MDM2 E3 ubiquitin ligase mediates UT-A1 urea transporter ubiquitination and degradation

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Chen G, Huang H, Fröhlich O, Yang Y, Klein JD, Price SR, Sands JM. MDM2 E3 ubiquitin ligase mediates UT-A1 urea transporter ubiquitination and degradation. Am J Physiol Renal Physiol 295: F1528–F1534, 2008. First published September 10, 2008; doi:10.1152/ajprenal.90482.2008.—UT-A1 is the primary urea transporter in the apical plasma membrane responsible for urea reabsorption in the inner medullary collecting duct. Although the physiological function of UT-A1 has been well established, the molecular mechanisms that regulate its activity are less well understood. Analysis of the UT-A1 amino acid sequence revealed a potential MDM2 E3 ubiquitin ligase-binding motif in the large intracellular loop of UT-A1, suggesting that UT-A1 urea transporter protein may be regulated by the ubiquitin-proteasome pathway. Here, we report that UT-A1 is ubiquitinated and degraded by the proteasome but not the lysosomal proteolytic pathway. Inhibition of proteasome activity causes UT-A1 cell surface accumulation and concomitantly increases urea transport activity. UT-A1 interacts directly with MDM2; the binding site is located in the NH2-terminal p53-binding region of MDM2. MDM2 mediates UT-A1 ubiquitination both in vivo and in vitro. Overexpression of MDM2 promotes UT-A1 degradation. The mechanism is likely to be physiologically important as UT-A1 ubiquitination was identified in kidney inner medullary tissue. The ubiquitin-proteasome degradation pathway provides an important novel mechanism for UT-A1 regulation.

Protein degradation is an important mechanism by which cells regulate the levels of cellular proteins (3). Several proteolytic systems are responsible for the bulk of protein degradation in eukaryotic cells. Specific targeting of intracellular and membrane proteins is largely achieved by the covalent conjugation of ubiquitin to the substrate protein. This process involves several enzymes that activate and transfer ubiquitin to the target protein. The critical step in this process is performed by the E3 ubiquitin ligases, which function as the substrate recognition component of the conjugation machinery (23). In the case of membrane proteins, some newly synthesized molecules undergo polyubiquitination and destruction by the endoplasmic reticulum (ER)-associated protein degradation (ERAD) system whereas the bulk of them traffic to the membrane. Mature membrane proteins on the cell surface can be modified by the addition of either monoubiquitin or polyubiquitin chains; both modifications serve as internalization signals. Once internalized, the proteins have three fates. Monoubiquitinated proteins are generally targeted to the lysosomes for degradation whereas polyubiquitinated proteins become substrates for the proteasome. Alternatively, some of the ubiquitin moieties may be removed by deubiquitinating enzymes and the proteins undergo recycling back to the membrane. Accumulating evidence indicates that levels of many membrane proteins are regulated by the ubiquitin-proteasome pathway. For example, NEDD4-2, a member of the HECT family of E3 ligases, ubiquitinates the ENaC epithelial sodium channel (20, 30). Aldosterone acts indirectly to reduce the interaction between NEDD4-2 and ENaC, thus stabilizing the channel and increasing sodium reabsorption. Analysis of the UT-A1 amino acid sequence revealed a potential binding site in its intracellular loop for MDM2, a RING finger E3 ligase that ubiquitinates p53 and other proteins (17). The presence of the MDM2 interaction site is consistent with the possibility that UT-A1 is regulated by the ubiquitin-proteasome system. Indeed, during the preparation of this manuscript, Stewart et al. (31) reported that several UT-A urea transporter isoforms undergo ubiquitination and proteasomal degradation. Their studies, however, used a high concentration of the proteasome inhibitor MG132 that does not distinguish between lysosomal and proteasomal proteolytic systems in renal cells (8).
ently, we tested the hypothesis that MDM2 mediates the turnover of UT-A1. Our results provide conclusive evidence that UT-A1 urea transporter activity is regulated by a mechanism involving MDM2-mediated protein degradation in the kidney inner medulla.

