Menin Missense Mutants Associated with Multiple Endocrine Neoplasia Type 1 Are Rapidly Degraded via the Ubiquitin-Proteasome Pathway

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MEN1 is a tumor suppressor gene that is responsible for multiple endocrine neoplasia type 1 (MEN1) and that encodes a 610-amino-acid protein, called menin. While the majority of germ line mutations identified in MEN1 patients are frameshift and nonsense mutations resulting in truncation of the menin protein, various missense mutations have been identified whose effects on menin activity are unclear. For this study, we analyzed a series of menin proteins with single amino acid alterations and found that all of the MEN1-causing missense mutations tested led to greatly diminished levels of the affected proteins in comparison with wild-type and benign polymorphic menin protein levels. We demonstrate here that the reduced levels of the mutant proteins are due to rapid degradation via the ubiquitin-proteasome pathway. Furthermore, the mutants, but not wild-type menin, interact both with the molecular chaperone Hsp70 and with the Hsp70-associated ubiquitin ligase CHIP, and the overexpression of CHIP promotes the ubiquitination of the menin mutants *in vivo*. These findings reveal that MEN1-causing missense mutations lead to a loss of function of menin due to enhanced proteolytic degradation, which may be a common mechanism for inactivating tumor suppressor gene products in familial cancer.

Multiple endocrine neoplasia type 1 (MEN1) is a dominantly inherited familial cancer syndrome characterized by the combined occurrence of tumors of the parathyroid gland, the pancreas, and the pituitary gland (2, 38). The responsible gene, *MEN1*, has been localized to chromosome 11q13 (21) and identified by positional cloning (7). The loss of heterozygosity of the *MEN1* locus in tumors suggests that *MEN1* is a tumor suppressor gene. *MEN1* contains 10 exons and encodes a 610-amino-acid protein, called menin (7), which shows no similarity to any known protein. Previous studies have revealed that menin is found predominantly in the nucleus, contains two independent nuclear localization signals in the C terminus (amino acids 479 to 497 and 588 to 608) (14), and binds to transcription factors, including JunD (1), Smad3 (18) and NF- κ B (15), all of which suggest a role related to transcriptional regulation. Menin has also been shown to interact with the putative tumor metastasis suppressor/nucleoside diphosphate kinase nm23 (27), the homeobox protein Pim (23), GFAP and vimentin (24), and the 32-kDa subunit (RPA2) of replication protein A (33). However, the exact molecular mechanism for tumor suppression by menin is largely unknown.

More than 400 independent germ line mutations distributed throughout the *MEN1* coding region have been identified for familial and sporadic cases of MEN1 (13, 36). The majority of these are nonsense mutations or frame shifts, but missense mutations and short in-frame deletions-insertions have also

been identified in about 30% of cases. Germ line *MEN1* mutations are also found in a small subset of familial isolated hyperparathyroidism (FIHP), a syndrome consisting of genetically heterogeneous diseases that are characterized by dominantly inherited parathyroid adenomas causing hyperparathyroidism with no evidence of other accompanying tumors (20, 34). Hyperparathyroidism is the earliest and most prevalent sign of MEN1, and FIHP caused by a *MEN1* mutation is considered a mild form of MEN1. Germ line *MEN1* mutations identified for FIHP cases are mostly missense mutations or in-frame deletions-insertions (29, 36), in contrast to the preponderance of frameshift and nonsense mutations in typical MEN1 cases, suggesting that some missense mutations identified in FIHP cases may not completely impair the tumor suppressor function of the *MEN1* gene and may cause FIHP as a milder form of MEN1.

The loss of function of both *MEN1* alleles is considered an important step in tumor formation. The effect of nonsense and frameshift mutations is obvious because they clearly predict a truncated and presumably inactive menin protein. However, little is known about the mechanism by which missense mutations causing single amino acid alterations lead to the inactivation of menin. One possibility is that such mutations affect functionally critical amino acid residues involved in the tumor suppressor activity of menin. Indeed, several missense mutations were reported to disrupt protein interactions or transcriptional regulation (1, 33). Nevertheless, the identified missense mutations are scattered over the entire coding region, with no apparent hot spots and no correlation between genotype and MEN1 phenotype (13, 36). These findings suggest that at least some missense mutations may not directly disrupt menin's active sites. In a previous study, we found that protein levels of

