Ubiquitin-interacting Motifs Inhibit Aggregation of PolyQ-expanded Huntingtin*

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Received for publication, December 5, 2006, and in revised form, January 26, 2007 Published, JBC Papers in Press, February 2, 2007, DOI 10.1074/jbc.M611151200

Several neurodegenerative diseases, including Huntington disease (HD) and Machado-Joseph disease, result from the expansion of a CAG trinucleotide repeat in the disease gene. In the case of HD, expansion of the CAG repeat in Htt results in a polyQ tract in the extreme NH2 terminus of the protein that destabilizes the protein, leading to the formation of SDS-insoluble protein aggregates also known as inclusion bodies in vitro and in vivo (1, 2). Adjacent to the polyQ repeat is a Pro-rich region that recruits WW domain- and Src homology 3 domain-containing proteins followed by 36 predicted HEAT repeats. Although the role of Htt in disease pathology is still unclear, the age of disease onset is directly correlated with the length of the polyQ tract (i.e. the longer the polyQ tract, the earlier the onset of the disease). Indeed, an expansion of 70 or more glutamines results in the juvenile form of the disease (3).

Ubiquitin is one of several proteins that is localized to insoluble aggregates formed by polyQ-expanded proteins, such as mutant Htt. This interaction has been hypothesized to contribute to the pathogenesis of HD, possibly through the recruitment or sequestration of ubiquitin-binding proteins into these aggregates (4). Recently, several domains have been demonstrated to bind to ubiquitin, including the ubiquitin-interacting motif (UIM), a short peptide sequence consisting of a highly conserved ΦXXAXXXXSXXAc core, where Φ represents a hydrophobic residue and Ac is an acetic residue (5). This motif not only binds noncovalently to ubiquitinated proteins but also has the ability to promote ubiquitination of a region located NH2-terminal to the UIMs (6–9).

Given these properties of the UIM and the presence of ubiquitinated proteins within polyQ-containing aggregates, we examined whether UIMs were able to alter aggregation of mutant Htt. In this study, we demonstrate that a subset of UIMs co-localized with mutant Htt but with different patterns of localization. Interestingly, the ability to bind ubiquitin was necessary but not sufficient for Htt-Q65 interaction. Using a cell-based aggregation assay, we demonstrate that UIMs from ataxin-3 and S5a specifically inhibited aggregation of mutant Htt. These data suggest that isolated UIMs may be efficacious inhibitors of Htt aggregation, thus attenuating Htt-induced toxicity in vivo.

MATERIALS AND METHODS

Cells and Reagents—Human embryonic kidney cells (293 and 293T) were cultured as previously described (8). COS cells were cultured similar to 293T cells. Antibodies used for these studies include the following: anti-GST and anti-Hsp70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); full-length A.V. polyclonal antibody, Living Colors peptide antibody to GFP and its variants (Clontech); monoclonal antibody against ubiquitin (Covance); and anti-His and anti-small ubiquitin-like modifier (SUMO) -1 (Boston Biochem).

Constructs—pEFG-UIM, pGEX-UIM, and YFP-UIM constructs were described previously (6). Htt-Q65-CFP, cyan fluorescent protein, was kindly provided by Dr. Peter Reinhardt and consists of 17 amino acids upstream of the polyQ region fol-
followed by 65 Qs and 130 amino acids following the polyQ region containing the proline-rich region of the full-length Htt. The following separate UIM constructs were created by PCR amplification of the pEFG S5A UIM or ataxin-3 UIM described previously: S5a UIM1 with 5’HTS5_UIM and 3’SSA-UIM1 (GAGGATCTGGCCCGGGGCTCTGCTCT); S5a UIM2 with 5’SSA-UIM2 (GAGCAGATCTGATGACCTAGACGTAGGCA-GAGTATT) and 3’SSA-UIM2 (GAGCAGATCTGCTGCTGATTCCGCTGGCGCAAA); ATAXIN-3 UIM1 with 5’ATAVIN-3correct and 3’ATAVIN-3-UIM1 (CAGCATCTGCAATTCTTGGCGA); ATAXIN-3 UIM2 with 5’ATAVIN-3-UIM2 (GAGCGATCGTGAAGAGCAGATCCTGCA) and 3’ATAVIN-3-UIM2 (GAGCGATCTGCTGCTGATTCCGCTGGCGCAAA); and ATAXIN-3 UIM3 with 5’ATAVIN-3-UIM3 (GAGCGATCGTGAAGACATGCTTCA) and 3’ATAVIN-3-UIM3 (GACGGATCCGTGCTGATTCCGCTGGCGCAAA).

