Ubiquitin-interacting Motifs Confer Full Catalytic Activity, but Not Ubiquitin Chain Substrate Specificity, to Deubiquitinating Enzyme USP37

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Background: The role of ubiquitin-interacting motifs (UIMs) in the deubiquitinating enzyme USP37 is unknown.

Results: Inactivation of the UIMs in USP37 resulted in lower isopeptidase activity toward ubiquitin chains.

Conclusion: The UIMs in USP37 are required for the full catalytic activity of the enzyme.

Significance: This study reveals a novel mechanism to increase the catalytic activity of deubiquitinating enzymes.

Ubiquitin-specific proteases (USPs) consist of a family of deubiquitinating enzymes with more than 50 members in humans. Three of them, including USP37, contain ubiquitin-interacting motifs (UIMs), an ~20-amino acid α-helical stretch that binds to ubiquitin. However, the roles of the UIMs in these USP enzymes remain unknown. USP37 has three UIMs, designated here as UIM1, UIM2, and UIM3, from the N-terminal side, between the Cys and His boxes comprising the catalytic core. Here, we examined the role of the UIMs in USP37 using its mutants that harbor mutations in the UIMs. The nuclear localization of USP37 was not affected by the UIM mutations. However, mutations in UIM2 or UIM3, but not UIM1, resulted in a significant decrease in USP37 binding to ubiquitinated proteins in the cell. In vitro, a region of USP37 harboring the three UIMs also bound to both Lys48-linked and Lys63-linked ubiquitin chains in a UIM2- and UIM3-dependent manner. The level of USP37 ubiquitination was also reduced by mutations in UIM2 or UIM3, suggesting their role in ubiquitination of USP37 itself. Finally, mutants lacking functional UIM2 or UIM3 exhibited a reduced isopeptidase activity toward ubiquitinated proteins in the cell and both Lys48-linked and Lys63-linked ubiquitin chains. These results suggested that the UIMs in USP37 contribute to the full enzymatic activity, but not ubiquitin chain substrate specificity, of USP37 possibly by holding the ubiquitin chain substrate in the proximity of the catalytic core.

Conjugation of ubiquitin, referred to as ubiquitination, is a post-translational modification of intracellular proteins that regulates the target protein functions in multiple ways (1). Deubiquitinating enzymes (DUBs) are proteases that hydrolyze the isopeptide or peptide bonds between ubiquitin and target proteins or between ubiquitin molecules in ubiquitin chains. DUBs have several important cellular functions, among which the major one is to reverse the ubiquitination-dependent regulation of protein functions (2). Namely, by removing the ubiquitin that serves as a regulatory signal tagged on target proteins, DUBs cancel the target proteins’ fate or function that was once decided by ubiquitination.

Based on the amino acid sequences of the isopeptidase catalytic core, DUBs are divided into five families: USP (ubiquitin-specific protease), UCH (ubiquitin C-terminal hydrolase), OTU (ovarian tumor-related protease), Ataxin-3/Josephin, and JAMM (Jab1/MPN/Mov34) (2). Although the JAMM DUBs are metalloproteases, DUBs in the other four families are Cys proteases. The human genome encodes ~90 DUBs, among which the USP family is the largest, comprising more than 50 members. USP enzymes harbor the conserved Cys box and His box motifs (3). Although these motifs are located apart in the primary sequences in all USPs, like other Cys proteases they form a single catalytic core in tertiary structures (4,5). In addition to the catalytic domain, most USPs contain multiple unique domains, which are considered to determine the characteristics, such as subcellular localization and substrates, of individual USPs (6).

Among the ~50 USPs in humans, three members (USP25, USP28, and USP37) harbor ubiquitin-interacting motifs (UIMs). A UIM is an ~20-amino acid ubiquitin-binding α-helical stretch with a consensus sequence of AcAcAc-Φ-AlaΦ-Ser-Ac, where Ac and Φ represent acidic (Asp or Glu) and bulky hydrophobic (Leu or Ile) residues, respectively (7). Because most USPs do not contain UIMs, it is unlikely that the UIMs in USP25, USP28, and USP37 are critical for their catalytic activity. USP25 and USP28 are paralogs of each other and have the same domain structure. The two UIMs in USP25 have been shown to be required for its own ubiquitination, although whether it is the only role for the UIMs is unknown (8). In contrast, the role of the UIMs in USP37 is totally unknown.

