ALS-linked mutations in ubiquilin-2 or hnRNPA1 reduce interaction between ubiquilin-2 and hnRNPA1

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Abstract

Amyotrophic lateral sclerosis (ALS)-linked mutations in UBQLN2 and some members of the heterogeneous nuclear ribonucleoproteins (hnRNPs) family cause ALS. Most mutations in UBQLN2 are missense mutations that occur in and around a PXX repeat motif located in the central domain of the encoded protein. However, neither the function of the PXX motif nor the mechanism by which mutations in UBQLN2 cause ALS is known. We screened a yeast two-hybrid library using the central domain of ubiquilin-2 hoping to identify proteins whose binding is affected by the UBQLN2 mutations. Three such interactors were identified—hnRNPA1, hnRNPA3 and hnRNPU—all members of the hnRNP family. The interacting region in each of these proteins was their glycine-rich domain, the domain most frequently mutated in hnRNP-related proteins that cause ALS. We focused on hnRNPA1, because a mutation in the protein causes ALS. We confirmed the interaction between wild-type (WT) ubiquilin-2 and hnRNPA1 proteins in vitro and in cells. In contrast, all five ALS mutations in ubiquilin-2 that we examined had reduced binding with WT hnRNPA1. In addition, hnRNPA1 carrying the D262V missense mutation that causes ALS failed to bind WT ubiquilin-2. Overexpression of ubiquilin-2 containing the ALS mutations increased cell death and, for several of the mutants, this correlated with increased translocation of hnRNPA1 to the cytoplasm. Knockdown of ubiquilin-2 led to increased turnover of hnRNPA1, indicating ubiquilin-2 functions to stabilize hnRNPA1. The discovery that ubiquilin-2 interacts with hnRNP proteins and that mutation in either protein disrupts interaction suggests a connection between proteostasis and RNA metabolism.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder associated with progressive demise of upper and lower motor neurons (1–3). The disease is characterized by rapid neuromuscular deterioration, culminating in fatal paralysis ~2–5 years after diagnosis. Like many other neurodegenerative diseases, there is no effective treatment to slow or prevent ALS.

ALS typically strikes in the fifth decade of life. Approximately 90% of all ALS cases are sporadic, occurring in individuals with no prior family history of the disease. The remaining 10% can be traced to inheritance of mutant genes (1). Mutations in ~20 different genes have been identified as the cause of familial ALS, but the precise mechanism(s) by which any of them cause disease still remains unresolved.

Clues into the etiology of ALS are emerging by examining the functions of the mutated genes. The genes can be categorized into two large groups: those that affect RNA metabolism and protein homeostasis (proteostasis), suggesting that perturbation of these processes can cause ALS. However, several other genes do not fall into these groups, but they are instead involved in diverse cellular functions, suggesting that defects in a wide variety of cell targets can cause ALS. The genes in which problems in RNA metabolism are directly or indirectly linked to ALS include C90rf72, TARDBP, ANG, FUS, TAF15, SETX and HNRNPA1 (2–4).
The genes linked to dysregulation of proteostasis include UBQLN2, SQSTM1, VCP, CHMP2B and UBQLN1 (3). At present, it is unclear whether the two groups are separate or connected in some way.

UBQLN2 mutations were first identified as a cause of X-linked dominant inheritance of ALS by Deng et al. (5). They reported on five families each of whom carried separate missense mutations (P497H, P497S, P506T, P509S and P525S mutations) in UBQLN2, an intron-less gene located on the X chromosome. Individuals who carried some of these mutations, particularly the P506T and P497S mutations, developed unusual and aggressive forms of ALS. Many had early-onset ALS, some beginning as early as their late teens and early twenties, and several of them also had dementia. Additional mutations and/or variants in UBQLN2 have now been found in other families, and some of them also appear particularly aggressive (6,7). Screening of ALS repositories indicates that the frequency of UBQLN2 mutations in general is rare (8,9).

Interestingly, all of the mutations in UBQLN2 that cause ALS map in, or near, a highly unusual PXX repeat that is only present in the ubiquilin-2 protein. Ubiquilin-2 is one of four ubiquitin proteins found in humans. The proteins have been implicated in regulating proteostasis by facilitating protein clearance through the proteasome and autophagy degradation pathways (10,11). The proteins are characterized by having a short N-terminal ubiquitin-like domain (UBL), followed by a longer more variable central domain, and a short C-terminal ubiquitin-associated domain (UBA) (12–14). The UBL domain binds subunits of the proteasome, while the UBA domain binds polyubiquitin chains typically conjugated onto proteins targeted for degradation. The location of the PXX motif where most of the UBQLN2 mutations cluster is in the central domain of the protein, away from its UBL and UBA domains that function in proteasome degradation. The central domain contains sites where chaperones and proteins that regulate ubiquitin function bind, such as Stch, erasin and UBXD8 (15–17). These bindings suggest that the central domain might serve as an adaptor to regulate different ubiquitin function(s) in cells. The exact mechanism by which the UBQLN2 mutations cause ALS is not known, but some initial studies suggest that they affect protein degradation (5,17).

To explore the mechanisms of how mutations in UBQLN2 might cause ALS, we conducted a yeast two-hybrid screen to search for proteins that bound to the central domain of wild-type ubiquilin-2, but whose interaction was altered by the ALS mutations. By this screen, we identified three different heterogeneous nuclear ribonucleoproteins (hnRNPs) (18,19). Here we describe the evidence that demonstrates one of them, hnRNP1A1, binds wild-type ubiquilin-2 and that ALS-causing mutations in either of the proteins reduce this binding. The identification of this interaction and its disruption by the ALS mutations could have important implications for understanding ALS pathogenesis.

