Studies of the Role of Ubiquitination in the Interaction of Ubiquilin with the Loop and Carboxyl Terminal Regions of Presenilin-2†

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ABSTRACT: Ubiquilin was originally identified as a presenilin-interacting protein. We previously reported that ubiquilin interacts with both the loop and carboxyl terminus of presenilin proteins and that the ubiquitin-associated (UBA) domain of ubiquilin, which binds poly ubiquitin chains, is important for mediating this interaction. In the present study, we examined whether ubiquitination of presenilin-2 (PS2) is required for interaction with ubiquilin-1 by mutating lysine residues that may be targets for ubiquitination in the presenilin loop and carboxyl terminus regions. Mutation of two lysine residues in the PS2-loop region suggested that ubiquitination is not required for interaction with ubiquilin-1 and may, in fact, even negatively regulate the interaction. Similarly, we found that ubiquitination of the PS2 carboxyl terminus (PS2-C-terminus) is not required for interaction with ubiquilin-1, although our results suggest that it could play some role. Instead, we found that the mutation of either one of the two lysine residues in the carboxyl terminus of PS2 or the proline residues in the highly conserved PALP motif in this region results in destabilization of the mutant PS2 polypeptides because of increased degradation by the proteasome. Furthermore, by GST-pull-down assays we found that the mutant polypeptides were unable to bind ubiquilin, suggesting that loss of ubiquilin interaction leads to destabilization of presenilin polypeptides. Paradoxically, however, knockdown of ubiquilin expression by RNA interference did not alter the rate of turnover of PS2 proteins in cells. Instead, we found that PS2 synthesis was reduced, and PS2 fragment production was increased, suggesting that ubiquilin expression modulates biogenesis and endoproteolysis of presenilin proteins.

Ubiquilin proteins are present in all eukaryotes examined (*1*) and are characterized by an N-terminal ubiquitin-like (UBL[1]) domain, a central more variable domain, and a C-terminal ubiquitin-associated (UBA) domain. There are three human ubiquilin isoforms: ubiquilin-1 is expressed in all cells and tissues examined, ubiquilin-2 is expressed with a more restricted tissue expression pattern compared to that of ubiquilin-1, and ubiquilin-3 is expressed only in the testis (*1*−*3*). The three proteins differ from each other primarily by the presence or absence of a series of different inserts in the central region of the protein.

Ubiquilin was originally identified in a yeast 2-hybrid screen as an interactor of presenilin proteins (*1*). The homologous presenilin-1 (PS1) and presenilin-2 (PS2) proteins, along with amyloid precursor protein (APP), are the only gene products in which dominant mutations are linked to early onset Alzheimer's disease (AD) (*4*, *5*). While the early onset cases represent only a small fraction (∼1%) of all AD cases and are chiefly caused by mutations in presenilins, the cause of the majority of late onset cases has remained obscure, with evidence suggesting that the ApoE4 allele may strongly predispose individuals to AD (*6*, *7*). Another candidate that has emerged for late-onset AD is ubiquilin-1. Bertram and colleagues first reported a genetic association of variants in the ubiquilin-1 gene with late-onset AD in family based studies (*8*). Since then, other groups have confirmed the existence of such an association (*9*, *10*), but several others have been unable to detect the association (*11*−*13*).

Ubiquilin interacts with the cytosolic loop region of the multi-transmembrane-spanning presenilins as well as with its cytosolic carboxyl terminus (*1*). The carboxyl terminus of presenilins is highly conserved across species, and contains a proline−alanine−leucine−proline sequence, known as a PALP motif, located at the proximal end of the polypeptide as it emerges out of the membrane (*14*). The PAL portion of the PALP motif is conserved in presenilin homologues, suggesting that it may perform some important function (*15*−*17*). Several functions have been proposed for the PALP motif, including acting as a binding site for a cellular factor involved in PS stabilization, playing a role in γ-secretase activity, acting as an SH3 ligand, maintaining conformation of the carboxyl terminus and membrane topology, and as an ER-retention motif (*14*, *18*, *19*).

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[1] Abbreviations: AD, Alzheimer's disease; FL, full length; PS2, presenilin-2; PS2-C, PS2-C-terminus, presenilin-2 carboxyl terminus; NTF, PS NH_2-terminal fragment; UBA, ubiquitin-associated domain, UBL, ubiquitin-like domain; ubqln, ubiquilin; wt, wild-type.

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Ubiquilin has been shown to affect presenilin protein accumulation and biogenesis because overexpression of ubiquilin results in increased accumulation of full-length presenilin proteins and a concomitant decrease in the production of presenilin N- and C-terminus fragments (*1*, *20*, *21*). In addition to interacting with presenilins, ubiquilin has also been reported to interact with numerous other proteins that are apparently unrelated in sequence (*1*, *3*, *22*−*34*). In most cases, ubiquilin has been found to alter the stability of the interacting protein, although it is not yet clear how ubiquilin functions in this capacity (*1*, *20*, *25*, *28*, *29*, *32*, *33*, *35*).

One hypothesis is that ubiquilin may function as a shuttle factor to deliver polyubiquitinated proteins to the proteasome for degradation (*35*−*39*). This proposed role is analogous to that of Rad23, a yeast protein with which ubiquilin shares similar structural organization (*40*−*43*). In this scenario, the UBA domain of ubiquilin would bind ubiquitinated proteins through interaction with their conjugated polyubiquitinated chains and bind the proteasome via interaction of the UBL domain of ubiquilin with the S5a subunit of the 26S proteasome (*20*, *37*, *43*−*45*). In this study, we investigated the requirements of ubiquilin interaction with the PS2-loop and -carboxyl terminus using site-directed mutagenesis to alter key residues in PS2. We report that mutants that prevent ubiquitination of the PS2-loop residues interact even more strongly with ubiquilin-1 than the wild-type (wt) loop, suggesting that ubiquitination of these residues may negatively regulate ubiquilin-1 interaction. In contrast, we found that mutants that prevent ubiquitination of the PS2-C-terminus display reduced interaction with ubiquilin-1. Further studies of these and additional mutants in the PALP motif revealed that many of the mutant polypeptides were unstable and degraded rapidly, which correlated with a reduction in interaction and binding of the proteins with ubiquilin. To directly test whether loss of ubiquilin interaction with PS proteins leads to destabilization of PS proteins, we compared PS2 protein accumulation and turnover in cells expressing normal and reduced amounts of ubiquilin. Consistent with this hypothesis, we found that reduction of ubiquilin levels by RNA interference decreased the accumulation of full length (FL) PS proteins, but unexpectedly, this effect was independent of the turnover of FL PS proteins. We discuss the importance of ubiquilin in the regulation of PS2 protein biogenesis and turnover.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Liquid Assay. Yeast strain EGY48 was transformed with PS2 constructs in bait vector pEG202, ubiquilin-1 in prey vector pJG4-5, and lacZ reporter plasmid pSH18-34. PS2-loop and PS2-C constructs corresponded to amino acids 270−319 and 410−449, respectively, which we previously showed to interact with ubiquilin-1 (*1*). Site directed mutagenesis was used to generate the K271R, K306R, and K271RK306R mutants in PS2-loop, and the K410R, K411R, K410RK411R, P414L, and P417S mutants in PS2-C. All of the constructs were sequenced and found to contain only the desired changes. Yeast transformants were selected for by plating on the appropriate drop-out plates. Interaction between ubiquilin and the PS2 constructs was measured by assaying for β-galactosidase enzyme activity in liquid cultures using ONPG as a substrate (*46*). All interactions were normalized to pEG202 negative control. Assays were performed at least three times in triplicate.

