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Analysis of the Role of Ubiquitin-interacting Motifs in Ubiquitin Binding and Ubiquitylation*[S]

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The ubiquitin-interacting motif (UIM) is a short peptide motif with the dual function of binding ubiquitin and promoting ubiquitylation. This motif is conserved throughout eukaryotes and is present in numerous proteins involved in a wide variety of cellular processes including endocytosis, protein trafficking, and signal transduction. We previously reported that the UIMs of epsin were both necessary and sufficient for its ubiquitylation. In this study, we found that many, but not all, UIM-containing proteins were ubiquitylated. When expressed as chimeric fusion proteins, most UIMs promoted ubiquitylation of the chimera. In contrast to previous studies, we found that UIMs do not exclusively promote monoubiquitylation but rather a mixture of mono-, multi-, and polyubiquitylation. However, UIM-dependent polyubiquitylation does not lead to degradation of the modified protein. UIMs also bind polyubiquitin chains of varying lengths and to different degrees, and this activity is required for UIM-dependent ubiquitylation. Mutational analysis of the UIM revealed specific amino acids that are important for both polyubiquitin binding and ubiquitin conjugation. Finally we provide evidence that UIM-dependent ubiquitylation inhibits the interaction of UIM-containing proteins with other ubiquitylated cellular proteins. Our results suggest a new model for the ubiquitylation of UIM-containing proteins.

The UIM[1] was first described as a peptide sequence consisting of a highly conserved Φ-*X*-*X*-A-*X*-*X*-*X*-S-*X*-*X*-Ac core where Φ represents a hydrophobic residue and Ac is an acidic residue (1). It was identified based on the ubiquitin binding region of the RPN10 subunit of the 26 S proteasome (2, 3). The presence of UIMs in numerous proteins ranging from the Machado-Joseph disease protein (MJD1/ataxin3) to USP25, a member of the deubiquitylating enzyme family, suggests that this region is involved in regulating protein function. Indeed our previous studies and those of others have demonstrated an important role for UIMs in both ubiquitylation and in ubiquitin binding (2–11).

Ubiquitylation is a post-translational modification resulting in the covalent attachment of ubiquitin through its COOH-terminal Gly to the ϵ-NH_2 group of a Lys residue in a target protein. This process involves a multienzyme cascade that begins with the activation of ubiquitin in the presence of ATP and an E1 ubiquitin-activating enzyme. Subsequently the ubiquitin is transferred through a thiol-ester bond to a ubiquitin-conjugating enzyme (E2) and through the action of an E3 ubiquitin ligase is attached to the substrate by an isopeptide bond. Polyubiquitylation, the attachment of multimeric chains of ubiquitin, leads to the proteolytic destruction of proteins when Lys^{48} of ubiquitin is the site of chain formation. However, ubiquitin chains formed through Lys^{63} are not involved in protein degradation but rather a variety of processes including DNA repair, translation, IκB kinase activation, endocytosis, and protein transport (for a review, see Ref. 12). In contrast to polyubiquitylation, some proteins are modified by the attachment of a single ubiquitin, termed monoubiquitylation (13). A variation on this process is the attachment of single ubiquitin molecules to multiple lysines within a protein, hereafter referred to as multiubiquitylation. Although the precise function of mono- and multiubiquitylation is still unclear, studies indicate that these modifications are important for endocytosis, transcriptional regulation, and trafficking of receptors to the lysosome (for a review, see Ref. 12). Thus, the ubiquitylation pathway controls protein function and cell fate in a number of ways.

Although the precise function of UIM-directed ubiquitylation is still unclear, a number of studies suggest that this modification is physiologically important. Genetic studies of photoreceptor development in *Drosophila* have identified an epsin ortholog, termed Liquid facets (Lqf), as an important mediator of photoreceptor cell fate (14) and also uncovered a role for a deubiquitylating enzyme, Fat facets (Faf), in regulating Lqf function. Loss-of-function mutations in Faf result in a phenotype similar to loss-of-function mutations in Lqf (14) and biochemically result in Lqf polyubiquitylation and degradation (15). However, in mammalian cells, epsin is not targeted for polyubiquitylation and degradation but rather is monoubiquitylated (8, 9). A recent study from DeCamilli and colleagues (16) provides compelling evidence for a rapid Ca^{+2}-dependent deubiquitylation of epsin mediated by the mammalian counterpart of Faf. This deubiquitylation restores the ability of epsin to interact with phospholipid vesicles as well as compo-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1–3.

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[1] The abbreviations used are: UIM, ubiquitin-interacting motif; HA, hemagglutinin; Ub, ubiquitin; UbWT, wild type ubiquitin; UbΔK, ubiquitin devoid of lysine residues; YFP, yellow fluorescent protein; GFP, green fluorescent protein; GST, glutathione *S*-transferase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; aa, amino acid(s); MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; MJD, Machado-Joseph disease protein; HEK, human embryonic kidney; WT, wild type; CUE domain, coupling of ubiquitin conjugation to ER degradation.

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nents of the clathrin coat thereby providing the first direct evidence for a physiological role for UIM-dependent ubiquitylation. Although less direct, genetic studies in yeast have demonstrated that mutations in UIMs of proteins also lead to defects in protein trafficking (10, 11). Although the phenotypes of these mutations were ascribed to loss of ubiquitin binding by the UIMs, these mutations would also block ubiquitylation of the UIM proteins. Thus, the contribution of these two activities of the UIMs, *i.e.* ubiquitylation and ubiquitin binding, cannot be discerned from these experiments.

Given previous findings and the observation that UIMs are present in a wide variety of proteins, we initiated studies of the UIMs from numerous proteins to evaluate whether these motifs play a general role in ubiquitylation. In addition, we performed a structure-function analysis to gain insight into the mechanism of UIM-dependent ubiquitylation. The results presented here demonstrate that UIMs, in general, bind polyubiquitin chains and promote ubiquitylation. Additionally ubiquitylation of UIM proteins appears to inhibit their interaction with ubiquitylated targets. Finally our data suggest a new mechanism for UIM-directed ubiquitylation.

MATERIALS AND METHODS

Cells and Reagents—Human embryonic kidney (293T) cells were cultured as described previously (8). Antibodies used for these studies include monoclonal anti-hemagglutinin (HA) (Babco); anti-glutathione *S*-transferase (GST) conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.); full-length A.V. polyclonal antibody, Living Colors peptide antibody to GFP, and its variants (Clontech); monoclonal antibody against ubiquitin (Covance); and anti-FLAG M5 monoclonal antibody (Sigma).

Constructs—The pCGN-epsin, pKU-Hrs, and pMT3-MEKK1 constructs were described previously (8, 17, 18). pCGN-MJD was constructed by PCR amplification of A431 cDNA with 5′MJDFULL (GACGGATCCATGGAGTCCATCTTCCACGAGA) and 3′MJDFULL (GACGGATCCGATATCTTATTTTTTTCCTTCTGTTTT). The fragment was subsequently digested with BamHI and EcoRV and subcloned into the pCGN-Hyg expression vector (a gift of M. Ostrowski) digested with NotI, blunt-ended with Klenow fragment, and then digested with BamHI. pCGN-KIAA1386 was constructed by digesting pBluescript-KIAA1386 (a gift of K. Nagase) with BglII and HpaI. The resulting fragment was subcloned into pCGN-Hyg digested with BamHI. pCGN-HSJ1 was constructed by PCR amplification of HSJ1B (16) with 5′HSJFULL (GACGGATCCATGGCATCCTACTACGAGATCCTA) and 3′HSJFULL (GACGGATCCTCAGGATACTCCCCTCTTCTCTTCCCA), digestion of the product with BamHI, and subcloning into pCGN-Hyg digested with BamHI.