**MATERIALS AND METHODS**

**Cells and culture medium.** UT-A1 Madin-Darby canine kidney (MDCK) cells were described before (8, 9). HEK293 cells were purchased from Stratagene. The HA-MDM2-HEK293 cell line was obtained from Dr. Hua Lu (12). Cell lines were cultured in DMEM supplemented with 10% FCS and 100 U/ml penicillin/100 µ/ml streptomycin. DMEM, FCS, and nonserum Opti-Medium were purchased from Invitrogen.

**Chemicals.** Cycloheximide (CHX), MG132, and isopropyl thiob-β-d-galactopyranoside (IPTG) were purchased from Calbiochem. Lactacystin and rhodamine phalloidin were from Biomol. Methylamine, chloroquine, dimethyurea, nutlin-3, and forskolin were from Sigma. LipofectAMINE and G418 were from Invitrogen. [14C]urea and chloroquine, dimethylurea, nutlin-3, and forskolin were from Sigma.

**Plasmids.** A rat cDNA encoding the entire UT-A1 coding sequence was cloned into pcDNA3 vector in HindIII/Ncol sites. MDM2 (1-491 and 1-160) in pGEX serial vectors were obtained from Drs. Lori LipofectAMINE and G418 were from Invitrogen. [14C]urea and 35S-methionine were from Amersham. The following antibodies were used in this study: UT-A1 antibody (21); MDM2 antibody (Santa Cruz Biotechnology); actin antibody (Abcam); secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham); and secondary FITC-conjugated goat anti-rabbit IgG (Sigma).

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antibody overnight at 4°C, followed by protein A beads for 2 h. After being washed with lysis buffer, the precipitated proteins were eluted in Laemmli sample buffer and processed for Western blot analysis.

RESULTS

**UT-A1 is degraded by the proteasome.** An in silico analysis of UT-A1 revealed the presence of a potential MDM2-binding sequence in the intracellular loop. The sequence was highly conserved among rat, human, mouse, dog, cattle, and platypus (Fig. 1), suggesting that ubiquitination/degradation may serve as an important mechanism for UT-A1 regulation. To determine whether UT-A1 is degraded by the proteasome, UT-A1-MDCK cells were treated with 100 µg/ml CHX to block protein synthesis, and UT-A1 protein was monitored by Western blot analysis of total cell lysates. In cells without proteolytic inhibitors, the half-life of UT-A1 was ~10 h (Fig. 2A, top). When UT-A1-MDCK cells were treated with CHX plus the proteasome inhibitor MG132 (10 µM), the half-life of UT-A1 was significantly longer (Fig. 2A, bottom). The increase in UT-A1 was dependent on both the time and dose of MG132 (Fig. 2B and C). Note that the higher-molecular-weight forms of polyubiquitinated UT-A1 became more apparent with higher concentrations of MG132. The level of unmodified UT-A1 also increased in parallel with modified UT-A1. The higher amount of unmodified UT-A1 may result from inhibition of ERAD, which involves the proteasome. This would reduce the degradation of immature UT-A1 and allow more UT-A1 to be fully processed. The specificity of MG132 and the presence of ubiquitinated UT-A1 were confirmed by treating UT-A1-MDCK cells with a second inhibitor of the proteasome (lactacystin) or two lysosomal inhibitors (methylamine or chloroquine) for 8 h. UT-A1 was immunoprecipitated from cell lysates, and ubiquitinated UT-A1 was detected by Western blot analysis.
of the precipitated protein using an ubiquitin antibody. As expected, polyubiquitinated UT-A1 was only detected in lysates of cells treated with proteasome inhibitors. The absence of polyubiquitinated UT-A1 in cells treated with lysosomal inhibitors indicates that lysosomal proteolysis is not significantly involved in UT-A1 turnover.

