigen (PSA) expression, an endogenous AR target gene, and Mdm2 further enhanced cAkt-mediated suppression of androgen-induced PSA expression (Figure 7B). We also demonstrated that C464A and S166A/S186A did not affect AR transcriptional activity (Figure 7C). Taken together, these results indicate that Mdm2-mediated AR degradation reduces AR
Fig. 4. Mdm2 forms a complex with Akt and AR in vivo and interacts with AR in vitro. (A) LNCaP cells were cultured in 10% FBS, harvested and lysed for the co-immunoprecipitation assay. (B) COS-1 cells were transfected with plasmids, as indicated, and harvested for co-immunoprecipitation. (C) GST–Mdm2 or GST–C464A was incubated with AR for 2 h and subjected to SDS–PAGE, followed by autoradiography. (D) GST–Mdm2 was incubated with wtAR or mtAR for 2 h (left panel). GST–Mdm2 was incubated with AR in the presence or absence of alkaline phosphatase (AP) for 2 h (right panel). (E) GST–Mdm2 was incubated with full-length AR or AR deletion mutants for 2 h. (F) GST–Mdm2 or GST–Mdm2 deletion mutants were incubated with AR for 2 h. (G) LNCaP cells were transfected with vector, Flag-AR-D (amino acids 483–651) or Flag-M3 (amino acids 341–491) for 36 h, followed by harvesting for the co-immunoprecipitation assay.
transcriptional activity, and this effect requires Akt-mediated Mdm2 phosphorylation and Mdm2 E3 ligase activity.

**Mdm2 attenuates androgen–AR-mediated suppression of colony formation**

Androgen–AR signaling controls prostate cancer cell growth and apoptosis (Heisler et al., 1997; Grossmann et al., 2001). Overexpression of AR in PC-3 prostate cancer cells inhibits cell growth and induces apoptosis in response to androgen treatment (Heisler et al., 1997; Yeh et al., 2000; Lin et al., 2001). Our above results have demonstrated that Mdm2 promotes AR ubiquitylation and degradation, resulting in the repression of AR transcriptional activity. To determine whether Mdm2 can regulate androgen–AR-mediated cell growth, we employed the colony formation assay using PC-3(AR)6 as a cell model, which stably expresses AR, and colonies were counted by crystal violet staining after 14 days of culturing. As shown in Figure 7D, androgen suppressed colony formation up to

![Figure 5](image)
90%. As expected, both cAkt and Mdm2 could reverse the effect of androgen on colony formation, supporting results showing that AR protein levels were down-regulated by cAkt or Mdm2, as determined by immunocytochemistry (Figure 7E). However, C464A and S166A/S186A did not prevent the androgen suppression and showed no reduction of AR protein levels (Figure 7D and E), implying that Mdm2 E3 ligase activity and phosphorylation of Mdm2 by Akt are required for Mdm2 function.

Discussion

The ubiquitin–proteasome pathway has been studied intensively in recent years and represents a very common degradation pathway for a large variety of short-life proteins (Lee and Goldberg, 1998; Pickart, 2001). Protein phosphorylation usually serves as an early step for initiating protein ubiquitylation. In the present study, we have identified that AR phosphorylation by Akt is required for AR ubiquitylation and subsequent degradation by the 26S proteasome. In support of this hypothesis, we demonstrated that an AR mutant that no longer responds to Akt-mediated phosphorylation has significantly diminished ubiquitylation and degradation. We further demonstrated that Mdm2 is involved in Akt-mediated AR ubiquitylation and degradation, as shown by two lines of evidence. First, Akt failed to induce AR ubiquitylation or reduce AR protein levels in Mdm2-deficient MEFs, while it induced AR ubiquitylation and reduced AR protein levels in Mdm2-intact cells. Secondly, C464A or S166A/S186A, each of which acts as a dominant-negative mutant to block endogenous Mdm2 function (Mayo and Donner, 2001), inhibited the effect of cAkt on AR ubiquitylation and degradation. Together, these results suggest that
phosphorylation-dependent ubiquitylation and degradation of AR by Akt require involvement of Mdm2 E3 ligase activity.