The purified pDEST566 His-maltose-binding protein (MBP) Htt-Q65 was created by PCR amplification of Htt-Q65-CFP with 5’HTT (CACCAGATGTGGCCGCTTGGCAGCAGATCCTGCA) and 3’HTT-STOP (CACCAGATGTGGCCGCTTGGCAGCAGATCCTGCA) and 3’ATAVIN-3-UIM (GACGGATCCGTGCTGATTCCGCTGGCGCAAA). The resulting blunt-end fragment was then cloned into the pENTR/TEV/D-TOPO vector (Invitrogen) and verified by DNA sequencing. Next, the pENTR/TEV/D-TOPO Htt-Q65 was mixed with the plasmid pENTR/TEV/D-TOPO Htt-Q65-CFP. Following precipitation with GSH beads, the precipitate was fractionated on SDS-polyacrylamide gels and immunoblotted with α-GST to detect expression of GST-UIMs or with Living Colors peptide antibody for evaluating binding of the Htt-Q65-CFP. Supernatant recovered after precipitation with GSH beads was then immunoprecipitated with the full-length A.V. polyclonal antibody, fractionated on a gel, and probed with Living Colors peptide antibody to evaluate expression of the Htt-Q65-CFP.

Cell Aggregation Assay—Htt-Q65-CFP was co-expressed with GST or GST-UIMs at various doses ranging from 0 to 3 μg in 293 HEK cells. Two days following transfection, 20 fluorescence images were acquired for each DNA amount using an Olympus 1X70 microscope system. An observer blind to dose quantified the percentage of aggregate formation by counting the number of cells with fluorescent aggregates and dividing by the total number of fluorescent cells. Relative aggregate formation was calculated by normalizing the percentage of aggregates to the aggregate percentage at a dose of 0 μg. For each experiment done in triplicate, 800–1000 cells were counted for each dose. Cells were then lysed with PLC-LB as described previously (8). Lysates were precipitated with GSH beads for several hours and spun down, and then the supernatant was immunoprecipitated with the full-length A.V. polyclonal antibody. Precipitates were washed five times with PLC-LB, fractionated by SDS-PAGE, and then transferred to Immobilon-P membranes. Membranes were either probed with α-GST to verify increased expression of the GST/GST-UIMs with increased dose or with Living Colors peptide antibody to detect similar expression of the Htt-Q65-CFP.

In Vitro Binding Assay—Binding assays were performed as described previously (6). Briefly, GST-UIM constructs were expressed in Escherichia coli and purified using GSH beads. 200 pmol of the GST fusion proteins were incubated with 200 pmol of His-MBP-tagged Htt-Q65, 1 μL of Lys63-linked polyubiquitin chains ranging from 2–7 ubiquitins, purified SUMO-1, or purified His-tagged NEDD8 at a concentration of 0.25 μg/μL (Boston Biochemical) for 2 h at 4 °C. Beads were pelleted and washed twice. Given that GST nonspecifically bound the mutant Htt, samples were treated with thrombin as described previously for 3 h at 22 °C (8). Samples were spun down, and the supernatant was transferred to another tube to obtain the soluble fraction. After the addition of sample buffer, the bead portion and soluble portion were fractionated on gels, and the GST or UIMs were detected via Gel Code® Blue Coomassie stain. Alternatively, gels were transferred to an Immobilon-P filter and probed with the His antibody for detection of His-MBP Htt-Q65 or NEDD8, α-ubiquitin for detection of Lys63-linked ubiquitin chains, or α-SUMO-1 for detection of SUMO.

RESULTS

SSA, ataxin-3, Hrs, and Eps15 UIMs Are Recruited to Mutant Htt Aggregates—To evaluate the interaction of UIMs with polyQ-expanded proteins, we expressed individual YFP-UIM chimeras in COS cells in the absence or presence of the first exon of Htt containing a polyQ expansion of 65 Qs fused to CFP (Htt-Q65-CFP) (Fig. 1). When expressed alone, most UIMs localized to both the cytoplasm and nucleus. However, the Eps15 UIM localized exclusively to the cytoplasm. Co-expression of Htt-Q65-CFP resulted in recruitment of ataxin-3,
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Eps15, S5a, and Hrs UIMs into inclusion bodies. In contrast, the distribution of KIAA1594, epsin, USP25, HSJ1, and KIAA1386 UIMs was unaffected by the presence of mutant Htt-Q65 (Fig. 1; data not shown). Although interaction of ataxin-3 UIMs with poly-Q-expanded aggregates correlated with ubiquitin binding (4), the observed difference in UIM recruitment could not be accounted for by the affinity of the particular UIMs for ubiquitin. For example, KIAA1594 binds Lys48-linked polyubiquitin chains as well as if not better than ataxin-3 (6) but, in contrast to ataxin-3, did not co-localize with Htt-Q65-CFP (data not shown).

