

The N-Terminal 81-aa Fragment is Critical for UT-A1 Urea Transporter Bioactivity

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Abstract: The serine protease, furin, is involved in the activation of a number of proteins most notably epithelial sodium channels (ENaC). The urea transporter UT-A1, located in the kidney inner medullary collecting duct (IMCD), is important for urine concentrating ability. UT-A1's amino acid sequence has a consensus furin cleavage site (RSKR) in the N-terminal region. Despite the putative cleavage site, we find that UT-A1, either from the cytosolic or cell surface pool, is not cleaved by furin in CHO cells. This result was further confirmed by an inability of furin to cleave *in vitro* an ³⁵S-labeled UT-A1 or the 126 N-terminal UT-A1 fragment. Functionally, mutation of the furin site (R78A, R81A) does not affect UT-A1 urea transport activity. However, deletion of the 81-aa N-terminal portion does not affect UT-A1 cell surface trafficking, but seriously impair UT-A1 urea transport activity. Our results indicate that UT-A1 maturation and activation does not require furin-dependent cleavage. The N-terminal 81-aa fragment is required for proper UT-A1 urea transport activity, but its effect is not through changing UT-A1 membrane trafficking.

Keywords: Proteolysis, membrane protein, urea transport, trafficking.

1. INTRODUCTION

Urea transport in the kidney is important for the production of concentrated urine. Four mammalian UT-A protein isoforms (UT-A1, A2, A3, and A4) have been identified in kidney medulla [1, 2]. UT-A1 is the largest and complete form. The others are structurally truncated forms of UT-A1. UT-A1 is expressed in the apical membrane of epithelial cells in the inner medullary collecting duct (IMCD) and plays an important role in urea reabsorption and the development of the corticomedullary osmolarity gradient. UT-A1 is physiologically significant in the kidney since the UT-A1 knock-out mouse has seriously impaired urine concentration ability [3].

The proposed structure of UT-A1 has 12 membrane-spanning domains with two large extracellular loops, each containing a N-glycosylation site, and a large intracellular loop connecting the two symmetric parts corresponding to UT-A3 (N-terminal half of UT-A1) and UT-A2 (C-terminal half of UT-A1). It also possesses relatively short amino and carboxyl termini (for structures and nomenclature, see [2]). UT-A1 from kidney inner medulla has two different glycosylated forms, 97 kDa and 117 kDa. Both are derived from an 88 kDa non-glycosylated protein [4, 5]. UT-A1 shows only one 97 kDa band when expressed in transfected cells or *Xenopus* oocyte [5, 6].

Regulation of UT-A1 is complicated [1], but surprisingly UT-A1 shares many of the same regulatory mechanisms as epithelial sodium channels (ENaC). Trafficking to the surface membrane of both are stimulated by increases in

intracellular cAMP mediated by V2 receptors [7-9]; the activity of both transporters is increased by activation of cAMP-stimulated non-PKA pathways [6,10]; both transporters are polyubiquitinated [11,12]; and both transporters are phosphorylated by protein kinases A and C (although the role of the phosphorylation in both transporters is not completely clear) [7-9,13,14]. On the other hand, recent work on ENaC has demonstrated an important role for proteolytic cleavage by several proteases in activating ENaC [15,16]. One of these is furin. ENaC activation requires furin-dependent cleavage at two sites in the α -subunit and at a single site within the γ -subunit. The cleavage produces increased channel activity due to an increase in open probability of the cleaved subunits [15, 17]. Interestingly, the furin-released small cleavage fragments from either the α or γ subunit inhibit ENaC activity [17].

Furin is a ubiquitous subtilisin-like proprotein convertase that is mainly localized in the trans-Golgi network (TGN) and shuttles between the TGN and plasma membrane [18]. It is the major endoprotease that processes proproteins of the secretory pathway. Furin has a broad range of substrates including blood coagulation factors, growth factor receptors such as the insulin-like growth factor receptor, pro-hormones, neuropeptides, enzymes, adhesion molecules, viral envelope glycoprotein precursors, and bacterial toxins [18-20]. Furin cleaves the extracellular domains of ENaC subunits, but can cleave other proteins in either their intracellular and extracellular domains depending upon the location of the proteins in trafficking pathways.

