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Characterization of the *MEN1* Gene Product, Menin, by Site-specific Polyclonal Antibodies

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The gene associated with multiple endocrine neoplasia type 1 (MEN 1), designated *MEN1*, has recently been identified. This gene shows no homology to other known genes, and its expression is not restricted to endocrine organs as estimated by northern blotting. Expression of the *MEN1* gene product, menin, has been studied only in a few tissues. In this report, expression of menin in various cells and mouse tissues was studied using two polyclonal antibodies against menin. Expression of menin as a 76 kDa single protein was observed in all cell lines examined, regardless of origin. Two nuclear localization signals of the menin have been reported, but through the study of mutant menin in lymphocytes from subjects with MEN 1, impaired nuclear localization of the mutant menin was observed even though the mutant retained one of the two nuclear localization signals (NLSs). Menin was stable *in vitro* with a half-life of over 24 h at 37°C. In the cell, the half-life of wild-type menin was about 10 h, while that of the mutant was about 2 h. The mutant rapidly disappeared from the nucleus.

Key words: Multiple endocrine neoplasia type 1 — Menin — Antibody — Western blotting — Nuclear localization

Multiple endocrine neoplasia type 1 (MEN 1) is a hereditary disease characterized by hyperplastic and neoplastic disorder of endocrine organs.[1] The gene associated with MEN 1, designated *MEN1*, has recently been identified.[2, 3] Germline mutations of *MEN1* have been identified in most subjects with familial and sporadic MEN 1[2–8] and in some kindreds with familial hyperparathyroidism.[9–11] Somatic *MEN1* gene mutations have also been identified in some sporadic parathyroid adenomas,[12] endocrine pancreas tumors[13] and lung carcinoid tumors.[14] Mutation of the *MEN1* gene along with the loss of the unaffected allele in the tumor suggests that this gene is a tumor suppressor gene.[12–14] Germline and somatic mutations have been identified throughout the coding region of the *MEN1* gene and no apparent hot spots or genotype-phenotype correlation were observed, providing little clue as to the function or domain structure of the translation product of the *MEN1* gene, designated menin.[2]

The physiological function of menin is little understood. *Men1* mRNA is widely expressed in various mouse tissues and is not restricted to target organs of MEN 1.[15] Also, expression of *Men1* mRNA in the early stage of mouse embryogenesis suggests that this gene may play a role in fetal development.[15] Menin has been demonstrated to be a nuclear protein and has two nuclear localization signals in the carboxyl-terminus of the protein.[16] Recently, Agarwal *et al.* reported that menin interacts with JunD and suppresses JunD-activated transcription.[17] Thus, the function of menin involves the regulation of transcription, though this is not sufficient to explain the clinical features of MEN 1.

To elucidate the role of menin in cell physiology and tumorigenesis, we have developed polyclonal antibodies against peptides derived from the amino acid sequence of menin. These antibodies specifically recognized menin expressed in various cultured cells. We found that two nuclear localization signals may be necessary for efficient transport of menin to the nucleus. We also found that wild-type menin was stable in the nucleus while the frame-shift mutant disappeared rapidly.

MATERIALS AND METHODS

Plasmids pCMVsportMenin, containing a full-length human menin cDNA, was generously provided by Dr. Chandrasekharappa.[16] The coding region of pCMVsport-Menin was amplified by polymerase chain reaction and subcloned into the *Bam*HI-*Eco*RI site of pcDNA3.1/HisC (Invitrogen, Carlsbad, CA) in-frame to produce pcDNA/HisMenin. The sequence of this plasmid was confirmed by dideoxy sequencing. pcDNA/HisMenin516FS was created by the insertion of a cytosine residue at the corresponding position identified in patients with MEN 1.[7]

Development of anti-menin antibodies Two peptides, HGKGNEDRRGQTVNC and AREGRRRGPRRESKPEE, corresponding to amino acids 199–212 and 475–491 of menin, respectively, were synthesized and conjugated to

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keyhole limpet hemocyanin. These amino acid sequences are identical in human and mouse menin.[15] Male rabbits were immunized with each derivative four times, and the sera were collected 10 days after the final immunization and purified using FMP-activated cellulofine (Seikagaku, Tokyo) which was conjugated with the corresponding peptide.