**Results**

**Recovery of three different hnRNP proteins by their interaction with ubiquilin-2 in a yeast two-hybrid screen**

We reasoned that the central domain of ubiquilin-2, where all of the ALS mutations map, binds proteins and that the mutations alter this binding. To search for these binding proteins, we used the central domain of human ubiquilin-2 including the PXX repeat sequence as bait to screen a human brain cDNA library for interactors (Fig. 1A). The UBL and UBA domains were excluded from the bait to avoid isolating proteasome subunits and ubiquitinated substrates, which are known to bind these end domains (20–22). Several positive clones were identified. Sequence analysis revealed that three of them were related in sequence, corresponding to hnRNPA1, hnRNPA3 and hnRNPU proteins (18,19).

Interestingly, the region recovered for each hnRNP protein was their C-terminal end [amino acid (aa) 219 to the C-terminus in hnRNPA1, aa 262 to the C-terminus in hnRNPA3 and aa 724 to the C-terminus in hnRNPU], all of which contained a glycine-rich domain (Fig. 1B). The glycine-rich domain is present in different locations in most hnRNPs, but its precise function is not known (23). Interestingly, it is the site where most ALS mutations map in the hnRNP-related proteins TDP43 and FUS (23–25). Because mutations in RNA-binding proteins are known to cause ALS, we focused our studies on these proteins, but especially on hnRNPA1, because mutations in it cause ALS (4).

We next examined whether the ALS mutations in ubiquilin-2 affect hnRNP protein binding by measuring the strength of interaction in yeast of the recovered hnRNP A1, A3 and U preys with ubiquilin-2 baits containing either the wild-type sequence or each of the five different ALS mutations described by Deng et al. (5). For each prey, interaction was also measured with the empty vector and with a random unrelated lamin-C bait, as controls. As shown in Figure 2, all of the hnRNPs proteins failed to interact with either of the control baits, as expected. However, all three hnRNP proteins interacted with the wild-type ubiquilin-2 baits, but with different strengths according to the β-galactosidase assays. hnRNPA3 interacted strongly, followed by hnRNPA1 and then hnRNPU. Comparison of the interaction of the hnRNP preys with the different wild-type and mutant ubiquilin-2 baits showed a general trend of all three hnRNP proteins interacting more weakly with the baits containing the ALS mutations than with wild-type ubiquilin-2 (Fig. 2A). However, there was one notable exception, the P506T mutant, which appeared to interact more strongly with hnRNPA1 and hnRNPU proteins. Immunoblot of the yeast lysates indicated that the P506T bait was not expressed to any higher extent than the other baits (Supplementary Material, Fig. S1), suggesting that the increased interaction seen with the bait was not from a simple increase in the expression of the bait. At present, we do not know why this bait interacted more strongly with the preys, but it could be related to the use of truncated proteins in the assay. Our communoprecipitation results done with the full-length proteins (described below) are likely to be more accurate.

**Confirmation of binding of recombinant full-length hnRNPA1 and hnRNPU proteins with ubiquilin-2 by GST-pull-down assays**

To confirm the specificity and interaction of hnRNP proteins with ubiquilin-2, we conducted GST-pull-down assays incubating in vitro translated [35S]-methionine radiolabeled hnRNPA1 and hnRNPU proteins with either GST-ubiquilin-1 or GST-ubiquilin-2 fusion proteins, or with GST protein alone. The hnRNPA3 protein could not be assayed as it was poorly expressed in this and our other experiments and was therefore not studied further. The assays revealed that hnRNPU protein was preferentially pulled down by GST-ubiquilin-2, but not by GST-ubiquilin-1 or by the control GST alone (Fig. 3A and B). In contrast, hnRNPA1 was pulled down to different degrees by the different GST proteins, including by the control GST alone. The background binding of hnRNPA1 to GST alone persisted despite repeated washes with different detergents and increasing salt concentrations in these and all subsequent experiments, suggesting it is quite...
Sticky. Subtraction of this background binding revealed that GST-ubiquilin-2 pulled down five times more hnRNPA1 than GST alone, whereas GST-ubiquilin-1 protein pulled down approximately half as much as GST-ubiquilin-2 (Fig. 3B). To establish whether the binding between hnRNPA1 and ubiquilin-2 proteins is direct and to further confirm their binding, we repeated the GST-pull-down experiment. The glycine-rich region (GRR) in each hnRNP protein is highlighted in red. Note the region found to interact with ubiquilin-2 in the Y2H clones was mainly with the GRR in each hnRNP.

Figure 1. Ubiquilin-2 binds to hnRNPs. (A) Schematic of human ubiquilin-2 (top) and the baits used for yeast two-hybrid studies (Y2H) (bottom). Six bait proteins were generated in total with one containing the WT sequence (B1) and five carrying the P497H or P497S or P506T or P509S or P525S mutations in the PXX domain. (B) Sequence alignments of three clones from the Y2H cDNA library screen showing the region of overlap with the open reading frames of hnRNPA1, hnRNPA3 and hnRNPU. The glycine-rich region (GRR) in each hnRNP protein is highlighted in red. Note the region found to interact with ubiquilin-2 in the Y2H clones was mainly with the GRR in each hnRNP.

