The Effects of the Polyglutamine Repeat Protein Ataxin-1 on the UbL-UBA Protein A1Up*

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Brigit E. Riley‡§¶, Yifan Xu†, Huda Y. Zoghbi‡, and Harry T. Orr‡§¶**

From the Departments of ‡Biochemistry, Molecular Biology, and Biophysics and ¶Laboratory Medicine and Pathology, ¶Institute of Human Genetics, University of Minnesota, Minneapolis, Minnesota 55455 and ‡Department of Molecular and Human Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030

The ataxin-1 interacting ubiquitin-like protein (A1Up) contains an amino-terminal ubiquitin-like (UbL) region, four stress-inducible, heat shock chaperonin-binding motifs (STI1), and an ubiquitin-associated domain (UBA) at the carboxyl terminus of A1Up. Although proteins that have both an UBL and UBA domain are thought to play a crucial role in proteasome-mediated activities, few are characterized, except for hHR23A/B. Similar to other UBL-containing proteins, the UBL of A1Up is essential for the interaction of A1Up with the S5a subunit of the 19S proteasome. Importantly, the interaction with the 19S proteasome was disrupted in the presence of the polyglutamine repeat protein, ataxin-1. The UBL domain of A1Up is ubiquitinated by both Lys48-linked and Lys63-linked chains. Intact A1Up is stable, suggesting that ubiquitination of A1Up is important for degradation-independent targeting of A1Up to the 19S proteasome. The UBA domain of A1Up binds polyubiquitin chains and has a role in the stability of A1Up and in the subcellular localization of A1Up. When the UBA domain was deleted, the localization of A1Up was entirely cytoplasmic, and it co-localized with the proteasome. Interestingly, the interaction between A1Up and mutant ataxin-1-(82Q) increased the half-life of A1Up, whereas nonpathogenic wild-type ataxin-1-(30Q) or ataxin-1-(82Q)-A776 did not.

Spinocerebellar ataxia type 1 (SCA1)† is an inherited progressive neurodegenerative disease that is characterized by motor deterioration and loss of cerebellar Purkinje cells. SCA1 is caused by the expansion of a CAG trinucleotide repeat tract in the SCA1 gene that results in an abnormally long polyglutamine tract within the protein. Thus, SCA1 is one of nine characterized polyglutamine repeat diseases; the other eight are spinocerebellar ataxias 2, 3, 6, 7, and 17; dentatorubral-pallidoluysian atrophy; Huntington’s disease; and spinobulbar muscular atrophy (1, 2). Accumulation of mutant ataxin-1 into nuclear inclusions is a hallmark of disease. These nuclear inclusions stain positive for components of the ubiquitin proteasome pathway and chaperones (3). The role these nuclear inclusions play in pathogenesis is complex. Data suggest that nuclear inclusions represent a cellular response to protect against the expanded polyglutamine, but whether sequestration of normal proteins into the inclusions contributes to the pathology of SCA1 remains to be determined (4). Recently, ataxin-1 was reported to be phosphorylated at serine 776, and mutation of this serine to an alanine decreased the ability of mutant ataxin-1-(82Q) to form nuclear inclusions (5, 6). Furthermore, transgenic mice expressing ataxin-1-(82Q)-A776 had reduced disease pathology. In contrast, when transgenic animals overexpressing mutant ataxin-1-(82Q) were crossed with animals lacking the E3 ligase, E6AP (Ube3A gene), there was also a decrease in nuclear inclusions, but there was increased disease pathology (7). These latter experiments suggest that modifications of ataxin-1 and modulation of the ubiquitin proteasome system in SCA1 are both critical for pathogenesis.

Previously, a two-hybrid screen was performed in search of ataxin-1 binding partners. From this screen, ataxin-1 interacting ubiquitin-like protein (A1Up) was identified (8). A1Up belongs to the UBL-UBA family of proteins that have a ubiquitin moiety fused to their amino terminus (ubiquitin-like domain, UBL) and a carboxyl terminus ubiquitin-associated domain (UBA) (9). Studies of the amino-terminal UBL family of proteins, such as hHR23A/B, hPLIC1/2, Dsk-2, BAG-1, and Parkin, have demonstrated that the UBL domain of these proteins is capable of binding to the 19S proteasome, more specifically, to the polyubiquitin receptor Rpn10/S5a subunit (10–14). Another subunit of the proteasome, Rpn1, has also been shown to bind to the UBL domain of the yeast homolog of hHR23, Rad23 (15). The residues in hPLIC-1 shown to be critical for interaction of hPLIC-1 with the Rpn10/S5a subunit of the proteasome are Ile79, Ala81, Ile84, Val105, and Ile106 (11). Furthermore, mutagenesis studies showed that Thr39 and Ile69 in the UBL of hHR23A bind to S5a (16, 37).