Whether furin also activates UT-A1 like ENaC is not known; however, rat UT-A1 does contain a consensus furin site in the putative N-terminal domain (<http://elm.eu.org/>) that implies that UT-A1 activity could be regulated by furin proteases. Here, we explored the possible roles of furin

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cleavage in UT-A1 maturation, trafficking, and activity. We found UT-A1 is not cleaved by furin. On the contrary, the supposed furin cleaved N-terminal 81-aa fragment is critical for UT-A1 urea transport activity.

2. MATERIALS AND METHODS

2.1. Cell Culture

Parental and furin manipulated (furin-deficient, furin-deficient + furin) CHO cells were kindly provided by Dr. Stephen Leppla (National Institute of Dental Research, Bethesda, MD). The cells were cultured in alpha-MEM (GIBCO32561) supplemented with 10% FCS and penicillin/streptomycin.

2.2. Constructs

The coding region of full-length rat UT-A1 was PCR amplified by using pcDNA3-UT-A1 as a template and cloned into the EcoRI/KpnI sites of pEGFP-C2 vector (Clontech) to generate N-terminal GFP tagged UT-A1. To generate the 126-aa fragment of N-terminus of UT-A1 (N-A1), a 378 nt cDNA fragment was PCR amplified and cloned in-frame with the GAL4 DNA binding domain into the pGBKT7 vector (Clontech). pGBKT7-N-A1 was originally used for our yeast two-hybrid experiment. It incorporates a c-myc epitope into the fusion protein. Xenopus ENaC- α subunit cDNA was in pcDNA 3.1 (Invitrogen).

Rat UT-A1 was cloned into the Xenopus oocyte expression pGH19 vector. The two UT-A1 mutants of the furin cleavage site (R78A and R81A) were generated using the Stratagene Quick-Change mutagenesis kit. N-terminal 81-aa deleted UT-A1 (Δ 81A1) was PCR amplified and cloned into pGH19. All constructs were confirmed by DNA sequencing.

2.3. Transient Transfection and Western Blot

CHO cells were grown in 6-well plates to ~80% confluency and transfected with pEGFP-UT-A1 using Lipofectamine for 48 h. Cells were then processed for cell surface biotinylation and lysed with RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Total cell lysates and biotinylated samples were analyzed for UT-A1 expression with both antibodies to GFP (Invitrogen) and UT-A1 [21].

2.4. Cell Surface Biotinylation Assay

Cell surface biotinylation assay was performed as described previously [5].

2.5. *In Vitro* Furin Cleavage

The full-length and a 126 N-terminal fragment of UT-A1 were synthesized *in vitro* by using the TNT T7-coupled rabbit reticulocyte translation system (Promega). The translation reaction in a final volume of 50 μ l included 25 μ l of reticulocyte lysate, 2 μ l of 10x reaction buffer, 1 μ l of 1 mM amino acid mixture (minus methionine), 2 μ l of 10 mCi/ml [³⁵S] methionine (Amersham), 1 μ g of DNA template (pcDNA3-UT-A1 or pGBKT7-N-A1), and 1 μ l of T7 RNA polymerase. The mixtures were incubated for 60 min at 30°C. Xenopus α -ENaC subunit was also prepared in the same way and used as a furin cleavage control.

The reaction was then immunoprecipitated with different antibodies to UT-A1, c-myc (Clontech) or xENaC- α , followed by Protein-A beads. The precipitated products were processed for furin digestion. The reaction was carried out in 30 μ l of total volume with different concentrations of recombinant furin (New England Biolabs), and 100 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM CaCl₂, and 1 mM 2-mercaptoethanol at 30°C for 2 or 16 h. The proteins were eluted by boiling in 2x SDS loading buffer, run on SDS-PAGE, and detected by autoradiograph.