Transient transfection COS cells were grown to subconfluence in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD) containing 10% fetal bovine serum in 6 cm culture dishes. Transfection was performed using Lipofectamine (Gibco) according to the manufacturer's instructions. Two micrograms of plasmid was used for each dish. Forty-eight hours after transfection, whole cell extract was prepared as described below and used for immunoblotting.

Immunoprecipitation of *in vitro* translated menin [^{35}S]Methionine-labeled tag-menin and tag-menin516FS were synthesized with the TNT T7-coupled Reticulocyte Lysate System (Promega, Madison, WI) using pcDNA/HisMenin or pcDNA/HisMenin516FS, respectively, as a template. For immunoprecipitation, programmed lysate was incubated with 400× diluted HGK or ARE in 20 m*M* Tris-Cl pH 7.6 containing 150 m*M* NaCl, 3 m*M* $MgCl_2$, 2 m*M* ethylenediaminetetraacetic acid (EDTA), 2 m*M* ethylene glycol-bistetraacetic acid (EGTA), 0.1% TritonX-100, 0.1% Nonidet P-40 and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) for 2 h at 4°C. Protein A Sepharose (Sigma, St Louis, MO) was added and incubated for an additional 1 h at 4°C. After incubation, protein A Sepharose was collected by centrifugation and washed 5 times. Protein bound to Sepharose was separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel followed by autoradiography. [^{35}S]Methionine-labeled luciferase was prepared by using T7 luciferase DNA (Promega) and was used as a control.

Preparation of cell extracts Cell lines were obtained from either ATCC (Rockville, MD) or RIKEN (Wako) and cultured according to the distributor's instruction. Cells cultured to subconfluence were collected by trypsinization and suspended in SMT buffer (0.32 *M* sucrose, 1 m*M* $MgCl_2$ and 10 m*M* Tris-Cl, pH 7.6) containing 1 m*M* PMSF and proteinase inhibitors (10 μg/ml aprotinin, 1 μg/ml pepstatin and 5 μg/ml leupeptin). The cell suspension was homogenized in a glass-Teflon homogenizer, followed by centrifugation. The supernatant was collected and used as a cytosolic fraction. The pellet was incubated in NMT buffer (400 m*M* NaCl, 1 m*M* $MgCl_2$ and 10 m*M* Tris-Cl, pH 8.0) containing 1 m*M* PMSF and proteinase inhibitors for 1 h on ice with vortexing every 10 min. After centrifugation, the supernatant was collected and used as a nuclear fraction. To prepare whole cell extract, cells were suspended in NMT containing PMSF and proteinase inhibitors, subjected to a freeze-thaw procedure three times, incubated on ice for 1 h with vortexing every 10 min, and centrifuged to collect the supernatant. For the study on the stability of menin, whole cell extract was prepared and incubated at 37°C without proteinase inhibitors. Tissues obtained from adult mouse were minced in SMT containing PMSF and proteinase inhibitors. Nuclear extracts were prepared as described above. Peripheral blood was drawn from patients with MEN 1 and a normal control after informed consent had been obtained. Lymphocytes were isolated using Lymphoprep (Nycomed Pharma, Oslo, Norway) and stimulated with phytohemagglutinin-M (Gibco) for 3 days in RPMI 1640 medium (Gibco) with 20% fetal bovine serum. Nuclear and cytosolic fractions were prepared as described above.

Immunoblotting Samples were separated on a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond-C extra, Amersham, Buckinghamshire, UK). Aliquots of 10 μg each for nuclear fraction and whole cell extract, and 30 μg each for cytosolic fraction were applied. The membrane was blocked in TBS-T (Tris-buffered saline with 0.05% Tween 20) containing 5% skim milk for 1 h, followed by incubation with the 2,000× diluted anti-menin antibody described above in TBS-T containing 1% skim milk for 2 h at room temperature with gentle agitation. The membrane was washed in TBS-T several times, and the bound antibody was detected with peroxidase-coupled anti-rabbit immunoglobulin-G (IgG) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL western blotting detection reagents (Amersham). For the detection of anti-nucleoporin p62 antibody (Transduction Laboratories, Lexington, KY) and anti-α-tubulin antibody (Zymed, South San Francisco, CA), peroxidase-