**FIGURE 1.** Ataxin-3, S5a, Eps15, and Hrs UIMs are recruited to polyQ-expanded Htt aggregates. YFP-UIM (green) chimeras, including ataxin-3, Eps15, S5a, and Hrs, were expressed alone (left column, UIM alone) or together with Htt-Q65-CFP (red) in COS cells. Confocal images were acquired as described under "Materials and Methods." Regions of co-localization are indicated in yellow. The white bar represents 10 μm. The arrows highlight enlarged areas. The UIMs of epsin, HSJ1, KIAA1386, KIAA1594, and USP25 did not co-localize with Htt-Q65-CFP (data not shown).

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UIMs Are Not Recruited to Htt-Q65 Aggregates through Binding to Htt-Q65, Hsp70, Lys63-linked Polyubiquitin Chains, or Ubiquitin-like Proteins—To determine the mechanism responsible for localization of specific UIMs to Htt-Q65 aggregates, we evaluated the binding of UIMs to several proteins present in poly-Q-expanded aggregates, including mutant, expanded Htt-Q65. HEK293T cells were transiently co-transfected with GST or GST-UIMs along with Htt-Q65-CFP. GST fusion proteins were then purified from lysates using GSH-agarose beads. Western blot analysis of purified GST proteins revealed no interaction between any UIM and Htt-Q65-CFP (Fig. 2A). Furthermore, an in vitro binding assay using purified GST or GST-UIMs and purified His-MBP Htt-Q65 failed to detect a specific interaction between Htt-Q65 and any GST-UIM. Since GST alone nonspecifically bound mutant Htt, samples were treated with thrombin to determine if this association was mediated by the GST or UIM portion of the chimeric protein. Upon thrombin cleavage, His-MBP-Htt-Q65 remained bound to the GST-containing beads (Fig. 2B, left). There was no evidence of binding between any UIMs and His-MBP-Htt-Q65 (Fig. 2B, right).

Although the interaction of the UIMs with Htt-Q65 did not correlate with the affinity for Lys48-linked polyubiquitin chains, recent data suggest that Lys63-linked chains may be important in aggregate formation (10). Using an in vitro ubiquitin binding assay (6), we evaluated the ability of different UIMs to bind purified Lys63-linked polyubiquitin chains (Fig. 3). Although different UIMs bound Lys63-linked ubiquitin chains to varying degrees, there was no correlation between the recognition of Lys63-linked chains and the association with Htt-Q65-CFP.

In addition to ubiquitin, there exist several additional ubiquitin-like proteins in the human proteome, including the SUMO and NEDD8. Like ubiquitin, SUMO is also present in mutant Htt aggregates, and sumoylation of mutant Htt leads to increased neurodegeneration in a Drosophila model of Htt-induced neurodegeneration (11). To determine whether an interaction with SUMO contributed to UIM recruitment to polyQ aggregates, we evaluated the binding of UIMs to SUMO using an in vitro assay. GST-UIM fusion proteins bound to purified SUMO to varying degrees (Fig. 4A). However, the ability to bind SUMO did not depend on the number of UIMs; nor did it correlate with aggregate recruitment, similar to results with Lys48- and Lys63-linked polyubiquitin chains. We also determined whether UIMs bound the ubiquitin-like protein NEDD8, which has been found in aggregates formed by polyQ expansion of the androgen receptor in spinal bulbar muscular atrophy (12). In contrast to ubiquitin and SUMO, none of the UIMs tested bound NEDD8 (Fig. 4B).