Immunoprecipitations from Yeast Cell Lysates. Yeast transformed with plasmids for two-hybrid assays were used to prepare cultures for immunoprecipitation studies. For these assays, 5 mL of yeast liquid culture was pelleted and resuspended in RIPA buffer containing 0.2% NP40 and 0.1 mM DTT. After adding 0.2 g of acid-washed glass beads, samples were then vortexed 6 times for 30 s, with 30 s intervals of incubation on ice. The mixture was then centrifuged at 13,000*g* for 10 min at 4 °C. A fraction of the supernatant was saved as a representative of the total cell lysate, and the rest was added to 30 μL of protein A sepharose beads equilibrated in RIPA buffer. Then, 20 μL of rabbit anti-LexA antibody was added, and samples were then incubated at 4 °C for 90 min with gentle rotation. Beads were washed 4 times with RIPA buffer prior to adding 40 μL of 2× Laemmli sample buffer and boiling for 15 min. Immunoprecipitate reactions and total cell lysate samples were separated on an 8.5% SDS−PAGE gel.

Western Blot Analysis. For immunoblot analysis, 5 mL of a yeast liquid culture was pelleted and resuspended in 100 μL of 2× Laemmli sample buffer. Acid-washed glass beads (0.2 g) were used to break up yeast cells as described above. Thirty microliter samples were separated on an 8.5% SDS−PAGE gel. Proteins were transferred to nitrocellulose membranes, which were blocked in milk prior to probing with antibody. Antibodies used were rabbit anti-LexA diluted 1:1000 (Upstate Biotechnology), rabbit anti-PS2-N-terminus (093) or anti-PS2-loop (096) (*20*) diluted 1:1000, goat anti-actin diluted 1:500 (Santa Cruz Biotechnology), mouse anti-ubiquilin diluted 1:500 (Zymed), and mouse anti-ubiquitin diluted 1:1000 (Chemicon). Secondary antibodies conjugated to horse radish peroxidase were used at a concentration of 1:3000, except for bovine anti-goat, which was used at 1:1000. Band intensity was quantitated using IPGel software.

Proteasome Inhibition Using MG132 in Yeast Cultures. To inhibit the proteasome in yeast cultures, MG132 was used as follows: 2 mL yeast overnight cultures were expanded by adding 7 mL of fresh drop-out media and 1 mL of glycerol. Cultures were vortexed prior to adding MG132 in DMSO to a final concentration of 50 μM. An equal volume of DMSO was added to control cultures. Cultures were vortexed once more, then grown at 30 °C with shaking at 250 rpm for 4 h. Cultures were then collected as described above.

Transcription/Translation in Vitro and GST-Pull-Down Binding Assay. LexA-PS2C fusion DNA was cloned into pBlueScript KS vector for each of the PS2 carboxyl terminus mutants used in yeast, as described above. To generate [^{35}S]-methionine-labeled LexA-PS2C fusion proteins, *in vitro* transcription and translation were carried out according to the manufacturer's protocol (Promega). GST-ubiquilin-1 fusion protein was induced and purified as previously described (*1*). For the GST-pull-down binding assays, 25 μL of *in vitro* translation product was preincubated with 0.8% BSA, protease inhibitors, GST, and glutathione agarose beads for 30 min at 4 °C. After centrifugation at 13,000*g* for 10 min, the supernatant was recovered and added to fresh glutathione agarose beads containing either purified GST-ubiquilin-1 or GST. Ten microliters from these reactions were saved as a representative indicator of the mixtures. Reactions

were incubated with gentle rotation at 4 °C for 2 h. Beads were then washed twice in pull-down buffer (0.5% NP40 in 1× PBS), once with 200 mM KCl in pull-down buffer, then twice more in pull-down buffer. After adding sample loading buffer and boiling for 15 min, samples were separated by SDS−PAGE. Gels were dried and exposed to autoradiography film. Relative band intensity was quantified using ImageQuant software (Amersham Biosciences).

Cell Culture and DNA Transfection. PS2-inducible HEK293 (P3) cells (*21*) were grown at 37 °C in DME supplemented with 10% FBS and containing Hygromycin B (100 μg/mL). For ubiquilin knock-down, P3 cells were transfected with 10 nM SMARTpool siRNA constructs in 6-well dishes using Dharmafect-1 reagent according to the manufacturer's protocol (Dharmacon) (*21*). Twenty-four hours following transfection, cells were induced with 10 nM PonasteroneA for 20 h to induce expression of PS2 prior to treating with 1 mM cycloheximide. Pulse−chase analysis of PS2 turnover was conducted as previously described (*1*, *21*).

RESULTS

Ubiquilin-1 Can Interact with the PS2-Loop and PS2-C-Terminus Independent of Ubiquitination of the Proteins. Ubiquilin interacts with PS proteins, although the molecular mechanism of this interaction is not understood (*1*, *22*). Because ubiquilin contains a ubiquitin-associated (UBA) domain that is involved in binding presenilins (*1*), and because the UBA domain binds polyubiquitin chains (*20*, *37*, *47*), we reasoned that ubiquilin might bind PS proteins through interaction with polyubiquitinated chains conjugated onto the proteins. To test this idea, we mutated key lysine residues in PS that could be a target for ubiquitin conjugation: to arginine (a conservative change), an amino acid onto which ubiquitin cannot be conjugated. Specifically, because ubiquilin has been shown to interact with the large cytoplasmic loop of PS2 as well as the cytoplasmic PS2-carboxyl terminus, we mutated all of the potential lysines residues in these regions that could be targeted for ubiquitin conjugation, singly and in combination, to arginine. There are two lysines in the PS2-loop and two in the PS2-C-terminus; therefore, the baits used were PS2-loop (wt), PS2-loop-K271R, PS2-loop-K306R, PS2-loop-K271RK306R, PS2-C-wt, PS2-C-K410R, PS2-C-K411R, and PS2-C-K410RK411R. The wild-type and mutant PS2 constructs were transformed in yeast, and interaction with ubiquilin was monitored by yeast 2-hybrid β-gal assays.

According to yeast 2-hybrid assays, ubiquilin-1 interaction with the PS2-loop did not decrease when the lysine residues were mutated either singly or in combination (Figure 1A). In fact, conservative mutation of the lysine residues within the PS2-loop appeared to increase interaction with ubiquilin. The strength of interaction between ubiquilin and the K271RK306R double mutant was greater than either of the single mutants, and nearly 2-fold greater than that of the wt-PS2-loop. In contrast, mutation of either lysine residue within the carboxyl terminus (K410R or K411R) led to a decrease in interaction with ubiquilin-1 (Figure 1B). Surprisingly, however, the strength of interaction between ubiquilin and the PS2-C K410RK411R double mutant was greater than that of either one of the single mutants (Figure 1B).