The PI3K–Akt pathway in AR ubiquitylation and degradation

AR is a phosphoprotein, and its activity can be regulated by several growth factors via induction of AR phosphorylation (Yeh et al., 1999; Wen et al., 2000; Lin et al., 2001). It is known that growth factors such as IGF-1 and epidermal growth factor (EGF), and cytokines such as interleukin-6 (IL-6) can activate the PI3K–Akt and mitogen-activated protein kinase (MAPK) pathways via distinct mechanisms (van der Geer et al., 1994; Qiu et al., 1998a,b). We have shown previously that Akt and MAPK could phosphorylate AR at distinct sites (Yeh et al., 1999; Lin et al., 2001). However, it remains unclear whether growth factors can regulate AR protein degradation via induction of AR phosphorylation. In the present study, we have shown that IGF-1 could promote AR degradation via activation of the PI3K–Akt pathway. In addition, we found that IL-6 could also down-regulate AR protein levels via the PI3K–Akt pathway, but we did not observe any change in protein levels by activation of the MAPK pathway (data not shown). Thus, PI3K–Akt, but not MAPK, may represent the major pathway for growth factor-induced AR protein degradation. While the mechanism responsible for this distinct effect on AR protein degradation is currently unclear, it is likely that phosphorylation of AR at distinct sites by these two pathways may result in different AR conformations, which may then contribute differentially to protein stability. In support of the role of PI3K–Akt in AR degradation, an AR mutant, which is defective in Akt-mediated AR phosphorylation, is remarkably stable compared with the wtAR (Figure 2C). Recently, several reports demonstrated that the PI3K–Akt pathway is involved in protein degradation. For example, PI3K–Akt has been associated with p27Kip1 and insulin receptor substrate-1 (IRS-1) stability, and blockage of the PI3K–Akt pathway causes the accumulation of p27Kip1 protein and IRS-1 (Gesbert et al., 2000; Lee et al., 2000; Mamillapalli et al., 2001). Therefore, the PI3K–Akt pathway may represent a central pathway for the degradation of several proteins, including AR.

Mdm2 is an E3 ligase for AR and is involved in AR ubiquitylation and degradation

It is thought that protein phosphorylation is required for the ubiquitylation of several proteins such as p27Kip1, IκB and cyclin E (Brown et al., 1995; Carrano et al., 1999; Koepp et al., 2001). In the case of p27Kip1, phosphorylation

Fig. 7. Mdm2-mediated AR degradation reduces AR transcriptional activity. (A) PC-3 prostate cancer cells were transfected with AR in combination with vector or Mdm2 in the presence or absence of cAkt in 10% CDS medium for 16 h, treated with ethanol or 10 nM DHT for 16 h and harvested for a luciferase assay. At least three independent experiments were performed and the data are presented as means ± SD. (B) LNCaP cells were transfected with vector or Mdm2 in the presence or absence of cAkt in 10% CDS medium for 16 h, treated with ethanol or 10 nM DHT for 24 h and then harvested for a northern blot assay. (C) PC-3 cells were transfected with AR in combination with plasmids as indicated in 10% CDS medium for 16 h, treated with ethanol or 10 nM DHT for 16 h and harvested for the luciferase assay. (D) PC-3(AR)6 cells were transfected with plasmids for 16 h as indicated and treated or not with 10 nM DHT. After 2 weeks of culture, the cells were stained with crystal violet, and colony formation was counted by microscope. (E) PC-3(AR)6 cells were transfected with plasmids for 16 h as indicated, treated with 10 nM DHT for 16 h and fixed for immunocytochemistry.
Thus, protein phosphorylation may provide the target site for E3 binding. Given that the AR mutation, which abolishes Akt-mediated phosphorylation, prevented Mdm2-induced AR ubiquitylation and degradation, it is possible that AR phosphorylation by Akt can enhance the interaction between AR and Mdm2. In support of this idea, we have shown that Akt enhanced the interaction of Mdm2 with AR. Furthermore, wtAR interacted well with Mdm2, whereas mtAR only interacted weakly with Mdm2 (Figure 4B and D). Thus, we propose that AR phosphorylation by Akt may help to recruit Mdm2 to AR, leading to AR ubiquitylation (Figure 8).