We recently demonstrated that the UIMs of epsin interacted with heat shock protein Hsp/Hsc70 (13). This protein has been implicated in aggregate formation due to its localization to aggregates, ability to transiently move in and out of polyQ...
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FIGURE 2. UIMs do not bind mutant Htt. A, Htt-Q65-CFP was expressed in HEK-293T cells along with the GST-UIM chimeras. Following incubation of cell lysates with GSH beads, purified proteins were fractionated on a gel, transferred to a membrane, and probed with α-GFP (top) or α-GST (middle). The expression levels of the transfected Htt-Q65-CFP are indicated in the bottom panel. B, bacterially expressed GST-UIMs were incubated with purified, bacterially expressed His-MBP-tagged Htt-Q65. Following purification, samples were treated with thrombin to cleave the UIM and any bound proteins from the GST portion. The immobilized bead fraction (GST) and the soluble UIM fraction were fractionated on gels and stained with Gel Code® blue for detection of GST (bottom left) and UIMs (bottom right). Identical samples were transferred to a membrane and probed with α-His to detect binding of mutant Htt to either the GST portion (top left) or UIM portion (top right). IP, immunoprecipitation.

FIGURE 3. Binding of UIMs to Lys63-linked ubiquitin chains. A, bacterially expressed GST-UIMs were incubated in an in vitro assay with Lys63-linked polyubiquitin chains ranging from 2 to 7 ubiquitins. Proteins were fractionated on gels and stained with Gel Code® blue for detection of GST (bottom) or UIM portion (top). A sample of the input ubiquitin chains used in this experiment is shown to the right. B, 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, ubiquitin.

aggregates, and effect on aggregate formation and/or neurodegeneration (14). Taken together, these data suggest that UIMs may be recruited to aggregates via an interaction with Hsp70. To determine whether the interaction with Hsp70 mediates the recruitment of UIMs to polyQ-expanded proteins, we examined the interaction of the various UIMs with endogenous Hsp70. As expected, the UIMs of epsin bound very well to Hsp70. In addition, the S5a, ataxin-3, and Eps15 UIMs also bound to Hsp70 but to a lesser degree as compared with the epsin UIM chimera. However, we did not observe a correlation between the ability of a UIM protein to bind Hsp70 and its recruitment to polyQ aggregates, suggesting that recruitment to the aggregate is not mediated by Hsp70 binding (Fig. 4C).

Expression of Ataxin-3 and SSA UIMs Decreased the Total Number of Aggregates Formed by Htt-Q65—Given the recruitment of S5a, ataxin-3, Hrs, and Eps15 UIMs to inclusion bodies, we examined whether the UIMs had any effect on aggregate formation by Htt-Q65-CFP using a cell-based assay. HEK293T cells were co-transfected with GST or GST-UIMs at varying doses along with Htt-Q65-CFP. Forty-eight hours following transfection, cells were examined for aggregate formation. Expression of either ataxin-3 or S5a UIMs resulted in a dose-dependent decrease in the percentage of CFP-positive cells containing visible aggregates, whereas expression of GST alone did not affect aggregate formation (Fig. 5A). This effect on aggregate formation was specific to a subset of UIMs and did not result from altered expression of Htt-Q65 (Fig. 5B). Although both Eps15 and Hrs UIMs co-localized to Htt-Q65 aggregates (Fig. 1), expression of these UIMs did not affect aggregate formation (Fig. 5A). Similarly, UIMs that did not co-localize with Htt-Q65 aggregates had no significant effect on the formation of aggregates.

Individual UIMs of S5a and Ataxin-3 Have Distinct Properties—Further analysis was undertaken to determine whether the individual UIMs of S5a and ataxin-3 were capable of localizing to the polyQ aggregates. S5a possesses two UIMs, whereas ataxin-3 has three UIMs with the second and third UIM separated by a polyQ region (Fig. 6A). Each of the separate UIMs for S5a and ataxin-3 was co-transfected into COS cells along with Htt-Q65-CFP. The second UIM of S5a co-localized with Htt-Q65 aggregates, whereas the first UIM did not (Fig. 6B). Similarly, the second UIM of ataxin-3 co-localized with Htt-Q65 aggregates, whereas the first and third UIMs did not (Fig. 6D). In both cases, however, the localization pattern of the individual UIMs was distinct from that of the full UIM region. Whereas the full UIM constructs from S5a and ataxin-3 were localized throughout the aggregate, UIM2 of ataxin-3 localized in a ring pattern surrounding the aggregate. In the case of UIM2 of S5a, two distinct patterns of localization were observed: a ring pattern...
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Figure 4. Interaction of UIMs with SUMO, NEDD8, and Hsp70. A, bacterially expressed GST or GST-UIMs were subjected to an in vitro binding assay with purified SUMO-1. 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-SUMO (top). B, bacterially expressed GST or GST-UIMs were subjected to an in vitro binding assay with purified His tagged NEDD8. 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-His (top). No evidence of binding was detected even on longer exposures. C, GST-UIM chimeras or GST were transiently expressed in 293 HEK cells. Following purification of the GST proteins from cell lysates with GSH beads, purified proteins were fractionated on a gel, transferred to a membrane, and probed with α-GST (bottom) or α-Hsp70 (top).