The 26S proteasome consists of the 20S core that contains the catalytic activity and the 19S regulatory particle that controls entry of ubiquitinated substrates into the 20S. The 19S subunit can associate with one or both ends of the 20S core particle. The 18-subunit 19S particle can be further divided into a base, which contains the AAA-ATPases (regulatory particle trio A proteins, Rpts 1–6), the non-ATPase regulatory particle proteins (Rpn1, Rpn2, and Rpn10), and a lid that is comprised of non-ATPases (Rpn 3–13) (9). Rpn10/S5a, part of the base, can also bind to the lid and is thought to stabilize the interaction between the base and lid (9). Moreover, Rpn10/S5a is the only proteasome subunit that can be found in large abundance dissociated from the intact proteasome (18). Furthermore, it has been suggested that excess free Rpn10/S5a can inhibit proteasome degradation by sequestering polyubiquitinated substrates from reaching the proteasome (9, 19). Rpn10/S5a has been shown to bind monoubiquitin and polyubiquitin

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** To whom correspondence should be addressed: Department of Laboratory Medicine and Pathology, Institute of Human Genetics, University of Minnesota, MMC 206, Minneapolis, MN 55455. Tel.: 612-625-3647; Fax: 612-626-7031; E-mail: orrxx002@umn.edu

† The abbreviations used are: SCA1, spinocerebellar ataxia type 1; HA, hemagglutinin; E3, ubiquitin-protein isopeptide ligase; PLIC, proteasome ligation interacting component; ER, endoplasmic reticulum.

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through the ubiquitin-interacting motifs of Rpn10/S5a, with preference for the latter (10, 20). Polyubiquitin chains consisting of four Lys48-linked ubiquitins function as the canonical degradation signal, and Rpn10/S5a binds these chains primarily through hydrophobic interactions (9, 11). However, Rpn10/S5a has also been shown to bind Lys6 and Lys11 chains with equal affinity to Lys48 chains (21). The exact role of these linkages has not been studied in depth; however, in the case of the UbL protein BAG-1, Lys11 chain formation facilitates degradation-independent targeting of BAG-1 to the proteasome by association with Rpn10/S5a (32). Rpn10 is nonessential in yeast, suggesting that more than one factor is involved in recognition and delivery of polyubiquitinated substrates to the proteasome (18, 23).

The UbL-UBA proteins were hypothesized to be “shuttle factors” capable of delivering polyubiquitinated substrates to the proteasome via polyubiquitin chain binding through their UBA domain and subsequent Rpn10/S5a binding via their UbL domain (12, 15, 24–26). However, recent findings contradict this shuttle hypothesis. Overexpression of UbL-UBA proteins inhibits the degradation of model substrates in yeast rather than stimulating their degradation (12, 24, 27–30). Furthermore, overexpression of UbL-UBA proteins is toxic to yeast and results in an accumulation of high molecular mass ubiquitin species (12, 31). UbL-UBA proteins have also been shown to exert stabilizing effects; however, the exact means by which this occurs has not been determined. It has been hypothesized to be the result of one or more of the following: sequestering Lys48-linked polyubiquitins; blocking access of deubiquitinating enzymes to polyubiquitin chains; capping monoubiquitin chains; and inhibiting ubiquitin chain assembly (24, 27, 28, 30).

To further explore the biological implications of the A1Up-ataxin-1 association, we defined the biochemical properties of A1Up and characterized the effects of interaction of A1Up with ataxin-1 on these properties. Here we show that the ability of A1Up to interact with S5a, the subcellular localization of A1Up, and the half-life of A1Up all depend on the integrity of the UbL and UBA domains of A1Up. We show that A1Up has the ability to stabilize a specific substrate in vivo, namely, ataxin-1, and that the ability of A1Up to stabilize ataxin-1 is dependent on the polyglutamine length of ataxin-1.

EXPERIMENTAL PROCEDURES

Expression Constructs and Antibodies—A1Up (1), ∆UBA, and ∆UBA were cloned into the mammalian expression vector pCDNA3.1 (Invitrogen). The primers for ∆UBA, residues 1–600 was 5′-ggagattcatttctcc-ctea-3′ and 5′-ggagattcatttctcc-tcctc-3′. The primer to construct ∆UBA (residues 1–600) was 5′-ggagattcatcttgagggttac-3′. Plasmids for pCDNA1Amp-ataxin-1(30Q) and pCDNA1Amp-ataxin-1(82Q) have been described previously (32). The plasmid for pCDNA-ataxin-1(82Q)-A776 was also described previously (6). The single lysine ubiquitin plasmids (with other lysines mutated to arginines including G, 11, 27, 29, and 33) pER-HA-Ub (or Lys6 or Lys29) were a gift from Dr. Zhijian J. Chen (33, 34). Expression plasmids for pCMV4-HA-E6AP and pCMV4-HA-E6AP (C833A) were kindly provided by Dr. Peter M. Howley (35). Mouse monoclonal Anti-Xpress antibody (Invitrogen) was used to detect A1Up, ∆UBA, and ∆UBA. Mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology) was used to detect E6AP and E6AP (C833A). Ubiquitylated proteins were detected with rabbit polyclonal antibody to ubiquitin (clone PB2; Affiniti, UK). The antibody used to immunoprecipitate ataxin-1 was 11NQ (32). The 20S proteasome was detected using rabbit polyclonal antibody to 20S α/β subunits (Affiniti, UK).