Mdm2, a p53 target gene, regulates p53 activity by inducing p53 ubiquitylation and subsequent degradation by the proteasome (Honda et al., 1997; Fang et al., 2000; Weissman, 2001). Thus, induction of Mdm2 expression by p53 provides a negative regulation loop to shut off p53 signaling. Recently, two groups demonstrated that Akt phosphorylates Mdm2 at Ser166 and Ser186, and mutations of these two sites prevent Mdm2 phosphorylation and nuclear localization, thus inhibiting its ability to block p53 activity (Mayo and Donner, 2001; Zhou et al., 2001). These results therefore suggest that Mdm2 phosphorylation by Akt is essential for Mdm2 nuclear localization and function. As with p53, we have observed that S166A/S186A did not induce AR ubiquitylation and degradation, suggesting that phosphorylation of Mdm2 by Akt is indispensable for the effect of Mdm2 on the induction of AR ubiquitylation and degradation. While it is currently unclear how this mutant loses its activity to regulate AR function, it is very likely that Mdm2 nuclear localization may be important for its activity. Alternatively, this mutant may simply lose its E3 ligase activity, abolishing its ability to regulate AR protein stability.

From our GST pull-down assay, we have demonstrated that Mdm2 can bind to the AR N-terminal or DBD region via its RING finger domain. It seems that Mdm2 may use its RING finger domain to interact with the AR DBD region in the cells. To support this idea, we have observed that the RING finger domain of Mdm2 and the AR DBD were able to disrupt the interaction of Mdm2 with AR in vivo (Figure 4G). The RING finger domain is thought to be involved in protein–protein interactions (Borden, 2000). Given that the AR DBD interacts with Mdm2 and contains two zinc finger domains, it is very likely that Mdm2 may use its RING finger domain to bind to the zinc finger domains of AR. However, the detailed information as to how these two proteins interact may need further investigation.

Several lines of evidence suggest that Mdm2 may be an E3 ligase for AR and involved in the regulation of AR ubiquitylation and degradation. First, we have demonstrated that Mdm2 interacted with AR in vitro and in vivo via the GST pull-down assay and co-immunoprecipitation. Secondly, Mdm2 markedly induced AR ubiquitylation and degradation in COS-1 cells and MEFs. Thirdly, C464A did not induce AR ubiquitylation. Moreover, AR ubiquitylation is significantly impaired in Mdm2-deficient MEFs compared with Mdm2-intact MEFs, and AR protein levels are higher in Mdm2-deficient MEFs than in Mdm2-intact MEFs. Thus, control of Mdm2 protein levels via regulation of its gene expression or changes in protein stability can influence AR protein stability. As p53 can induce Mdm2 expression, it is conceivable that p53 may control AR protein levels and activity via induction of Mdm2 expression. Since it has been reported that p53 is frequently mutated in a subset of advanced stage prostate cancers (Bookstein et al., 1993; MacGrogan and Bookstein, 1997), it is possible that loss of p53 function may reverse the inhibition of AR activity via enhancement of AR protein levels, contributing to prostate cancer progression. In support of this hypothesis, Shenk et al. (2001) demonstrated that p53 can suppress AR transactivation. It remains to be determined whether Mdm2 is involved in p53-mediated inhibition of AR signaling.

In summary, our data indicate that Akt mediates AR ubiquitylation and degradation by the proteasome through AR phosphorylation, and this event requires Mdm2. The phosphorylation of AR by Akt recruits Mdm2 to AR, resulting in AR ubiquitylation and subsequent degradation by the proteasome (Figure 8). Thus, identification of Mdm2 as an E3 ligase for AR may help us to understand better how AR protein activity is regulated and further provides insight into how AR protein is degraded, which may aid in the development of drug targets for the treatment of prostate cancer.

Materials and methods

Materials

cAkt and dAkt were described previously (Lin et al., 2001). LY294002 and MG132 were purchased from Calbiochem, and DHT was from Sigma. The anti-AR monoclonal antibody was purchased from PharMingen, the anti-HA antibody from Roche, Akt and phospho (S473) Akt antibodies and alkaline phosphatase from New England Biolabs, Mdm2 antibody from Santa Cruz Biotechnology, Texas Red-conjugated secondary anti-rabbit antibody from ICN, and the TNT in vitro protein expression kit from Promega. Mdm2 and C464A were kindly provided by Drs A.M.Weissman and K.H.Vousden. The HA-Ub construct was a gift from Dr D.Bohmann. The Mdm2 wild-type MEFs and Mdm2-

![Fig. 8. The model for AR degradation. IGF-1 or IL-6 activates PI3K, resulting in activation of Akt. Akt phosphorylates AR and Mdm2 and increases the binding between AR and Mdm2, leading to AR ubiquitylation by Mdm2. The 26S proteasome recognizes the polyubiquitylated AR, leading to AR degradation.](image-url)
null MEFs were kindly provided by Dr G.Lozano. To construct Flag-AR-D (amino acids 483–651) or Flag-M3, an AR fragment with an EcoRI site at the 5' end and a KpnI site at the 3' end, or an Mdm2 fragment with a BamHI site at the 5' end and an XhoI site at the 3' end was obtained using PCR from pSG5-AR or Mdm2 and subcloned into the pFlag-CMV vector and the pCDNA3-Flag vector, respectively.