similar to UIM2 of ataxin-3 and a punctate pattern (Fig. 6B). Interestingly, in the S5a UIM2 expressing cells, the localization of Htt-Q65-CFP was punctate and cytosolic, suggesting that UIM2 may inhibit or disrupt aggregation, resulting in smaller polyQ aggregates (Fig. 6B).

Expression of combinations of ataxin-3 UIMs resulted in different patterns of localization with Htt-Q65-CFP compared with the individual UIMs. Although UIM2 localized in a ring pattern to Htt-Q65-CFP, the presence of UIM1 blocked the interaction of UIM1-2 with Htt-Q65. UIM2-3 localized in a ring pattern within the Htt-Q65 aggregate (Fig. 6D).

Given the effect of S5a and ataxin-3 UIMs on Htt aggregation, we next examined whether the individual UIMs or combinations of UIMs retained the ability to inhibit polyQ aggregate formation. Expression of UIM2 of S5a resulted in a 50% decrease in the number of cells with aggregates similar to the effect of expressing both UIMs of S5a (Fig. 6C). In contrast, the first UIM did not affect aggregate formation (Fig. 6C). In the case of ataxin-3, expression of the second UIM resulted in a 20% decrease in the fraction of cells with aggregates, whereas expression of the first or third UIMs was without effect (Fig. 6E). Since the individual UIMs of ataxin-3 did not reduce aggregate formation to the same extent as all three UIMs together, we transfected combinations of the UIMs of ataxin-3 with the Htt-Q65-CFP. Expression of UIM1-2 led to a similar decrease as UIM2 alone. However, UIM2-3 had no significant effect on aggregate formation (Fig. 6E). Thus, in the case of ataxin-3, all three UIMs are required for the maximal reduction in aggregate formation, whereas the isolated UIM2 of S5a is sufficient for maximal inhibition.

**DISCUSSION**

Recent work has demonstrated that ubiquitin binding proteins are recruited to aggregates formed by polyQ-expanded proteins (4, 15–17). In the case of ataxin-3, the ubiquitin binding activity of UIMs appeared necessary for recruitment to the aggregates, since mutation of conserved residues in the UIM that are necessary for ubiquitin binding inhibited co-localization of ataxin-3 with the polyQ aggregates (4). Similarly, we found that the UIMs of S5a, ataxin-3, Hrs, and Eps15 localized to polyQ-expanded Htt, although the pattern of localization varied between different UIMs (Fig. 1). This difference in co-localization may reflect when the UIM interacts with the misfolded polyQ protein during aggregate formation. In the early stages of aggregate formation, the polyQ monomer undergoes misfolding to form a nucleus (i.e. the core of the aggregate). Following a fast extension phase, additional monomers are added to the nucleus to form the insoluble aggregate (18). Recent data suggest that aggregates consist of a central core region containing the insoluble protease-resistant mutant Htt fibrils and a nonprotease resistant shell region containing soluble mutant Htt (19, 20). Our data support the hypothesis that the aggregate consists of different regions with specific characteristics that allow recruitment of different UIMs. Both ataxin-3 and S5A UIMs are found throughout Htt aggregates. However, the Hrs and Eps15 UIM chimeras are not found within the central region of the aggregate but rather localize in a ring pattern surrounding the aggregate. These different patterns of localization may signify the early recruitment of the S5a and ataxin-3 versus a later recruitment of the Hrs and Eps15 UIMs during aggregate formation. This difference in recruitment may account for the differential inhibition of aggregate formation by ataxin-3 and S5a UIMs versus Hrs and Eps15 UIMs.