Human USP37 is composed of 979 amino acids, and harbors three UIMs between the Cys box and His box in the primary sequence. Although the first and second UIMs are separated by ~80 amino acids, the second and third are positioned in tan-
with only an 8-amino acid spacer between them. Other than the catalytic domain and the UIMs, USP37 has no known domain structures. Cyclin A, a cell cycle regulator essential for the S phase entry, undergoes ubiquitination via the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase complex, which targets it for proteasomal degradation during the G1 phase (9). USP37 is expressed at the highest level in the G1/S transition and promotes S phase entry of the cell by deubiquitinating and stabilizing cyclin A (10). USP37 has also been shown to deubiquitinate and stabilize other proteins such as the pro-myelocytic leukemia zinc finger/retinoic acid receptor α fusion protein (11) and the cyclin-dependent kinase inhibitor p27 (12). Consistent with the role for USP37 in counteracting proteasomal degradation of ubiquitinated substrates, USP37 cleaves Lys48-linked ubiquitin chains, a major ubiquitin chain type that directs target proteins for proteasomal degradation, more efficiently than Lys63-linked ubiquitin chains (10). Proteasomal degradation of USP37 is promoted by Lys11-linked polyubiquitination of itself at the early G1 phase and G2/M transition (13). However, the role of the UIMs in USP37 function has not been clarified.

In this study, we biochemically characterized USP37 mutants with mutations in the three UIMs and showed that two of them play an important role in ubiquitin binding, ubiquitination of USP37, and maximal catalytic activity of USP37.

**EXPERIMENTAL PROCEDURES**

**cDNA Expression Constructs**—The cDNA for human USP37 was obtained from Thermo Fisher Scientific and inserted into the N-terminally FLAG-tagged mammalian expression vector pME-FLAG. The region of USP37 containing the three UIMs (amino acids 694–855) was inserted into pGEX 6P-2 (GE Healthcare) to construct an expression vector for the GST-UIM fusion protein. Introduction of point mutations and deletions into USP37 cDNA was performed using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA).

**Cell Culture and DNA Transfection**—HeLa and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. DNA was transfected into the cells using the FuGENE 6 transfection reagents (Roche Diagnostics) for 48 h.

**Cell Lysate Preparation**—Cell lysates were prepared by solubilizing cells in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A in the presence or absence of 10 mM N-ethylmaleimide and collecting the supernatants after centrifugation. To prepare lysates using the hot SDS lysis method, cells were solubilized in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% SDS, and 1 mM EDTA for 10 min at 100 °C. After centrifugation, supernatants were diluted 4-fold with 1.33% Triton X-100.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation and immunoblotting of the cell lysates were performed using standard procedures. M2 anti-FLAG antibody (1 µg; Sigma-Aldrich) was used for immunoprecipitation. Primary antibodies for immunoblotting were: FK2 anti-ubiquitin (1 µg/ml; Medical & Biological Laboratories, Co., Ltd, Nagoya, Japan), P4G7 anti-ubiquitin (5 µg/ml; Covance, Princeton, NJ), M2 anti-FLAG (4 µg/ml), anti-GST (10 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-tubulin (0.02 µg/ml; Abcam, Cambridge, UK) antibodies. Secondary antibodies were peroxidase-conjugated anti-mouse IgG and rabbit IgG antibodies (GE Healthcare). Blots were detected using ECL Western blotting detection reagents (GE Healthcare).

**Immunofluorescence Staining**—Cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked in 5% fetal bovine serum in PBS. Cells were then incubated with rabbit anti-FLAG (2 µg/ml; Sigma-Aldrich) and FK2 anti-ubiquitin (1 µg/ml) antibodies. Secondary antibodies were Alexa Fluor 488- and Alexa Fluor 594-conjugated anti-mouse IgG and rabbit IgG antibodies (1:1,000; Invitrogen). Nuclei were stained with TO-PRO-3 iodide (642/661) (50 µm; Invitrogen) during incubation with secondary antibodies. Fluorescence images were captured with a laser-scanning confocal microscope (Axiovert 200M, Carl Zeiss, Oberkochen, Germany).