**Cell culture and transfections**

PC-3, COS-1, MCF-7, Mdm2-intact MEFs and Mdm2-null MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing penicillin (25 U/ml), streptomycin (25 μg/ml) and 10% fetal calf serum (FCS). LNCaP cells were maintained in RPMI-1640 with 10% FCS, and PC-3(AR)6 cells were cultured in RPMI-1640 with 10% charcoal-stripped serum (CDS). Transfections were performed using the calcium phosphate precipitation method in MEFs as previously described (Yeh and Chang, 1996) or SuperFect™ in LNCaP, COS-1 and PC-3 cells according to standard procedures (Qiagen).

**Luciferase reporter assays**

Cells were transfected with plasmids in 10% CDS medium for 16 h and then treated with ethanol or 10 nM DHT for 16 h. The cells were lysed and luciferase activity was detected by the dual luciferase assay according to standard procedures and normalized by the Renilla luciferase activity.

**GST pull-down assay**

To construct GST–Mdm2 deletion mutants, Mdm2 fragments with a BamHI site at the 5' end and an XhoI site at the 3' end were obtained using PCR from pGEX-KG (Amersham Pharmacia). The GST pull-down assay was described previously (Yeh et al., 2000).

**Immunoprecipitation and western blot analysis**

Immunoprecipitation and western blotting were performed as previously described (Lin et al., 2001).

**Northern blot assay**

COS-1 cells or MEFs were transfected with plasmids with or without HA-Ub for 16 h in DMEM with 10% CDS. The cells were treated with 10 nM DHT for 16 h, and then harvested using RIPA buffer. AR proteins were immunoprecipitated with an anti-AR antibody and subjected to SDS–PAGE, followed by western blot assay with an HA antibody.

**Ubiquitylation assay**

COS-1 cells were plated on 12 mm coverslips and incubated overnight. Cells were transfected with wtAR or mtAR in combination with vector, COS-1 cells were plated on 12 mm coverslips and incubated overnight. The cells were transfected with plasmids in 10% CDS medium for 16 h and then treated with ethanol or 10 nM DHT for another 16 h. The cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min on ice or 10 nM DHT for another 16 h. The cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min on ice and permeabilized with 100% methanol for 15 min on ice. The following steps were performed at room temperature. The coverslips were rinsed with PBS twice and incubated in 5% bovine serum albumin for 30 min. The primary antibody against AR was added for 1 h and washed with PBS four times. The secondary antibody was added for 1 h and then washed four times with PBS, followed by application of the counting medium containing 4,6-diamino-2-phenylindole. Texas Red anti-rabbit antibody was used as the secondary antibody. Coverslips were examined with a confocal microscope.

**Colony formation assays**

PC-3(AR)6 cells (1 × 10⁶) were plated in triplicate in 35 mm dishes, allowed to attach to the dish and transfected with pCDNA3, cAkt, Mdm2, C464A or S166A/S186A. The transfected cells were selected by 100 μg/ml G418 (Gibco-BRL) and treated with ethanol or 10 nM DHT every 2 days. The cells were incubated at 37°C and supplied with fresh 10% CDS medium containing 300 μg/ml G418 every 4 days. After 14 days, the cells were fixed with 10% formalin, stained with crystal violet, and the colonies visible to the naked eye were counted.

**Pulse-chase experiments**

COS-1 cells were transfected with AR in combination with vector or Mdm2 in 10% CDS medium for 36 h. Cells were grown under serum starvation conditions for 2 h in methionine/cysteine-deficient medium, and then the cells were pulsed for 45 min with 200 μCi/ml [35S]methionine/cysteine (NEN). Cells were washed with DMEM twice and incubated with DMEM containing 0.2% CDS along with 10 nM DHT. The cells were lysed using RIPA buffer in the presence of protease inhibitors, followed by immunoprecipitation using an AR antibody. The immunocomplexes were subjected to 8% SDS–PAGE and visualized by autoradiography.

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