Cell Transfection, Immunoprecipitation, and Native Electrophoresis—COS-1 cells were plated the day before transfection at 5 × 10⁵ cells/60-mm plate. The following day, the cells were transfected using LipofectAMINE Plus (Invitrogen). Twenty-four or 48 h later, the proteasome was inhibited using clasto-lactacystin β-lactone (20 μM; Boston Biochemicals) by treating transfected cells for 6–7 h. To harvest extracts under non-denaturing conditions, the cells were washed in ice-cold phosphate-buffered saline and lysed in 50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 100 mM NaCl, 0.5% Triton X-100, 1 × protease inhibitors (Roche Biochemicals), and phosphatase inhibitor cocktails I and II (Sigma) with rocking at 4 °C for 15 min. For immunoprecipitations under denaturing conditions, the cells were washed as described above but lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS containing protease inhibitors as described above, with rocking as described above (36). All extracts were sheared with a 21-gauge needle (10 times), followed by a 25-gauge needle (4 times). Cell debris was pelleted by centrifugation at 14,000 × g for 10 min at 4 °C. For immunoprecipitation, extracts were preclarified with protein G-Sepharose (Amersham Biosciences) and nonspecific antibody of the same isotype (0.5 μg/ml) for a time period ranging from 1 h to overnight at 4 °C on a rugged rotor, followed by centrifugation at 12,000 rpm for 20 s at 4 °C. The protein concentration was then determined using protein dye reagent (Bio-Rad). Protein extract (500 μg), Xpress monoclonal antibody (1 μl; Invitrogen) or 11NQ (1:2 μl), and protein G (50 μl) were incubated at 4 °C on a rugged rotor for a time period ranging from 4 h to overnight.

For native gel electrophoresis, COS-1 cells were transfected as described above, and cells were harvested in 50 mM NaHPO₄, 300 mM NaCl, 10 mM imidazole, and 0.5% Triton X-100. Tris–glycine gels (6%, 8%, and 10%; Invitrogen) were used for electrophoresis, and samples were prepared in Tris-glycine native sample buffer and loaded without heating (Invitrogen). To estimate molecular mass of unknowns, the high molecular mass calibration kit for native electrophoresis was used (Amersham Biosciences), and staining was performed with Porcine Staining Solution (Sigma). Sigma technical bulletin MKR-137 was also used for reference using this procedure.

Immunofluorescence—COS-1 cells were plated onto coverslips at 2 × 10⁶ cells/well plate. The following day, they were transfected using LipofectAMINE Plus (Invitrogen). Twenty-four to 48 h after transfection, cells were treated with proteasome inhibitor as described above and then immunostained as described previously (32). A1Up, ∆UBA, and ∆UBA were visualized using mouse monoclonal Anti-Xpress antibody (1:100; Invitrogen). The 20S α and β subunits of the proteasome were detected using rabbit polyclonal anti-20S α/β antibody (1: 2500; Affiniti, UK). Cells were incubated with three secondary antibodies: anti-mouse antibody conjugated with Cy3 (1:100; Jackson Immunoresearch); anti-rabbit antibody conjugated to Alexa488 (1:100; Molecular Probes); and 4′,6-diamidino-2-phenylindole (1:1,000; Sigma) to visualize the nucleus for 1 h at 37 °C. The coverslips were mounted on slides, and analysis was performed using confocal microscopy (Bio-Rad).

In Vitro Binding Assays—Transfected COS-1 cells (48 h) were incubated (500 μg/ml) with purified GST-S5a–agarose beads (30 μl; Affiniti, UK). The mixture was incubated overnight on the rotating wheel at 4 °C. The beads were collected by centrifugation and washed three times with lysis buffer, followed by one wash with Tris-HCl (pH 7.5). The agarose beads were then heated for 10 min at 70 °C in gel loading dye plus reducing agent (Invitrogen), subjected to SDS-PAGE using 4–12% NuPAGE Bis-Tris gels (Invitrogen), transferred to nitrocellulose, and Western blotted with monoclonal Xpress antibody or monoclonal S5a (Affiniti, UK), or the gels were stained with Simply Blue (Invitrogen) to determine total 19S/S5a in each lane.

Pulse-Chase Experiments—COS-1 cells were transfected as indicated above (48 h). Metabolic labeling was carried out by first starving the cells in glutamine and methionine-free media supplemented with 5% dialyzed fetal bovine serum and 1-glutamine. One hour later, these media were removed, and the cells were pulsed in the same media containing 50 μCi/ml [35S]methionine for 1 h. Cells were washed two times and incubated in media supplemented with 10% fetal bovine serum and 180 μg/ml unlabeled methionine. Cells were then chased for various times and collected by washing twice in ice-cold phosphate-buffered saline followed by cell lysis and immunoprecipitation as described above.