In order to determine if the PS2 baits used in these assays get ubiquitinated, we immunoprecipitated the baits from yeast lysates and probed them for anti-ubiquitin immunoreactivity. By this analysis, we found that the LexA-PS2-wt-loop and -carboxyl terminus constructs get ubiquitinated as expected (Figure 2A and B). Mutation of either lysine residue in the PS2-loop, alone or together, dramatically reduced or abolished ubiquitination (Figure 2A). The loss of ubiquitin immunoreactivity was not from a failure to immunoprecipitate the proteins, as LexA immunoreactivity revealed immunoprecipitation of all of the baits. These results indicate that ubiquitination of the PS2-loop is not required for interaction with ubiquilin and, in fact, suggests that ubiquitination of the PS2-loop may even negatively regulate interaction with ubiquilin-1.

By the same analysis, we also found that mutation of either lysine residue in the PS2-C terminus decreased ubiquitination, whereas mutation of both lysine residues in combination completely abolished ubiquitination (Figure 2B). Again LexA immunoreactivity revealed that the loss of ubiquitination was not from a failure to immunoprecipitate the proteins. Interestingly, on first inspection, there seemed to be a direct correlation between the reduction in ubiquitination of the PS2 K410R mutant and the reduced strength of its interaction with ubiquilin (6%) compared to those of the wt PS2-C bait. However, an inspection of the K410RK411R double mutant revealed that it was even more poorly ubiquitinated than the PS2 K410R single mutant, yet the K410RK411R double mutant interacted with ubiquilin-1 to nearly 70% the strength of the wt PS2-C terminus construct. Taken together, these results suggest that ubiquitination of the PS2-loop, and most likely that of the PS2 C-terminus too, is not essential for interaction with ubiquilin.

Although these results do not entirely rule out the possibility that ubiquitination regulates interaction between ubiquilin-1 and PS2 to some extent, they suggest that interaction between the proteins is more likely governed by some other mechanism.

Mutation of the PALP Motif in PS2 Reduces Interaction of the Ubiquilin-1 with the PS2-C-Terminus in Yeast. Because the carboxyl terminus of presenilins is highly conserved, we questioned whether mutation of residues within this region of the protein has deleterious effects on PS2 interaction with ubiquilin. To address this question, we focused on the PALP motif in the PS2-C terminus, which is highly conserved not only in presenilins but also in presenilin homologues and signal peptide peptidases, which share structural and mechanistic similarities with presenilins (see Introduction). Mutations of the PALP motif in presenilins or its homologues are known to have detrimental effects in humans and other organisms. Thus, missense mutation of the first proline in the motif to a leucine in the *Caenorhabditis elegans* presenilin homologue Spe-4 results in a protein that although still able to localize to its correct subcellular compartment causes a defect in spermatogenesis (*15*). In *Drosophila*, the same missense mutation leads to the failure of presenilin undergoing endoproteolysis and of their incorporation into high molecular complexes (*48*). In contrast, mutation of the last proline in the PALP motif to a serine is found in some AD pedigrees, and this mutation also affects Notch cleavage (*14*, *49*, *50*). We generated mimics of these two PALP mutants in PS2, PS2-C-P414L, and PS2-C-P417S and examined them for interaction with ubiquilin-1 by yeast 2-hybrid assays.

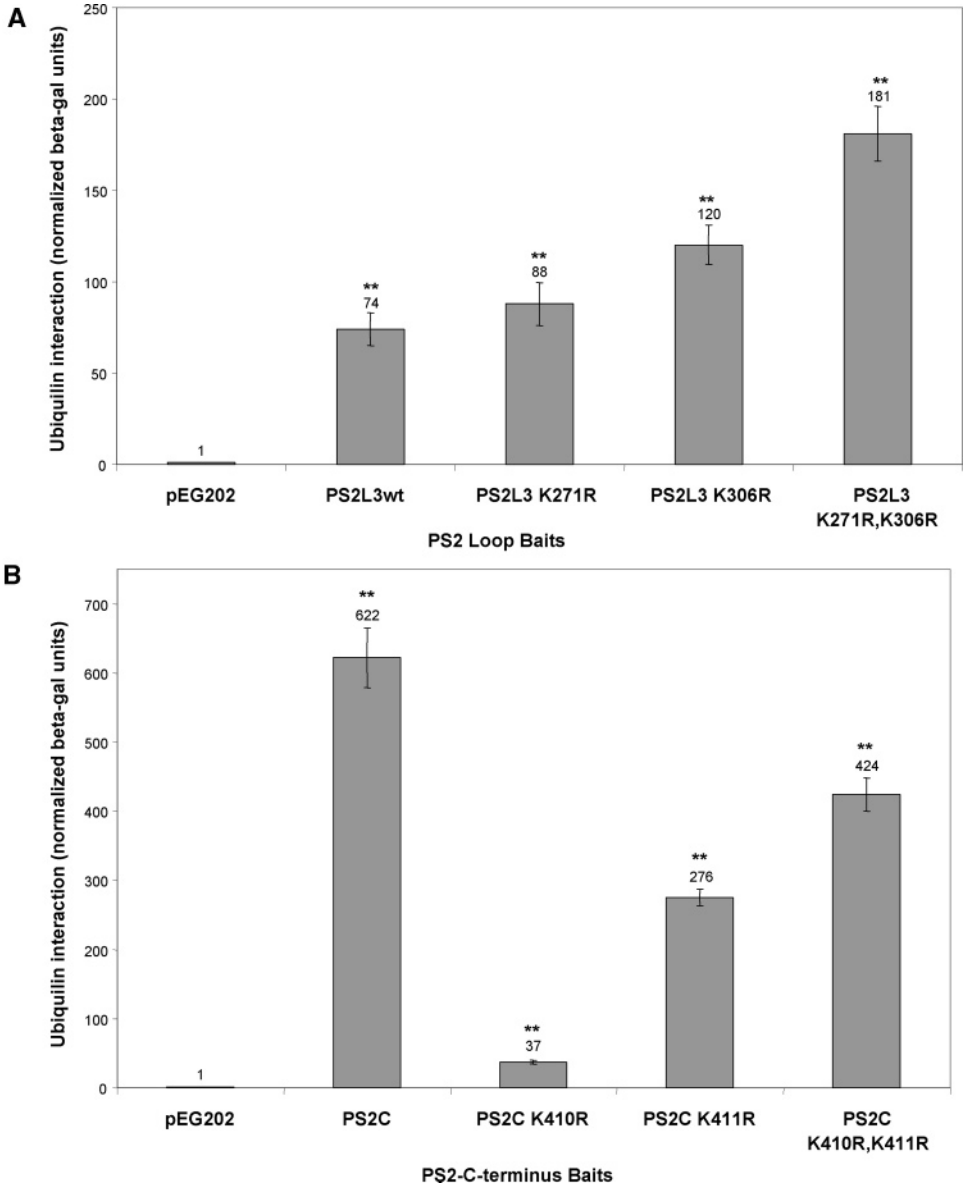

FIGURE 1: Ubiquilin interaction with PS2-loop and PS2-C-terminus proteins. β-galactosidase yeast two-hybrid liquid culture interaction of ubiquilin-1 as prey and PS2-loop (A) or PS2-C constructs (B) as bait. The PS2-loop constructs used were PS2-loop wt, PS2-loop K271R, PS2-loop K306R, or PS2-loop K271RK306R. The PS2-C constructs used were PS2-C wt, PS2-C K410R, PS2-C K411R, or PS2-C K410RK411R. The β-galactosidase units were normalized to pEG202, the empty bait vector. Each assay was performed in triplicate. The error bars indicate standard deviation, and ** indicates $p < 0.01$.