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FIG. 1. MEN1 disease-associated single amino acid mutations reduce steady-state protein levels, but not RNA levels. FLAG-tagged menin constructs were transfected into two 100-mm-diameter dishes of 293T cells. The cells from one dish were used to prepare the total protein, and equal amounts of protein were used for Western blot analyses of menin and tubulin (as a loading control) with anti-FLAG and anti- β -tubulin antibodies, respectively. Cells from the other dish were used to prepare the total RNA, and equal amounts of RNA were subjected to Northern blot analysis with a MEN1 probe. The endogenous menin mRNA signal was not detected with a short exposure. The blot was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a loading control.

menin mutants with single amino acid alterations associated with MEN1 were markedly decreased compared to levels of wild-type (WT) menin in transiently transfected cells (40), raising the alternative possibility that single amino acid mutations lead to decreased expression or stability of the RNA or protein rather than a reduced specific catalytic activity, and consequently, to the loss of function of menin. Here we show that single amino acid alterations associated with MEN1 disease reduce protein stability due to rapid degradation via the ubiquitin-proteasome pathway. We also show that the mutants, but not WT menin, interact both with the chaperone Hsp70 and with the ubiquitin ligase CHIP (C terminus of Hsp70-interacting protein) and that CHIP promotes the ubiquitination of the mutants *in vivo*. These findings reveal a new alternative mechanism for mutant menin inactivation by ubiquitin-mediated proteolysis.

MATERIALS AND METHODS

Plasmids and antibodies. The construction of expression plasmids for FLAG-tagged human menin variants (WT, P12L, H139D, and P320L) was described previously (40). Other expression plasmids for FLAG-tagged menin variants were generated by use of a QuikChange site-directed mutagenesis kit (Stratagene), and their sequences were confirmed by DNA sequencing. For the generation of constructs for N-terminally green fluorescent protein (GFP)-fused proteins, the cDNA constructs were inserted into the pEGFP-C1 expression vector (Clontech). The CHIP cDNA was cloned from human skeletal muscle by reverse transcription-PCR and then was subcloned into the vector pCMV-Tag3 (Stratagene) to allow for expression of N-terminally Myc-tagged CHIP. An expression plasmid for human ubiquitin was kindly provided by Dirk Bohmann.

The rabbit antimenin polyclonal antibody used was described previously (17). A mouse antimenin monoclonal antibody was raised against a keyhole limpet hemocyanin-conjugated synthesized peptide (KLQLTAQSQVQMKKQKVST) corresponding to human menin positions 576 to 594 and was purified from ascites fluid by protein A affinity chromatography. All other antibodies were purchased commercially, including mouse anti-FLAG (M2; Sigma), mouse anti-c-myc (9E10; Clontech), rabbit antiubiquitin (SPA-200; Stressgen), mouse anti-Hsp70/Hsc70 (SPA-820; Stressgen), mouse anti-Hsp90 (F-8; Santa Cruz), and mouse anti- β -tubulin (D-10; Santa Cruz) antibodies.

Cell culture and transfection. COS-7 cells, human embryonic kidney cells (293T cells), and mouse pituitary tumor cells (AtT20 cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin-streptomycin. Cells were transiently transfected by the use of FuGENE 6 according to the manufacturer's instructions (Roche) and were used in experiments 30 h after transfection. The total amount of plasmid DNA used for transfection was kept constant by the addition of an appropriate amount of vector plasmid.

Northern blotting. Total RNAs were isolated from transfected cells with an RNeasy total RNA isolation kit (Qiagen). Five micrograms of each RNA sample was resolved in a 1% formaldehyde-agarose gel and then transferred to a Hybond-N membrane (Amersham Biosciences) by standard techniques. The membranes were hybridized with a 32 P-labeled cDNA probe for human menin or human glyceraldehyde-3-phosphate dehydrogenase, washed, and then subjected to autoradiography.

Immunoprecipitation and Western blotting. Immunoprecipitation assays were performed as described previously (28). Briefly, transfected 293T cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and one Complete protease inhibitor mixture tablet [Roche]) and sonicated briefly. The cell lysates were centrifuged at $15,000 \times g$ for 5 min, and the supernatants were incubated with anti-FLAG M2 affinity gel for 1 h at 4°C with gentle rocking. After three washes with phosphate-buffered saline (PBS), the immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. For an examination of protein levels, whole-cell extracts were collected in lysis buffer supplemented with 0.1% SDS and were sonicated briefly. The immunodetection of proteins separated by SDS-PAGE was performed as described previously (28), using the ECL Plus Western blotting detection system (Amersham Biosciences).