2.6. Xenopus Oocyte Experiments

UT-A1 urea transport activity was measured in *Xenopus* oocytes by measuring urea flux. Capped complementary RNAs of UT-A1, UT-A1 furin site mutants (R78A and R81A), and truncated UT-A1 (Δ 81) were transcribed with T7 polymerase using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion). For the oocyte isolation and microinjection, a female *Xenopus laevis* was anesthetized with 0.2% tricaine (Sigma). The oocytes were defolliculated by two treatments with 2 mg/ml collagenase I-V (Sigma) and the cells were grown in OR3 medium. Stage V-VI oocytes were used for cRNA injection. Five ng of UT-A1 were injected into each oocyte. Urea transport activity was measured as described previously [22].

Oocyte cell biotinylation was performed with some modification according to Harris *et al.* [16]. Oocytes (15/group) were pre-incubated with ND96 for 1 h at 4°C. Cells were then labeled with biotin in a biotinylation buffer containing 10 mM triethanolamine, 150 mM NaCl, 2 mM CaCl₂, and 1.5 mg/ml EZ-link sulfo-NHS-SS-Biotin (Pierce) for 30 min. Excess biotin reagent was quenched by 0.1 M lysine in OR3 medium for 10 min. After washing, the cells were lysed in 700 μ l of lysis buffer containing 1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, and protease inhibitor cocktail (Sigma). The lysates were centrifuged for 10 min at 10,000 rpm. Fifty μ l of supernatant were saved as total protein for Western blotting and 550 μ l of supernatant were added to 20 μ l of immunopure immobilized streptavidin beads (Pierce) for precipitating the membrane biotin-labeled proteins. After overnight incubation at 4°C, the beads were washed and the biotin conjugated proteins were eluted and processed for Western blot with UT-A1 antibody. To ensure that equal amounts of biotinylated membrane protein were loaded, the total biotin labeled protein was detected with HRP conjugated avidin-biotin complex (ABC) from Vector Laboratories.

3. RESULTS

3.1. UT-A1 is not Cleaved by Furin in CHO Cells

Rat UT-A1 has a consensus furin cleavage site (⁷⁸RSKR⁸¹) in its N-terminus. An amino acid homology search shows that this site is highly conserved in mammalian UT-A1 among human, rat, mouse, dog, and monkey, but not in cattle (Fig. 1A). We examined UT-A1 expression in three different types of CHO cells, wild type, furin-deficient, and furin-deficient plus furin re-introduced cells. We first transiently transfected CHO cells with pcDNA3-UT-A1 and detected UT-A1 with antibody specific to the UT-A1 C-terminus. However, only a single band corresponding to the full-length UT-A1 was observed among the three CHO cells

(not shown). To clearly identify a possible furin cleaved 81 aa N-terminal fragment, a N-terminal GFP tagged UT-A1 (pEGFP-UT-A1) was prepared and transfected into furin manipulated CHO cells. UT-A1 expression was examined by both GFP antibody and UT-A1 antibody, respectively. With the UT-A1 C-terminus antibody, we detected the same size UT-A1 bands among these three different types of CHO cells, without any sign of a furin-cleaved (reduced size) UT-A1 (Fig. 1C). With the GFP antibody, the expected 81-amino acid furin-induced proteolytic fragment was not observed (Fig. 1B). We also examined the possibility of whether a small fraction of UT-A1 that trafficks to the cell membrane could be cleaved by furin. Cell surface proteins were biotinylated and cell surface UT-A1 was detected with UT-A1 antibody. Again, we did not find any furin cleaved UT-A1 (Fig. 1C).

3.2. Furin Does not Cleave UT-A1 *In Vitro*

To exclude the possibility that other unknown factors might inhibit furin cleavage in cells, recombinant UT-A1 was prepared from rabbit reticulocyte lysate and purified with UT-A1 antibody and protein A beads. The purified UT-A1 was incubated with recombinant furin. ENaC α subunit was used as positive cleavage control since it is known to be cleaved by furin [15]. Fig. (2A) shows that purified UT-A1

could not be cleaved by furin *in vitro*, while ENaC α produces the two expected small bands.

UT-A1 is a 929-aa large transmembrane protein. To eliminate the possibility that its tertiary structure might impede furin's access to the cleavage site, we also prepared a 126 aa short N-terminal fragment and performed *in vitro* furin digestion. However, this N-terminal fragment of UT-A1 was also not cleaved by furin (Fig. 2B).