Remarkably, mutation of either one of the proline residues in the PALP motif dramatically reduced interaction of the PS2-C terminus with ubiquilin-1 (Figure 3). Mutation of the first proline reduced interaction by ~95% compared to that of the wt protein, whereas mutation of the second proline reduced interaction by almost 80%. Taken together, our results suggest that conservation of the PALP motif is important for PS2-C interaction with ubiquilin-1.

Mutation of the PALP Motif Leads to Destabilization of PS2-C-Terminus Proteins in Yeast. We next wanted to determine whether the decrease in interaction of the PALP mutants with ubiquilin-1 was due to an inability of the proteins to interact with each other or a secondary consequence of lower expression of the PS2-C terminus mutants. To examine this and other possibilities, we immunoblotted protein lysates from the different yeast cultures for the different PS2-C proteins. These blots revealed that all of the mutant LexA-PS2-C fusion proteins were expressed at considerably lower steady-state levels than the LexA-PS2-C-wt fusion protein (Figure 4A). Moreover, when compared to the wild-type PS2C-terminus fusion protein, the levels of mutant PS2C-terminus fusion proteins correlated roughly with the strength of interaction of the proteins with ubiquilin-1 seen in β-galactosidase liquid assays.

We reasoned that the decrease in expression of mutant fusion LexA-PS2-C proteins might be due to increased proteasomal degradation of the mutant constructs compared to that of the LexA-PS2-C-wt fusion protein. To examine this possibility, we grew yeast cultures in the absence or presence of proteasome inhibitor MG132 and afterward probed equal amount of proteins from these lysates with the anti-LexA antibody. The immunoblot revealed that the levels of LexA-PS2-C mutant fusion proteins were profoundly increased after MG132 treatment, suggesting that the PS2-C terminus mutant proteins turn over rapidly in yeast because of increased degradation by the proteasome (Figure 4B and

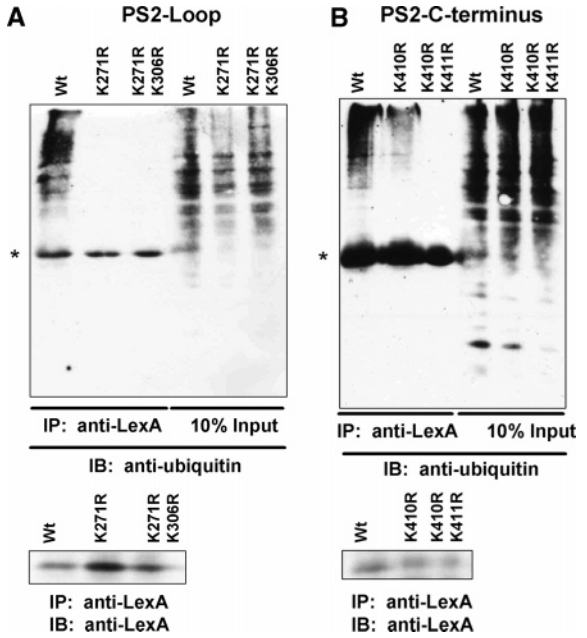

FIGURE 2: Mutation of lysine residues in the PS2-loop and PS2-C-terminus reduces ubiquitination of the proteins in yeast. LexA-PS2-loop (A) and LexA-PS2-C-terminus (B) fusion proteins were immunoprecipitated from certain yeast cultures described in Figure 1 using an anti-LexA antibody. An equivalent fraction of the immunoprecipitates and a 10% fraction of total yeast lysates were separated by SDS−PAGE, transferred to nitrocellulose membranes, after which they were probed with anti-ubiquitin (upper blot) or anti-LexA antibodies (lower blot). Note that the anti-LexA blots confirmed that the LexA-PS2-loop and LexA-PS2-C fusion proteins were indeed immunoprecipitated in all of the reactions. The asterisk indicates the cross-reaction of the IgG heavy chain used in the immunoprecipitations with the anti-ubiquitin antibody.

data not shown). These results suggested that the reduction in interaction between the PALP PS2-C mutant constructs and ubiquilin could stem from reduced accumulation of the mutant PS2 baits in yeast.

Mutations in the PS2 C-Terminus Decrease Interaction with Ubiquilin-1 in Vitro. The preceding yeast two-hybrid results suggested that the decrease in interaction between ubiquilin-1 and the PS2-C mutants was a result of increased proteasomal degradation of the PS2-C terminus mutant baits. However, these assays do not distinguish between the two possible etiologies of this effect. One possibility is that the mutations in the PS2-C terminus may result in proteins that are physically less able to interact with ubiquilin-1. A second possibility is that the PS2-C mutants are so unstable that they are rapidly degraded by the proteasome before they have an opportunity to interact with ubiquilin-1.

To distinguish between these possibilities, we performed *in vitro* pull-down assays examining binding between GST-ubiquilin-1 and ^{35}S-labeled LexA-PS2-C fusion proteins. Briefly, *in vitro*-translated LexA-PS2-C fusion proteins were incubated with GST-ubiquilin-1 or GST and glutathione-agarose beads. Following incubation, the beads were washed several times to remove nonspecific binding. Samples were then separated by SDS−PAGE prior to autoradiography to detect the binding of ^{35}S-LexA-PS2-C fusion proteins to the GST proteins. Pull-down samples were compared to 10% input fractions to obtain a ratio of ^{35}S-LexA-PS2-C that bound to GST-ubiquilin-1 to input ^{35}S-LexA-PS2-C. The ratio of each of the mutants was compared to the ratio for the ^{35}S-LexA-PS2-C-wt fusion protein retained by GST-ubiquilin-1.

Considering the two possible scenarios outlined above, if the first possibility is correct, then we should observe differences in the amount of mutant proteins that are pulled down in these assays. But, if the second scenario is correct, then we would expect the ratio of bound ^{35}S-LexA-PS2-C mutant fusion protein input to be comparable to the ratio of the ^{35}S-LexA-PS2-C-wt. As shown in Figure 5A, mutation of either proline in the PALP motif of PS2-C disrupted interaction with GST-ubiquilin *in vitro*, strongly suggesting that the mutant PS2-C terminus fusion proteins are intrinsically compromised in their ability to bind ubiquilin-1 protein.