Protein stability assays. To measure the rate of degradation of menin proteins, we transfected FLAG-tagged menin constructs in the absence or presence of the proteasome inhibitor MG132 (final concentration, 25 μ M) (Calbiochem) into 293T cells that had been treated with 20 μ g of cycloheximide (CHX)/ml to prevent further protein synthesis. Whole-cell extracts were prepared from samples taken at different time points, and the amounts of menin variants were determined by Western blotting with an anti-FLAG antibody. The Western blots were scanned with a model GS-800 densitometer, and protein bands were quantified with Quantitation One software (Bio-Rad). For pulse-chase experiments, transfected cells (293T or AtT20) were preincubated for 60 min in methionine-free, cysteine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum, pulsed with 250 μ Ci of [35 S]methionine-cysteine/ml for 60 min, and chased at different time points. Whole-cell extracts were prepared, and the fate of radiolabeled FLAG-menin variants was analyzed by immunoprecipitation using anti-FLAG M2 affinity gel. Endogenous menin was immunoprecipitated from untransfected cells with an anti-menin monoclonal antibody. The radioactive bands separated by SDS-PAGE were visualized by autoradiography and quantified with a bioimaging analyzer (BAS2500; Fuji-film).

Immunofluorescence. COS-7 cells grown on coverslips were transfected, treated with MG132 or dimethyl sulfoxide, fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked in 3% bovine serum albumin in PBS for 30 min. The cells were incubated with

FIG. 2. MEN1 disease-associated single amino acid mutations reduce the half-lives of mutant proteins. (A) To determine the half-lives of menin variants, we transfected 293T cells with the indicated FLAG-tagged menin constructs and treated them with 20 μ g of CHX/ml to block any further protein synthesis. Whole-cell lysates prepared at the time points indicated were immunoblotted with an anti-FLAG antibody. The relative levels of each menin species, specifically the WT (circles), PMs (squares), FIHP mutants (diamonds), and MEN1 mutants (triangles), were quantitated by densitometrical scanning. (B) 293T cells were labeled with [35 S]methionine-cysteine and then incubated in unlabeled culture medium for indicated times. Endogenous menin (Endo-menin) was immunoprecipitated (IP) from untransfected cells with an anti-menin antibody or normal mouse serum (NMS) as a control. Transiently expressed FLAG-tagged menin proteins were immunoprecipitated from transfected cells with an anti-FLAG antibody or normal mouse serum as a control. The radioactivities of the bands corresponding to full-length endogenous menin (arrowhead) and the FLAG-tagged menin proteins in each lane were quantitated with a BAS 2500 bioimaging analyzer (Fuji). The data shown are representative of three experiments. (C) Determination of half-lives of menin proteins in AtT20 cells by pulse-chase analysis.

A

B

C

anti-Hsp70/Hsc70 or anti-c-myc antibodies overnight at 4°C and then were incubated for 60 min at room temperature with a tetramethyl rhodamine isocyanate-conjugated goat anti-mouse secondary antibody (Zymed Laboratories Inc.). For the detection of FLAG-tagged menin proteins, an anti-FLAG M2 monoclonal antibody-fluorescein isothiocyanate conjugate was used, and DAPI (4',6'-diamidino-2-phenylindole) was used as a nuclear counterstain. Cells were analyzed with a fluorescence microscope (BX51; Olympus) equipped with a digital camera system (DP70; Olympus). For colocalization experiments, cells were observed under a laser scanning confocal microscope (Radiance 2100; Bio-Rad). Images were collected by using Bio-Rad LaserSharp 2000 software and were processed with Adobe Photoshop 6.0.

Ubiquitination assay. 293T cells were transfected with combinations of ubiquitin, FLAG-tagged menin variants, and Myc-tagged CHIP. Cells were treated with MG132 or vehicle and subjected to immunoprecipitation with anti-FLAG M2 affinity gel as described above, except that the cells were lysed with lysis buffer supplemented with 0.1% SDS. The immunoprecipitates were subjected to Western blot analysis with polyclonal antimenin and antiubiquitin antibodies.