3.3. Mutations of the Furin Site do not Alter Functional UT-A1 Expression in Xenopus Oocytes

Although our results so far made it seem highly doubtful that furin cleaved UT-A1 in CHO cells, we thought we should confirm that UT-A1 activity was not altered when cleavage was not possible. Using site-specific mutagenesis, we prepared two UT-A1 mutants in which the two key arginine residues in the putative furin site were replaced by alanine and then investigated whether the mutations would result in altered UT-A1 urea transport activity. Fig. (3A) shows that urea transport activity measured in oocytes was unchanged by either mutation. Meanwhile, analysis of UT-A1 protein expression by immunoblotting showed that the mutations did not alter the pattern of UT-A1 expression (Fig. 3B).

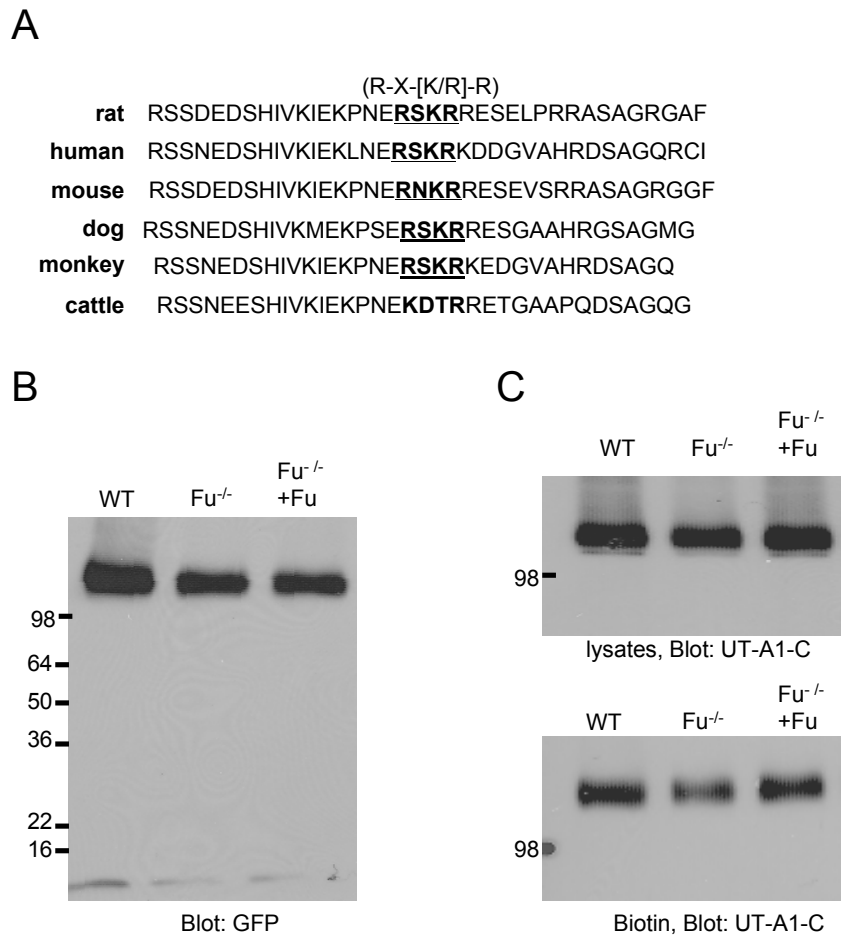


Fig. (1). **A**) A consensus furin cleavage site is conserved in rat, human, dog, monkey, but not in cattle. **B, C**) UT-A1 expression in furin manipulated CHO cells. pEGFP-UT-A1 was transiently transfected into wild type CHO, furin-deficient CHO, and furin-deficient + furin CHO cells for 48 hr. Total cell lysates or cell surface biotinylated proteins were analyzed by immunoblotting with anti-GFP (N-term, **B**) or anti-UT-A1 (C-term, **C**) antibodies.