In contrast to the S5a, Hrs, Eps15, and ataxin-3 UIMs, other UIMs were not recruited to aggregates, although several of these UIMs bind avidly to polyubiquitin chains (Fig. 1) (6). Previously, we demonstrated that UIMs bind purified Lys⁴⁸-linked polyubiquitin chains but with varying affinities (6). Similar results were seen when evaluating the ability of UIMs to bind purified Lys⁶⁵-linked polyubiquitin chains (Fig. 3). The UIMs
from KIAA1594 bound to Lys\(^{48}\)- and Lys\(^{63}\)-linked polyubiquitin chains as well as or better than the UIMs from S5a, ataxin-3, Eps15, and Hrs. Similarly, KIAA1594 UIMs bound to ubiquitinated cellular proteins as well as or better than the UIMs from Hrs, ataxin-3, and Eps15 (6). However, the KIAA1594 UIMs were not recruited to Htt-Q65 aggregates. These data demonstrate that although ubiquitin binding may be necessary for recruitment of UIMs to polyQ-expanded aggregates (4), this property is not sufficient for recruitment to the aggregates.

Comparison of the binding pattern of UIMs to Lys\(^{48}\)-linked versus Lys\(^{63}\)-linked ubiquitin chains revealed both similarities and differences in UIM recognition of these different species. As with recognition of Lys\(^{48}\)-linked chains, the degree of binding to Lys\(^{63}\)-linked chains did not directly correspond to the number of UIMs in a given protein. For example, S5a bound Lys\(^{63}\)-linked chains better than ataxin-3, although it has one less UIM than ataxin-3. However, binding to shorter Lys\(^{63}\)-linked chains inversely corresponded to the number of UIMs; the more UIMs present, the greater the binding to shorter Lys\(^{63}\)-linked chains. In support of this observation, the single UIM-containing chimeras (Hrs, KIAA1386, or USP25) bound to di-, tri-, or tetraubiquitin chains to a far lesser degree than the multiple UIM-containing chimeras. This phenomenon is most likely due to the difference in structure between Lys\(^{48}\)- and Lys\(^{63}\)-linked chains. The Lys\(^{48}\)-linked diubiquitin chain has a closed conformation with the hydrophobic binding surface located on the interdomain interface (21). However, the structure of the Lys\(^{63}\) diubiquitin chain has an open extended conformation with no interaction between the hydrophobic binding surfaces of the two ubiquitins. Thus, the presence of multiple UIMs may lead to increase contact with the single ubiquitins located within the Lys\(^{63}\)-linked chains.

To determine the protein(s) responsible for recruitment of UIMs to Htt-Q65 aggregates, we examined the interaction of the UIMs with a variety of proteins, including mutant Htt-Q65, Hsp70, and several ubiquitin-like proteins, and found no direct correlation between binding and recruitment (Figs. 2 and 4). Recently, Regan-Klapisz et al. (22) indicated that Eps15 and Hrs are recruited into cytoplasmic aggregates through an interaction with ubiquilin, another ubiquitin-like protein. This interaction may be responsible for localization of these two UIMs into the shell region of the polyQ aggregate. However, the recruitment differences between S5a, ataxin-3, Hrs, and Eps15 UIMs suggest ubiquilin is not responsible for the recruitment of S5a and ataxin-3 UIMs to the central region of the aggregate. These data also imply that there are distinct differences between mutant Htt aggregates and aggregates induced upon proteasome inhibition, since Eps15 localized differently in these aggregates (22).

We demonstrate for the first time that a subset of UIMs...
(epsin, ataxin-3, S5a, Hrs, and USP25) interact with SUMO in addition to ubiquitin (Fig. 4). Recent studies have uncovered a short peptide motif that binds to SUMO (23). Interestingly, the SUMO-interacting motif (SIM)-SUMO binding surface is separate and distinct from the UIM-ubiquitin interface (24). These observations raise the intriguing possibility that SUMO may be able to either simultaneously interact with a subset of UIM- as well as SIM-containing proteins or that UIM- and SIM-containing proteins compete for binding to SUMO. Future studies will be needed to address these possibilities.

In contrast to the interaction with ubiquitin and SUMO, we did not observe binding of any of the UIMs tested to the ubiquitin-like protein NEDD8. This result suggests that UIMs exhibit some selectivity in the interactions with different ubiquitin-like proteins. A recent study by Yarden and co-workers (25) suggests that the UIMs of Hgs binds the ubiquitin-like protein NEDD8; however, this interaction was not shown to be a direct UIM-NEDD8 interaction. Thus, it is possible that the UIM-dependent interaction of Hgs with NEDD8 may be indirect and mediated by one or more proteins.