The following UIM constructs were constructed by PCR amplification of A431 cDNA: Hrs UIM (aa 252–288) with 5′HRS_UIM (GACGGATCCAAGAGGGACGAGACGGCCCTG) and 3′HRS_UIM (GACGATATCAGTGTACGTGGACTTCTGTCT), USP25 UIM (aa 88–117) with 5′USP_UIM (GACGGATCCGATACAAATGTGATTGATCTCA) and 3′USP25_UIM (GACGATATCAATGGCTTGTTCCTCATCAGTT), MJD1 UIM (aa 216–356) with 5′MJDcorrect (GACGGATCCAATGATGGCTCAGGAATGTT) and 3′MJD_UIM (GACGATATCTGTTTTCAAATCATTTCTGACA), RPN10 UIM (aa 203–329) with 5′HS5_UIM (GACGGATCCGACTTTGAATTTGGAGTAGAT) and 3′HS5_UIM (GACGATATCGGCATCAATGTCTGTGATT), KIAA1386 UIM (aa 974–1010) with 5′K1386_UIM (GACGGATCCTTGGATGAAGACGATCCCAATATA) and 3′K1386_UIM (GACGATATCTAAGGATGCTTCATTACTGAGGAA), HSJ UIM (aa 235–294) with 5′HSJ_UIM (GACGGATCCCAGGTCCAGCAGACCCCTGCCT) and 3′HSJ_UIM (GACGATATCCTGGATCTTGGTGCTGGGCCTT), KIAA1594 UIM (aa 646–799) with 5′K1594_UIM (GACAGATCTGAATTGGAAAACTCAGGATTT) and 3′-K1594_UIM (GACGATATCGGAGTTGTTAAACTCTTGAAGA), and MEKK1 UIM (aa 1162–1186) with 5′MEKK1_UIM (GACGGATCCATGGAGGCTGAGGAAGAGGAGGCGCT) and 3′MEKK1_UIM (GACGATATCTATGGGGAGGGCGTCCTGAGAAGCCGA). Each resulting product except KIAA1594 was digested with BamHI and EcoRV. The KIAA1594 product was digested with BglII and EcoRV. The fragments were then subcloned into pEFG (8) digested with BamHI and EcoRV, pEYFP-C1 (Clontech) digested with BglII and SmaI, and pGEX (Amersham Biosciences) digested with BamHI and EcoRV. GST-epsin (UIM1–3) and GST-Eps15 (UIMwt) were described previously (8). pGEX epsin, pGEX eps15, yellow fluorescent protein (YFP)-epsin, and YFP-eps15 were made by digesting GST-epsin and GST-eps15 with BamHI and SmaI and subcloning into pEYFP digested with BglII and SmaI and pGEX digested with BamHI and EcoRV.

Mutant versions of Hrs and USP25 UIMs were created using Qiagen multisite-directed mutagenesis kits with the following primers: HrsL265R with HRSmutL-R (GAGGAGGAGCTGCAGCGGGCCCTGGCGCTGTC), USP25R104L with USPmutR-L (GATGATCTTCAGCTAGCAATTGCCTTG), USP25L110Q/A111S with USPmutLA-QS (AATTGCCTTGAGTCAGGCCGAATCAAACA), and Hrs Q271L/S272A with HRSmutQS-LA (GCCCTGGCGCTGTCATTGGCAGAGGCGGAGGA). Hrs triple mutant and USP25 triple mutant were created using both corresponding primers. The pGEX versions of these constructs were created by PCR amplifying the UIMs from the YFP mutated versions using 5′HRS_UIM, 3′HRS_UIM, 5′USP_UIM, and 3′USP_UIM. Fragments were then digested with BamHI and EcoRV and subcloned into pGEX digested with BamHI and EcoRV.

The UIMs of Hrs, MJD, and RPN10 were PCR-amplified from the YFP-UIM DNA using the 3′ UIM primers from above and the following 5′ primers: RPN10-YFP with 5S5a-YFP (GACAAGCTTACCACCATGGACTTTGAATTTGGAGTAGAT), Hrs-YFP with 5HRS-YFP (GACAAGCTTACCACCATGGACGAGACGGCCCTGCAGGA), and MJD-YFP with 5MJD-YFP (GACAAGCTTACCACCATGGCAAATGATGGCTCAGGAAT). Each primer included the addition of a start site at the beginning of the sequence. The resulting products were digested with BamHI and EcoRV and subsequently subcloned into the pEFYP-N1 vector (Clontech) digested with BglII and SmaI.

The HA epitope-tagged ubiquitin expression construct pMT123 used in the GST fusion protein experiments has been described previously (19) and was kindly provided by Dr. Dirk Bohmann. For determining the type of ubiquitylation and YFP fusion protein experiments, FLAG-tagged ubiquitin was created by digesting the pcDNA3 HA-ubiquitin construct kindly provided by Dr. Cam Patterson with BamHI and EcoRI. The fragment was then subcloned into the pFLAG-CMV-2 vector digested with SmaI and XbaI (Sigma). The UbΔK construct was created by first mutating lysines 6, 11, 27, and 33 of the pCDNA3 HA-UbK29R/K48R/K63R mutant construct kindly provided by Dr. Cam Patterson using the Qiagen multisite-directed mutagenesis kit and the following 5′ phosphorylated primers: Lysine 6, CAGATCTTCGTGAGGACCCTTACCGGC; Lysine 11, ACCCCTTACCGGCAGGACCATCACCCTT; Lysine 27, CATCGAAAATGTGAGGGCCAGGATCCAGG, and Lysine 33, CAAGATCCAGGATAGAGAAGGCATCCCTC. The construct was then digested with NheI and EcoRV and subcloned into the pFLAG-CMV-2 vector digested with SmaI and XbaI. FLAG-UbI44A was created by mutating isoleucine 44 to alanine of the pFLAG-CMV-2 Ub construct using the Qiagen multisite-directed mutagenesis kit and the following primer: UB I44A, CAGCAGAGGCTCGCGTTTGCAGGCAAG. All constructs were verified by DNA sequence analysis.

Ubiquitylation Assays—Assays were performed essentially as described previously (8). Briefly HEK 293T cells were transiently transfected with 2 μg of HA-tagged epsin, MJD1, HSJ1, Hrs, KIAA1386, or MEKK1 and 4 μg of FLAG-tagged UbWT, UbΔK, or UbI44A. Fusion proteins were purified from lysates (1 mg of protein) using HA affinity beads (Affiniti). Precipitates were washed five times with PLC-LB as described previously (8), fractionated by SDS-PAGE, and then transferred to Immobilon P membranes. Membranes were probed with either anti-HA to detect expression of the full-length proteins or anti-FLAG to detect ubiquitylation. Western blot analysis of 30–40 μg of lysates verified equal expression of the UbWT, UbΔK, and UbI44A.

Similarly HEK 293T cells were transiently transfected with 2 μg of various YFP constructs along with 4 μg of FLAG-tagged UbWT, UbΔK, or UbI44A. Lysates were immunoprecipitated with the full-length A.V. polyclonal antibody (Clontech), fractionated by SDS-PAGE, and then immunoblotted with anti-FLAG antibody to detect ubiquitylation. Expression of the YFP-UIM or UIM-YFP proteins was detected by immunoblotting with Living Colors peptide antibody (Clontech). The GST pull-down experiments were done as described previously (8).

Treatment with harsher lysis conditions was done by adding $\frac{1}{10}$ volume of 10% sodium deoxycholate and $\frac{1}{100}$ volume of 10% SDS to the lysate prior to immunoprecipitation. The immunoprecipitates were then washed twice with PLC-LB supplemented with 1% sodium deoxycholate and 0.1% SDS and twice with PLC-LB containing no sodium deoxycholate or SDS.

Ubiquitin Binding Assays—The wild type or mutant GST-UIM constructs were expressed in *Escherichia coli* and purified using GSH beads. The GST fusion proteins were diluted to 200 pmol/50 μl of a 50% slurry of the following lysis buffer: 25 mM HEPES, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 0.5%