RESULTS

The Ubl of A1Up Is Required for A1Up to Interact with the Proteasome—Examination of the sequence alignment of amino-terminal UbL domain-containing proteins with A1Up indicated that the residues responsible for the interaction with the proteasome are also conserved in the UbL of A1Up (11). These correspond to residues Ile65, Ala67, Ile69, Val81, and Ile82 in

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A1Up. Furthermore, the residues confirmed by mutagenesis studies to be responsible for the interaction of the UbL domain with the Rpn10/S5a proteasomal subunit are also conserved in the UbL of A1Up and correspond to residues Thr9 and Ile55 (16, 37). To characterize whether A1Up could interact with the proteasome, a series of deletion mutants were constructed (Fig. 1A). The ability of these forms of A1Up to interact with the proteasome was examined by pull-down analyses of cellular extracts using purified 19S-(S5a)-GST agarose beads. Fig. 1B shows that an interaction with 19S/S5a was detected with either full-length A1Up or A1Up lacking the UBA (but only in the presence of a proteasome inhibitor) (Fig. 1B, lanes 1 and 3).
A1Up lacking the UbL domain did not interact with the 19S proteasome (Fig. 1B, lane 2). These results are consistent with a role of the UbL domain in mediating the interaction between A1Up and the proteasome.

Besides detecting A1Up at 63.8 kDa, the monoclonal anti-Xpress antibody also detected a series of bands of increasing molecular mass of ~10 kDa, suggestive of ubiquitin chain addition to A1Up (Fig. 1B, lane 1). To verify that these polypeptides were ubiquitin-modified A1Up, cellular extracts expressing A1Up and a HA-tagged ubiquitin were used in denaturing immunoprecipitations. Fig. 1C, lane 2 (asterisks) shows that A1Up was covalently modified by the addition of ubiquitin. Examination of the primary sequence of A1Up shows that the UbL domain is the only region that contains lysine residues and, thus, is capable of being ubiquitinated (8). Consistent with this, the distinct high molecular mass bands reflecting the ubiquitination of A1Up (Fig. 1C, lane 2, asterisks) were not detected when the ΔUbL form was used (Fig. 1C, lane 3). However, we did not observe ubiquitination of A1Up lacking the UBA domain (Fig. 1B, lane 3; Fig. 1C, lane 4), suggesting that ubiquitination of A1Up requires both the UbL domain and UBA domain.

To further characterize the ubiquitin chains, we utilized single-lysine mutants of HA-tagged ubiquitin that contain one lysine at residue 48 (Lys48) or at residue 63 (Lys63) with all other lysine residues mutated to arginine (33, 34, 38). We observed polyubiquitination of A1Up in the presence of both of these single-lysine mutants, suggesting that Lys48 and Lys63 of ubiquitin are capable of supporting polyubiquitin chain formation on A1Up (Fig. 1D, lanes 3 and 4). There was a decrease in overall A1Up ubiquitination as compared with wild-type HA-ubiquitin in the presence of Ub(K48) or Ub(K63), suggesting that both Lys48- and Lys63-linked chains contribute to A1Up ubiquitination (Fig. 1D, lanes 3 and 4 compared with lane 2). Overall, the data show that A1Up is covalently modified by formation of complex polyubiquitin chains involving linkages previously shown to be important for proteasome targeting and cellular signaling, and this promotes the interaction of A1Up with the S5a subunit of the 19S proteasome.

Previous studies of the hHR23A protein showed that overexpression of the E3 ligase E6AP enhanced ubiquitin modification of hHR23A and that catalytically inactive (CS33A) E6AP failed to promote ubiquitination of hHR23A (39). To determine whether E6AP could also promote ubiquitination of A1Up, cellular extracts expressing A1Up and E6AP or the dominant negative E6AP (CS33A) were immunoprecipitated with anti-Xpress antibody, and the level of A1Up ubiquitination was determined. E6AP and the mutant E6AP (CS33A) did not affect the ability of A1Up to be ubiquitinated or the total levels of A1Up (Fig. 1E, lanes 2 and 3). Furthermore, co-immunoprecipitation of E6AP with A1Up was not detected (data not shown). Therefore, we conclude that E6AP is not the E3 ligase responsible for the observed ubiquitination of A1Up.

To investigate whether ataxin-1 affects the ability of A1Up to interact with the proteasome, the pull-down analysis with 19S-(S5a)-GST agarose beads was performed using cellular extracts expressing A1Up and either wild-type ataxin-1-(30Q), mutant ataxin-1-(82Q), or ataxin-1-(82Q)-A776 were used in pull-down analysis with 19S-(S5a)-GST agarose beads. The asterisk denotes ubiquitinated A1Up. B, A1Up ubiquitination was disrupted in the presence of ataxin-1. Denaturing immunoprecipitation of A1Up from cells coexpressing A1Up and the different forms of ataxin-1.

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Fig. 2. Ataxin-1 affects the ability of A1Up to interact with the S5a subunit of the proteasome. A, cellular extracts expressing A1Up and either wild-type ataxin-1-(30Q), mutant ataxin-1-(82Q), or ataxin-1-(82Q)-A776 were used in pull-down analysis with 19S-(S5a)-GST agarose beads. The asterisk denotes ubiquitinated A1Up. B, A1Up ubiquitination was disrupted in the presence of ataxin-1.