Mass spectrometric analysis. Proteins resolved by SDS-PAGE were stained with GelCode Blue staining reagent (Pierce), and the protein bands were excised from the gel, destained with 25 mM ammonium bicarbonate–50% acetonitrile, and incubated with 0.1 µg of sequence-grade modified trypsin (Promega)/µl in digestion buffer (50 mM Tris-HCl [pH 8.0] and 1 mM CaCl₂) overnight at 37°C. The digested samples were extracted with 5% trifluoroacetic acid–50% acetonitrile and analyzed by mass spectrometry. An electrospray mass spectrometer (LCQ-DECA; ThermoQuest Co.) coupled online to a capillary high-performance liquid chromatography column (Magic 2002; Michrom BioResources) was used to acquire tandem (MS/MS) spectra of peptides. Peptides were subjected to a Magic C₁₈ column (5-µm diameter, 200 Å pore size, 0.2 by 50 mm) and were eluted with a gradient of 5 to 95% buffers B (90% acetonitrile, 0.1% formic acid) and A (2% acetonitrile, 0.1% formic acid) for 30 min at a flow rate of 3 µl/min. The flow was split with a Magic precolumn capillary splitter assembly (Michrom BioResources) at 80 µl/ml. The mass spectrometer was operated in autodetection mode to acquire MS/MS spectra during high-performance liquid chromatography. Proteins were identified by searching sequence databases with Mascot software.

RESULTS

MEN1 disease-associated single amino acid mutations reduce the stability of menin protein. We previously found low expression levels of menin proteins with missense mutations (P12L, H139D, and P320L) that were identified in patients with MEN1 in comparison to levels of WT menin (40). Decreased protein levels of missense mutants in transfection assays were also mentioned by Agarwal et al. (1). Thus, we speculated that *MEN1* gene mutations causing single amino acid alterations affect menin mRNA or protein stability, thereby resulting in a reduced steady-state protein level. To test this hypothesis, we first examined the mRNA and protein levels of WT menin and 11 variants with single amino acid alterations (substitutions or deletions) in transiently transfected 293T cells: 7 mutants were identified in patients with MEN1 (MEN1 mutants P12L, K119del, H139D, A309P, P320L, D418N, and S555N), 2 mutants were described for FIHP families (FIHP mutants E255K and Q260P), and 2 variants described as benign polymorphisms (PMs; R171Q and A541T) were used as controls. Thirty hours after transfection with FLAG-tagged menin constructs, total RNAs and whole-cell extracts were prepared and analyzed by Northern and Western blotting, respectively. Surprisingly, all of the MEN1 mutants tested for this study were expressed at extremely low levels compared to the WT protein (Fig. 1). In addition, one FIHP mutant, Q260P, also showed a decreased level of protein, whereas the level of the other FIHP mutant, E255K, was similar to that of the WT protein. Both of the PM proteins were expressed at similar levels to the WT. On the other hand,

FIG. 3. The ubiquitin-proteasome pathway mediates degradation of the menin mutants. (A) Effects of the proteasome inhibitor MG132 on the degradation of menin proteins. 293T cells transfected with FLAG-tagged menin constructs (WT or P12L) were treated with the proteasome inhibitor MG132 (25 µM) or vehicle during a CHX chase. Whole-cell lysates prepared at the time points indicated were immunoblotted with an anti-FLAG antibody. (B) Protein levels of menin variants after a 2-h treatment with MG132 or vehicle during the CHX chase. (C) Effects of MG132 on distribution of menin variants. COS-7 cells transfected with FLAG-tagged menin constructs (WT or P12L) were treated with MG132 or vehicle for 6 h. Menin localization was detected with a fluorescein isothiocyanate-conjugated anti-FLAG antibody under a fluorescence microscope. Cell nuclei were visualized with DAPI staining. (D) Representative immunofluorescence images for menin variants after a 6-h treatment with MG132.

menin mRNAs were equally expressed in all transfections, indicating that mutational effects at the RNA level are not an explanation for the decreased levels of the protein.