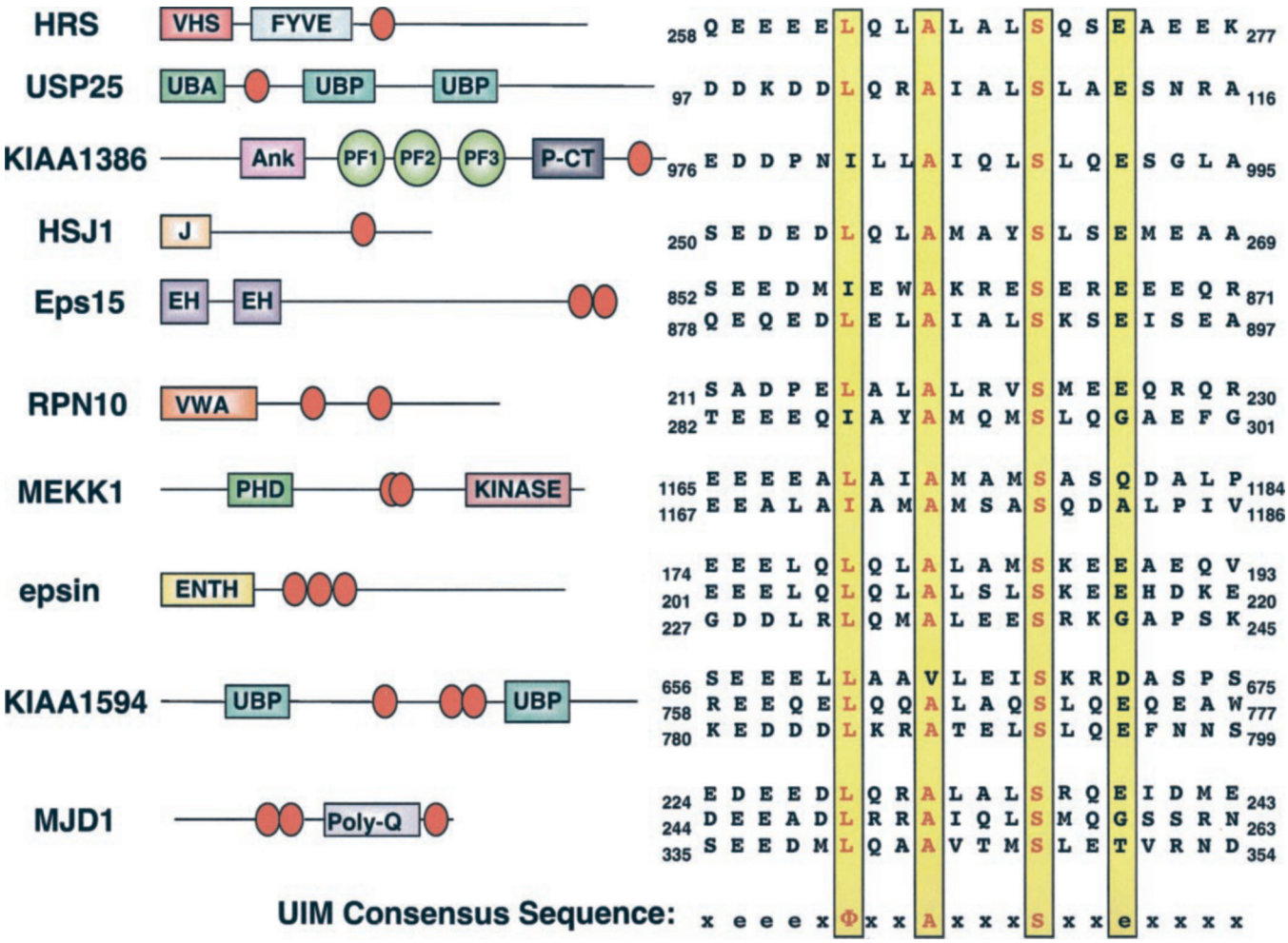

FIG. 1. **The UIM is present in numerous proteins with varying functions.** These proteins range from endocytic adaptor proteins epsin and Eps15 to USP25, a deubiquitylating enzyme linked to Down syndrome. UIMs are represented by *red ovals*. Each UIM consists of a conserved core sequence, *X*eee*X*Φ*XX*A*XXX*S*XX*e where e is a negatively charged residue, Φ is a hydrophobic residue, and *X* is any amino acid. Conserved residues are highlighted in *yellow*. Sequences conserved in >50% of the UIMs as described by Hoffman and Falquet (1) are highlighted in *red*. Note that the UIMs in MEKK1 are overlapping. *Ank*, ankyrin repeat region; *EH*, Eps15 homology domain; *ENTH*, epsin NH_2-terminal homology domain; *FYVE*, FYVE finger domain; *J*, dnaJ chaperone domain; *KINASE*, protein kinase catalytic domain; *P-CT*, parkin COOH-terminal domain; *PF1–3*, parkin-type complex zinc finger domains 1–3; *Poly-Q*, polyglutamine repeat region; *UBA*, ubiquitin-associated domain; *UBP*, bipartite ubiquitin protease catalytic domain; *VHS*, Vps27-HRS-Stam domain; *VWA*, von Willebrand factor type A domain; *PHD*, plant homeodomain.

Triton X-100, 1 mM dithiothreitol plus protease inhibitors (as described in Ref. 8) supplemented with 1 mg/ml bovine serum albumin. 50 μl of the GST proteins in 450 μl of lysis buffer were mixed with 1 μl of ubiquitin or polyubiquitin ranging from two to seven ubiquitins (Affiniti) at a concentration of 0.25 μg/μl. Samples were incubated at 4 °C for 2 h. Beads were pelleted and washed twice with 500 μl of lysis buffer without bovine serum albumin. Samples were fractionated on a gel and detected via Gel Code© blue stain. Alternatively gels were transferred to an Immobilon P filter and preincubated with denaturing buffer (6 M guanidine HCl, 20 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) as described in Ref. 20. After washing extensively with phosphate-buffered saline, the filter was immersed in 100% methanol for 5 min, placed on filter paper, dried for 15 min, and then probed with α-ubiquitin.

RESULTS

Ubiquitylation of UIM-containing Proteins—To gain understanding into the function of UIMs in ubiquitylation and ubiquitin binding, we evaluated the biochemical properties of UIMs from a variety of proteins (Fig. 1). These proteins were chosen based on their involvement in endocytosis, signal transduction pathways, and various human diseases. RPN10 is a subunit of the 26 S proteasome that specifically binds polyubiquitin chains and was the founding member of the UIM family of proteins (2, 3). Hrs is a tyrosine kinase receptor substrate that has been implicated in the endocytosis of growth factor-receptor complexes (21), while USP25 is thought to be a deubiquitylating enzyme linked to Down syndrome (22). KIAA1386 and KIAA1594 are uncharacterized proteins (23). Similar to USP25, KIAA1594 contains two bipartite ubiquitin protease catalytic domains suggesting it may function as a deubiquitylating enzyme. KIAA1386 contains three parkin-type complex zinc finger domains and one parkin COOH-terminal domain suggesting a link to Parkinson's disease. HSJ1 is a neuron-specific protein involved in clathrin uncoating (24), while MJD1 is a polyglutamine tract protein that undergoes expansion of its polyglutamine region leading to the neurodegenerative disorder Machado-Joseph disease (25). MEKK1 is a Ser/Thr kinase involved in activation of the mitogen-activated protein kinase pathway and also contains a plant homeodomain domain that functions as a non-canonical RING finger E3 ligase (26, 27). Previous studies have shown that epsin, Eps15/Eps15R, and Hrs are monoubiquitylated in a UIM-dependent manner (6–9). We tested the possibility that UIM-containing proteins in general might undergo a similar post-translational modification. HA epitope-tagged MJD1, Hrs, HSJ1, epsin, KIAA1386, or MEKK1 were transiently co-expressed in 293T HEK cells along with FLAG epitope-tagged ubiquitin (UbWT). Western blot analysis of HA immunoprecipi-