To test whether the lysine mutants that we had generated in the PS2 C-terminus were also compromised in binding ubiquilin-1, we performed additional GST-pull-down assays, incubating GST-ubiquilin-1 with ^{35}S-LexA-PS2-C-wt, ^{35}S-LexA-PS2-C-K410R, ^{35}S-LexA-PS2-C-K411R, or ^{35}S-LexA-PS2-C-K410RK411R. Similar to the ^{35}S-LexA-PS2-C PALP mutant fusion proteins, we found that all of the lysine mutants bound ubiquilin-1 *in vitro* with reduced affinity compared to that of wt PS2-C terminus fusion protein (Figure 5B). The different lysine mutants displayed varied binding with GST-ubiquilin-1, ranging from 2% to 29% strength compared to that of the LexA-PS2-C-wt fusion protein (Figure 5C). Together, our data reveal that mutation of several different residues in the highly conserved PS2 C-terminus disrupts LexA-PS2-C interaction with GST-ubiquilin. Furthermore, in conjunction with the data from yeast, it appears that the interaction between ubiquilin and the carboxyl terminus of PS2 is critical for conferring PS2-C protein stability in yeast.

Reduction of Ubiquilin Levels in Human Cells by RNA Interference Results in a Reduction of Accumulation of Full-Length PS2 Protein but Does Not Affect the Rate of Turnover of the Protein. In order to determine the significance of the ubiquilin interaction with PS2 *in vivo*, we examined the effect of reducing ubiquilin levels on PS2 turnover in human cells. Using HEK293 cells stably transfected with an inducible form of PS2 (*21*), we knocked-down ubiquilin-1 alone or together with ubiquilin-2 by siRNA-mediated RNA interference. Twenty-four hours after transfection, ponasteroneA was added to the cultures for 20 h to induce PS2 expression. The induced cells were then treated with cycloheximide to inhibit new protein synthesis, and protein lysates were collected at 2-h intervals thereafter. Equal amounts of protein from these lysates were immunoblotted for PS2, ubiquilin, and actin proteins. When compared to cells transfected with nonsense siRNA, the cells transfected with siRNAs that induced the knockdown of ubiquilin-1 protein contained lower amounts of full-length PS2 protein at the 0 h time point of the experiment, whereas the combined reduction of ubiquilin-1 and -2 proteins led to an even greater reduction in PS-2 levels (Figure 6A). Surprisingly, the rate of turnover of the full-length PS2 protein, after normalization for actin loading, was approximately the same in the three sets of lysates, suggesting that the reduction of ubiquilin levels does not affect the turnover of full-length PS2 protein (Figure 6B). Because we previously reported that the knock down of ubiquilin protein expression results in increased PS fragment production (*21*), we examined if this effect is observed in our current experiments. In accord with our previous findings, we found that more PS2 fragments accumulate over time following

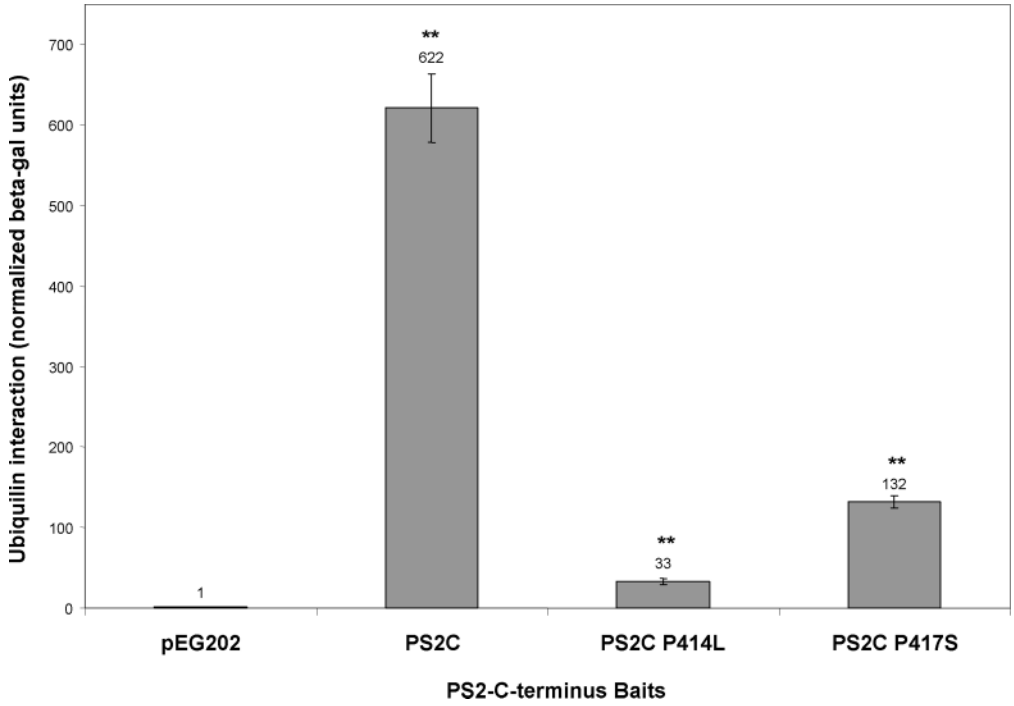

FIGURE 3: Mutations of proline residues in the PALP motif of the PS2 C-terminus disrupts the interaction of ubiquilin with the PS2-C-terminus. β-galactosidase yeast two-hybrid liquid culture interaction of ubiquilin-1 with PS2-C-terminus constructs containing mutations in the PALP motif. Yeast were co-transformed with ubiquilin-1 as prey and PS2-C-terminus wt, PS2-C P414L, or PS2-C P417S as bait. β-galactosidase liquid assays were used to test for interaction. All experimental values were normalized to yeast co-transformed with ubiquilin-1 prey and pEG202. The error bars indicate standard deviation, and ** indicates $p < 0.01$. Each assay was performed in triplicate. Mutation of the first and fourth proline within the highly conserved PALP motif leads to a 95% and 80% decrease, respectively, in ubiquilin-1 interaction with the PS2-C-terminus.

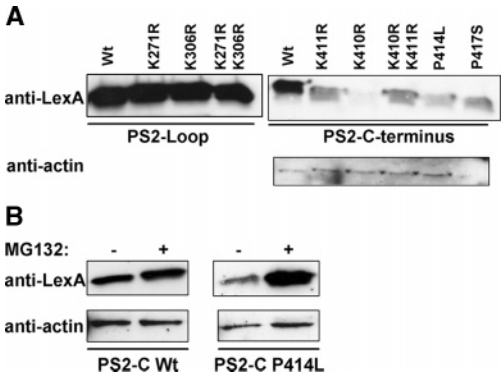

FIGURE 4: Mutation of the PS2-C-terminus leads to destablilization of the protein in yeast. (A) Equivalent amounts of protein lysate isolated from yeast transformed with some of the PS2-loop or PS2-C-terminus baits used in the β-galactosidase liquid assays described in Figures 1 and 3 were immunoblotted with either an anti-LexA antibody (upper panels) or an anti-actin antibody (lower panel). Note that all of the mutations in the PS2-C-terminus lead to destablization of the expressed protein in yeast. (B) Equivalent amounts of protein lysate from yeast transformed with the PS2-C-terminus wt or -P414L mutant construct and treated with MG132 in DMSO or mock-treated with DMSO alone were immunoblotted with either an anti-LexA antibody (upper panels) or an anti-actin antibody (lower panel). Note that proteasome inhibition by MG132 leads to increased accumulation of the PS2-terminus proteins. Similar results were observed for the LexA-PS2-C-terminus-K410R, -K411R, and -P417S mutants (data not shown).