Figure 2. Mutant ubiquilin-2 constructs have reduced interaction with hnRNPs. (A–C) Y2H β-galactosidase liquid culture interaction assays comparing hnRNPA1, hnRNPA3 and hnRNPU with different baits (the first six baits are WT and the five ubiquilin-2 mutants, followed by PEG202, the empty vector control and another lamin C control). The β-galactosidase units were normalized to the WT bait. Several of the ubiquilin mutants interacted less strongly with the hnRNP preys. (A) One-way ANOVA (F7,23 = 10.17, P ≤ 0.0001), (B) one-way ANOVA (F7,23 = 16.74, P ≤ 0.0001) and (C) one-way ANOVA (F7,23 = 6.25, P = 0.0012). Tukey’s post hoc tests: **P < 0.01; ***P < 0.0001. Data shown are the mean ± SEM.
assays, but this time used purified bacterially expressed His-hnRNPA1 as ligand. These pull-down assays revealed preferential binding of His-hnRNPA1 with GST-ubiquilin-2, but not with GST-ubiquilin-1 or GST alone (Fig. 3C and D).

**Demonstration that endogenous ubiquilin and hnRNP proteins bind in cells**

To determine whether endogenous ubiquilin and hnRNP proteins found in cells bind in a complex, we immunoprecipitated ubiquilin from HeLa cells using a pan-ubiquilin-specific antibody and examined the precipitates for the presence of hnRNPA1 and hnRNPU proteins by immunoblotting. As shown in Figure 4A, both hnRNPA1 and hnRNPU were coimmunoprecipitated with ubiquilin, but not by the control immunoprecipitation lacking the ubiquilin antibody. Binding to hnRNPA3 could not be assessed because of lack of a suitable antibody to detect its presence. The results confirmed binding between endogenous ubiquilin proteins and hnRNPA1 and hnRNPU proteins.

Because multiple ubiquilin proteins are expressed in HeLa cells (ubiquilin-1, 2 and 4 proteins), we next investigated whether hnRNPA1 binds ubiquilin-2. To address this, HeLa cells were either mock transfected or transfected with expression plasmids encoding either full-length HA-tagged ubiquilin-2 by itself or together with full-length Myc-tagged hnRNPA1. The expressed HA-tagged ubiquilin-2 protein was immunoprecipitated from the cells using an anti-HA antibody and the precipitates examined for binding of endogenous and/or the exogenously expressed hnRNPA1 protein. The blots showed endogenous hnRNPA1 coimmunoprecipitates with HA-tagged ubiquilin-2 (Fig. 4B). The specificity of this coimmunoprecipitation can be seen by the almost complete absence of the hnRNPA1 signal in the cells in which HA-ubiquilin-2 was not expressed. We also probed the precipitates for nucleolin to see whether ubiquilin-2 binds any protein containing a glycine-rich sequence. Nucleolin contains 34 glycine residues in a 52 amino acid stretch near its C-terminus. However, the immunoblots indicated that only trace amounts of nucleolin were coprecipitated with ubiquilin-2, which was in contrast to the higher amounts of hnRNPA1 that was coinmunoprecipitated (Supplementary Material, Fig. S2). The result suggests that ubiquilin-2 does not bind indiscriminately to any protein with a glycine-rich sequence. Further confirmation that
hnRNPA1 forms a complex with HA-ubiquilin-2 was seen by the coprecipitation of Myc-tagged hnRNPA1 protein with HA-ubiquilin-2 (Fig. 4B, last lane). Taken together, these in vitro and cell binding studies provide strong evidence that ubiquilin-2 binds hnRNPA1 protein.

Ubiquilin-2 proteins containing ALS mutations have reduced binding to hnRNPA1 protein

We next investigated whether ubiquilin-2 proteins containing ALS mutations were altered in their ability to bind hnRNPA1 protein. To examine this, we transfected HeLa cell cultures with constructs encoding HA-tagged ubiquilin-2 WT or each of the P497H, P497S, P506T, P509S and P525S mutations. The HA-tagged ubiquilin-2 proteins were immunoprecipitated from each culture and the precipitates examined for binding of endogenous hnRNPA1 protein by immunoblotting (Fig. 4C). The ratio of hnRNPA1 protein coprecipitated relative to HA-ubiquilin-2 protein precipitated was then calculated for each immunoprecipitation. The quantification revealed that ubiquilin-2 proteins containing all of the ALS mutations analyzed bind significantly less hnRNPA1 protein than does WT ubiquilin-2 (Fig. 4C and D).

The D262V hnRNPA1 mutant that causes ALS has reduced binding to ubiquilin-2

We next asked whether the converse is also true, does the D262V mutation in hnRNPA1 that causes ALS (4) affect hnRNPA1 binding to ubiquilin-2? To assess this, we purified recombinant His-tagged hnRNPA1 proteins encoding either the WT or D262V mutant protein and used equivalent amounts of each to examine binding to WT ubiquilin-2 by GST-pull-down assays (Fig. 5). Again, the bindings were corrected for the background binding to GST alone and were then normalized to WT hnRNPA1 binding. The results indicate that GST-ubiquilin-2 binds WT hnRNPA1, as expected, but fails to bind hnRNPA1 containing the D262V mutation. The results support the idea that ALS mutations in either
hnRNPA1 or ubiquilin-2 have the same effect: they reduce interaction between the two proteins (Fig. 5B and C).