Interactions (Fig. 2A, lanes 2 and 3, arrow). Interestingly, ataxin-1-(82Q)-A776 significantly interfered with A1Up/S5a interaction, even more so than wild-type ataxin-1-(30Q) (Fig. 2A, lane 4).

To determine whether the ability of wild-type ataxin-1-(30Q) or ataxin-1-(82Q)-A776 to decrease the association of A1Up with S5a was dependent on the ability of ataxin-1 to decrease the ubiquitination of A1Up, cellular extracts expressing A1Up and the different forms of ataxin-1 were immunoprecipitated under denaturing conditions with anti-Xpress antibody, and ubiquitinated forms of A1Up were determined by immunoblotting. Consistent with polyubiquitination of A1Up promoting the ability of A1Up to interact with S5a, there was dramatic-
cally reduced polyubiquitin chain formation on A1Up detected in the presence of ataxin-1-(82Q)-A776 (Fig. 2c) compared with A1Up (Fig. 2B, lane 2) or A1Up transfected with an empty vector (Fig. 2B, lane 8). In contrast, only the highest molecular mass polyubiquitin band was reduced in the presence of wild-type ataxin-1-(30Q) (Fig. 2B, lane 5 versus lane 8). There was no effect on the level of ubiquitinated A1Up in the presence of mutant ataxin-1-(82Q) (Fig. 2B, lane 6 versus lane 8). Overall, the 19S-(S5a)-GST pulldowns and ubiquitination data support a role for wild-type ataxin-1-(30Q) in maintaining A1Up in a configuration that has limited accessibility to the S5a subunit of the 19S proteasome. Ataxin-1-(82Q)-A776 seems to have an accentuated ability to induce such a conformation in A1Up. In contrast, ataxin-1-(82Q)-S776 seems to keep A1Up at S5a, thus the UbL of A1Up maintains continued accessibility to the S5a subunit of the 19S proteasome.

The UBA Domain of A1Up Directs Subcellular Localization of A1Up and Regulates A1Up Stability—To determine whether the UbL and UBA domains of A1Up influence the subcellular localization of A1Up, immunofluorescence was carried out using cells expressing A1Up, ΔUbL, and ΔUBA, all in the presence of proteasome inhibitor. A significant difference in localization of A1Up or ΔUbL was not observed in the presence or absence of proteasome inhibitor (data not shown). There was very little ΔUBA staining in the absence of proteasome inhibitor (data not shown). Full-length A1Up was uniformly distributed between the nucleus and cytoplasm, with large accumulations in both compartments (Fig. 3A, a). In both the nucleus and cytoplasm, A1Up co-localized with the 20S proteasome (Fig. 3A, c). A1Up lacking the UbL (ΔUbL) was also nuclear and cytoplasmic (Fig. 3A, d). However, accumulations of the protein were predominately nuclear (Fig. 3A, d). A1Up lacking the UbL did not co-localize with the 20S proteasome (Fig. 3A, f). In contrast, A1Up lacking the UBA domain co-localized with the 20S proteasome (Fig. 3A, i). Interestingly, the distribution of A1Up lacking the UBA was entirely cytoplasmic upon proteasome inhibition, with the nucleus devoid of staining, and the core subunits of the 20S proteasome were redistributed to the cytoplasm in the presence of A1Up lacking the UBA domain (Fig. 3A, g). These results illustrate that the UBA domain of A1Up has a role in the subcellular localization of A1Up.

To determine whether the presence of the UbL and UBA domains of A1Up impact the stability of A1Up within a cell, the half-life of the various forms of A1Up was determined. COS-1 cells were transfected with A1Up, ΔUbL, or ΔUBA and pulse-chased, and the protein was immunoprecipitated. Intact A1Up was found to be stable over the time course studied (Fig. 3B). A1Up lacking the UbL domain was also stable (Fig. 3B), with both forms of A1Up having a half-life of >4 h (Fig. 3C). This correlates with the results observed after proteasome treatment, that is, no increase detected in total protein levels of A1Up or ΔUbL (data not shown). In contrast, deletion of the UBA domain of A1Up resulted in a half-life of 60 min (Fig. 3C), thus this correlates with the immunofluorescence data showing complete co-localization of ΔUBA and the 20S proteasome and further indicates that the UBA domain is important for the stability of A1Up.