CHX treatment of the cells transfected with siRNAs that induced genetic interference of ubiquilin-1 and -2 proteins compared to the cells transfected with nonsense siRNAs that were designed not to induce genetic interference of any known gene (Figure 6C).

We next examined whether ubiquilin knockdown produces a similar effect on endogenous full-length PS2 levels by studying the turnover of PS2 in SH-SY5Y neuronal cells using classical ^{35}S-methionne pulse−chase analyses. Similar to the results seen with the PS2-inducible cell line, we found that ubiquilin knockdown resulted in a lower accumulation of endogenous full-length PS2 proteins in the SH-SY5Y cells (Figure 7A). Again, the rate of turnover of the full-length PS2 protein was calculated to be similar in the ubiquilin knockdown and normal lysates (Figure 7B), although the rates were difficult to calculate because of extremely low amounts of the full-length PS2 species present in the cells. Nevertheless, the results obtained with endogenous PS2 proteins were similar to those seen with the inducible PS2 cell line.

DISCUSSION

We previously reported that ubiquilin modulates the accumulation and endoproteolysis of presenilin proteins (*1*, *20*, *21*), the genes for which are most frequently mutated in early onset cases of AD. It is likely that the modulation of presenilin protein biogenesis and processing by ubiquilin are governed by physical interaction between the proteins, considering that the proteins have been reported to interact with each other both *in vivo* and *in vitro* (*1*, *20*−*22*, *51*). In our previous studies, we demonstrated that ubiquilin interacts with both the loop and C-terminus regions of presenilins, possibly through the UBA domain of ubiquilin, which is a domain that is known to bind ubiquitin moieties (*1*, *37*, *47*, *51*). Here, we investigated whether ubiquitination of the PS2-loop and PS2-C-terminus is required for interaction with ubiquilin. Surprisingly, we found that ubiquitination of the

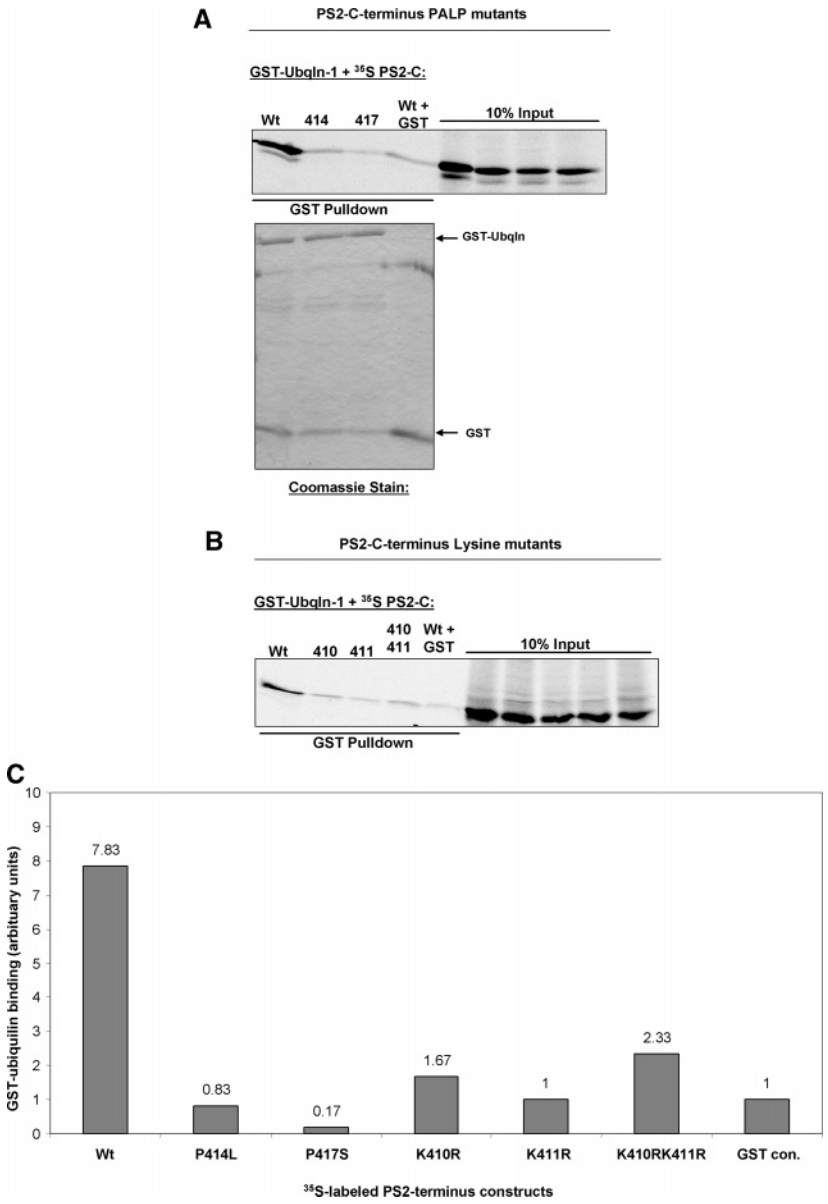

FIGURE 5: Evidence that mutation of the PS2 C-terminus leads to the disruption of the interaction with ubiquilin *in vitro*. PS2-C constructs were transcribed *in vitro* and translated in the presence of ^{35}S-methionine using cDNA templates encoding LexA-PS2-C-teminus -wt, -P414L, -P417S, -K410R, -K411R, or -K410RK411R proteins. A portion of each of the ^{35}S-labeled reactions was incubated with 2.5 μg of purified recombinant ubiquilin-1-GST (GST-Ubqln-1) protein *in vitro*, after which the ubiquilin-1-GST protein was affinity purified from the mixtures using glutathione-agarose beads. The beads from these pull-down assays were resuspended in protein sample buffer and association of the different ^{35}S-labeled PS2-C-terminus proteins with GST-ubiquilin-1 was analyzed by Coomassie blue staining and autoradiography after SDS−PAGE. (A) Coomassie blue (lower gel) and GST-ubiquilin-1 pull-down reactions (upper gel) with LexA-PS2-C PALP mutants. (B) GST-ubiquilin-1 pull-down reactions with LexA-PS2-C K → R mutants. (C) Quantification of the ^{35}S-labeled PS-2 proteins that were pulled down in the experiments shown in A and B. Note that all of the PS2-C-terminus mutations lead to reduced interaction with GST-ubiquilin-1.

PS2-loop was not required for ubiquilin interaction and, in fact, may even inhibit the interaction. This conclusion is based on our findings that mutation of the two-lysine residues in the PS2-loop, which could theoretically serve as potential acceptors for ubiquitin conjugation, to arginine residues, which cannot support ubiquitin conjugation, enhanced interaction between the PS2-loop and ubiquilin in yeast.