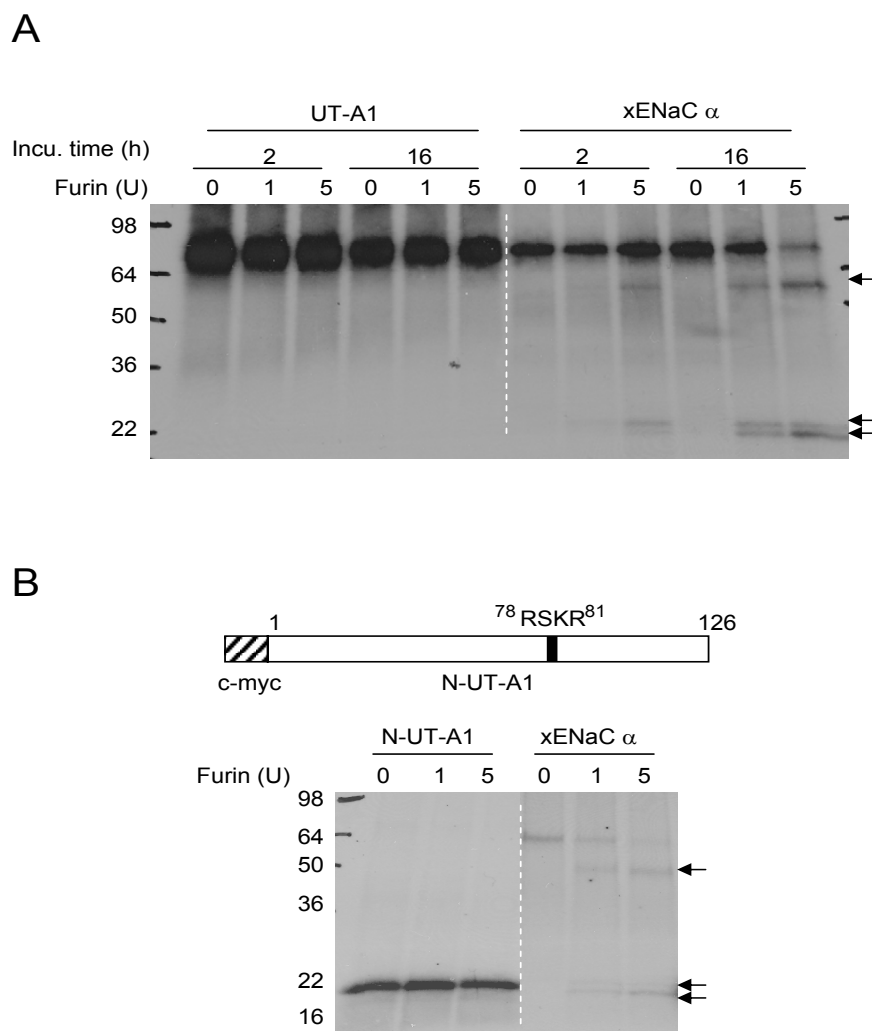


Fig. (2). *In vitro* furin cleavage. The whole UT-A1 (**A**) or a 126 N-terminal fragment (**B**) was prepared from rabbit reticulocyte lysates and radioactively labeled with ³⁵S-methionine. The recombinant purified products were then processed for enzyme digestion with 1 and 5 units of furin at 30°C for 2 or 16 h. The samples were run on 4-20% SDS-PAGE and detected by autoradiograph. Arrows indicate the cleaved products.

3.4. Deletion of N-Terminal 81-aa Reduces UT-A1 Activity

Finally, we examined the effect on urea transport of the potential 81-aa furin cleavage fragment (since, if UT-A1 is like ENaC the furin cleavage product will alter transporter activity). We deleted the 81-aa fragment from UT-A1, and tested whether this deletion altered UT-A1 transport activity. Fig. (4A) shows that loss of the N-terminal 81 aa significantly reduced UT-A1 urea transport activity. This suggests that the 81 aa fragment contains some important motifs, like phosphorylation sites, which are required for urea transport activity. Interestingly, without the 81-aa fragment, UT-A1 is still able to move to the cell membrane (Fig. 4B), suggesting that UT-A1 trafficking does not involve the N-terminal end.