Cells expressing mutant ubiquilin-2 proteins have altered subcellular distribution of hnRNPA1 protein

Several hnRNP proteins, including hnRNPA1, shuttle between the cytoplasm and nucleus (19, 26). Because ubiquilin-2 proteins also segregate between the two compartments (13), we reasoned that changes in the binding between the ubiquilin-2 mutants and hnRNPA1 proteins might alter the subcellular distribution of one or both proteins. To examine this possibility, we biochemically fractionated HeLa cell cultures transfected with cDNAs encoding either HA-tagged WT ubiquilin-2 or each of the five ALS mutations into a cytosol (C), soluble nuclear and mitochondrial (N), and an insoluble pellet (P) fraction using differential centrifugation and variation in Triton-X100 extraction. The identity of the pellet fraction is not known but could represent aggregated protein. Equal volumes of the three fractions were probed by immunoblotting to determine the relative proportion of hnRNPA1 and HA-tagged ubiquilin-2 protein in each of them (Fig. 6A). Immunoblots for caspase-3, Tom-20 and lamin B were used as controls to monitor the quality and consistency of the fractionations. The controls partitioned differently, but in a manner consistent with their distinct subcellular locations and/or their biochemical properties. Importantly, the profile of the fractionation of the controls was the same across all sets, demonstrating the reproducibility of the fractionations. The experimental HA-tagged ubiquilin-2 WT and mutant proteins also had similar subcellular fraction patterns, with highest amounts of the proteins being present in the cytoplasm and slightly lesser amounts in the insoluble fraction and the least amount in the soluble nuclear fraction (Fig. 6B – sorbitol, for simplicity only the cytoplasm fraction is shown). The similarity suggests the ubiquilin-2 mutants partition biochemically in a similar manner to WT ubiquilin-2 by this assay. Instead, fractionation of endogenous hnRNPA1 protein was found to be altered in the cells transfected with four of the five ubiquilin-2 mutants, as evidenced by a larger amount of mutant protein in the cytoplasm compared with that seen in mock-transfected cells, or those transfected with WT ubiquilin-2 (Fig. 6C – sorbitol).

Ubiquilin-2 mutants are more sensitized to osmotic shock-induced cell death

We next examined whether expression of the ALS ubiquilin-2 mutants also affects hnRNPA1 shuttling. hnRNPA1 protein

Figure 5. hnRNPA1 carrying the ALS D262V mutation has reduced binding to ubiquilin-2. (A) Coomassie stain gel showing the purified recombinant proteins used in the pull-down studies described in B. Left-hand panel are the GST proteins (lane 1, GST alone; lane 2, GST-WT-ubiquilin-2) and the right-hand panel the HIS-tagged hnRNPA1 proteins (lane 1, WT HIS-hnRNPA1; lane 2, HIS-hnRNPA1 carrying the D262V). The arrows indicate the positions of the full-length fusion proteins in the different lanes. (B) GST-pull-down studies comparing binding of WT HIS-hnRNPA1 and HIS-hnRNPA1 D262V with GST alone and GST-ubiquilin-2. Equal amounts of the HIS-tagged proteins were analyzed for binding the GST proteins. The blots shown are similar to those described in Figure 3C. Although both hnRNP proteins displayed non-specific binding to the GST proteins, HIS-tagged hnRNPA1 bound more strongly to GST-ubiquilin-2 than GST alone. (C) Quantification of the binding studies shown in C after subtracting the background binding to GST alone. The D262V mutation abolishes binding to ubiquilin-2. Unpaired t-test t(4) = 5.879, P = 0.0042, ** P < 0.01. Data shown are the mean ± SEM.
shuttles from its predominantly nuclear location to the cytoplasm in cells exposed to osmotic shock (i.e. treatment with 600 mM sorbitol) (27). The translocation is thought to be part of a protective response to the insult. We therefore quantified the extent of hnRNPA1 translocation to the cytoplasm in the transfected cultures following treatment with 600 mM sorbitol for 2 h, comparing it with those seen in the untreated cultures (Fig. 6A + sorbitol). The change in distribution of the HA-tagged ubiquilin-2 proteins was also quantified (Fig. 6A and B). Sorbitol treatment resulted in an increase in distribution of ubiquilin-2 in the soluble nuclear fraction and a concomitant reduction in the cytoplasm fraction compared with untreated cells, but there was no statistical difference in the change between cells transfected with WT and the ubiquilin-2 mutants. In contrast, hnRNPA1 distribution changed in an opposite manner. It increased in the cytoplasm following sorbitol treatment, but the overall accumulation was little different in cells transfected with the WT and mutant ubiquilin-2 constructs. However, net translocation was reduced for the same four mutants that displayed increased cytoplasmic translocation when cultured under normal growth conditions.

We also quantified cell death in the transfected cultures grown under normal conditions and following 2 h of sorbitol treatment (Fig. 7). The transfection efficiency in these cultures typically ranged between 85 and 95%. Thus, any estimate of cell death would be a slight underestimate considering a small percentage of cells were untransfected. Nevertheless, the quantification revealed that overexpression of all of the ubiquilin-2 mutants, but not the WT, increased cell death compared with mock-transfected cells, irrespective of whether they were treated with sorbitol or not (Fig. 7B). The increase in cell death induced by expression of the mutants was confirmed for the non-sorbitol-treated cultures using an independent assay for cell death (Supplementary Material, Fig. S3). As expected, sorbitol treatment increased the amount of cell death, consistent with osmotic shock causing greater insult to the cells.