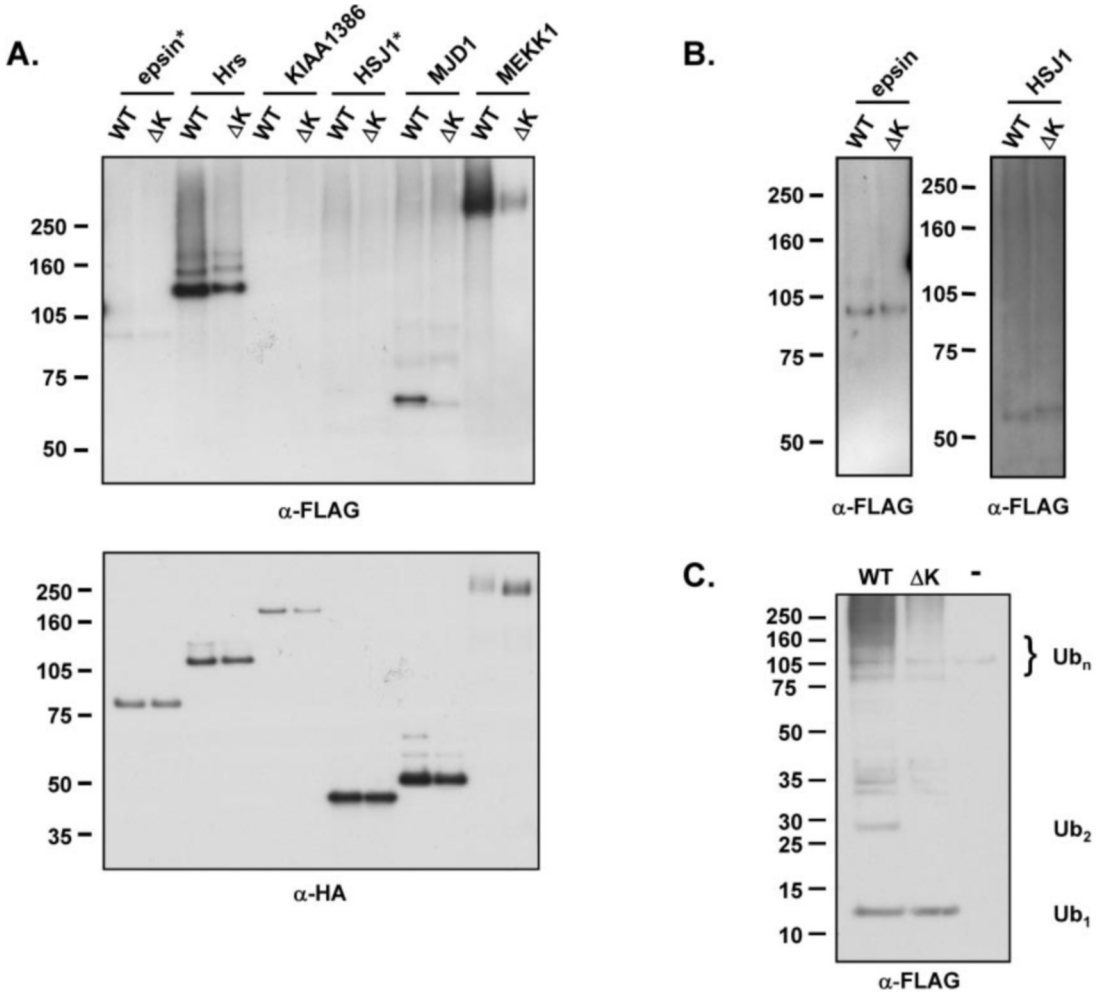

FIG. 2. **Ubiquitylation of the full-length proteins.** *A*, full-length HA-epitope tagged UIM-containing proteins were co-expressed with either FLAG-tagged wild type ubiquitin (*WT*) or ubiquitin with all lysines mutated to arginines (Δ*K*) in 293T HEK cells. Following immunoprecipitation with HA affinity beads in the presence of SDS and deoxycholate, proteins were fractionated on gels, transferred to Immobilon P filters, and probed with either α-FLAG (*top*) or α-HA (*bottom*). *, longer exposures (*B*) revealed ubiquitylation of epsin and HSJ1 in both lanes, although the levels were lower in the UbΔK samples. Although the levels of UbWT and UbΔK expression were equivalent with a given protein, the expression of these two vary between the different UIM proteins. *C*, to verify equal expression and lack of ubiquitin chain formation by the mutant ubiquitin, FLAG-UbWT and -UbΔK were expressed in 293T cells, and equivalent amounts of cell lysates were analyzed by Western blot using the FLAG antibody.

tates revealed that all the full-length proteins were expressed at similar but not identical levels (Fig. 2*A*, *bottom panel*). In addition, there were slower migrating HA-reactive bands in the Hrs and MJD1 samples that were shifted by 13-kDa increments consistent with the attachment of FLAG ubiquitin. Indeed these shifted forms reacted with antibodies to tagged ubiquitin (Fig. 2*A*, *top panel*). In the case of MJD1, the first shifted band in the HA Western blot did not react with antibodies to FLAG ubiquitin indicating that the altered mobility was not due to ubiquitylation. Although MEKK1 did react with α-FLAG antibody indicating it was ubiquitylated, it was not possible to resolve a molecular weight shift due to the size of MEKK1. In contrast, KIAA1386 was not ubiquitylated. Multiple shifted forms of MJD1, Hrs, epsin, HSJ1, and MEKK1 were detected with antibodies to FLAG-ubiquitin suggesting that these UIMs promoted polyubiquitylation. To determine whether these higher molecular weight forms represented polyubiquitylation, multiubiquitylation, or a combination of these two possibilities, the HA-tagged full-length proteins were transiently co-expressed with FLAG epitope-tagged ubiquitin devoid of Lys (UbΔK) and thus no longer capable of forming chains. Ubiquitylation of the full-length proteins was decreased in cells expressing UbΔK indicating that epsin and Hrs as well as MJD1, MEKK1, and HSJ1 were at least partially polyubiquitylated. Longer exposures uncovered shifted bands with the expression of UbΔK suggesting that these proteins may also be modified by the attachment of multiple monoubiquitins (Fig. 2 and data not shown). Furthermore a decrease in the level of monoubiquitylation of several of these proteins was observed with the UbΔK as compared with UbWT suggesting that polyubiquitin chains are required for monoubiquitylation. These differences in ubiquitylation were not due to differences in the steady state levels of the mono-UbWT and mono-UbΔK as both were expressed equally (Fig. 2*C*). Treatment of cells with proteasome inhibitor (MG132) did not alter the levels of the ubiquitylated or non-ubiquitylated UIM-containing proteins (data not shown) consistent with previous studies (6–9).

Our analysis of the UIM-containing proteins also indicated that these proteins associated with ubiquitylated cellular proteins consistent with a role for the UIMs in binding ubiquitin (Supplemental Fig. 1). These high molecular weight FLAG-reactive bands corresponded to ubiquitylated cellular proteins rather than polyubiquitylated UIM proteins since their presence was dramatically reduced by the inclusion of SDS and deoxycholate in the lysis buffer (Fig. 2 and Supplementary Fig. 1). Furthermore these high molecular weight FLAG-reac-

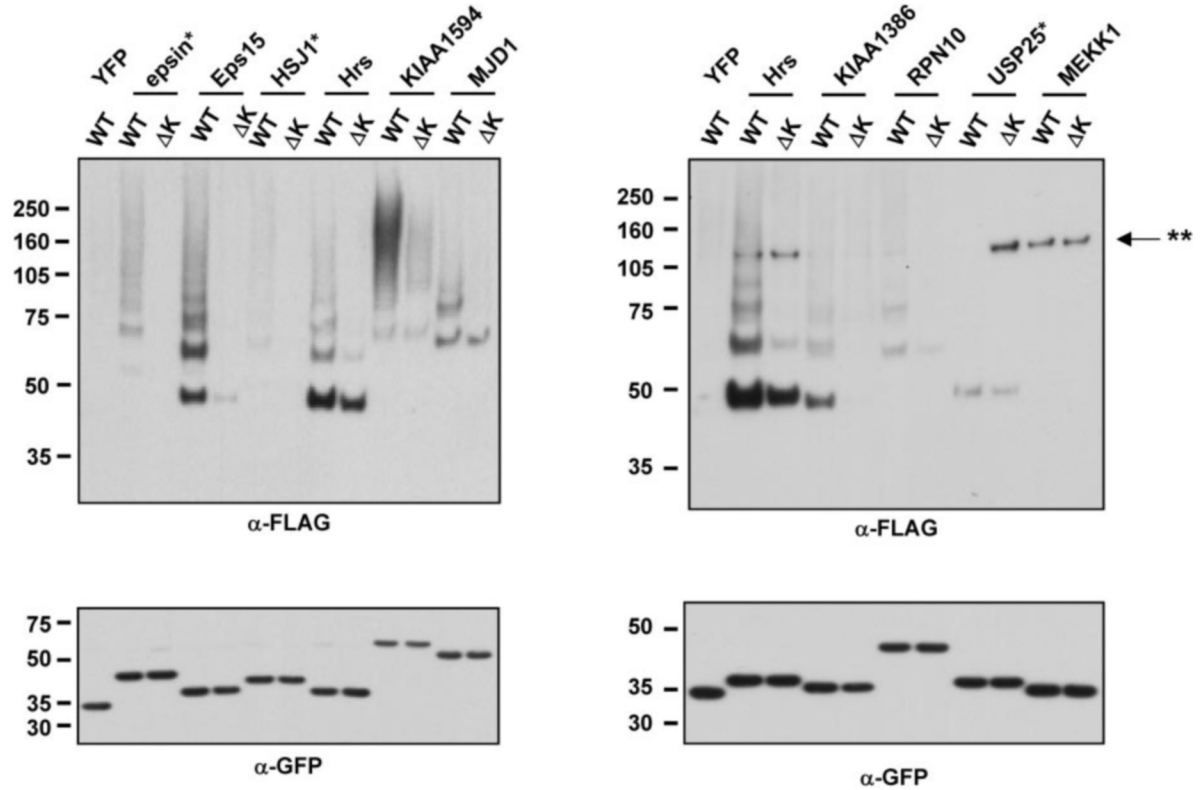

FIG. 3. **The isolated UIMs are sufficient to promote ubiquitylation of a chimeric YFP fusion protein.** The YFP-UIM constructs or YFP alone were co-expressed with FLAG-tagged UbWT or UbΔK in 293T HEK cells. Following immunoprecipitation with GFP antibody in the presence of SDS and deoxycholate, proteins were fractionated on gels, transferred to Immobilon P filters and probed with α-FLAG (*top*) or α-GFP (*bottom*). *, longer exposures (data not shown) revealed ubiquitylation of epsin, HSJ1, and USP25 in both lanes, although the levels were lower in the UbΔK samples. ** represents a contaminating band. Expression of UbWT and UbΔK were the same as in Fig. 2.

tive bands were not visible on longer exposure of the HA Western blot of the HA immunoprecipitates (data not shown).

UIMs of Various Proteins Are Sufficient to Promote Ubiquitylation of a Chimeric Fusion Protein—To determine whether the isolated UIMs of the aforementioned proteins promoted ubiquitylation, YFP-UIM chimeras were expressed in 293T cells along with FLAG-tagged ubiquitin. Western blot analysis demonstrated UIM-dependent ubiquitylation of these heterologous fusion proteins to varying degrees with the highest level by Eps15 and Hrs and the lowest level by USP25 and HSJ1 (Fig. 3). The MEKK1 UIM exhibited no ubiquitylation activity, although full-length MEKK1 was highly ubiquitylated (Fig. 2).