4. DISCUSSION

UT-A1 is a large 929 amino acid, transmembrane protein. The furin site, RSKR, is located between amino acids 78 and 81. We originally hypothesized that, like other proteins which are activated by releasing a small fragment at the N-terminus, it is possible that cleavage of the 81aa N-

terminal fragment by furin could affect UT-A1 maturation and activation. However, based on our results our hypothesis appears to be false, since UT-A1 does not undergo furin proteolysis in CHO cells (Fig. 1) or *in vitro* (Fig. 2). Here, we found the 81aa N-terminal fragment is critical for the UT-A1 urea transport activity.

One of the well studied membrane proteins that is regulated by furin proteolysis is ENaC. The α -ENaC subunit undergoes endogenous cleavage producing an 80-kD fragment and a 65-kD fragment at the cell surface [16]. These cleaved products are not seen in the non-biotinylated intracellular pool. Furin cleavage of α -ENaC occurs in the late Golgi or at the cell surface. In fact, we were able to use α -ENaC as a positive control to demonstrate furin cleavage. To answer the question of whether, like α -ENaC, a small portion of UT-A1 at the cell surface could be cleaved by furin, we also examined proteolytic processing of UT-A1 at the cell surface. Even in the biotinylated cell surface pool of UT-A1, we did not detect any furin-cleaved UT-A1 either in furin-containing or furin-deficient CHO cells (Fig. 1C). Mutation of the two key residues of the potential furin cleavage site

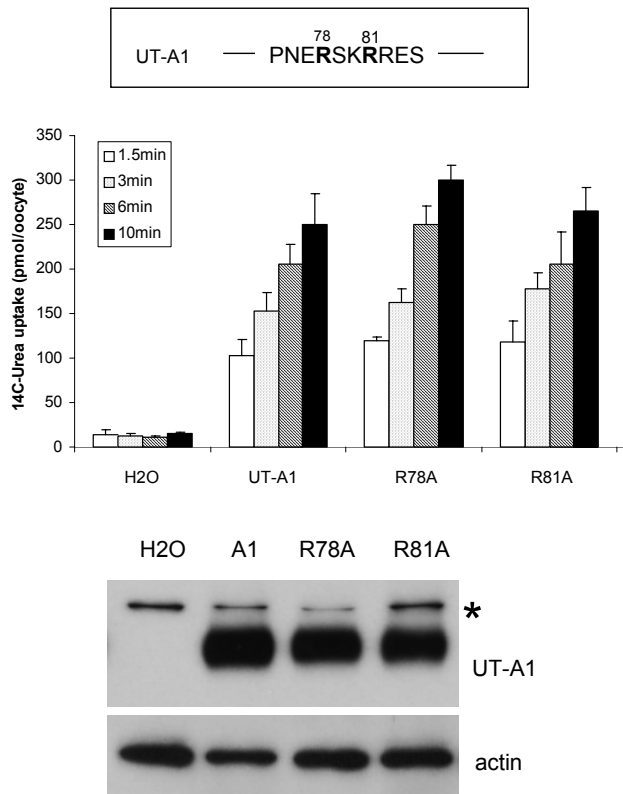


Fig. (3). Disruption of UT-A1 furin sites does not affect UT-A1 activity. Oocytes were injected with cRNAs encoding UT-A1, or UT-A1 mutants (R78A, R81A) for 3 days. Top) urea transport activity was measured by ^{14}C urea flux ($n=5\sim 6$ oocytes). Bottom) UT-A1 protein was analyzed by immunoblotting with antibody to UT-A1. Asterisk indicates a non-specific band. The same membrane were striped and re-probed with actin.

affected neither UT-A1 urea transport activity nor protein expression pattern in the cell membrane in the *Xenopus* oocyte expression system. This result strongly implies that maturation and trafficking of UT-A1 does not involve furin proteolytic cleavage. On the contrary, removal of the entire N-terminal 81-aa fragment significantly reduced UT-A1 urea transport activity, although UT-A1 trafficking to the cell surface is normal.