The UBA of A1Up Binds Polyubiquitin Chains—To further define the role of A1Up, the ability of A1Up to bind ubiquitin was examined. Immunoprecipitations under non-denaturing conditions were used to characterize the ubiquitin binding ability of Xpress-tagged intact A1Up and the UbL and UBA deletion mutants using mouse monoclonal anti-Xpress antibody, followed by Western blot with monoclonal antibody to polyubiquitinated and monoubiquitinated proteins. Full-length A1Up and the amino-terminal truncation lacking the UbL were both able to co-immunoprecipitate polyubiquitin (Fig. 4A, lanes 2 and 3). In contrast, A1Up that lacked the UBA domain was considerably less able to co-immunoprecipitate polyubiquitin (Fig. 4A, lane 4). A1Up lacking the UBA did not completely eliminate polyubiquitin chain binding, suggestive of another region on A1Up besides the well-defined carboxyl-terminal UBA domain that contributes to the ubiquitin binding of A1Up. Sequence alignment of hHR23A/B internal UBA domains (residues 163–200 and 190–227, respectively) with the internal sequence of A1Up (residues 235–269) shows that the residues determined to be important for ubiquitin binding in hHR23A/B are in fact moderately conserved in the internal sequence of A1Up (Fig. 4B (17, 40). Moreover, the leucine at the end of the UBA domain of hHR23A/B (residues 198 and 225, respectively), which was originally determined to be important for ubiquitin binding but recently identified by NMR to be important for the overall structural integrity of the α helix that defines the UBA domain, is conserved in the sequence of A1Up (residue 272) (27, 40, 41). We propose that the sequence of A1Up contains an internal UBA domain (Fig. 4B).

Using the two-hybrid system, A1Up was shown to self-associate (8). Another UBL-UBA protein, Rad23, the yeast homolog of hHR23, has been shown to form homodimers (42). However, the mammalian homolog, hHR23A, behaves as a monomer, with intramolecular interactions between UBL and UBA domains (16). To determine the molecular mass of A1Up in mammalian cells, cellular extracts expressing A1Up, ΔUbL, and ΔUBA were electrophoresed through non-denaturing 6%, 8%, and 10% Tris-glycine gels. Using molecular mass standards, Ferguson plots were generated by plotting the electrophoretic mobility versus the gel concentration for each standard (43) (Fig. 4C). The slope of each protein standard is the retardation coefficient, and the logarithm of the negative slope is plotted against the logarithm of each molecular mass standard (Fig. 4C (44–46). A linear plot was produced, and the molecular mass of A1Up, ΔUbL, and ΔUBA was calculated. A1Up was determined to have a molecular mass of 425 kDa, a significantly greater size than that expected if it were behaving as a monomer (63.8 kDa) (Fig. 4C, lanes 1 and 2). A1Up lacking the UBA domain was able to form even higher molecular mass species suggestive of further multimerization (Fig. 4C, lane 5). Deletion of the UbL domain diminished the ability of A1Up to form higher molecular mass oligomers, with a size of 153 kDa, approximately the expected size for a trimer. Our data indicate that the UbL domain plays an essential role in the ability of A1Up to form multimers in mammalian cells.

A1Up Exerts a Stabilizing Effect on Ataxin-1 That Is Polyglutamine Tract-dependent—There are now several reports of the yeast homolog of hHR23A/B, Rad23, inhibiting degradation by sequestering Lys48-linked polyubiquitin chains, inhibiting access of deubiquitinating enzymes to substrate-linked ubiquitin chains, and inhibiting ubiquitin chain assembly (24, 27, 28, 30). To determine whether A1Up affects degradation of an interacting protein, the half-life of ataxin-1 was determined in the presence and absence of A1Up. Using a pulse-chase radiolabeling approach employing transfected cells, there was not a significant difference in the clearance of wild-type ataxin-1-(30Q) versus mutant ataxin-1-(82Q); both had a half-life of ~8 h (Fig. 5B). However, the half-life of ataxin-1-(82Q)-A776 was decreased, with a half-life of ~6 h (Fig 5B). This result confirms the data reported by Chen et al. (5) that the regulatory molecule 14-3-3 preferentially interacts with phosphorylated mutant ataxin-1-(82Q) at Ser275 and, as a result, increases the steady-state level of mutant ataxin-1-(82Q). Interestingly, in
the presence of A1Up, the half-life of wild-type ataxin-1-(30Q) increased to >11 h. To a lesser extent, A1Up also increased the half-life of ataxin-1-(82Q)-A776 to 8 h (Fig. 5B). The ability of A1Up to stabilize wild-type ataxin-1-(30Q) was only partially independent on the UbL of A1Up because A1Up lacking the UbL domain also stabilized wild-type ataxin-1-(30Q) (Fig. 5B). In contrast, A1Up was not able to influence the half-life of mutant ataxin-1-(82Q) (Fig. 5, A and B).
The Half-life of A1Up Is Compromised in the Presence of Ataxin-1—Since we found that ataxin-1-(82Q)-A776 and wild-type ataxin-1-(30Q) decreased the ubiquitination of A1Up and S5a proteasomal association, the half-life of A1Up was determined in the presence of wild-type ataxin-1-(30Q), mutant ataxin-1-(82Q), and ataxin-1-(82Q)-A776. COS-1 cells were