To determine whether the lowered protein level of the mutants was due to rapid protein degradation, we performed a CHX chase analysis of menin variants in 293T cells and estimated their turnover rate. Thirty hours after transfection with FLAG-tagged menin variants, CHX was added to inhibit de novo protein synthesis. Whole-cell extracts were prepared from the cells at different times after the CHX addition, and the rate of menin degradation was monitored by Western blot analysis. The half-lives of WT, PM, and E255K menin were estimated to be 6 to 8 h in 293T cells (Fig. 2A). In contrast, the mutants that were expressed at lower levels (Fig. 1) turned out to be highly unstable (Fig. 2A); all of the MEN1 mutants and the Q260P FIHP mutant rapidly disappeared, with half-lives of <2 h. These results were confirmed by a pulse-chase analysis. Cells transfected with a FLAG-tagged WT or mutant (P12L) menin construct were pulsed for 60 min with [³⁵S]methionine-cysteine, and after a chase in unlabeled medium, anti-FLAG antibody immunoprecipitates were subjected to SDS-PAGE. Again, we observed that the mutant protein was much more unstable than WT menin (Fig. 2B). Moreover, we confirmed that the half-life of transiently expressed FLAG-tagged WT menin was comparable to that of endogenous menin in 293T

FIG. 3—Continued.

cells. Finally, we examined the half-lives of the WT and mutant proteins in a pituitary cell line, AtT20, which may be a pertinent model for MEN1-related endocrine cells, and again we observed a decreased protein stability of the P12L mutant (Fig. 2C).

Menin mutants are targeted for degradation via the ubiquitin-proteasome pathway. In eukaryotic cells, the major mechanism for the targeted degradation of unstable proteins is the ubiquitin-proteasome pathway. To test whether the mutants are targeted for degradation via the ubiquitin-proteasome pathway, we investigated the effects on the stability of mutants of the proteasome inhibitor MG132 during a CHX

chase. As shown in Fig. 3A, whereas treatment with MG132 for 2 or 6 h had little effect on the stability of the WT protein, it decreased the amount of mutant P12L protein that was degraded at each time point compared with untreated cells, indicating that degradation of the P12L mutant was inhibited and that P12L accumulated in the cells. An MG132 treatment for 2 h also stabilized all other MEN1 mutants and the Q260P FIHP mutant, which were otherwise unstable (Fig. 3B).

To confirm the accumulation of the mutants, we performed an immunofluorescence analysis with COS-7 cells transfected with FLAG-tagged menin (WT or P12L) variants. Consistent with previous findings (14), the transiently expressed menin

WT protein was found to be predominantly localized to the nucleus with a diffuse staining pattern which was not affected by an MG132 treatment for 2 h (Fig. 3C). A relatively weak but similar staining for the mutant P12L protein was observed in the absence of MG132, and only a small subset of cells were labeled, with each having a punctate distribution of the fluorescent signal. However, this changed markedly when the proteasome was inhibited; a remarkable accumulation at multiple foci was observed throughout not only the nucleus, but also the cytoplasm, and a fraction of the cells showed strong perinuclear staining. A similar accumulation was found for all of the other MEN1 mutants and the Q260P FIHP mutant, but not for the PMs and the E255K mutant (Fig. 3D). Furthermore, similar images were obtained for 293T cells (data not shown).

Menin mutants are ubiquitinated in vivo. To determine whether menin mutants could be ubiquitinated, we performed an in vivo ubiquitination assay. 293T cells were cotransfected with constructs expressing ubiquitin and FLAG-tagged menin (WT or P12L), and lysates were immunoprecipitated with an anti-FLAG antibody. Western blotting with antimenin and antiubiquitin antibodies detected higher-molecular-weight bands, which is indicative of ubiquitinated menin, in the mutant P12L immunoprecipitate, the intensity of which was enhanced when the ubiquitin-proteasome pathway was blocked by MG132 (Fig. 4A). These bands were detected after reprecipitation with the anti-FLAG antibody after boiling in 1% SDS, verifying that menin itself was ubiquitinated (data not shown). Whereas treating cells with MG132 for 2 h led to a marked accumulation of polyubiquitinated forms of the P12L mutant protein, the ubiquitination of WT menin was barely detectable even after 6 h of treatment with MG132. The other unstable mutants (all of the MEN1 mutants and the FIHP Q260P mutant) also showed a high level of ubiquitination after 2 h of treatment with MG132, whereas the FIHP E255K mutant and PM menin proteins were not ubiquitinated, similar to WT menin (Fig. 4B).