With several of the YFP-UIM fusion proteins, we detected a ladder of ubiquitin conjugates. As with the full-length proteins in Fig. 2, co-expression of UbΔK resulted in a dramatic reduction in these ladders indicating that the proteins were polyubiquitylated. Longer exposures revealed multiple shifted forms with the UbΔK indicating that a portion of these shifted bands resulted from the attachment of multiple monoubiquitins. Furthermore expression of UbΔK also resulted in reduction in the monoubiquitylated form of the YFP chimeras similar to results with the full-length proteins. Treatment with MG132 did not alter the levels of either the unmodified or ubiquitylated fusion proteins (data not shown). The YFP-UIM proteins associated with ubiquitylated cellular proteins as evidenced by a high molecular weight smear of proteins that reacted with the FLAG antibody and were dramatically reduced when SDS and deoxycholate were included in the lysis buffer (Fig. 3 and Supplementary Fig. 2).

To address whether the UIM was the site of ubiquitylation, GST-tagged versions of the isolated UIMs were co-expressed along with HA-Ub in 293T HEK cells as described previously (8). Many of the GST chimeric fusion proteins were ubiquitylated but to varying degrees similar to the YFP-UIM fusion proteins (Supplementary Fig. 3). Purification of the GST fusion proteins from cells followed by thrombin cleavage revealed that the GST and not the UIM portion of the fusion protein was the site of ubiquitin attachment even though a number of these UIMs contain lysine residues (Supplemental Fig. 3). However, in the case of KIAA1594, we detected ubiquitylation of the UIM portion of the fusion protein suggesting that one or more of the lysines present in the region between the UIMs may function as ubiquitylation sites.

UIM-dependent Ubiquitylation Occurs in an Orientation-dependent Manner—Our previous results with epsin suggested that ubiquitylation occurs within the epsin NH_2-terminal homology domain (8). Given the preponderance of UIMs in the central and COOH terminus of proteins (1), we tested the possibility that UIMs promoted ubiquitylation in an orientation-specific manner. YFP was fused to either the NH_2 terminus or COOH terminus of the UIMs of Hrs, RPN10, and MJD and then co-expressed with epitope-tagged ubiquitin. As seen previously, the YFP-UIM proteins (N1 and N2) were ubiquitylated; however, the UIM-YFP fusion proteins (C1 and C2) were significantly reduced in their ubiquitylation even though both forms of the proteins were expressed equally (Fig. 4). In addition, the UIM-YFP chimeric proteins all bound to high molecular weight ubiquitylated proteins to a greater extent than the YFP-UIM proteins. Lysis of cells in buffer containing 0.1% SDS and 1% sodium deoxycholate dramatically reduced levels of these high molecular weight bands without affecting the level of the YFP fusion proteins. Thus, these proteins represent ubiquitylated UIM-associated proteins and not polyubiquitylated forms of the YFP fusion protein.

Mutational Analysis of the UIM—Given the differences in ubiquitylation between UIMs, we tested whether specific

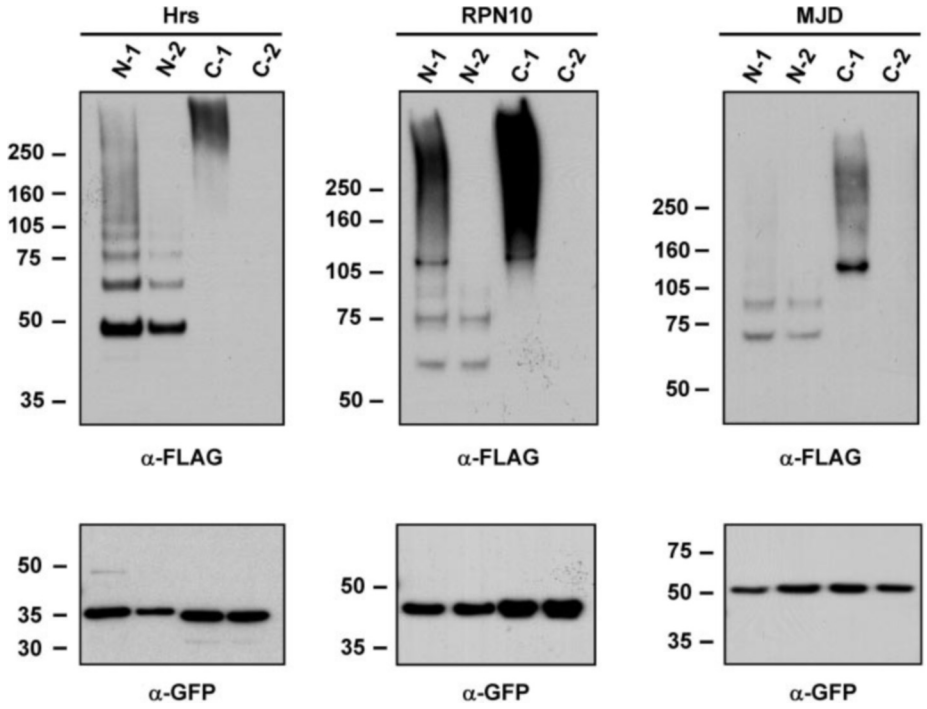

FIG. 4. **Isolated UIMs promote ubiquitylation in an orientation-dependent manner.** The isolated UIMs from Hrs, RPN10, or MJD were fused with YFP as either NH_2-terminal or COOH-terminal chimeras and then co-expressed with UbWT into 293T cells. Following immunoprecipitation with anti-GFP, the proteins were fractionated on gels, transferred to Immobilon P filters, and probed with α-GFP (*bottom*) or α-FLAG (*top*). To distinguish between ubiquitylation of UIM proteins *versus* the association of ubiquitylated proteins, lysis buffer lacking (*1*) or supplemented with (*2*) 0.1% SDS and 1% sodium deoxycholate was used.

amino acid mutations might alter ubiquitylation efficiency by a particular UIM. Hrs and USP25 were chosen for comparison because each contains a single UIM, and they are representative of the range of ubiquitylation promoted by the various UIMs. Sequence comparison revealed two significant differences between the UIMs from Hrs and USP25 that might influence UIM function: a leucine *versus* arginine (amino acid 265 of Hrs) and glutamine-serine *versus* leucine-alanine (amino acids 271 and 272 of Hrs) (Fig. 1). We mutated these regions either alone or in combination to determine their importance for UIM function. YFP fusions of either wild type or mutant Hrs and USP25 UIMs were co-expressed in 293T cells along with UbWT. HrsL265R was impaired in ubiquitylation (94% decrease relative to Hrs wild type), while the corresponding mutation in USP25 (R104L) enhanced ubiquitylation by more than 7-fold as compared with the wild type USP25 UIM (Fig. 5). In contrast, ubiquitylation of Hrs Q271L/S272A was similar to wild type Hrs. The Hrs triple mutant demonstrated a 49% decrease in ubiquitylation, whereas USP25L110Q/A111S and USP25 triple mutants demonstrated no ubiquitylation. Thus, the choice of amino acid at these three positions plays an important role in dictating whether a UIM will promote ubiquitylation.

Ubiquitin Binding by the UIM—Although the UIM promotes ubiquitylation, several groups have demonstrated that UIMs bind ubiquitin (2–4, 9–11, 28, 29). In addition, ubiquitin binding activity appears necessary for the ability of yeast epsins ENT1p and ENT2p to promote receptor internalization (11). Using an *in vitro* ubiquitin binding assay, we found that most UIMs bound polyubiquitin chains to varying degrees (Fig. 6*A*). In contrast, the MEKK1 UIM did not bind polyubiquitin. As with the ubiquitylation assays, the ability to bind polyubiquitin did not correlate with the number of UIMs present in the protein. In addition, different UIMs exhibited preferences for different length ubiquitin chains (Table I). We did not observe detectable binding of any UIM to monoubiquitin (data not shown), although others have reported low affinity binding (9, 10, 30). Thus, UIMs appear to preferentially bind polyubiquitin chains *versus* monoubiquitin. This selectivity was not due to an additive effect since there was selectivity in the recognition of different length ubiquitin chains by particular UIMs (see Table I).

The dual role for the UIM in binding ubiquitin and promoting ubiquitylation prompted us to test whether mutations that affected ubiquitylation also affected ubiquitin binding. GST fusion proteins of the Hrs and USP25 UIM mutants described above were purified and used in an *in vitro* ubiquitin binding assay as described above. Quantitation of the binding data indicated that the same mutations that altered ubiquitylation of the YFP-UIM proteins also altered polyubiquitin binding (Fig. 6, *B* and *C*). The HrsL265R mutation dramatically decreased polyubiquitin binding by 88%. Conversely the USP25R104L mutant had a 2–3-fold increase in polyubiquitin binding relative to the wild type UIM. Similar to their ubiquitylation properties, USP25L110Q/A111S and USP25 triple mutants possessed very weak polyubiquitin binding activity. Indeed of six experiments only one resulted in any measurable binding. In contrast to their ubiquitylation pattern, Hrs Q271L/S272A exhibited a 2-fold binding increase, whereas the Hrs triple mutant showed binding similar to the WT Hrs.