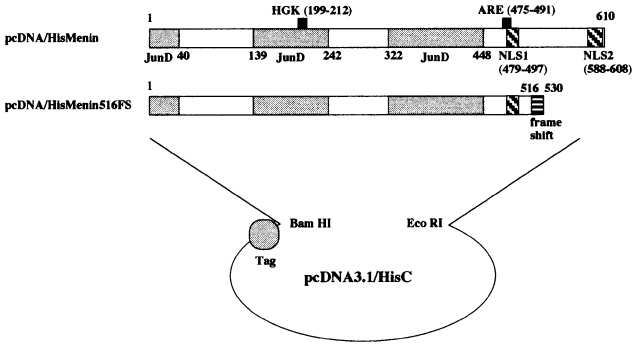

Fig. 1. Schematic presentation of pcDNA/HisMenin constructs. The full-length human *MEN1* cDNA was inserted in-frame between the *Bam*HI and *Eco*RI sites of pcDNA3.1/HisC. pcDNA/HisMenin516FS was created as described in "Materials and Methods." The amino-terminal tag consists of 35 amino acid residues. Positions of critical domains for menin-JunD interaction (shaded boxes),[17] nuclear localization signals (NLS-1 and NLS-2) (hatched boxes),[16] and amino acid residues utilized to produce antibodies in this report (HGK and ARE) are indicated.

coupled anti-mouse IgG antibody (Santa Cruz Biotechnology) was used as a secondary antibody.

RESULTS

Development of site-specific anti-menin antibodies To examine the expression and function of menin in the cell and to elucidate its role in tumorigenesis, we have developed polyclonal antibodies against peptides corresponding to partial amino acid sequences of menin. Two peptides corresponding to amino acids 199–212 and 475–491 of menin were synthesized and male rabbits were immunized with each peptide. The antibody against peptide 199–212 was designated HGK, and the other antibody against peptide 475–491 was designated ARE. To examine the specificity of these antibodies, COS cells were transfected with either pCMVsportMenin, pcDNA/HisMenin or pcDNA/HisMenin516FS (Fig. 1). pcDNA/HisMenin expresses menin tagged with a 35 amino acid polypeptide at its amino terminus and pcDNA/HisMenin516FS expresses tagged mutant menin. This mutation, designated either 1650insC[4] or 1657insC,[7,8] has been identified in patients with MEN 1, and causes a frame shift at amino acid 516 with a new termination codon after amino acid 530. Whole cell extracts of transfected cells were subjected to immunoblotting. As shown in Fig. 2A, HGK recognized expressed menin with the size of 76 kDa (lane 2). This size is larger than the predicted molecular weight of 68 kDa, but agrees with the report by Huang *et al*.[18] HGK also recognized tag-menin (lanes 3 and 5) and tag-menin-516FS (lanes 4 and 5). The same result was obtained using ARE (data not shown). Despite the fact that equal amounts of plasmids were used for transfection, the signal of tag-menin516FS was always weaker than that of tag-menin (lane 5). This was not due to lower efficiency of antibodies to recognize tag-menin516FS, since both antibodies recognized *in vitro*-translated tag-menin and tag-menin516FS with similar efficiency (Fig. 2B). These antibodies were able to immunoprecipitate menin. As shown in Fig. 2C, both HGK and ARE immunoprecipitated *in vitro*-translated tag-menin and tag-menin516FS, but not a control protein, luciferase. Protein A did not precipitate expressed proteins (right 3 lanes). These results demonstrate that both HGK and ARE specifically recog-