Evidence that ubiquilin-2 stabilizes hnRNPA1 protein

To understand the significance of the ubiquilin-2-hnRNPA1 interaction, we knocked down ubiquilin-2 in NSC-34 mouse motor neuron cells using siRNA specific for mouse ubiquilin-2...

Figure 6. Overexpression of ubiquilin-2 mutants results in increased subcellular localization of hnRNPA1 in the cytoplasm. (A) HeLa cell cultures were either mock transfected or transfected with HA-tagged WT or mutant ubiquilin-2 cDNAs and 24 h later were fractionated to proteins in the cytoplasm (C), nuclear and mitochondria (N) and insoluble pellet (P) fractions. The fractions were adjusted to the same volume and equal volumes of the three fractions were immuno-blotted with the antibodies shown (upper two of the set of the four panels shown). Antibodies against caspase-3, Tom20 an outer mitochondrial protein (for lack of a good nuclear extractable protein) and lamin B were used as controls to ensure proper fractionation in the cultures (second set of three panels). The experiment was repeated, but this time the cell cultures were treated for 2 h with 600 mM sorbitol to induce osmotic shock and immuno-blotted for HA and hnRNPA1 (lower two of the set of four panels shown). (B and C) Graphs showing quantification of HA-tagged ubiquilin-2 (B) and endogenous hnRNPA1 (C) proteins in the cytoplasm, which was calculated by dividing the signal in the C fraction by the total signal (C + N + P). The results revealed increased amounts of hnRNPA1 in the cytoplasm in the cultures transfected with the ubiquilin-2 mutants, but not with the WT ubiquilin-2 construct, compared with the mock-transfected control. One-way ANOVA an increase *P < 0.05. Statistical analysis revealed that HA-ubiquilin-2 shows no alteration in distribution of the WT and mutant proteins in the different fractions. Data are shown as mean ± SEM.
for different periods and examined the cells for cell viability and hnRNPA1 accumulation (Fig. 8). NSC-34 cells were used, because ubiquilin-2 expression was easily detectable and knockdown was robust. Cells transfected with the ubiquilin-2-specific siRNAs showed a time-dependent knockdown of ubiquilin-2, compared with cells that were mock transfected or transfected with a scrambled siRNA (Fig. 8A and B). Interestingly, knockdown of ubiquilin-2 led to a statistically significant reduction in hnRNPA1 levels. Furthermore, cycloheximide-chase analysis revealed that ubiquilin-2 knockdown increased hnRNPA1 turnover, indicating...
ubiquilin-2 functions to stabilize hnRNPA1 protein (Fig. 8D and E). The ubiquilin-2 knockdown did not affect cell viability up to the 72 h time point (data not shown).

Discussion

Here we provide evidence that ubiquilin-2 interacts with hnRNP proteins. Focusing on its interaction with hnRNPA1, we show that mutations in either UBQLN2 or HNRNPA1 that cause ALS have the same affect: they reduce interaction between the encoded proteins. The loss of interaction caused by mutation in either protein suggests that the interaction we have uncovered has some functional importance and raises the possibility that the loss of the interaction may be mechanistically linked to disease. Further exploration of whether the interaction affects ALS is clearly warranted.

Because ubiquilin proteins function in protein clearance pathways, an obvious possibility was that the ubiquilin-2 interaction with hnRNPA1 enhances hnRNPA1 degradation, while loss of interaction seen with the ubiquilin-2 mutants would be predicted to increase hnRNPA1 levels. Quantification of hnRNPA1 levels in cells overexpressing either WT ubiquilin-2 or
the ubiquilin-2 mutants showed little, if any, difference in hnRNPA1 accumulation compared with untransfected cells (see blot in Fig. 4A). Paradoxically, however, we found that knockdown of ubiquilin-2 in NSC-34 motor neuron cells led to a decrease in hnRNPA1 levels due to increased turnover of the protein. The results indicate that ubiquilin-2 is required to stabilize hnRNPA1 protein. How ubiquilin-2 stabilizes hnRNPA1 protein is not known. The central domain of ubiquilins, which was the region of ubiquilin-2 used to identify interaction with hnRNPA1, has been speculated to play a more regulatory role compared with the UBL and UBA end domains that bind the proteasome and ubiquitin chains conjugated onto proteins destined for degradation, respectively. Consistent with its regulatory role, the central domain of ubiquilins is the site of interaction of heat-shock chaperone proteins and factors involved in ERAD (15–17). It will be exciting to discover how binding with the central domain of ubiquilin-2 regulates hnRNPA1 stability and function.