**Ubiquitin Pulldown Assay**—GST-UIM fusion proteins were purified from transformed Escherichia coli cells using glutathione-Sepharose beads (GE Healthcare). Each fusion protein (4 µg) was immobilized on glutathione-Sepharose beads (10 µl) and incubated with Lys48 (1 µg)- or Lys63 (0.2 µg)-linked ubiquitin oligomers (dimer-heptamer, Affiniti Research Products, Exeter, UK) in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, and 1 mM dithiothreitol for 16 h at 4 °C. The beads were washed with the same buffer, and bound ubiquitin oligomers were detected by immunoblotting.

**DUB Activity Assay**—FLAG-tagged USP37 and its mutants were expressed in COS-7 cells, immunoprecipitated from the cell lysates using agarose beads conjugated with anti-FLAG antibody (anti-FLAG M2 affinity gel, Sigma-Aldrich), and eluted from the beads by incubation with 120 µl of PBS containing the FLAG peptide (150 µg/ml, Sigma-Aldrich). The purity and concentration of eluted USP37 proteins were assessed by Coomassie Brilliant Blue staining using purified bovine serum albumin as a standard. Immunopurified USP37 proteins (∼5 ng; ∼2.3 nM) were incubated with 0.4 µg (∼560 nM) of Lys48- or Lys63-linked ubiquitin chains in 20 µl of PBS containing 5 mM MgCl2 and 2 mM dithiothreitol at 37 °C for 10 or 120 min. Reaction products were separated by SDS-PAGE and detected by immunoblotting.

**RESULTS**

**Construction of USP37 Mutants Lacking Functional UIMs**—Amino acid sequences of the three UIMs in human USP37, together with the consensus UIM sequence, are aligned in Fig. 1A. To study the role of the UIMs (designated as UIM1, UIM2, and UIM3 from the N-terminal side (Fig. 1B, left)) in USP37, we introduced point mutations to each UIM individually (∆UIM1, ∆UIM2, and ∆UIM3) or in combination (∆UIM123). In the mutants, the invariant Ala/Val and Ser residues (Fig. 1A, indicated with a dot in the consensus sequence) in each UIM were replaced by Gly and Ala, respectively, because this combination of mutations has been shown to fully abolish the ubiquitin binding ability of UIMs in other proteins (14, 15). We also introduced a catalytically inactivating point mutation CA, which replaces the invariant Cys residue (Cys350) in the Cys box with
UIMs Are Dispensable for Nuclear Localization of USP37—We first expressed FLAG-tagged versions of USP37 WT, USP37CA, USP37ΔUIM1, USP37ΔUIM2, USP37ΔUIM3, and USP37ΔUIM123 in HeLa cells and examined the cells by immunofluorescence staining. As reported previously (13), USP37WT was localized to the nucleoplasm (Fig. 2A). This pattern of subcellular localization was not affected in any of the UIM mutants nor in USP37CA, indicating that neither UIMs nor DUB activity are required for the nuclear localization of USP37 (Fig. 2, B–F).

To exclude the possibility that the ubiquitin binding of each UIM was not fully abolished by the point mutations, we also examined the localization of deletion mutants of USP37 in which the whole UIM sequence (amino acids 705–719 for UIM1, 807–821 for UIM2, and 830–843 for UIM3) was deleted individually or in combination. As a result, all the UIM-deleted mutants localized normally to the nucleus (data not shown).

Co-staining of the cells with FK2, a monoclonal anti-ubiquitin antibody that recognizes ubiquitin-protein conjugates and polyubiquitin chains but not free ubiquitin monomer (16), showed that overexpression of USP37WT caused a decrease in the steady state pool of ubiquitinated proteins in the nucleoplasm, suggesting that USP37 exhibits DUB activity in the nucleus (Fig. 2A', arrowheads). As expected, overexpression of catalytically inactive USP37CA was without such an effect (Fig. 2B'). When the four UIM point mutants were overexpressed, protein ubiquitination in the nucleus was decreased to a similar level to that in USP37WT-transfected cells (Fig. 2, C'–F'). Therefore, in this experimental condition where overexpressed USP37 was highly localized in the nucleus, the difference in catalytic activity was hardly observed between WT and the UIM mutants of USP37.