Proteasome inhibition increases cell surface UT-A1 and urea transport activity. The activity of a membrane protein depends, in part, on the level of its expression on the cell surface. We next examined whether proteasome inhibition affects UT-A1 cell surface expression. UT-A1-MDCK cells were treated with increasing concentrations of MG132 for 8 h followed by biontylation and affinity purification of labeled cell surface proteins. Western blot analysis for UT-A1 revealed that inhibition of the proteasome increased the amount of UT-A1 protein on the cell membrane (Fig. 3A). The result was confirmed by immunohistochemical detection and confocal microscopy. Cell surface UT-A1 protein was significantly increased in the apical membrane after MG132 treatment (Fig. 3B). To determine whether the increased amount of urea transporter protein at the plasma membrane corresponded to elevated function, urea transport activity was measured by transepithelial urea flux in Transwell inserts. MG132 increased urea transport activity in a concentration-dependent fashion under both basal conditions and after forskolin induction (Fig. 3C). The urea transport inhibitor dimethylurea (DMU) was used to demonstrate that the elevated urea flux was specifically mediated by UT-A1 transporter protein. When treated with DMU, urea flux was inhibited, which excluded the possibility that MG132 treatment compromised epithelial cell integrity. This finding confirms that proteasome inhibition results in the accumulation of functional UT-A1 protein on the apical surface of cells.

MDM2 interacts with UT-A1. To test whether the MDM2 E3 ligase interacts with UT-A1, radiolabeled recombinant UT-A1 was incubated in vitro with wild-type or truncated forms of GST-MDM2. As seen in Fig. 4A, UT-A1 interacted with the NH2-terminal 100 amino acids of MDM2. This region contains the p53-binding site. Although the NH2-terminal domain of MDM2 is the major p53-binding site, recent studies suggest that the central acid domain of MDM2 also may interact with p53 (15). We investigated whether UT-A1 binding to MDM2 also involved the second binding site, the central acidic domain. Deletion of the central acidic domain (200–300) of MDM2 did not affect UT-A1 binding to MDM2 (Fig. 4A).

MDM2 mediates UT-A1 ubiquitination. To determine whether MDM2 mediates UT-A1 ubiquitination, purified re-
Western blotting with UT-A1 antibody. Lysates and streptavidin-purified (i.e., biotinylated) proteins were analyzed by blotting with nutlin-3 for 8 h, then processed for cell surface biotinylation. Total cell total protein and membrane protein pools. UT-A1-MDCK cells were treated with equal amounts of in vitro translated UT-A1. Glutathionine bead-precipitated samples were subjected to immunoblotting with UT-A1 antibody. With nutlin-3, a MDM2 inhibitor, increases UT-A1 protein abundance in both the total protein and membrane protein pools.

**DISCUSSION**

The ubiquitin-proteasome pathway plays an important role in diverse cellular functions by selectively degrading a variety of cellular proteins. A number of membrane transporter proteins and ion channels including CFTR (33), AQP1 (16), and ENaC (30) are regulated, in part, via this proteolytic pathway. Our results demonstrated that the UT-A1 urea transporter undergoes ubiquitination by the MDM2 E3 ligase and subsequent degradation by the proteasome. This mechanism enables the cell to change the amount of cell surface UT-A1 and thus modulate the transport of urea across the membrane.

Some membrane proteins are capable of being degraded by either the proteasomal or lysosomal pathways, depending on the type of ubiquitination (i.e., monoubiquitin vs. polyubiquitin), the state of the protein complex (e.g., phosphorylation), and/or the cell type. For example, endogenous ENaC in renal A6 cells is degraded by the proteasome complex but heterologously expressed ENaC subunits are degraded by both lysosomal and proteasomal systems in MDCK cells (20). ENaC undergoes ubiquitination in both cell types. While it is notable that polyubiquitin chains more than four residues in length are required for 26S proteasome recognition, it would have been very difficult technically to determine whether UT-A1 was modified by a single chain rather than by multiple, single ubiquitin molecules. Therefore, we relied on protease inhibitors to determine whether UT-A1 was degraded in lysosomes or by the proteasome. Although MG132 is well known to be a nonspecific inhibitor of both proteasomes and lysosomal proteases at high concentrations, we found that 0.5$\mu$M MG132 as well as lactacystin caused accumulation of higher-molecular-weight species of UT-A1 whereas inhibition of lysosomal proteolysis using two different compounds did not affect the degradation of ubiquitinated UT-A1. Importantly, we also detected multiple higher-molecular-weight species of UT-A1 in vitro and in vivo, consistent with the hypothesis that the transporter has been modified by the addition of multiple ubiquitin moieties.