Menin mutants interact with the molecular chaperone Hsp70. The results presented above prompted us to examine whether WT menin and the mutants have different partners that may affect protein stability. To test this possibility, we immunoprecipitated WT or mutant menin complexes from 293T cells, resolved the immunoprecipitates by SDS-PAGE, and visualized them by staining with GelCode Blue (Coomassie G-250). To minimize the difference in steady-state protein levels, we transfected a smaller amount of WT menin plasmid DNA. Two very close bands at 70 kDa were visible on the gels for every mutant immunoprecipitate, but not for the WT immunoprecipitate (Fig. 5A). Mass spectrometry revealed that these bands corresponded to two forms of the heat shock protein Hsp70: constitutively expressed Hsc70 and stress-inducible Hsp70. This interaction was confirmed by Western blotting analysis (Fig. 5B). All of the MEN1 mutants and the FIHP Q260P mutant, which had been found to be unstable (Fig. 2), were associated with Hsp70, whereas WT, PM, and E255K menin variants showed no or only very weakly detectable bands.

Next, we performed a confocal immunofluorescence analysis to examine the colocalization of the mutant protein and Hsp70 in COS-7 cells transfected with constructs encoding GFP fused to WT menin (GFP-WT) or mutant menin (GFP-P12L). In

FIG. 4. Menin mutants are ubiquitinated in vivo. (A) 293T cells transfected with ubiquitin and FLAG-tagged menin (WT or P12L) were treated with MG132 for 0 to 6 h before being harvested. The lysates were immunoprecipitated (IP) with an anti-FLAG antibody and subjected to Western blot analysis (WB) with antimenin and antiubiquitin antibodies. (B) Ubiquitination of menin variants after a 2-h MG132 treatment. Note that half the amount of plasmid for the WT, PMs, and the E255K mutant relative to that for the mutants was transfected for the expression of approximately equal amounts of protein.

nontransfected cells, Hsp70 was expressed predominantly in the cytoplasm (data not shown), as was previously shown (26). When GFP-WT was expressed, this localization pattern of Hsp70 remained the same and did not overlap with that of the WT protein in either the presence or absence of MG132 (Fig. 5C). In contrast, we observed a colocalization of GFP-P12L with Hsp70 staining. When GFP-P12L was expressed in the nucleus, Hsp70 was translocated into the nucleus and overlapped with GFP-P12L. In the presence of MG132, Hsp70 showed substantial colocalization with nuclear and cytoplasmic aggregates containing mutant proteins.

The Hsp70 cofactor CHIP interacts with menin mutants and promotes their degradation. Molecular chaperones such as Hsp70 and Hsp90 recognize aberrantly folded proteins and promote adoption of the native conformation whenever possi-

ble (39). If the native conformation is not attainable, the misfolded protein is targeted for degradation. Since the mutant proteins associate with Hsp70 (Fig. 5), a single amino acid change might result in aberrant folding, which is more susceptible to proteasomal degradation. Recently, the Hsp70 cochaperone CHIP was shown to function as a ubiquitin ligase towards several substrates presented by Hsp70 and Hsp90 (8, 25), leading us to ask whether CHIP stimulates the mutant proteins for proteasomal degradation. First, we investigated the interaction of CHIP with menin variants in a coimmunoprecipitation assay. Myc-tagged CHIP was coexpressed with FLAG-menin variants in 293T cells, and the cell lysates were immunoprecipitated with an anti-FLAG antibody. Consistent with the binding to Hsp70, immunoprecipitation of MEN1 mutants and the Q260P FIHP mutant resulted in the coprecipitation of CHIP, whereas the WT, PM menin, or the E255K FIHP mutant did not (Fig. 6A). No association with Hsp90 was observed for any menin proteins, even after a long exposure time.

Next, we examined the colocalization of menin and CHIP by using COS-7 cells transfected with GFP-WT or GFP-P12L and Myc-CHIP. Overexpressed Myc-CHIP stained with an anti-Myc antibody exhibited cytoplasmic staining, as reported previously (3), and did not colocalize with GFP-WT menin (Fig. 6B). In the absence of MG132, coexpression with CHIP resulted in a much less intense signal for GFP-P12L than when P12L was expressed alone (data not shown). In cells expressing GFP-P12L and Myc-CHIP, the GFP signal partially overlapped with anti-Myc staining in the cytoplasm, predominantly in the perinuclear region. Also, the cytoplasmic colocalization was clearly visualized when a proteasome inhibitor was used. However, in contrast to the pattern for Hsp70, there was little colocalization in the nucleus, where the mutant GFP signal but no CHIP staining could be found.