Ubiquitin Binding Is Necessary for UIM-dependent Ubiquitylation—The ubiquitin binding properties of the mutant UIMs suggested that polyubiquitin binding may be necessary for UIM-dependent ubiquitylation. To address this question, we tested whether the UIMs were able to promote ubiquitylation when co-expressed with a mutant ubiquitin (I44A) no longer capable of binding to ubiquitin binding domains such as CUEs and UIMs (11, 31–33). Co-expression of either WT or I44A ubiquitin along with full-length proteins demonstrated that ubiquitin binding is important for ubiquitylation of UIM-containing proteins (Fig. 7). In contrast to previous experiments, we did not include SDS and deoxycholate in the lysis buffer so that we could examine both ubiquitylation of the UIM proteins as well as binding to ubiquitylated proteins by the UIM proteins. As with the full-length proteins, ubiquitylation of the chimeric YFP-UIM proteins was also dramatically decreased in the I44A ubiquitin-expressing cells suggesting that ubiquitin binding is necessary for UIM-directed ubiquitylation. As expected, the expression of I44A ubiquitin also resulted in a decreased association of the UIM proteins with ubiquitylated proteins (Fig. 7). These results were not due to differential

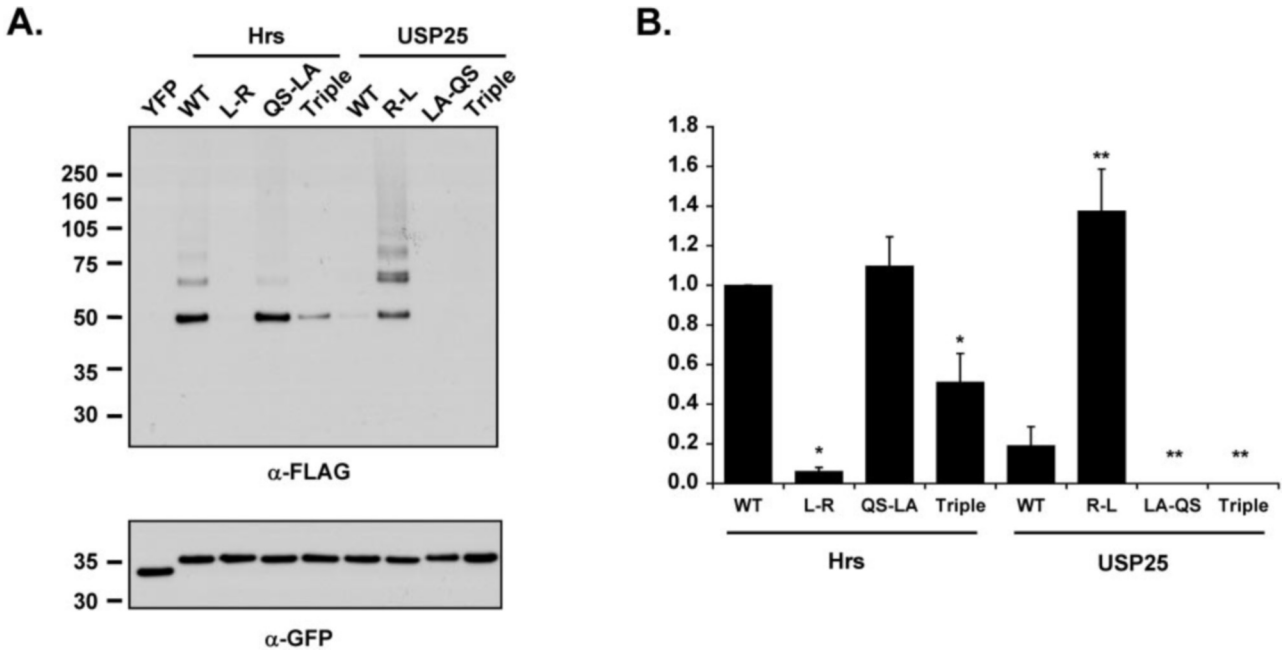

FIG. 5. **Non-conserved amino acids within the UIM determine the efficiency of ubiquitylation.** *A*, wild type and mutant Hrs and USP25 UIMs fused to the COOH terminus of YFP were co-expressed with UbWT in 293T HEK cells. Following immunoprecipitation with anti-GFP antibodies in the presence of SDS and deoxycholate, proteins were analyzed by Western blot with α-FLAG (*top*) or α-GFP (*bottom*) antibodies. *B*, the amount of ubiquitylation was quantitated by NIH Image analysis and then standardized to the amount of total YFP-UIM and ubiquitin in the lysates. The data in the graph represent the ratio between Hrs WT and the other UIMs ±S.E. from three independent experiments. The level of ubiquitin expression was equivalent between cell samples. Statistical significance was determined using Student's *t* test. * represents *p* values <0.05 when compared with WT Hrs. ** represents *p* values <0.05 when compared with WT USP25. *L-R*, L265R; *QS-LA*, Q271L/S272A; *R-L*, R104L; *LA-QS*, L110Q/A111S; *Triple*, all three mutations.

expression of WT or I44A ubiquitin as both were expressed equally (data not shown).

DISCUSSION

Recent studies have revealed an important role for UIMs in the monoubiquitylation of epsin, Hrs, and Eps15/Eps15R and in the binding of ubiquitin and ubiquitin-like proteins (2–11). Our current study characterized the activities of a panel of UIMs from diverse proteins to determine the sequence and structural requirements for their function. We have found that many but not all UIM-containing proteins are ubiquitylated to varying degrees and that the UIMs promote this modification. In addition to Eps15, Hrs, and epsin, we found that MJD1, MEKK1, and HSJ1 are ubiquitylated. However, our results with MEKK1 suggest that its UIMs are not functional: they neither bind ubiquitin nor promote ubiquitylation. Since MEKK1 possesses an E3 ligase domain in addition to its kinase domain, mutation or deletion of the UIMs will be necessary to determine whether these motifs play any role in the ability of MEKK1 to self-ubiquitylate.

In contrast to previous studies on UIM-dependent ubiquitylation, we found that UIMs promoted polyubiquitylation in addition to monoubiquitylation. A number of UIMs promoted polyubiquitylation of GST and YFP chimeric proteins (Ref. 8, and Fig. 3 and Supplementary Figs. 1–3). Indeed full-length epsin, Hrs, and MJD1 are modified by the attachment of multiple ubiquitins. These shifted forms were dramatically decreased upon expression of ubiquitin lacking all its lysines (UbΔK). Since the levels of these polyubiquitylated species were unaffected by inhibition of the proteasome, we predict that this modification alters the function of these proteins rather than targeting them for degradation. Several studies have indicated that the UIMs are important for endocytosis and sorting of ubiquitylated receptors and cargo proteins (6, 10, 11, 34). However, all of these studies utilized UIM mutations that would simultaneously block ubiquitin binding as well as ubiquitylation thus making it impossible to determine whether the observed effects were due to one or both of these activities. Once the site of ubiquitylation has been identified in these proteins and mutants have been constructed it will then be possible to assess whether this modification does indeed alter function.

Expression of a ubiquitin mutant lacking lysines for conjugation (UbΔK) resulted in a significant reduction in the levels of monoubiquitylated epsin, Hrs, and MJD1 suggesting that polyubiquitin chains are necessary for monoubiquitylation of these proteins. Since free polyubiquitin chains are present *in vivo* (35), it is possible that these chains are used for ubiquitylation of UIM proteins. This hypothesis is consistent with our observation that UIMs preferentially recognize polyubiquitin *versus* monoubiquitin and that polyubiquitin binding correlates with ubiquitylation (see below). Through a process analogous to glycosylation, we propose that UIM-containing proteins recruit free polyubiquitin chains through their UIMs leading to the ligation of these chains *en bloc* to a specific lysine(s) followed by trimming of the chains to a specific length by a deubiquitylating enzyme. Consistent with this idea, genetic and biochemical experiments suggest that epsin is regulated in part by the deubiquitylating enzyme Faf (14, 16), mutation of which increases the levels of polyubiquitylated epsin leading to an overall decrease in epsin due to proteasomal degradation (15). However, epsin does not appear to be regulated by proteasomal degradation in mammalian cells (8, 9). We speculate that *in vivo* epsin is transiently polyubiquitylated but is spared from degradation through rapid processing of the polyubiquitin chain by Faf leaving predominantly monoubiquitylated epsin, which has a specific function in the cell. That we do not observe any effect of MG132 on epsin or ubiquitylated epsin levels may be due to high levels of Faf expres-