UIM2 and UIM3 of USP37 Bind to Endogenous Ubiquitin-Protein Conjugates—We next examined the ubiquitin binding ability of WT and the UIM point mutants of USP37 by detecting endogenous ubiquitin-protein conjugates that were co-immunoprecipitated with transfected USP37 proteins. To prevent the cleavage of ubiquitin from USP37-bound ubiquitinated proteins by the isopeptidase activity of USP37, we used UIM mutants that also harbor the catalytically inactive CA mutation (Fig. 1B, right). The CA+UIM mutants were transfected to COS-7 cells and immunoprecipitated from their lysates with anti-FLAG antibody. Immunoblotting of the precipitates with anti-ubiquitin antibody showed that USP37CA co-precipitates...
endogenous ubiquitin-protein conjugates (Fig. 3A). Although USP37\textsuperscript{CA+\textalpha UIM1} precipitated a similar amount of ubiquitin-protein conjugates to USP37\textsuperscript{CA}, the amount was significantly reduced with USP37\textsuperscript{CA+\textalpha UIM2} or USP37\textsuperscript{CA+\textalpha UIM3} and completely lost with USP37\textsuperscript{CA+\textalpha UIM123} (Fig. 3A). We obtained the same results using the UIM-deleted mutants of USP37 that also harbor the CA mutation, further supporting a minor role for UIM1 in the ubiquitin binding of USP37 (Fig. 3B).

To confirm that the ubiquitin-positive high molecular weight smear detected in the USP37 immunoprecipitates was indeed endogenous ubiquitinated proteins and not polyubiquitinated forms of FLAG-USP37 itself, we lysed the transfected cells in the presence of 1% SDS for 10 min at 100 °C (hot SDS lysis) to strip associated endogenous ubiquitinated proteins from FLAG-USP37. After dilution of the lysates, FLAG-USP37 was immunoprecipitated with anti-FLAG antibody. Immunoblotting of the precipitates with anti-ubiquitin antibody showed that the ubiquitin-positive smear disappeared after the hot SDS lysis, suggesting that they were noncovalently associated endogenous proteins (Fig. 3C). These results suggested that USP37 binds to ubiquitin-protein conjugates through UIM2 and UIM3 in the cell. However, several bands that were slightly larger than unmodified USP37 still remained after the hot SDS lysis (Fig. 3C, see below).

**UIM2 and UIM3 of USP37 Bind to Lys\textsuperscript{48-} and Lys\textsuperscript{63-linked Ubiquitin Chains in Vitro**—To further study the mode of ubiquitin binding of the USP37 UIMs, we expressed the region of USP37 harboring all three UIMs as a GST fusion protein in E. coli cells (Fig. 4A, GST-UIM\textsuperscript{WT}). We also introduced the same point mutations as in Fig. 1B to the GST fusion protein (GST-UIM\textsuperscript{A1}, UIM\textsuperscript{A2}, UIM\textsuperscript{A3}, and UIM\textsuperscript{A123}). The GST-UIM proteins were purified and incubated with Lys\textsuperscript{48-} and Lys\textsuperscript{63-linked ubiquitin oligomers (dimer-heptamer) in pulldown experiments. Detection of ubiquitin oligomers that were pulled
UIMs Elevate the Deubiquitinating Enzyme Activity of USP37

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**A**

|    | GST | UIM |
|----|-----|-----|
| WT | 1   | 2 3 |
| Δ1 |     |     |
| Δ2 |     |     |
| Δ3 |     |     |
| Δ123 |   |   |

**B**

|    | K48 | K63 | input |
|----|-----|-----|-------|
| GST WT | Δ1 | Δ2 | Δ3 |
| GST Δ123 | Δ3 | Δ2 | Δ1 |

![IB: Ub](image)

![IB: GST](image)

**FIGURE 4. UIMs in USP37 bind to both Lys48-linked and Lys63-linked ubiquitin chains.** A, schematic structures of GST-USP37 UIM fusion proteins used in this study are shown. B, indicated GST-UIM fusion proteins were immobilized on glutathione beads and incubated with Lys48- and Lys63-linked ubiquitin oligomers (dimer-heptamer). After washing the beads, bound ubiquitin oligomers were detected by immunoblotting (IB) with anti-ubiquitin antibody (Ub, top). The amounts of GST fusion proteins used in the experiment were assessed by immunoblotting with anti-GST antibody (bottom).