While the majority of our studies have focused on the interaction between ubiquilin-2 and hnRNPA1, two additional hnRNP proteins, hnRNPA3 and hnRNPU1, were also isolated by their interaction with ubiquilin-2 in our yeast two-hybrid screen. Of the two additional proteins, binding was confirmed with hnRNPU by GST-pull-down and coimmunoprecipitation assays. Binding to hnRNPA3 could not be confirmed for technical reasons. Interestingly, according to our GST-pull-down assays, both hnRNPU and hnRNPA1 displayed stronger binding with ubiquilin-2 than with ubiquilin-1. An obvious reason for their preferential binding to ubiquilin-2 is that ubiquilin-1 and -2 proteins differ in sequence. In particular, ubiquilin-2 contains sequences not present in ubiquilin-1, such as the PXX domain in which most ALS mutations cluster, that might facilitate hnRN protein binding. It will be important to determine whether each of the four human ubiquilin isotypes display any preferential binding with different hnRNP proteins. Similarly, it would be important to determine whether ubiquilin-2 displays preferential binding to different hnRNP proteins. An indication that ubiquilin-2 can bind several different hnRNP proteins comes not only from our yeast two-hybrid results showing three different hnRNP5s interact with ubiquilin-2, but also from a recent report showing TDP43, a hnRNP-related protein, binds the protein (28). Mutations in TDP43 cause ALS, but there are exceptions to the effects of these mutations on ubiquilin-2 binding were not reported, and vice versa. The site of ubiquilin-2 interaction with TDP43 was shown to be with its glycine-rich domain (28). Examination of the portions of the hnRNP proteins recovered in our yeast two-hybrid screen revealed that they all contain the glycine-rich domain, suggesting it is likely the domain that is responsible for binding ubiquilin-2. Especially telling was the interaction seen with hnRNPU, where interaction was found with the C-terminal 1/10 end of the protein, exactly where the glycine-rich domain is located. The glycine-rich domain is found in many hnRNP proteins, but its exact function is not known. Our data would suggest that the domain binds ubiquilin-2. This novel interaction could be important as many mutations in FUS and TDP43 that cause ALS map to the glycine-rich domains of each protein (24). It will be interesting to see whether ubiquilin-2 binds FUS and TDP43 through the glycine-rich domains and whether ALS mutations in the domain affect interaction with ubiquilin-2.

A clue into the possible consequence of loss of interaction between the proteins was our findings that hnRNPA1 was mislocalized more in the cytoplasm in cells expressing several of the ubiquilin-2 mutations compared with its normal predominantly nuclear location. The reason for this increase in the cytoplasm is not clear. However, we speculate on several possible explanations for the increase.

One possibility is that loss of interaction/binding between ubiquilin-2 and hnRNPA1 in the nucleus leads to increased translocation of hnRNPA1 to the cytoplasm. The second possibility is that loss of ubiquilin-2 interaction in the nucleus leads to a failure in transport of hnRNPA1 to the nucleus. According to both of these scenarios, ubiquilin-2 binding to hnRNPA1 would be predicted to either retain or facilitate hnRNPA1 transport to the nucleus. To examine these possibilities, we immunoprecipitated HA-tagged ubiquilin-2 from equivalent portions of the cytoplasm and nuclear fractions of cells transfected with WT ubiquilin-2 and found that hnRNPA1 coimmunoprecipitated with ubiquilin-2 in both the nucleus and the cytoplasm, although binding was greater in the nuclear fraction compared with the cytoplasm fraction (Supplementary Material, Fig. S4; hnRNPA1 was 25% of HA-ubiquilin-2 immunoprecipitated in the nucleus compared with 15% in the cytoplasm). The results leave open both possibilities, although they tend to support the first explanation. Despite this uncertainty, the results clearly indicate that ubiquilin-2 interacts with hnRNPA1 in both the cytoplasm and nucleus. We attempted to pinpoint the sites of interaction in cells by double immunofluorescence microscopy but could not do so for technical reasons. This was because hnRNPA1 staining was predominantly localized and spread throughout the nucleus, whereas ubiquilin-2 staining was spread throughout the whole cell, and therefore, any overlap in the two staining patterns would naturally appear colocalized. Further complicating this effort is that the amount of the two proteins that are bound together at any one time is likely to be small. This is evident by comparing the distribution of ubiquilin-2 and hnRNPA1 in the cytoplasmic and nuclear fractions, which were quite discordant. Additionally, we found little variance in the fractionation patterns of WT ubiquilin-2 and ubiquilin-2 proteins containing the ALS mutations despite the differences in their capacity to coimmunoprecipitate hnRNPA1. The results suggest that only a small fraction of ubiquilin-2 and hnRNPA1 protein pools are bound to each other at any one time.

A third possibility is that the ubiquilin-2 mutants induce stress, which leads to increased translocation of hnRNPA1 from the nucleus to the cytoplasm. This idea stems from studies that have shown hnRNPA1 translocates to the cytoplasm under conditions of stress, which classically has been demonstrated following exposure to osmotic shock (27). We examined whether hnRNPA1 translocation was different in cells challenged with osmotic shock, but found little change in the overall translocation of the protein into the cytoplasm in cells transfected with the mutants compared with mock-transfected cells. The results suggest that the stress response is intact in the cells, but it does not eliminate the possibility that cells expressing the ubiquilin-2 mutants are more sensitized to stress. Indeed quantification of cell death in the cell cultures transfected with the ubiquilin-2 constructs indicated that overexpression of all of the mutants induced an increase in cell death compared with mock-transfected cells, which is supportive of the idea that the mutants are more toxic. Furthermore, cell death increased to higher levels in all of cultures transfected with the mutants after challenge to osmotic shock, suggesting that overexpression of the mutants sensitizes cells to stress.

One obvious consequence of the altered distribution of hnRNPA1 caused by the ubiquilin-2 mutants is the effect that it might have on hnRNPA1 function. hnRNPA1 has been implicated in mRNA export and splicing as well as in telomere biogenesis.
(19). The altered distribution might affect these functions, leading to disease. Additionally, our evidence that ubiquilin-2 is involved in stabilizing hnRNPA1 protein suggests that the ubiquilin-2 mutants that have reduced binding with hnRNPA1 could lead to changes in hnRNPA1 levels causing disturbances in hnRNPA1-related functions.