E3 ligases frequently serve as the substrate recognition component of the ubiquitin conjugation process. Our experiments identified MDM2 as an E3 ligase that catalyzes the modification of UT-A1. MDM2 was originally identified as the ubiquitin E3 ligase for the tumor suppressor protein p53 (11), and proteins that were larger than the 97- and 117-kDa glycosylated isoforms in immunoprecipitated homogenate. Ubiquitinated proteins expressed. It is notable that MDM2 is capable of autoubiquitination and degradation via the proteasome (5). Consistent with previous reports, MDM2 is rapidly degraded in cells treated with CHX alone (Fig. 5B); treating cells expressing MDM2 with MG132 prevented its disappearance.

**UT-A1 ubiquitination in rat kidney tissue.** To explore whether UT-A1 undergoes ubiquitination in vivo, a coinmunoprecipitation experiment was performed with a homogenate prepared from rat inner medulla. Proteins were immunoprecipitated with either UT-A1 or ubiquitin antibodies followed by immunoblot analysis with the ubiquitin antibody. The UT-A1 antibody detects 97- and 117-kDa glycosylated isoforms in immunoprecipitated homogenate. Ubiquitinated proteins were not detected in reactions lacking MDM2. The slower migrating protein of UT-A1 was detected in the presence of MDM2 and UbcH5a but not with UbcH1. The slower migrating protein was not detected in reactions lacking MDM2. The possibility that MDM2 facilitates UT-A1 degradation by catalyzing its ubiquitination was tested in HEK293 cells that were stably transfected to heterologously express MDM2. In the presence of CHX, the stability of transiently expressed UT-A1 was significantly less in cells expressing MDM2 (Fig. 5B). As in earlier experiments, inhibiting proteasome activity with MG132 prevented the loss of UT-A1 even when MDM2 is expressed.

**Fig. 4. MDM2 interacts with UT-A1.** A: UT-A1 binds to the NH2-terminal p53-binding region of MDM2. A series of truncated MDM2 were constructed in the pGEX vector. GST-MDM2 fusion proteins were prepared and incubated with equal amounts of in vitro translated UT-A1. Glutathione bead-precipitated samples were subjected to immunoblotting with UT-A1 antibody. B: nutlin-3, a MDM2 inhibitor, increases UT-A1 protein abundance in both the total protein and membrane protein pools. UT-A1-MDCK cells were treated with nutlin-3 for 8 h, then processed for cell surface biotinylation. Total cell lysates and streptavidin-purified (i.e., biotinylated) proteins were analyzed by Western blotting with UT-A1 antibody.

Combimod UT-A1 was incubated with biotinylated ubiquitin, several different E2 enzymes, and recombinant GST-MDM2. As seen in Fig. 5A, a higher-molecular-weight species of UT-A1 was detected in the presence of MDM2 and UbcH5a and UbcH5c but not with UbcH1. The slower migrating proteins were not detected in reactions lacking MDM2. The possibility that MDM2 facilitates UT-A1 degradation by catalyzing its ubiquitination was tested in HEK293 cells that were stably transfected to heterologously express MDM2. In the presence of CHX, the stability of transiently expressed UT-A1 was significantly less in cells expressing MDM2 (Fig. 5B). As in earlier experiments, inhibiting proteasome activity with MG132 prevented the loss of UT-A1 even when MDM2 is expressed. It is notable that MDM2 is capable of autoubiquitination and degradation via the proteasome (5). Consistent with previous reports, MDM2 is rapidly degraded in cells treated with CHX alone (Fig. 5B); treating cells expressing MDM2 with MG132 prevented its disappearance.