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down by the GST-UIM proteins using anti-ubiquitin antibody showed that GST-UIMWT binds to Lys48- and Lys63-linked ubiquitin chains at similar levels (Fig. 4B). Similarly to UIMs in other proteins (17), the UIM region of USP37 exhibited much higher affinity to long ubiquitin oligomers (tetramer –) than to a monomer-trimer of ubiquitin (Fig. 4B). Consistent with the results in Fig. 3A, introduction of mutations into UIM1 in GST-UIMΔ3 did not affect ubiquitin binding significantly, with some reduction in binding to the tetramer and, to a lesser extent, pentamer of both ubiquitin chains. In contrast, inactivation of UIM2 or UIM3 individually in GST-UIMΔ2 and GST-UIMΔ3 reduced ubiquitin binding almost to the background level, which was indistinguishable from that of GST-UIMΔ123 with all three UIms mutated. It should be noted that in the experiment shown in Fig. 4B, different amounts of Lys48 (1 μg) and Lys63 (0.2 μg) ubiquitin oligomers were incubated with the GST-UIM proteins. This was because the bands for long Lys48-linked chains (tetramer –) were less intense than those for Lys63 chains when the same amounts were immunoblotted with anti-ubiquitin antibody, probably due to the lower affinity of the antibody toward Lys48 chains (see Fig. 4B, input for comparison). The difference in the affinity of the anti-ubiquitin antibody for Lys48 and Lys63 chains makes it difficult to rigorously infer the relative affinity of the UIms for Lys48 and Lys63 chains in this experiment. Anyway, the results in Figs. 3 and 4 collectively suggested that USP37 is a ubiquitin-binding protein in which UIM2 and UIM3 are almost solely responsible for binding to Lys48- and Lys63-linked ubiquitin chains.

**UIM2 and UIM3 Are Required for Ubiquitination of USP37—**

When FLAG-tagged USP37CA, but not USP37WT, was expressed, several faint bands larger than the major ~120-kDa unmodified band were detected by anti-FLAG immunoblotting after immunoprecipitation from hot SDS lysate (Fig. 3C, second panel from top, asterisk; see also Fig. 5, middle panels, and Fig. 6A for the modification of USP37CA). These bands were clearly detected as two sharp bands when blotted with anti-ubiquitin antibody, suggesting that they correspond to ubiquitinated USP37 (Fig. 3C, top panel, asterisk). Consistent with a previous study (10), these results suggested that USP37 undergoes ubiquitination and exhibits DUB activity toward itself. Considering their sizes, they probably represent oligo-, rather than mono-, ubiquitinated forms.

Importantly, the size and intensity of these ubiquitin-positive bands were affected by introducing mutations into UIM2 or UIM3, but not UIM1, suggesting that UIM2 and UIM3 are required for the ubiquitination of USP37 itself (Fig. 3B). This was consistent with the requirement of UIms for ubiquitination of many other UIm-containing proteins (18). However, the effects of mutations in UIM2 and UIM3 were different. Although the sizes of the two ubiquitin-positive bands were reduced in USP37CA+ΔUIM2Δ3 without reduction in intensity, the intensity of the bands was further reduced in USP37CA+ΔUIM123, as well as in USP37CA+ΔUIM123, suggesting that UIM2 and UIM3 have distinct roles in USP37 polyubiquitination.

**UIM2 and UIM3 Are Required for Full Catalytic Activity of USP37 toward Endogenous Ubiquitin-Protein Conjugates—**

In addition to immunofluorescence staining of cells with anti-ubiquitin antibody (Fig. 2), we examined the total cellular level of protein ubiquitination in USP37-overexpressing cells by immunoblotting. COS-7 cells were transfected with FLAG-tagged WT and the UIM mutants of USP37, and their lysates were prepared in the presence of N-ethylmaleimide, an inhibitor of Cys protease-type DUBs including USPs, to prevent the progression of a deubiquitination reaction by overexpressed USP37 in the cell lysates. Immunoblotting of the lysates with anti-ubiquitin antibody showed that the cellular level of protein ubiquitination was not significantly different between cells transfected with USP37WT, USP37CA, and all the UIM mutants (Fig. 5A).