Finally, our discovery that ubiquilin-2 binds hnRNP proteins provides evidence that the two distinct gene clusters that are most frequently mutated in ALS, those involved in RNA metabolism and proteostasis, may be functionally connected as evidence by the direct binding of ubiquilin-2 with hnRNPA1 and with the other hnRNP proteins mentioned. The implications of our findings are that mutations in one group might affect the function of the other, and vice versa.

Materials and Methods

Yeast two-hybrid

The yeast two-hybrid (Y2H) procedure described previously was used (29). In the system we used, interaction between bait and prey results in activation of two reporter genes, leucine and β-galactosidase. A human fetal brain cDNA library was screened using the central domain of WT ubiquilin-2 (amino acids 108–556) fused to LexA as the bait. Interactors based on their growth in the absence of leucine and that displayed a substantial blue color change when grown on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates were selected for sequencing. Prey plasmids were isolated from the clones, and the DNA sequence of their cDNA inserts was determined by the Biopolymer-Genomic Core Facility at the University of Maryland, Baltimore. The sequences were compared with entries in the GenBank/EMBL/DDBJ databases using the BLAST search program. Sequences with homology were aligned to identify the overlap between the cDNAs and the corresponding full-length open reading frame that was retrieved from the BLAST search using MacVector software (MacVector Inc., Cary, NC, USA). Candidate clones were rescreened for their strength of interaction, in triplicate, with each of the baits mentioned below using liquid cultures of yeast. Fusions to the β-galactosidase assay by the enzymatic conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol and β-galactose by β-galactosidase. The baits used were the original WT ubiquilin-2 bait, and five identical baits to it, but containing the P497H, P497S, P506T, P509S or P525S ALS mutations, and negative control baits containing only the empty vector and an unrelated lamin C bait (16).

Immunoblotting

Cells were collected in our standard protein lysis buffer [0.5% SDS, 0.5% NP40, 0.5% sodium N-lauroylsarcosine, 50 mM Tris pH 6.8, 150 mM NaCl, 20 mM EDTA, 1 mM EGTA, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM Pefabloc (AEBSF, Roche, Indianapolis, IN), 1 mM leupeptin and 1 mM aprotinin] (30) and sheared by passing the lysates 20 times through a 25-gauge needle. Protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). Lysates were prepared with SDS sample buffer and heated for 5 min at 100°C prior to loading. Equal amounts of protein were separated on either 8.5 or 10% SDS–PAGE gels and the proteins then transferred onto a 0.45 µm PVDF membrane (Millipore, Billerica, MA, USA) for 3 h at 200 mA using the Mini-Trans Blot cell system (BioRad, Berkeley, CA, USA). The membranes were then incubated with appropriate primary antibodies. The following primary antibodies were used: mouse monoclonal antibodies to hnRNPA1, hnRNPU (both from Santa Cruz Biotech, Santa Cruz, CA, USA), ubiquilin (Invitrogen, Grand Island, NY, USA), HIS (GE Healthcare), lamin B (MatriTect, Cambridge, MA, USA), Myc (prepared in-house) rabbit antibodies to HA, GST, and ubiquilin-2 (all generated in-house), caspase-3 (Cell Signaling Technology, Beverly, MA, USA), LexA (EMD Millipore, Billerica, MA, USA), Tom20 (BD Biosciences, San Jose, CA, USA) and goat antibodies to actin (Santa Cruz). Binding of primary antibodies was detected by incubation with appropriate secondary horseradish peroxidase-conjugated antibodies using the SuperSignal West Pico system (Thermo Fisher Scientific), and the chemiluminescence signal was captured using the Fluoro-Chem M imager (Protein Simple, Santa Clara, CA, USA). The following secondary antibodies were used: goat anti-mouse (Thermo Fisher Scientific), goat anti-rabbit (Pierce biotechnology, Rockford, IL, USA) and rabbit anti-goat (Pierce biotechnology). The intensity of different bands was quantified using AlphaView software (Protein Simple).

Cell culture, turnover and cell death assays

HeLa and NSC-34 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) with 5% CO₂. Cells were transiently transfected with plasmid DNA using Lipofectamine LTX (Life Technologies) for 5–6 h in Opti-Mem reduced serum medium (Life Technologies). For cellular stress experiments, HeLa cells were treated with sorbitol for 2 h at a final concentration of 600 mM. For cell death quantification, HeLa cells were incubated with Hoescht 33342 at a concentration of 1 µg/ml for 15 min. Images were then captured using a Leica DM IRB microscope using a Leica PL APO ×10 lens. Quantification of cell death was performed using iVision-Mac software (BioVision Technologies, Exton, PA, USA) based on greater uptake of the Hoescht dye and abnormal nuclear morphology. Cell death was also quantified by the trypan blue exclusion method. In this second method, the medium was removed from the cultures 24 h after transfection and the cells were spun down and resuspended in 1 ml 1× PBS, and trypan blue was added to a final concentration of 0.4%. The number of dead cells was quantified using a hemocytometer. For analysis of siRNA-mediated knockdown of ubiquilin-2, NSC-34 cells were transfected with 20 nM siRNAs designed to specifically target mouse ubiquilin-2 (WJ-040102-11, Dharmacon, Inc., Chicago, IL, USA) using DharmaFect 1 reagent (Thermo Fisher Scientific).
Subcellular fractionation