Furin cleavage of ENaC does affect channel activity; but despite possessing a consensus furin cleavage site (R-X-K/R-R), UT-A1 is not cleaved and UT-A1 activity affected by furin. Structurally, the furin cleavage site(s) in ENaC (α , γ) is located in the extracellular loop. Though topology analysis shows UT-A1 N-terminus is in the cytosolic side, the exact structure of UT-A1 is still unclear. A number of studies also demonstrated that other serine proteases cleave and activate ENaC, including channel-activating serine protease CAP1 (or prostasin), CAP2, CAP3, and neutrophil elastase [15, 16]. However, it appears unlikely that UT-A1 can be cleaved by other proprotein convertases. In addition to the current study (in CHO cells and oocytes), we did not observe different sizes of UT-A1 protein in other experiments when we express UT-A1 in MDCK or HEK 293 cells where alternative proteases are likely to be present (not shown).

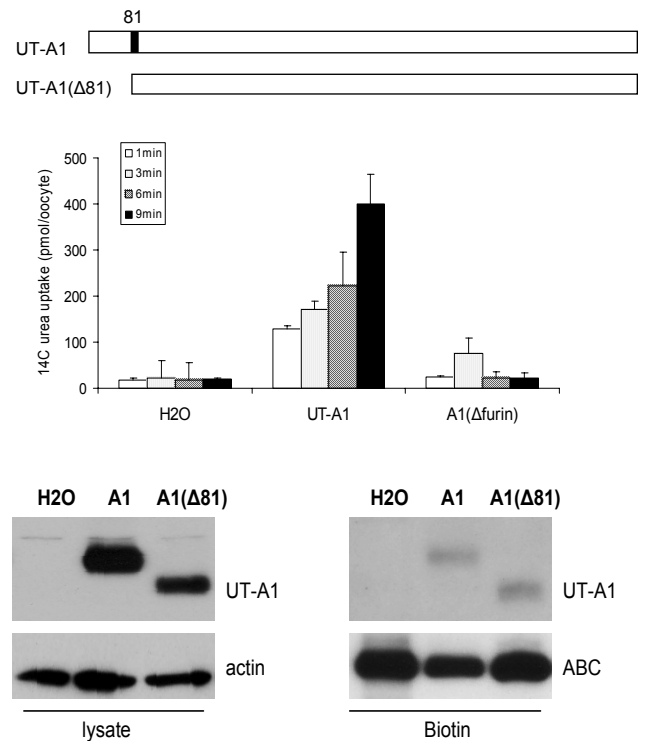


Fig. (4). Deletion of N-terminal 81-aa impairs UT-A1 activity. Oocytes were injected with cRNAs of UT-A1 or truncated UT-A1 ($\Delta 81\text{A1}$) for 3 days (top). Middle) urea transport activity was measured by ^{14}C urea flux ($n=5\sim 6$ oocytes). Bottom) Total and biotinylated UT-A1 were analyzed by immunoblotting with antibody to UT-A1. The same membranes were re-probed for actin or detected by ABC (avidin-biotin complex).

It is generally believed that the cleavage sequence for furin is Arg-X-X-Arg, but the enzyme prefers the site Arg-X-(Lys/Arg)-Arg [18]. UT-A1 has the sequence RSKR, which seems to perfectly fit the consensus sequence. However, our results showed that furin could not cleave UT-A1, either in cells or in purified UT-A1 *in vitro*. The literature reports that bone morphogenetic protein-4 (BMP-4) [23], SeMNPV virus envelope fusion protein [24], and Crimean-congo hemorrhagic fever virus glycoprotein precursor [25] all have a RSKR sequence and can be cleaved by furin. This suggests that other residues besides the simple consensus sequence may be required for efficient cleavage by furin. Krysan *et al.* [26] noticed that the ability of furin to cleave a particular protein substrate might also depend on the tertiary structure as well as the amino acids immediately surrounding the cleavage site. They found that an additional Arg at the P6 position appears to enhance cleavage. However, the three proteins listed above [23-25] do not possess Arg at the P6 position. Therefore, the consensus furin site (R-X-R/K-R) is the minimum required but may not be sufficient for furin cleavage. Additional information may be needed to clarify the nature of the furin cleavage site.

In summary, UT-A1 activation does not involve furin-dependent cleavage. The N-terminal 81-aa fragment is required for UT-A1 urea transport activity. Further study will be needed to clarify how the 81-aa fragment affects UT-A1

activity and the critical functional domains in the N-terminal end.

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