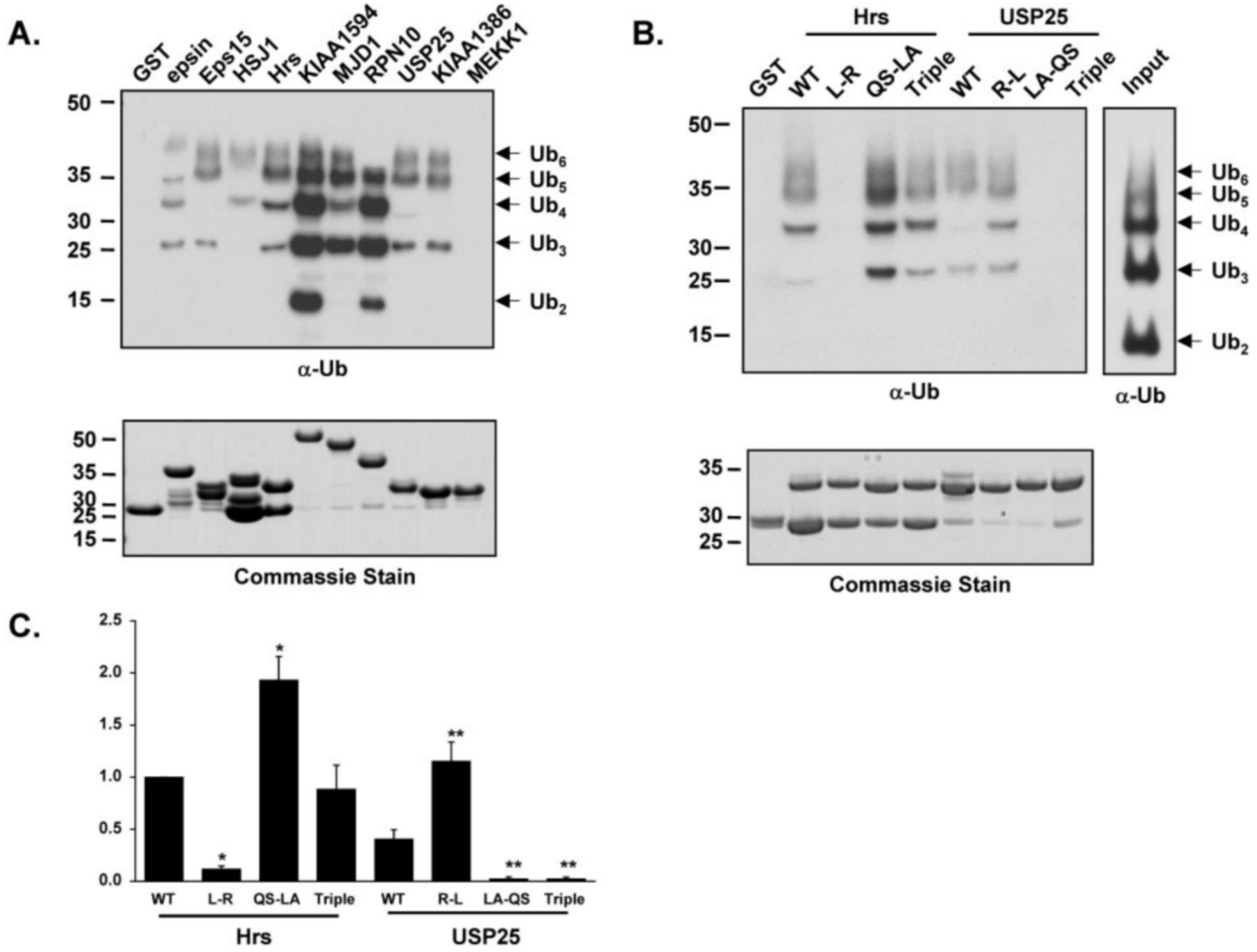

FIG. 6. **Polyubiquitin binding varies between UIMs and is dependent on non-conserved sequences within the UIM.** *A*, bacterially expressed GST-UIMs (~200 pmol) were incubated with 0.25 μg of polyubiquitin chains ranging from two to seven ubiquitins (Affiniti) for 2 h. Proteins were fractionated on gels and stained with Gel Code blue for detection of GST proteins (*bottom*) or transferred to a membrane and probed with α-ubiquitin (*top*). A sample of the input ubiquitin chains used in these experiments is shown in *B* on the *right*. *B*, GST fusions of wild type and mutant UIMs from Hrs or USP25 were tested for binding polyubiquitin chains *in vitro*. Western blots of bound ubiquitin chains were probed with α-ubiquitin (*top*). Protein gels were stained with Gel Code blue (*bottom*) to demonstrate input levels of the GST fusion proteins. The relative amount of each ubiquitin chain is indicated in the Western blot of the input ubiquitin on the *right*. *C*, ubiquitin binding was quantitated by NIH Image analysis and then standardized to the amount of total GST-UIM. The data in the graph represent the ratio between Hrs WT and the other UIMs ±S.E. from six independent experiments (five experiments for Hrs triple mutant). Statistical significance was determined using Student's *t* test. * represents p values <0.05 when compared with WT Hrs. ** represents p values <0.05 when compared with WT USP25. *L-R*, L265R; *QS-LA*, Q271L/S272A; *R-L*, R104L; *LA-QS*, L110Q/A111S; *Triple*, all three mutations.

TABLE I
UIMs demonstrate selectivity for polyubiquitin chains of varying lengths

Qualitative analysis of the binding of different length ubiquitin chains by UIMs. The levels of ubiquitin binding are represented by the following: −, no binding; −/+, weak binding; +, low binding; ++, moderate binding; +++, high binding.

	Ub_1	Ub_2	Ub_3	Ub_4	Ub_5	Ub_6
Epsin	−	−	−/+	+	+	+
Eps15	−	−	+	−/+	++	+
HSJ	−	−	−	+	−	+
Hrs	−	−	+	++	+++	+++
KIAA1594	−	+++	+++	+++	+++	+++
MJD	−	−	+++	+++	+++	+++
RPN10	−	+++	+++	+++	+++	−
USP25	−	−	+	−/+	++	++
KIAA1386	−	−	++	−/+	++	++
MEKK1	−	−	−	−	−	−

sion in 293 cells,[2] which may prevent the stable attachment of ubiquitin chains of sufficient length to target epsin to the proteasome. This idea reconciles the discrepant observations concerning epsin ubiquitylation in *Drosophila versus* mammalian cells, *i.e.* in Faf mutants, polyubiquitylated epsin would accumulate and thus be shunted toward degradation, whereas in mammalian cells, polyubiquitylated epsin would normally be rapidly processed and thus refractory to proteasomal degradation. Furthermore this model provides for multiple levels of epsin regulation thereby allowing for combinatorial control of epsin function.

The ability of UIMs to promote ubiquitylation as well as to bind ubiquitin is dependent on the sequence of the UIMs rather than the absolute number of UIMs in a given protein. The core UIM sequence of eee*X*Φ*XX*A*XXX*S*XX*e (where e is a negatively charged residue, Φ is a hydrophobic residue, and *X* is any amino acid) as proposed by Hofmann and Falquet (1) and extended by Swanson *et al.* (36) contains three highly conserved positions and four less well conserved negatively charged amino acids. Recent x-ray crystallographic and NMR structures of UIMs have revealed that the majority of these conserved residues lie at the interaction surface between the UIM and ubiquitin (or ubiquitin-like domain) (30, 36, 37). Indeed mutation of these conserved residues alters ubiquitin binding and ubiquitylation (6, 9–11, 30, 36, 37). Comparison of

[2] S. A. Wood, personal communication.