**Fig. 4.** The UBA domain of A1Up binds polyubiquitin chains. **A**, non-denaturing immunoprecipitation of Xpress-tagged A1Up and deletion constructs. The amount of polyubiquitinated chains pulled down by A1Up, ΔUBL, and ΔUBA was analyzed by Western blot with an antibody that recognizes mono- and poly-conjugated ubiquitin. Total protein was determined by Western blot with Xpress antibody. **B**, top, alignment of UBA1 of hHR23A and hHR23B with internal residues in A1Up. Residues shown in green are conserved among all three proteins. Residues that are conserved between two proteins are shown in dark gray. Light shading represents shared similarity between amino acids. Bottom, proposed schematic of A1Up showing UBA1 (residues 235–269). **C**, native PAGE of A1Up and deletion constructs. A1Up was lysed under native conditions and separated on an 8% Tris-glycine gel. A1Up and deletion constructs were visualized with anti-Xpress antibody. To determine the molecular mass of A1Up, ΔUBL, and ΔUBA indirectly, a Ferguson plot was generated. A1Up, ΔUBL, ΔUBA, and non-denatured protein molecular mass standards were electrophoresed through 6%, 8%, and 10% Tris-glycine gels. The standards were then transferred to nitrocellulose and stained with Ponceau Red.
Fig. 5. A1Up stabilizes wild-type ataxin-1-(30Q). A, autoradiograph of ataxin-1-(30Q), ataxin-1-(82Q), and ataxin-1-(82Q)-A776 with and without A1Up. After radiolabeling, immunoprecipitations were performed using anti-ataxin-1 (11NQ) antibody at the indicated time points during a chase with nonlabeled methionine. B, amount of radiolabeled ataxin-1 remaining after the indicated time points. Experiments with ataxin-1/without A1Up were replicated three to four independent times with similar results. Results with ataxin-1/with A1Up were replicated in two to three independent experiments with similar results.
transfected with A1Up and ataxin-1, pulse-chased and A1Up was immunoprecipitated. A1Up was a stable protein, with a half-life of 10 h (Fig. 6, A and B). In the presence of ataxin-1-(30Q) and ataxin-1-(82Q)-A776, the half-life of A1Up was decreased to <8 h (Fig. 6, A and B), with a substantial amount of A1Up cleared in the first 4 h in the presence of ataxin-1-(82Q)-A776 as compared with A1Up alone. Unlike wild-type ataxin-1-(30Q), the presence of mutant ataxin-1-(82Q) further stabilized A1Up over the time course studied, increasing the half-life of A1Up to >11 h (Fig. 6, A and B).

**DISCUSSION**

Analysis of the primary sequence of A1Up revealed that this protein contains both UbL and UBA motifs (8). Proteins that have both an UbL and UBA domain are thought to play a crucial role in proteasome-mediated activities. Similar to other UbL-containing proteins, the UbL of A1Up was shown to be essential for interaction of A1Up with the S5a subunit of the 19S proteasome. In addition, we demonstrated that the UBA of A1Up, similar to other UbL-UBA proteins, bound polyubiquitin chains. Thus, the UbL and UBA of A1Up have the basic biochemical properties characteristic of other UbL-UBA proteins. At a functional level, A1Up was also found to have similarities with other UbL-UBA proteins. Based on the ability of UBA domains to bind polyubiquitin chains, UBA domains can exert a stabilizing effect on specific target substrates. For example, hHR23A degradation is inhibited upon UV-induced DNA damage, which results in increased levels of hHR23A and subsequent stabilization of the DNA damage repair protein XPC (xeroderma pigmentosum group C) (33). Furthermore, the yeast homolog of hHR23A/B, Rad23, stabilizes the cell cycle protein Pds1, and the *Xenopus* homolog of Dsk2, XDRP1, stabilizes cyclin A (27, 28). Similar to these other UbL-UBA proteins, we demonstrated that A1Up stabilized its target substrate, wild-type ataxin-1-(30Q).

The PLIC proteins were identified based on their ability to
interact with the E3 ligase E6AP through their UbL domain (47). In addition, the levels of hHR23A/B are regulated in mammalian cells in a cell cycle-dependent manner by E6AP, which enhances hHR23A ubiquitination and degradation (39).

Based on the sequence homology between the UbL and UBA domains of A1Up containing only the UbL domain for ability to interact with E6AP, and it is possible that the UBA domain of A1Up masks the UbL domain, preventing an interaction of intact A1Up with E6AP. This seems unlikely, considering that full-length A1Up was ubiquitinated, and we were not able to detect even a modest interaction with full-length A1Up and E6AP, as was the case with PLIC proteins (47). Thus, although significant homology exists between the UbL and UBA domains of A1Up and the corresponding domains on hPLIC-1/2 and hHR23A/B, the observed ubiquitination of A1Up is not dependent on the ligase activity of E6AP. Another E3 ligase, CHIP (carboxyl terminus of hsc 70-interacting protein), has been shown to be responsible for the ubiquitination of BAG-1 and Hsp70 and the core subunits of the proteasome, not all A1Up molecules were found to co-localize with the proteasome. This suggests the role of A1Up with the proteasome is not only the function of A1Up, further indicating the importance of ubiquitination of A1Up by Lys48- and Lys63-linked chains and possibly other undetermined ubiquitin chain linkages. Another group reported on a ubiquitin-like protein, UBIN, that was identified on compartments and pathways, possibly the endoplasmic reticulum (48).