By Western blotting (compare Fig. 6A and Fig. 5B) and immunofluorescence analysis (data not shown), we found that co-overexpression with CHIP severely reduced the levels of the mutant menin proteins and that this reduction was inhibited by MG132, suggesting that CHIP promotes the ubiquitination of mutants and directs their degradation by the proteasome. To test this possibility, we investigated the effects of the overexpression of CHIP on the ubiquitination of the mutants. 293T cells cotransfected with ubiquitin and FLAG-tagged menin (WT or P12L), with or without Myc-CHIP, were treated with MG132 or a vehicle and subjected to an *in vivo* ubiquitination assay. Western blotting of total lysates again confirmed that CHIP reduced the protein levels of the mutant, but not WT, proteins and that the expression was restored by treatment with MG132 (Fig. 6C), suggesting that the effect of CHIP on mutant protein levels requires the proteasome pathway. Western blotting of the immunoprecipitates demonstrated that the ubiquitination of WT menin was undetectable in either the absence or presence of CHIP. In contrast, the overexpression of CHIP caused an increase in the ubiquitination of the mutants.

DISCUSSION

In the present study, we have shown that MEN1 disease-associated single amino acid mutations lead to greatly diminished levels of menin protein due to rapid degradation via the

ubiquitin-proteasome pathway. The question of how missense mutations contribute to the development of tumors in MEN1 patients has so far been addressed under the assumption that such mutations disrupt important active sites of menin. However, our results provide an alternative mechanism for the inactivation of menin with disease-associated missense mutations by ubiquitin-mediated proteolysis.

To our surprise, all seven MEN1 mutants tested for this study exhibited extreme instability at the protein level, with more-than-threelfold shorter half-lives than the WT and PM menin variants, leading us to predict a similar instability in other missense mutants associated with MEN1 disease. Such unstable mutants would be rapidly degraded, which would result in a reduced steady-state level. Although the actual functions of menin remain unclear, it is likely that a definite level of protein is required for its function(s) to be carried out. If this is the case, we expect these missense mutations to exert their transforming effect by destabilizing the menin protein. At present, it is difficult to establish the relationship between the rapid degradation of menin mutants and menin's function(s); however, a comprehensive analysis of steady-state levels or protein half-lives, using expressed menin proteins with a series of identified missense mutations, will yield valuable information about the molecular pathogenesis of MEN1. In addition, future investigations may demonstrate that some missense mutations do not affect the half-life of the protein, which would provide a clue as to which residues are directly involved in important functions or interactions. In this context, our interesting observations with two FIHP mutants may help us to determine the role of menin. We found that one FIHP mutant, Q260P, was unstable, similar to all of the MEN1 mutants, but that the other, E255K, showed a half-life similar to that of the WT, suggesting that the glutamic acid at codon 255 may be a functionally crucial residue and that its replacement with lysine may disrupt some catalytic activity of menin. Therefore, it will be interesting to determine the mechanism of the loss of function caused by the E255K mutation.

We have found by immunofluorescence microscopy that inhibition of the proteasome leads to an accumulation of mutant proteins in punctate aggregates. Similar aggregates are often observed for nonnative proteins (22, 26). Thus, we assume that a single amino acid alteration may result in impaired folding of the menin protein, which makes the protein more susceptible to degradation. This would explain why rapid degradation is a common consequence of different single amino acid mutations distributed across menin. A misfolded mutant protein is also consistent with the strong binding of the molecular chaperone Hsp70 to the mutants, but not to WT menin. It is well known that Hsp70 recognizes nonnative proteins via exposed hydrophobic surfaces and that by stabilizing aberrant conformations, it facilitates correct folding (6, 39). Therefore, it is likely that single amino acid mutations alter the conformation of the menin protein, leading to the exposure of a hydrophobic stretch that should be buried in the native structure, although confirmation of this will have to await determination of the three-dimensional structure of the menin protein.