In contrast, our results suggested that ubiquitination of the PS2 C-terminus, although not essential, could conceivably be involved in regulating interaction with ubiquilin because mutation of the two lysine residues in the highly conserved C-terminus of PS2 reduced ubiquitination as well as interaction with ubiquilin-1 in yeast compared to the wt PS2 C-terminus protein. However, the results of the strength of interaction of ubiquilin with the PS2 C-terminus mutants and the extent of ubiquitination of the various PS2 C-terminus mutants lead us to speculate that ubiquilin interaction with the PS2 C-terminus might not be regulated by ubiquitination but more likely by the structural conformation of the region. This is underscored by our results showing that although mutation of either one of the two lysine residues in the PS2 C-terminus leads to decreased interaction with ubiquilin, the K410R single mutant was partially ubiquitinated and interacted with 6% the strength of the wt protein, whereas the K410RK411R double mutant, which was even more poorly ubiquitinated than the K410R single mutant, interacted with

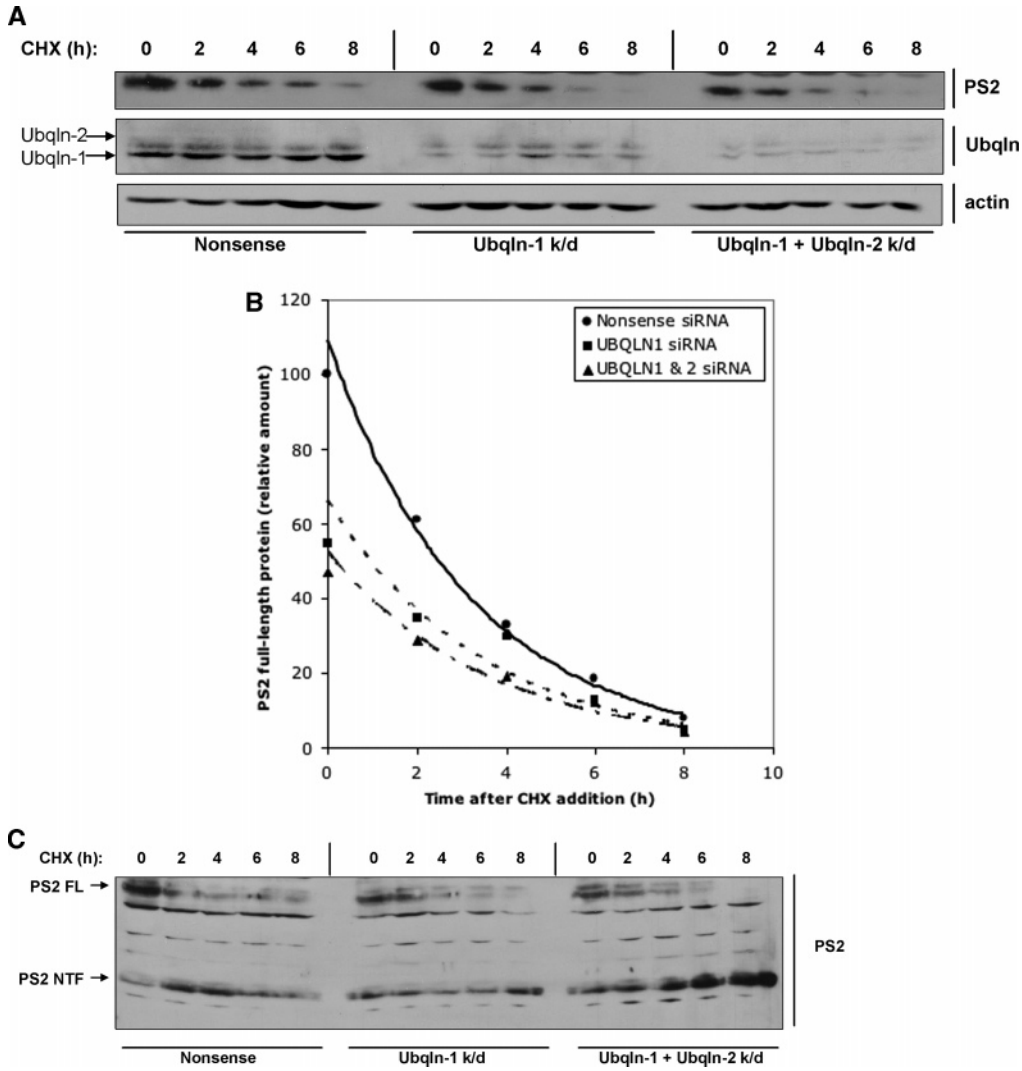

FIGURE 6: Reduction of ubiquilin expression by RNA interference is associated with a reduction in steady-state accumulation of full-length PS2 protein without affecting the turnover of the protein. PS2-inducible cultures were transfected with SMARTpool siRNAs directed against ubiquilin-1, ubiquilin-1 and ubiquilin-2, or nonsense siRNA. Twenty hours following transfection, the cultures were treated with PonasteroneA to induce PS2 expression. After an additional 24 h, the cultures were treated with cycloheximide to inhibit new protein synthesis, and lysates were collected at 2 h intervals, as indicated in the Figure. (A) Western blots showing immunoreactivity with anti-PS2 N-terminus 096 (upper panel), anti-ubiquilin (middle panel), and anti-actin (lower panel) antibodies. (B) Quantification of PS2 protein levels of the experiment shown in A, after normalization for actin loading. (C) Western blot showing expression of PS2-NTF using the anti-PS2 (093) antibody in an experiment similar to that described in A. Note that in cells transfected with ubiquilin 1 and 2 siRNA, PS2 NTFs accumulate to higher levels compared to those in cells transfected with nonsense siRNA.

ubiquilin to almost 70% the strength of the wt protein. From these results, it appears that ubiquitination of the PS2-C terminus, like that of the PS2-loop, may not be required for mediating the interaction with ubiquilin, although we cannot formally eliminate the possibility that it may play some role.

Instead, our results obtained from mutational analysis of the PS2-C terminus suggests that the structural integrity of this region might be more critical for modulating interaction with ubiquilin. Thus, mutation of any of the lysine residues in the PS2 C-terminus region or mutation of either proline residue in the PALP motif in the region led to destabilization of the mutant PS2-C protein baits in yeast, which we showed was due to increased proteasomal degradation of the baits. Using *in vitro* pull-down assays, we found that the mutant PS2-C constructs are inherently less able to interact with ubiquilin, suggesting that loss of ubiquilin interaction correlates with the decreased stability of the mutant polypeptides.