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tor (28), androgen receptor (19), IGF receptor (10), PSD-95 (4), arrestin (18), p300 (12), FOXO3a (35), anti-apoptotic protein ARC (7), and GRK2 (24). It can be difficult to obtain unequivocal proof that proteins are substrates for specific E3 ligases. In the case of UT-A1, there are several independent findings that support the specific nature of the UT-A1-MDM2 interaction. First, the interaction between MDM2 and UT-A1 was confined to NH2-terminal amino acids of MDM2 without E2 (1, 2), with UbcH1 (3, 4), UbcH5a (5, 6), UbcH5c (7, 8), and plus 0.1 μg of MDM2 (2, 4, 6, 8) for 1 h at 37°C. Samples 9 and 10 were incubated with UbcH5c and 0.1 μg of purified glutathione-S-transferase (GST) (8) or GST-MDM2 (10). The reaction was terminated by adding Laemmli sample buffer, and proteins were separated by SDS-PAGE and blotted to PVDF membrane. The incorporated biotin-ubiquitin was detected by Vector ABC and ECL. The same membrane was stripped and reprobed with UT-A1 antibody.

During the preparation of this paper, Stewart, et al. (31) reported that ubiquitination regulates the plasma membrane expression of UT-A1 and two other UT-A isoforms, UT-A2 and UT-A3. Although some of their conclusions were essen-

Fig. 5. MDM2 mediates UT-A1 ubiquitination. A: MDM2 directly mediates UT-A1 ubiquitination in vitro. Recombinant UT-A1 was prepared with reticulocyte lysate and purified by UT-A1 antibody immunoprecipitation. The purified UT-A1 was then incubated with ubiquitination buffer containing biotin-ubiquitin without E2 (1, 2), with UbcH1 (3, 4), UbcH5a (5, 6), UbcH5c (7, 8), and plus 0.1 μg of MDM2 (2, 4, 6, 8) for 1 h at 37°C. Samples 9 and 10 were incubated with UbcH5c and 0.1 μg of purified glutathione-S-transferase (GST) (8) or GST-MDM2 (10). The reaction was terminated by adding Laemmli sample buffer, and proteins were separated by SDS-PAGE and blotted to PVDF membrane. The incorporated biotin-ubiquitin was detected by Vector ABC and ECL. The same membrane was stripped and reprobed with UT-A1 antibody. The asterisk indicates unknown nonspecific ubiquitinated protein. B: overexpression of MDM2 mediates UT-A1 ubiquitination. Wild-type or MDM2 293 cells were transiently transfected with pcDNA3-UT-A1 for 48 h. Cells were collected at different times after CHX (100 μg/ml) treatment. Some cells were exposed to 6 μM MG132 for 8 h. UT-A1 was detected by immunoblotting with the UT-A1 antibody. The same membrane was re-probed with the MDM2 antibody.

Fig. 6. UT-A1 is ubiquitinated in rat inner medulla. A cleared homogenate of rat inner medulla was prepared, and immunoprecipitations were performed using equal amounts with protein and UT-A1 antibody or ubiquitin antibody. The precipitated proteins were subjected to Western blot analysis with the ubiquitin antibody. Arrows indicate the position of ubiquitinated, glycosylated forms of UT-A1.
typically correct for UT-A1, their experiments did not directly test 
whether ubiquitination of the urea transport proteins was re-
 sponsible for changes in their activity and/or cellular localization.
Instead, they showed that inhibition of the proteasome using MG132 (50 μM) altered UT-A1 stability and activity. In con-
trast, our studies demonstrate that MDM2 ubiquitinates 
UT-A1 and that overexpression of MDM2 promotes UT-A1 
degradation by the proteasome.

In summary, several different regulatory mechanisms have been 
elucidated for UT-A1. Vasopressin quickly increases the 
amount of UT-A1 that localizes in the cell membrane (14). 
UT-A1 also undergoes phosphorylation (1). Now, we report 
whether ubiquitination of the urea transport proteins was re-
dependent ubiquitination and degradation of androgen receptor by Akt 

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