Protein ubiquitination is mediated by the sequential actions of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (16, 37). E3s are recruited from large protein families and have a

FIG. 5. Menin mutants, but not WT menin, interact with Hsp70. (A) Identification of proteins interacting with menin mutants. WT or mutant FLAG-tagged menin variants were immunoprecipitated (IP) from transfected 293T cells with an anti-FLAG antibody, and the immunoprecipitates were separated by SDS-10% PAGE. A photographic image of a gel stained with GelCode Blue (Coomassie G-250) is shown. The proteins (indicated by arrows) that coimmunoprecipitated with mutants but not with the WT were excised from the gel, digested with trypsin, and subjected to mass spectrometric analysis. Predicted peptide sequences are shown. (B) Interaction of menin mutants with Hsp70. A vector plasmid (control) or FLAG-tagged menin constructs were transfected into 293T cells. Immunoprecipitates of menin proteins obtained with an anti-FLAG antibody, as well as total cell lysates, were analyzed by Western blotting with anti-FLAG and anti-Hsp70 antibodies. Note that half the amount of plasmid for the WT, PMs, and E255K mutant compared to that for the mutants was transfected for the expression of approximately equal amounts of protein. (C) Subcellular localization of Hsp70 and menin. COS-7 cells transfected with GFP-WT or GFP-P12L were treated with MG132 or vehicle for 2 h. The cells were fixed and analyzed by (laser scanning) confocal microscopy for the localization of GFP-menin (green) and endogenous Hsp70 (red) by use of an antibody to Hsp70. The colocalization of menin and Hsp70 appears in yellow. Areas marked by a rectangle are enlarged and shown as insets.

limited range of substrates, providing specificity to the system. Our finding that highly ubiquitinated mutant proteins accumulate when the proteasome is inhibited suggests that the mutants are unstable because they are targeted for degradation via the ubiquitin-proteasome pathway. If the mutant protein fails to attain its native conformation, resulting in a prolonged

interaction with Hsp70, which mechanism mediates the decision to abort an unsuccessful folding and instead to target the misfolded mutant for ubiquitin-mediated proteolysis? Little is known about the mechanism by which nonnative proteins are delivered to the ubiquitination machinery, and it has yet to be determined whether an E3 ubiquitin ligase specific for mis-

FIG. 5—Continued.

folded proteins exists. However, recent studies have shown that the chaperone-dependent E3 ubiquitin ligase CHIP stimulates the proteasomal degradation of known chaperone substrates, such as the glucocorticoid receptor (8) and the immature endoplasmic reticulum-localized forms of the cystic fibrosis transmembrane regulator (25), suggesting that CHIP switches the activity of molecular chaperones from protein folding to protein degradation. Given this finding, it is conceivable that CHIP can function as the E3 ubiquitin ligase of menin mutants presented by Hsp70 and thus direct them to degradation by the proteasome. This possibility is consistent with our findings that the mutants bind to both Hsp70 and CHIP and that the co-overexpression of CHIP reduces the levels of menin mutants in a proteasome-dependent manner and promotes their ubiquitination *in vivo*. However, thus far we have not been able to detect the ubiquitination of menin by CHIP *in vitro* (data not shown), suggesting that the CHIP-induced ubiquitination of menin mutants may require the presence of additional unknown cellular proteins or that the enhancement of menin ubiquitination by CHIP may be due to forced overexpression and the actual physiological ubiquitination may be mediated by another E3 ubiquitin ligase(s). Further experiments will be needed to distinguish between these possibilities.

We have found that menin mutants are predominantly located in the nucleus, similar to the WT, but that MG132 treatment results in a strong accumulation of mutant proteins

at multiple foci in not only the nucleus, but also the cytoplasm. Since in mammalian cells about one-third of newly synthesized proteins are reported to be degraded by the proteasome shortly after translation (30), the observed cytoplasmic accumulation of menin mutants may partially reflect the immediate proteasomal degradation of a fraction of newly synthesized mutants in the cytoplasm before translocation to the nucleus. However, it remains to be investigated where the nuclear mutant proteins are ubiquitinated and degraded. It was reported that some nuclear proteins are degraded in the nucleus, whereas others have to be exported from the nucleus to be degraded in the cytosol (9, 12, 35). Given that the mutants and CHIP colocalize not in the nucleus, but in the cytoplasm, if CHIP is an E3 ubiquitin ligase specific for menin mutants, the mutant proteins would be expected to be retrotranslocated to the cytoplasm to be ubiquitinated by CHIP and degraded by the proteasome. This possibility is unlikely, however, because our preliminary data indicate that preventing nuclear export by a treatment with leptomycin B does not affect the stability of the mutant protein (data not shown). Thus, it is likely that the nuclear mutant proteins are ubiquitinated by a nuclear E3 ubiquitin ligase and degraded in the nucleus by a pathway other than that for their cytoplasmic degradation.

An interesting question is whether the WT menin protein is degraded by the same pathway as the mutant proteins. The

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