To examine if loss of ubiquilin interaction affects PS2 protein stability in mammalian cells, we used RNAi to knock down ubiquilin proteins in PS2-inducible HEK cells as well as in human neuronal SH-SY5Y cells, which express endogenous amounts of PS2. By these studies, we found that reduction of ubiquilin proteins in cells results in lower accumulation of full-length PS2 proteins, a feature that was seen in both cells expressing either the inducible or the endogenous forms of PS2. Interestingly, the reduction in PS2 protein accumulation upon ubiquilin knockdown was not associated with a noticeable change in the rate of turnover of the full-length protein. Instead, we found that the levels of PS2 fragments increased upon ubiquilin knockdown, which is most likely due to increased endoproteolysis of the full-length protein, as we showed previously (*21*). A consistent picture now emerges of how ubiquilin regulates presenilins biogenesis and processing: overexpression of ubiquilin increases full-length presenilin protein accumulation

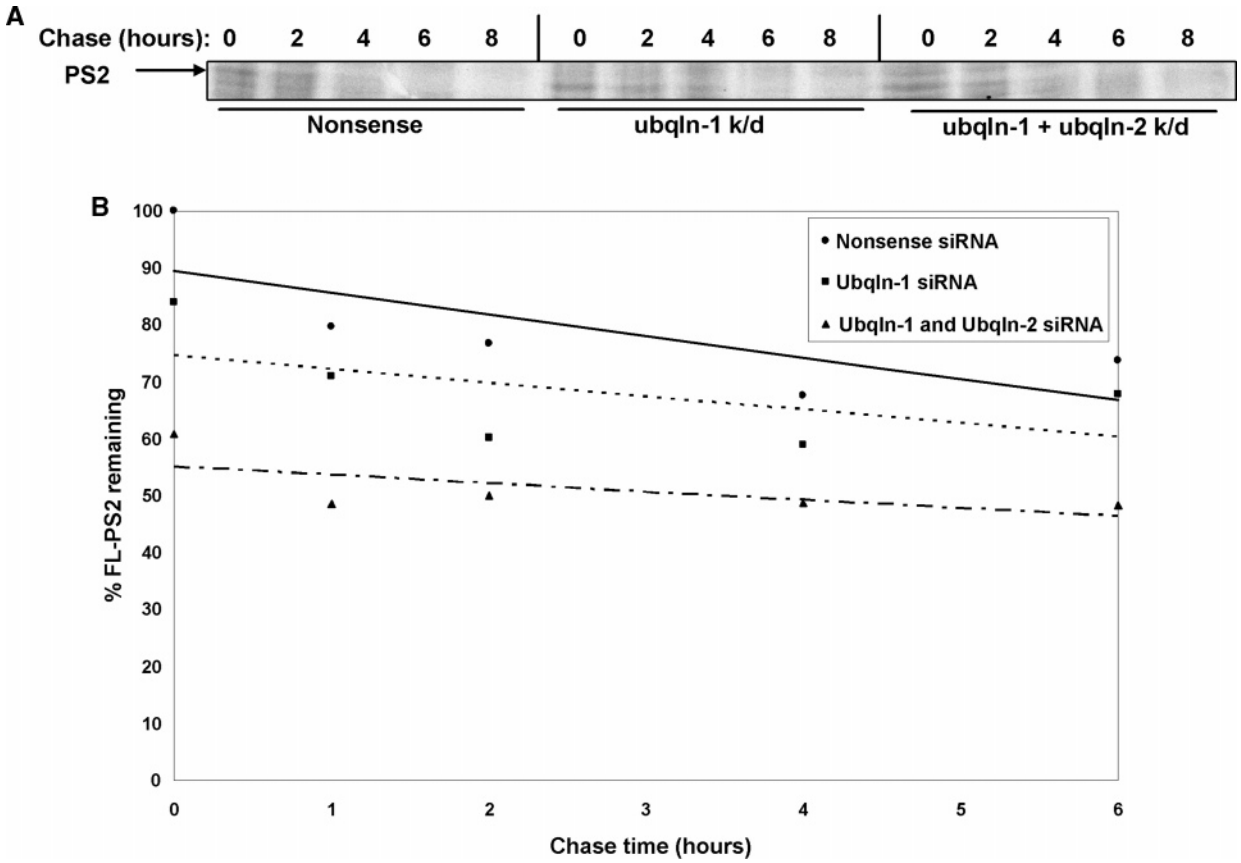

FIGURE 7: Reduction of ubiquilin expression by RNA interference leads to decreased synthesis of PS2 proteins but does not alter the turnover of PS2 proteins in neuronal SH-SY5Y cells. SH-SY5Y cell cultures were transfected with SMARTpool siRNAs directed against ubiquilin-1, ubiquilin-1 and ubiquilin-2, or nonsense siRNA. Forty-eight hours following transfection, the cultures were metabolically labeled with ^{35}S-methionine for 1 h. After the labeling period, the cultures were washed and incubated in non-radioactive medium, and protein lysates were collected at 2 h intervals as indicated in the Figure. (A) Autoradiogram showing full-length PS2. (B) Quantification of PS2 bands in A. Note that ubiquilin knockdown results in decreased synthesis of PS2 but has little affect on the turnover of the protein.

and decreases PS fragment production, whereas a reduction of ubiquilin expression produces the opposite effect.

Our results provide clues of the mechanism of how ubiquilin might regulate presenilin protein accumulation and endoproteolysis. With regard to its effect on the full-length presenilin protein, we considered two potential possibilities: first, overexpression of ubiquilin could increase presenilin synthesis, or second, it could decrease presenilin protein turnover. Surprisingly, our results have shown that the rate of turnover of the full-length PS2 protein is not affected by either overexpression (as shown previously; (*1*)) or underexpression (this article) of ubiquilin. Instead, and in accord with the second possibility, we reported previously that overexpression of ubiquilin increases by almost 2-fold the amount of new PS2 synthesized during the pulse-labeling period compared to the amount synthesized in the absence of ubiquilin overexpression (*1*). As we have shown in the present article, the converse is also true: a reduction of ubiquilin expression reduces net PS2 synthesis, compared to cells in which ubiquilin was not altered. These studies strongly suggest that ubiquilin might act as a chaperone to facilitate new presenilin protein synthesis.

However, the mechanism underlying the regulation of PS2 endoproteolysis by ubiquilin may have more to do with the binding of ubiquilin to the PS2-loop. One possibility we favor, is that when ubiquilin is expressed at high levels more of the protein interacts with the PS2-loop region preventing presenilin fragment production by either physically blocking access to the endoproteolytic activity or by altering trafficking of presenilins toward the endoproteolytic pathway (*21*). In contrast, a reduction of ubiquilin expression would lead to less binding to the PS2-loop and should produce the opposite effect. On the basis of the results shown here, we further propose that the binding of ubiquilin to the presenilin loop is negatively regulated by ubiquitination of the region.

Also of interest is what function is derived from the ubiquilin interaction with the C-terminus of presenilins. Although our results do not provide definitive clues of its function, they hint at a possible role in regulating protein turnover or stability of presenilins. In accord with this hypothesis, all of the PS2 mutants (both the lysine and the PALP mutants) we constructed in the carboxyl terminus led to destabilization of the mutant polypeptides in yeast, and we showed that this was due to increased degradation by the proteasome. Interestingly, the mutant polypeptides that turned over rapidly failed to interact with ubiquilin, suggesting that ubiquilin interaction may be important for regulating presenilin protein stability. Paradoxically, however, we found that the rate of presenilin protein turnover did not increase following the knockdown of ubiquilin in cells. These results lead us to speculate that, at least *in vivo*, ubiquilin may primarily function in regulating presenilin synthesis, which in our experiments may be more evident than its subtle role in regulating presenilin protein degradation.

In summary, our results have provided new information on the role of ubiquitination in the interaction of ubiquilin with the loop and carboxyl terminus of PS2. We have also provided additional evidence in support of our contention that the amount of ubiquilin expressed in cells regulates presenilin biogenesis and endoproteolysis (*1*, *21*).

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