However, we obtained a strikingly different result when the same experiment was performed in the absence of N-ethylmaleimide during cell lysis preparation. When compared with mock-transfected cells, the level of ubiquitinated proteins, particularly high molecular weight species, was reduced in USP37WT-transfected cells (Fig. 5B, open arrowhead), suggesting that overexpressed USP37 deubiquitinated endogenous ubiquitin-protein conjugates during ~1 h after cell lysis and before mixing the lysates with boiling SDS-PAGE sample buffer. In contrast, the ubiquitination level was elevated in the
lysate of USP37CA-transfected cells, suggesting that as reported for other DUBs, the catalytically inactive mutant served as a dominant-negative protein that inhibited the DUB activity of endogenous USP37. Interestingly, although overexpression of USP37WT/H9004UIM1 had a similar effect to that of USP37WT, the ubiquitination level was more elevated in the lysates of USP37WT/H9004UIM2- and USP37WT/H9004UIM3-transfected cells than in that of USP37WT-transfected cells. The level of protein ubiquitination was even higher in the lysate of USP37WT/H9004UIM123-transfected cells. These results suggested that although USP37WT/H9004UIM2, USP37WT/H9004UIM3, and USP37WT/H9004UIM123 retain DUB activity, they are less active than USP37WT in cell lysates.

UIM2 and UIM3 Are Required for Full Catalytic Activity of USP37 toward Ubiquitin Chains—To further investigate the possibility that UIMs play an important role in regulating the catalytic activity of USP37, we performed an in vitro DUB assay using immunopurified USP37 and its UIM mutants. FLAG-tagged USP37 proteins were expressed in COS-7 cells and immunoprecipitated with anti-FLAG antibody. Precipitated proteins were eluted from the antibody with the FLAG-competing peptide. Coomassie Brilliant Blue staining of aliquots of the eluted proteins after SDS-PAGE allowed us to estimate that 1–2 μg of each protein were recovered from cells in a 60-mm dish (Fig. 6A).

We then incubated the immunopurified USP37 proteins (∼5 ng; ∼2.3 nM) with Lys48- and Lys63-linked ubiquitin oligomers (0.4 μg; 560 nM) for 10 or 120 min at 37 °C. In this reaction, the molar ratio between the enzyme (USP37) and the substrate (ubiquitin oligomers) was ∼1:240. After the incubation, the reaction products were separated by SDS-PAGE, and ubiquitin oligomers and the monomer were detected by blotting with anti-ubiquitin antibody (Fig. 6B). When incubated with Lys48-linked ubiquitin oligomers, USP37WT cleaved them almost completely after 120 min of incubation as judged from the reduced level of ubiquitin oligomers (trimer−) and the appearance of ubiquitin monomer (Fig. 6B, top). The level of ubiquitin dimer was unchanged by the incubation, suggesting that the isopeptidase activity of USP37 is lower toward ubiquitin dimer than toward longer oligomers. In contrast, USP37WT exhibited no catalytic activity. Although USP37WT/H9004UIM1 was also as active as
USP37\textsuperscript{WT} in this assay, USP37\textsuperscript{ΔUIM2} and USP37\textsuperscript{ΔUIM3} were less active as judged by the amounts of ubiquitin oligomers (trimer $\sim$) remaining and the monomer produced after incubation. The catalytic activity was more severely affected in USP37\textsuperscript{ΔUIM123}. Incubation of purified FLAG-USP37 proteins with Lys\textsuperscript{63}-linked ubiquitin oligomers showed that, as reported previously (10), the isopeptidase activity of USP37 was lower on Lys\textsuperscript{63}-linked ubiquitin chains than on Lys\textsuperscript{48}-linked chains because USP37\textsuperscript{WT} did not reduce ubiquitin oligomers at detectable levels and barely produced ubiquitin monomer after 10 min of incubation with Lys\textsuperscript{63}-linked oligomers (Fig. 6B, bottom). After 120 min, however, the level of ubiquitin oligomers (trimer $\sim$) was reduced, and the monomer was clearly detected. Again, the level of ubiquitin dimer was unchanged. As for Lys\textsuperscript{48}-linked ubiquitin oligomers, USP37\textsuperscript{ΔUIM1} was as active as USP37\textsuperscript{WT} for Lys\textsuperscript{63}-linked chains. In contrast, USP37\textsuperscript{ΔUIM2} and USP37\textsuperscript{ΔUIM3} were less active as judged from the amounts of ubiquitin oligomers and the monomer after incubation for 120 min. The activity of USP37\textsuperscript{ΔUIM123} was below the detectable level in this assay because ubiquitin monomer was hardly detected even after 120 min of incubation.