Subcellular fractionation to obtain cytoplasm and nuclear fractions was performed as described by Li et al. (31). HeLa cells were collected in 1× PBS, centrifuged for 2 min at 10,000 rpm and resuspended in 500 µl HB buffer (10 mM Tris pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 protease inhibitor cocktail pellet, 5 mM NEM) for 15 min on ice. Triton X-100 was added to a final concentration of 0.2% before vortexing and centrifuging for 10 min at 1000g. The cytoplasmic fraction (supernatant) was collected and adjusted to a final salt concentration of 200 mM. The pellet was resuspended in 250 µl of buffer C (10 mM Tris pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 protease inhibitor cocktail pellet, 5 mM NEM, 400 mM NaCl, 0.4% Triton X-100), vortexed every 5 min at 4°C for 30 min total and then centrifuged for 15 min at 20000g. The soluble nuclear fraction (supernatant) was collected and diluted with equal volume of HB buffer. The remaining pellet (insoluble fraction) was re-suspended in 500 µl of our standard protein lysis buffer and sheared using a 25-gauge needle. Samples were mixed with SDS sample buffer and heated for 5 min at 100°C. Equal volumes of the three fractions were then separated by SDS-PAGE and proteins were detected by immunoblotting.

Cloning and bacterial expression of proteins

Full-length human WT ubiquilin-2 or human WT ubiquilin-1 cDNAs were each cloned in-frame downstream of GST. The recombinant plasmids were transformed into Escherichia coli BL21 (DE3), and the fusion proteins were induced with 0.4 mM IPTG for 4 h at 37°C. HIS-tagged hnRNPA1 protein was expressed in bacteria following cloning of the entire human hnRNPA1 isoform. Open reading frame (320 amino acids) in the pET-21a (Novagen, Billerica, MA, USA) vector. The D262V ALS mutation was introduced into the resulting plasmid by site-directed mutagenesis using standard PCR procedures. The mutation was verified by DNA sequencing. The plasmids were transformed into E. coli BL21(DE3), and protein expression was induced with 1.0 mM IPTG for 4 h at 37°C. Bacteria were lysed by sonication and frozen at −20°C until needed.

HIS-tag protein purification

Bacterial pellets containing induced HIS-hnRNPA1 proteins were thawed, resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 0.1% NP40) and sonicated at 30% output two times for 2 min. The lysate was centrifuged at 54000 g for 1 h at 15°C in the 70Ti rotor. The supernatant was collected and incubated with pre-equilibrated Nickel-NTA agarose beads for 4 h at 4°C with gentle rotation. The mixture was loaded onto a 10 ml column (Pierce Biotechnology), washed with two column volumes of Buffer A (50 mM Tris pH 8.0, 350 mM NaCl, 10% glycerol, 30 mM imidazole) and two column volumes of Buffer B (50 mM Tris pH 8.0, 10% glycerol, 30 mM imidazole), and the bound HIS-tagged proteins were eluted with 50 mM Tris pH 8.0, 10% glycerol, 250 mM imidazole. The eluted proteins were dialyzed against 20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol buffer and frozen in small aliquots at −80°C.

GST-ubiquilin purification and GST-pull-down assays

Bacterial pellets containing induced GST-ubiquilin fusion proteins were thawed, resuspended in lysis buffer (20 mM Tris pH 8.0, 350 mM NaCl, 10% glycerol, 1 mM EDTA) and sonicated to reduce their viscosity. The lysates were centrifuged at 54000 g for 1 h at 4°C in the 70Ti rotor, and the resulting supernatants incubated with pre-equilibrated glutathione agarose beads (Sigma) for 2 h at 4°C with gentle rotation. The mixture was poured onto a column and after extensive washing the GST proteins were eluted from the column with 20 mM Tris 8.0, 10% glycerol, 1 mM EDTA and 10 mM glutathione. The purified GST-fusion proteins were dialyzed in buffer composed of 20 mM Tris pH 8.0, 150 mM NaCl and 10% glycerol for 3 h at 4°C. Purified GST alone, GST-ubiquilin-1 and GST-ubiquilin-2 were stored until needed at −80°C. Equal amounts of each GST purified protein (4 µg) were incubated with the same amount of different ligands (either 35S-methionine in vitro transcribed and translated hnRNPA1 or hnRNPU proteins, or HIS-hnRNPA1, or HIS-hnRNPA1 containing the D262V mutation) in 800 µl 2% Chaps buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2 mM MgCl2, 10% glycerol, 2.0% Chaps). Seventy microlitres of equilibrated glutathione agarose beads were added, and the proteins were incubated for 2 h rotating at room temperature. The mixture was centrifuged again at 2300g for 5 min and the supernatant was discarded. The beads were washed four times with 1× PBS with 2% Tween-20 and 200 mM KCl and one time with 1× PBS, resuspended in the SDS sample buffer and heated for 5 min at 100°C. Equal volumes of the pull-down assays were separated by SDS-PAGE, and proteins were detected by immunoblotting or, in the case for detection of 35S-labeled proteins, the gel was dried and subjected to Phosphoimage analysis.

In vitro transcription and translation

The same procedure as described previously (16) was used to prepare in vitro transcribed and translated 35S-labeled hnRNPA1 and hnRNPU proteins. The plasmid template used for this synthesis contained cDNAs encoding the complete open reading frame for each of the hnRNPs proteins.

Statistical analysis

Experiments where error bars are included were performed a minimum of three times. Student’s t-test and ANOVAs with post hoc tests all performed using Microsoft Excel and GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Supplementary Material

Supplementary Material is available at HMG online.

Conflict of Interest statement

None declared.

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References