The in vitro binding data demonstrate striking differences between UIMs in their protein/protein interactions despite their small size and highly conserved core residues. These differences further highlight the importance of nonconserved amino acids in determining the protein/protein interactions of a UIM (6) and suggest that UIMs may be further classified by their interactions with SUMO, Hsp70, or ubiquitin.
The S5a and ataxin-3 UIMs not only co-localized to polyQ aggregates but also decreased aggregate formation. Surprisingly, the Eps15 and Hrs UIMs had no effect on aggregate formation despite their association with the aggregate (Fig. 5). This difference may be due to the specific localization of the UIM. Both ataxin-3 and S5a UIMs are uniformly distributed throughout the aggregate, suggesting that these UIMs are either recruited during the early phases of aggregate formation or penetrate the insoluble core of the aggregate. Evaluation of the separate UIMs of ataxin-3 further supports this hypothesis. The first and third UIMs of ataxin-3 when expressed alone did not co-localize to the mutant Htt aggregates and had no effect on aggregate formation. The second UIM displayed a ring pattern of co-localization similar to Eps15 but did not affect aggregate formation as significantly as UIM1 to -3 of ataxin-3 (Fig 6D). Thus, in the case of the ataxin-3, all three UIMs work in combination for reducing aggregate formation. This chimera was the only one that was recruited throughout the aggregate, further supporting the premise that an interaction within the central region of the aggregate is responsible for the extensive inhibition of aggregation. Although ataxin-3 contains a polyQ repeat between UIM2 and UIM3, this region does not appear to contribute to localization with polyQ-expanded proteins (4).

Evaluation of the separate UIMs of S5a revealed several differences from the ataxin-3 chimeras. Similar to the first and third UIMs of ataxin-3, the first UIM of S5a did not co-localize and had no effect on aggregate formation. However, expression of the second UIM of S5a decreased aggregate formation to a similar level as the two UIMs of S5a (Fig 6C). Thus, a single UIM was sufficient to decrease Htt-Q65 aggregates. Furthermore, the second UIM of S5a demonstrated two different patterns of recruitment. In the first pattern, the UIM localized in a ring surrounding the central portion of the aggregate. In the second pattern, the UIM was localized in a punctate pattern as if there were miniaggregates. Thirty-eight percent of aggregate positive cells exhibited this second pattern of localization. It has been suggested that quasiaggregates are formed prior to the appearance of mature aggregates (26, 27). These quasiaggregates are transported by microtubules to the microtubule organizing center, where they are assembled into a spherical aggregate known as the aggresome (27, 28). These data suggest that UIM2 of S5a may disrupt the transport of miniaggregates into the aggresome. However, it remains to be determined whether UIM2 prevents formation of the mature aggregates, disrupts mature aggregates once formed, or both. These data also suggest that the mechanism for decreasing aggregation may vary between UIMs.

A recent study by Bonini and co-workers (29) demonstrates that overexpression of wild type ataxin-3 decreased aggregation of polyQ-expanded proteins and suppressed polyQ-mediated neurodegeneration. These effects were dependent on the ubiquitin-binding activity of the UIMs. Thus, both isolated UIMs as well as UIMs in the context of the wild type protein inhibit polyQ aggregation. These findings raise the intriguing possibility that expression of isolated UIMs may alter the toxicity of polyQ-expanded proteins, such as Htt. Future studies will be necessary to evaluate this possibility.