**DISCUSSION**

In this study, we investigated the role of the three UIMs in USP37 using its mutants with point mutations in the UIMs. These mutants were expressed at similar levels to USP37\textsuperscript{WT}, normally solubilized by Nonidet P-40, and solely localized to the nucleus like USP37\textsuperscript{WT}, suggesting that they folded into a native conformation of USP37. Our results showed that UIM2 and UIM3, located in tandem with an 8-amino acid spacer in the region between the Cys and His boxes, play an essential role in the acquisition of full catalytic activity of USP37.

In ubiquitin chain binding experiments in vitro, mutations in UIM1 resulted in some decrease in its affinity to ubiquitin tetramer and pentamer, but not hexamer, of both Lys\textsuperscript{48}-linked and Lys\textsuperscript{63}-linked chains (Fig. 4). However, the activity of USP37\textsuperscript{ΔUIM1} in other experiments, as well as of the UIM1-deleted mutant in some experiments, was almost indistinguishable from that of USP37\textsuperscript{WT}. Therefore, the contribution of UIM1 to the UIM-dependent functions of USP37, if any, is expected to be very low in vivo. By contrast, UIM2 and UIM3 had a critical role in ubiquitin binding of USP37 to endogenous ubiquitin-protein conjugates (Fig. 3) and purified ubiquitin chains (Fig. 4). In both experiments, ubiquitin binding was severely affected when UIM2 or UIM3 was individually mutated, suggesting that the two UIMs have a synergistic, rather than additive, role in ubiquitin binding. Although not in the context of full-length proteins, such a cooperative role is reported for tandem UIMs in other proteins, Rap80 (19) and Ataxin-3 (19, 20). Individual inactivation of UIM2 or UIM3 also affected the ubiquitination level of USP37 itself (Fig. 3) and its catalytic activity (Figs. 5 and 6). We speculate that these effects originated from the severely reduced ubiquitin binding ability of USP37 when UIM2 or UIM3 is mutated.

USP5, also known as isopeptidase T, is a DUB of the USP family. It has two tandem ubiquitin-associated (UBA) domains located at similar positions to UIM2 and UIM3 in USP37 (namely, positioned $\sim$35 amino acids before the His box and spaced by a 26-amino acid sequence). The UBA domains in USP5 facilitate the binding of USP5 to ubiquitin oligomers (21). Therefore, although no sequence homology is found between the USP37 UIM region and the USP5 UBA region (data not shown), they possibly have the same biological function.

Consistent with the fact that many UIM-containing proteins undergo ubiquitination (monoubiquitination in most cases) in a UIM-dependent manner (18), ubiquitination of USP37 is observed when cells are synchronized at late mitosis or USP37 is catalytically inactivated by the CA mutation (10). The undetectable level of ubiquitinated forms when USP37\textsuperscript{WT} is expressed suggests a DUB activity of USP37 toward itself (10). In USP37, ubiquitinated forms were observed as a smear (10) or two bands that were larger than the expected size of monoubiquitinated USP37 in immunoblotting (Fig. 3 in this study), suggesting that USP37 is modified with poly/oligo-ubiquitin chains. So far, the requirement of UIMs in USP37 ubiquitination has not been studied. Here, we showed that USP37 ubiquitination was affected when either UIM2 or UIM3, but not UIM1, was further mutated in USP37\textsuperscript{CA}, suggesting that UIM2 and UIM3 are required for the ubiquitination of USP37. Unexpectedly, however, the electrophoretic patterns of ubiquitinated USP37\textsuperscript{CA+ΔUIM2} and USP37\textsuperscript{CA+ΔUIM3} were different (Fig. 3). Although the sizes of the two bands for ubiquitinated USP37\textsuperscript{CA+ΔUIM2} were reduced when compared with ubiquitinated USP37\textsuperscript{CA}, the intensity of the bands for ubiquitinated USP37\textsuperscript{CA+ΔUIM3}, as well as for USP37\textsuperscript{CA+ΔUIM123}, was further reduced. These results suggested distinct roles for UIM2 and UIM3 in USP37 ubiquitination; UIM3 is required for the initiation of ubiquitination, whereas UIM2 is important for elongation of the ubiquitin chain. Because a previous study showed that USP37 undergoes Lys\textsuperscript{11}-linked polyubiquitination, which directs it for proteasomal degradation (10), the UIMs possibly participate in the regulation of the cellular USP37 levels through its ubiquitination. On the other hand, although UIM2 and UIM3 were required for the full catalytic activity of USP37 (Figs. 5 and 6), UIM-dependent ubiquitination of USP37 is unlikely to be responsible for the role of the UIMs because USP37\textsuperscript{WT} exhibited higher catalytic activity than USP37\textsuperscript{ΔUIM2}, USP37\textsuperscript{ΔUIM3}, and USP37\textsuperscript{ΔUIM123} when not ubiquitinated at detectable levels (Fig. 6).