Ataxin-3 has been postulated to be a polyubiquitin chain receptor of the proteasome, as well as a deubiquitinating enzyme hypothesized to facilitate deubiquitination of polyubiquitinated substrates before entry into the proteasomal core (36, 51, 53). However, in either case, no differences were observed between wild-type ataxin-3 and expanded (mutant) ataxin-3, thus providing no insight into whether these activities of ataxin-3 have a role in the disease mechanism underlying SCA3.

The assessment of overexpression of a UbL-UBA protein and its toxicity caused by accumulation of higher molecular mass polyubiquitin conjugates suggest that control of accessibility of the UbL and UBA domains, as well as control of the levels of UbL-UBA protein in the cell, needs to be tightly regulated. Using NMR, intradomain interactions were reported for the first time for the UBA domains of hHR23A and hHR23B to the UbL domain (16, 54). These intradomain interactions could be important for controlling proteolysis of by hHR23 through sequestration of UBA domains by the UbL domain. These structural data correlate with the result observed in vitro that deletion of the UbL domain enhanced the ability of Rad23 to sequester Lys48-linked chains, indicating that deletion of the UbL does increase the accessibility of the UBA domain (16, 30).

The authors showed that UbL domain binding to S5a and ubiquitin binding to UBA domains facilitates hHR23A/B in an open conformation, and the residues responsible for UbL-UBA intradomain interactions are similar to those that participate in UbL binding to S5a and ubiquitin binding to the UBA domains (16, 40). Thus, it was suggested that interaction with S5a and ubiquitin in vivo is regulated by the accessibility of hHR23A/B to form intramolecular interactions. The residues in UBA1 and UBA2 of hHR23A/B shown to be important for this interaction with the UbL domain are conserved in A1Up, suggesting that A1Up could also form intramolecular interactions (16, 40, 54).

Depending on its interaction with ataxin-1, we suggest a model in which the distribution of A1Up among different states varies (Fig. 7). In the absence of ataxin-1, a steady state exists between "closed" A1Up via intra- or interdomain interactions and A1Up ubiquitinated through Lys48, Lys63, or other lysine-linked ubiquitin moieties (Fig. 7A). Ubiquitination of A1Up induces it to adopt an open conformation in which the UBA domain is free to bind polyubiquitinated substrates, and the UbL domain binds S5a, thereby targeting A1Up to the free subunit S5a of the 19S proteasome. Thus, this interaction with S5a need not be linked to A1Up degradation. The fact that the only region of A1Up in which lysines are found is the UbL is consistent with ubiquitination having a role in regulation of UbL-UBA interactions in A1Up. Direct evidence in support of this model is our data demonstrating that the UbL domain of A1Up is responsible for the interaction of A1Up with the proteasome. Furthermore, the in vitro association of A1Up with S5a was detected when A1Up was covalently modified by ubiquitin. The robust ubiquitination of intact A1Up and association with S5a were observed in both the presence and absence of proteasome inhibition, supporting the idea that ubiquitination and S5a association of intact A1Up were not degradation-dependent. In addition, intact A1Up was shown to be stable. In contrast, A1Up lacking the UBA domain associated with S5a independent of A1Up ubiquitination. However, this interaction was only detected in the presence of proteasome inhibitor, indicating that A1Up lacking the UBA domain was rapidly degraded. The ubiquitin pattern of A1Up was shown to be complex, consisting of Lys48- and Lys63-linked chains. Thus, ubiquitination of A1Up may also target A1Up to other cellular compartments and pathways, possibly the endoplasmic reticulum (48).

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The presence of nonpathogenic forms of ataxin-1 is proposed to disrupt the steady state, promoting a "closed" A1Up, thereby reducing A1Up ubiquitination and the UbL-S5a interaction (Fig. 7B). In support of this, we detected a decrease in A1Up ubiquitination in the presence of the two forms of ataxin-1 that do not cause disease, wild-type ataxin-1-(30Q) and ataxin-1-(82Q)-A776.
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(82Q)-A776, which cannot be phosphorylated at position 776. There was also a decrease in S5a association of A1Up in the presence of ataxin-1-(82Q)-A776 and wild-type ataxin-1-(30Q).

In terms of pathogenesis, a key point is the hypothesis that in the presence of nonpathogenic ataxin-1, the accumulation of A1Up along with other polyubiquitinated proteins would be low (Fig. 7B).

Conversely, the interaction between A1Up and mutant ataxin-1-(82Q) is proposed to allow an open conformation in A1Up along with other polyubiquitinated proteins would be low.

Previously, it was shown that in the presence of a nonpathogenic ataxin-1, the accumulation of A1Up, ubiquitination of A1Up, and interaction of A1Up with ataxin-1-(82Q)-A776, which cannot be phosphorylated at position 776.

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The Effects of the Polyglutamine Repeat Protein Ataxin-1 on the UbL-UBA Protein A1Up
Brigit E. Riley, Yifan Xu, Huda Y. Zoghbi and Harry T. Orr

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