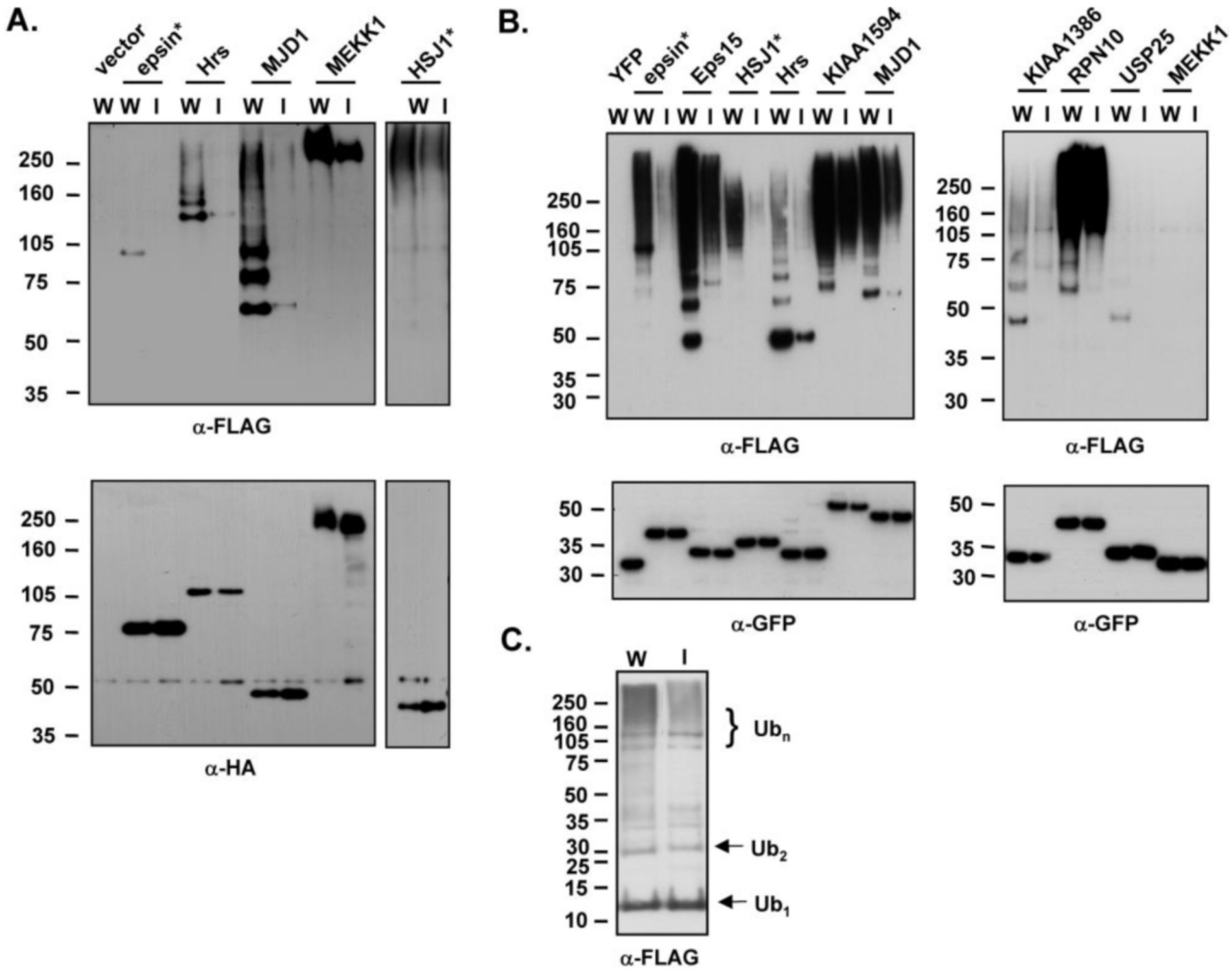

FIG. 7. **Ubiquitin binding by the UIM is necessary for UIM-dependent ubiquitylation.** *A*, HA epitope-tagged full-length proteins were co-expressed with FLAG-tagged UbWT (*W*) or UbI44A (*I*) in 293T HEK cells. Following immunoprecipitation with HA affinity beads in the absence of SDS and deoxycholate, the proteins were fractionated on a gel and probed with α-HA (*bottom*) or α-FLAG (*top*). *B*, similarly YFP-UIM constructs or YFP alone was co-transfected with UbWT or UbI44A. Lysates were then immunoprecipitated with the GFP antibody, fractionated on a gel, and probed with either α-GFP (*bottom*) or α-FLAG (*top*). *, longer exposure revealed ubiquitylation of these proteins was decreased by expression of UbI44A compared with UbWT (data not shown). *C*, lysates of cells expressing either FLAG-tagged wild type ubiquitin or ubiquitin I44A were probed with anti-FLAG antibodies to demonstrate equal levels of expression of the monomeric forms of both proteins. In addition, ubiquitin conjugates are present in both samples.

the single UIMs of Hrs and USP25, which have dramatically different activities in binding ubiquitin and promoting ubiquitylation, revealed two areas that contribute to these different activities (Fig. 1). The presence of a hydrophobic residue in the seventh position greatly enhanced both ubiquitin binding (3–5-fold) and ubiquitylation (10–20-fold) (Figs. 5 and 6, compare Hrs WT to L265R and USP25 WT to R104L). Examination of the UIM structures in complex with ubiquitin do not provide immediate insight into these effects given that this amino acid is on the helical face of the UIM opposite the ubiquitin binding interface. However, the comparable residue in the second UIM of RPN10 (Tyr^{289}) exhibited several interactions with amino acids in the ubiquitin-like domain of HHR23 suggesting that this residue does indeed contribute to ligand binding (37). Given the selectivity of UIMs for polyubiquitin chains, it is possible that this amino acid provides additional contacts with a second ubiquitin in a chain thereby stabilizing the overall interaction. Positions 13 and 14 of the UIM have been predicted to be an important affinity determinant, and indeed our mutational data support this prediction (36). Both ubiquitin binding and ubiquitylation are increased by the presence of aliphatic residues in these positions.

The analysis of the triple mutants in Hrs and USP25 are more difficult to interpret. Based on the results with mutations in position 7 or 13 and 14 of the UIM, we predicted that the activities of the Hrs triple mutant would more closely resemble those of the wild type UIM from USP25. Conversely we predicted that the activities of the USP25 triple mutant would resemble those of the wild type UIM from Hrs. Our results did not support those predictions and thus lead us to suggest that residues outside the core UIM sequence may also influence the activities of these UIMs, a possibility consistent with the results of Fisher and colleagues (30).

Several lines of evidence indicate that ubiquitin binding is an important prerequisite for UIM-directed ubiquitylation. The Hrs and USP25 mutants revealed quantitative changes in ubiquitylation that directly correlated with effects on polyubiquitin binding. Additionally expression of the ubiquitin I44A mutant, which is impaired in its ability to bind ubiquitin-associated, CUE, and UIM domains (for a review, see Ref. 38), is not permissive for UIM-directed ubiquitylation (Fig. 7). Although this mutant ubiquitin still forms polyubiquitin chains (31), its expression decreased ubiquitylation of UIM proteins.

It is unclear, however, whether the UIMs are required to

bind free ubiquitin chains or ubiquitin attached to E2s and/or E3s, although these two possibilities are not mutually exclusive. We have found that the UIMs of epsin recruit an E3 ligase complex leading to epsin ubiquitylation.[3] Since epsin has three UIMs, a subset of these may bind ubiquitin directly, while the remaining UIM(s) recruit the E2-E3 complex. This separation of functionality in multi-UIM proteins has been observed with Eps15 in which the first UIM is important for ubiquitylation but not ubiquitin binding, whereas the second UIM is important for both functions (9).

Previous studies have found that the UIMs from epsin, Eps15, and Hrs bind monoubiquitin with dissociation constants in the range of 150–300 μM (Ref. 30 and references therein). Although we were unable to detect interaction of UIMs with monoubiquitin, we did observe robust binding of polyubiquitin chains by most UIMs (Fig. 6). The higher affinity for polyubiquitin chains does not appear to be due to a cooperative effect from having multiple ubiquitins present in a chain since we observed that the affinity of UIMs for different length ubiquitin chains did not correlate with chain length (Fig. 5 and Table I). For example, the UIMs from Eps15 and KIAA1386 bound Ub_3, Ub_5, and Ub_6 chains with little binding to Ub_4. These observations suggest that a particular UIM discriminates between different length ubiquitin chains and that a longer chain does not necessarily confer increased binding. Given that ubiquitin chains adopt different conformations (39, 40), UIMs may select one conformation over another. This property may also dictate the specificity of interaction with various ubiquitylated proteins *in vivo*.

While it is clear that UIM-containing proteins as well as those with CUE domains are ubiquitylated, the role for this modification remains elusive. It has been proposed that ubiquitin and ubiquitin binding domains such as UIMs and CUEs may be comparable to tyrosine phosphorylation and Src homology 2 domains in that ubiquitylation may serve to regulate a network of protein-protein interactions (41). Our data support this notion. Results from this study as well as our previous work on epsin (8) demonstrate that the UIMs promote ubiquitylation of sites NH_2-terminal to the UIM. Furthermore UIM-YFP proteins associated with polyubiquitylated proteins more avidly than YFP-UIM proteins. This result suggests that UIM interactions with ubiquitylated cellular proteins are regulated through an intramolecular interaction in which the UIM binds to the ubiquitylation site in the UIM protein leading to a conformational change that blocks the interaction with exogenous ubiquitylated proteins. In the case of epsin, ubiquitylation inhibits the association with liposomes, clathrin, and AP2 (16). Although many proteins possess UIMs in the central or COOH-terminal portions of their sequence, examination of the SMART data base (42) revealed several uncharacterized proteins with UIMs located at the extreme NH_2 terminus. Based on our findings and given the absence of Lys acceptor sites NH_2-terminal to the UIMs in these proteins, we predict that these UIMs will function primarily to bind polyubiquitylated substrates and not to promote ubiquitylation.

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[3] Y. Timsit, S. L. H. Miller, and J. P. O'Bryan, unpublished observation.

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