The results in Figs. 5 and 6 suggest that UIM2 and UIM3 are required for USP37 to acquire full catalytic activity. Two possibilities can be raised on the role of the UIMs in regulating USP37 activity. They may elevate the activity by holding the ubiquitin chain substrate in the proximity of the catalytic core. Otherwise, binding of ubiquitin to the UIMs may allosterically activate the enzyme by inducing a conformational change of the catalytic domain. In the case of USP5, binding of monoubiquitin to the zinc finger domain, located adjacent to the catalytic core, elevates its DUB activity toward a synthetic substrate, ubiquitin-amidomethyl coumarin, suggesting that ubiquitin binding allosterically activates USP5 (21, 22). We, however, did not observe an elevated activity of purified USP37 toward ubiquitin-amidomethyl coumarin in the presence of monoubiquitin (data not shown). We therefore prefer the possibility that in
USP37, the UIMs elevate the activity by fixing the substrate in the proximity of the catalytic core, as illustrated in Fig. 7. Structural biology studies of USP37 in complex with a ubiquitin chain will be required to verify the model. On immunofluorescence staining, overexpression of USP37WT, but not catalytically inactive USP37Cys, caused a decrease in the protein ubiquitination level in the nucleus (Fig. 2), suggesting that consistent with the nuclear localization of its substrates such as cyclin A (9) and p27 (23), USP37 is localized to the nucleus and regulates the ubiquitination level of nuclear proteins. In cells overexpressing USP37ΔUIM2, USP37ΔUIM3, or USP37ΔUIM2Δ3, the nuclear ubiquitination level was reduced to a similar level to that in USP37WT-overexpressing cells, which may argue against our conclusion that UIM2 and UIM3 are required for the full DUB activity of USP37. We speculate that this inconsistency was due to the high concentration of ectopically expressed USP37 proteins in the nuclei of transfected cells, which overcame the reduced catalytic activity of the UIM mutants toward nuclear ubiquitinated proteins.

Although the tandem UIMs in Ataxin-3, a DUB of the Ataxin-3/Josephin family, bind equally to Lys48- and Lys63-linked ubiquitin chains, this DUB selectively cleaves Lys63-linked chains (24). When the UIMs are mutated, Ataxin-3 loses substrate specificity and cleaves Lys48 and Lys63 ubiquitin chains with similar efficiency, suggesting that in Ataxin-3, the UIMs play a role in rendering the ubiquitin chain substrate specificity, rather than increasing the catalytic activity, toward Lys63-linked ubiquitin chains (24). OTUD1 is a DUB of the OTU family harboring a single UIM positioned just C-terminal to the catalytic OTU domain. A recent study showed that its substrate specificity toward Lys63 ubiquitin chains is maintained in a bacterially expressed region composed of the OTU domain and the UIM, but is lost in the OTU domain alone, again suggesting a role for the UIM in determining DUB substrate specificity (25). Because USP37 prefers Lys48 (and Lys11)-linked ubiquitin chains to Lys63 chains as a substrate for its isopeptidase activity (10), it is possible that the substrate specificity of USP37 is also regulated by the UIMs. The relative affinity of the USP37 UIMs for Lys48 and Lys63 ubiquitin chains could not be rigorously inferred in this study due to the different affinity for different ubiquitin chains of the anti-ubiquitin antibody used (Fig. 4). However, mutations in UIM2 and UIM3 did not affect the ubiquitin chain substrate specificity of USP37 because the UIM mutants still cleaved Lys48 chains more efficiently than Lys63 chains (Fig. 6). Therefore, it is likely that the ubiquitin chain preference of the isopeptidase activity of USP37 is not determined by the UIMs, but by another region of the enzyme. Regions comprising the catalytic domain with the Cys and His boxes are likely candidates for such a determinant, as has been demonstrated for another USP enzyme CYLD.

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