**REFERENCES**

1. Wanker, E. E. (2000) **Biol. Chem.** 381, 937–942
2. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H., and Wanker, E. E. (1997) **Cell** 90, 549–558
3. Myers, R. H., MacDonald, M. E., Koroschetz, W. J., Duyao, M. P., Ambrose, C. M., Taylor, S. A., Barnes, G., Srinidhi, J., Lin, C. S., Whaley, W. L., Lazzarini, A. M., Schwartz, M., Wolf, G., Bird, E. D., Vonsattel, J.-P. G., and Gusella, J. F. (1993) **Nat. Genet.** 5, 168–173
4. Donaldson, K. M., Li, W., Ching, K. A., Batalov, S., Tsai, C. C., and Joazeiro, C. A. (2003) **Proc. Natl. Acad. Sci. U. S. A.** 100, 8892–8897
5. Hofmann, K., and Falquet, L. (2001) **Trends Biochem. Sci.** 26, 347–350
6. Miller, S. L., Malotky, E., and O’Bryan, J. P. (2004) **J. Biol. Chem.** 279, 33528–33537
7. Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., and Di Fiore, P. P. (2002) **Nature** 416, 451–455
8. Oldham, C. E., Mohney, R. P., Miller, S. L., Hanes, R. N., and O’Bryan, J. P. (2002) **Curr. Biol.** 12, 1112–1116
9. Klapzis, E., Sorokina, I., Lemeer, S., Pijnenburg, M., Verkleij, A. J., and Van Bergen En Henegouwen, P. M. (2002) **J. Biol. Chem.** 277, 30746–30753
10. Lim, K. L., Dawson, V. L., and Dawson, T. M. (2006) **Neurobiol. Aging** 27, 524–529
11. Steffen, J. S., Agrawal, N., Pallos, J., Rockabrand, E., Troutman, L. C., Slepkov, N., Illes, K., Lukacsovich, T., Zhu, Y. Z., Cattaneo, E., Pandolfi, P. P., Thompson, L. M., and Marsh, J. L. (2004) **Science** 304, 100–104
12. Stenoien, D. L., Cummings, C. J., Adams, H. P., Mancini, M. G., Patel, K., DeMartino, G. N., Marcelli, M., Weigel, N. L., and Mancini, M. A. (1999) **Hum. Mol. Genet.** 8, 731–741
13. Timists, Y. E., Miller, S. L., Mohney, R. P., and O’Bryan, J. P. (2005) **Biochem. Biophys. Res. Commun.** 328, 550–559
14. Kim, S., Nollen, E. A., Kitagawa, K., Bindokas, V. P., and Morimoto, R. I. (2002) **Nat. Cell Biol.** 4, 826–831
15. Doi, H., Mitsu, K., Kurosawa, M., Machida, Y., Kuroiwa, Y., and Nukina, N. (2004) **FEBS Lett.** 571, 171–176
16. Wang, G., Sawai, N., Kotliarov, S., Kanazawa, I., and Nukina, N. (2000) **Hum. Mol. Genet.** 9, 1795–1803
17. Davidson, J. D., Riley, B., Burright, E. N., Duivick, L. A., Zoghbi, H. Y., and Orr, H. T. (2000) **Hum. Mol. Genet.** 9, 2305–2312
18. Chen, S., Ferrone, F. A., and Wetzel, R. (2002) **Proc. Natl. Acad. Sci. U. S. A.** 99, 11884–11889
19. Qin, Z. H., and Gu, Z. L. (2004) **Acta Pharmacol. Sin.** 25, 1243–1249
20. Matsumoto, G., Kim, S., and Morimoto, R. I. (2006) **J. Biol. Chem.** 281, 4477–4485
21. Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002) **J. Mol. Biol.** 324, 637–647
22. Regan-Klapizis, E., Sorokina, I., Voortman, J., de Keizer, P., van Bergen en Henegouwen, M. D., Kochupukkkal, B. S., Katz, M., Lavi, S., Cesareni, G., and Yarden, Y. (2003) **Nat. Genet.** 33528–33537
23. Mistry, A., Dumont, X., and Caput, D. (2000) **J. Biol. Chem.** 275, 36316–36323
24. Hecker, C. M., Rahibller, M., Hagnlund, K., Bauer, P., and Dikic, I. (2006) **J. Biol. Chem.** 281, 16117–16127
25. Oved, S., Mosesson, Y., Zang, Y., Santonico, E., Shtigman, K., Marmor, M. D., Kochupukkkal, B.S., Katz, M., Lavi, S., Cesareni, G., and Yarden, Y. (2006) **J. Biol. Chem.** 281, 21640–21651
26. Garcia-Mata, R., Bubok, Z., Sorscher, E. J., and Sztul, E. S. (1999) **J. Cell Biol.** 146, 1239–1254
27. Garcia-Mata, R., Gao, Y. S., and Sztul, E. (2002) **Traffic** 3, 388–396
28. Johnston, J. A., Illing, M. E., and Kopito, R. R. (2002) **Cell Motil. Cytoskeleton** 53, 26–38
29. Warrick, J. M., Morabito, L. M., Bilen, J., Gordesky-Gold, B., Faust, L. Z., Paulson, H. L., and Bonini, N. M. (2005) **Mol. Cell** 18, 37–48

**Acknowledgments**—We thank Dr. Peter Reinhart for providing the CFP-tagged Htt constructs and members of the O’Bryan laboratory for many helpful comments and suggestions.