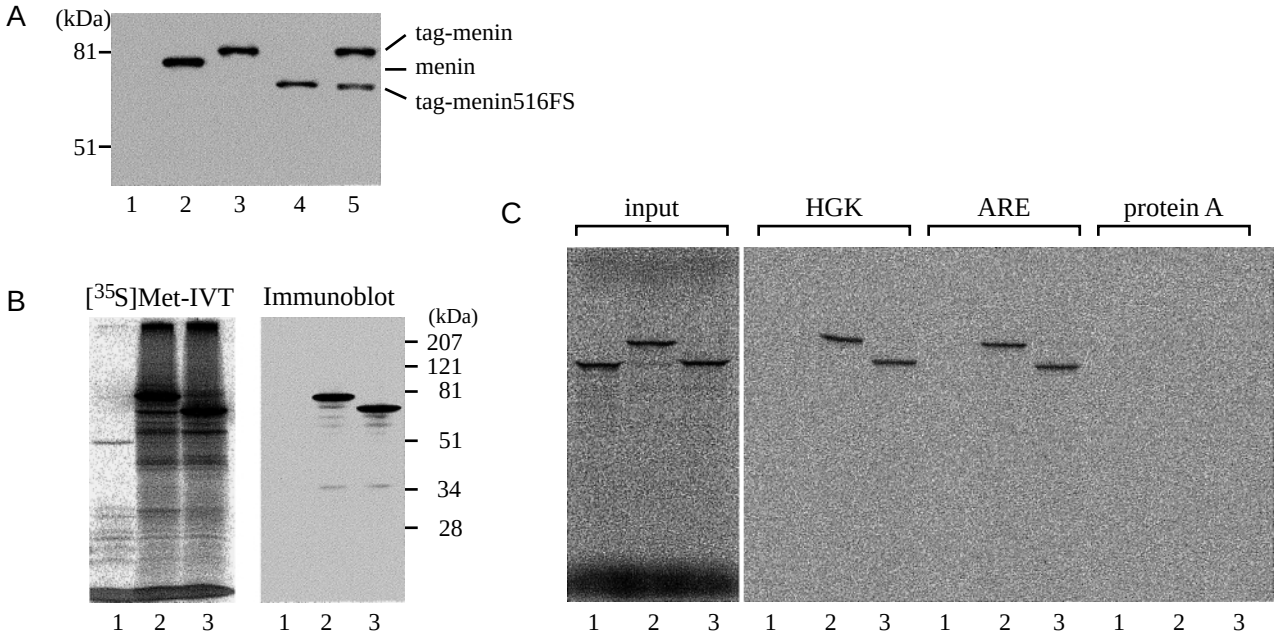

Fig. 2. A. Immunoblotting of whole cell extracts of COS cells transfected with pcDNA3.1/HisC (lane 1), pCMVsportMenin (lane 2), pcDNA/HisMenin (lane 3), pcDNA/HisMenin516FS (lane 4) and pcDNA/HisMenin plus pcDNA/HisMenin516FS (lane 5). Primary antibody, HGK; exposure, 15 s. B. Immunoblotting of *in vitro* translated menin. Five microliters of ^{35}S-labeled, programmed TNT lysate was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto Hybond-C extra for immunoblotting. Plasmids used for *in vitro* translation are, lanes 1, pcDNA3.1/HisC; lanes 2, pcDNA/HisMenin; lanes 3, pcDNA/HisMenin516FS. Primary antibody, HGK; exposure, 15 s. Similar results were obtained using ARE. C. Immunoprecipitation of *in vitro*-translated menin. ^{35}S-labeled, programmed TNT lysate was incubated with HGK, ARE or without antibody. Proteins bound to Protein A Sepharose were separated on 10% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film for autoradiography. Plasmids used for *in vitro* translation are, lanes 1, T7 luciferase DNA (Promega); lanes 2, pcDNA/HisMenin; lanes 3, pcDNA/HisMenin516FS.

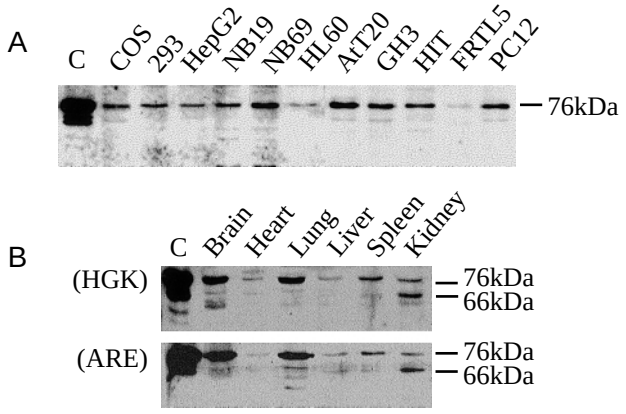

Fig. 3. A. Expression of menin in various cell lines. Nuclear extract was prepared from cultured cells and used for immunoblotting. The blot with HGK as a primary antibody is shown. Exposure, 2 min. Similar results were obtained with ARE. Lane C, nuclear extract of COS cells transfected with pCMVsportMenin. B. Expression of menin in mouse tissues. Nuclear extracts were prepared from adult mouse tissues and used for immunoblotting. Exposure, 10 min. Lane C, nuclear extract of COS cells transfected with pCMVsportMenin.

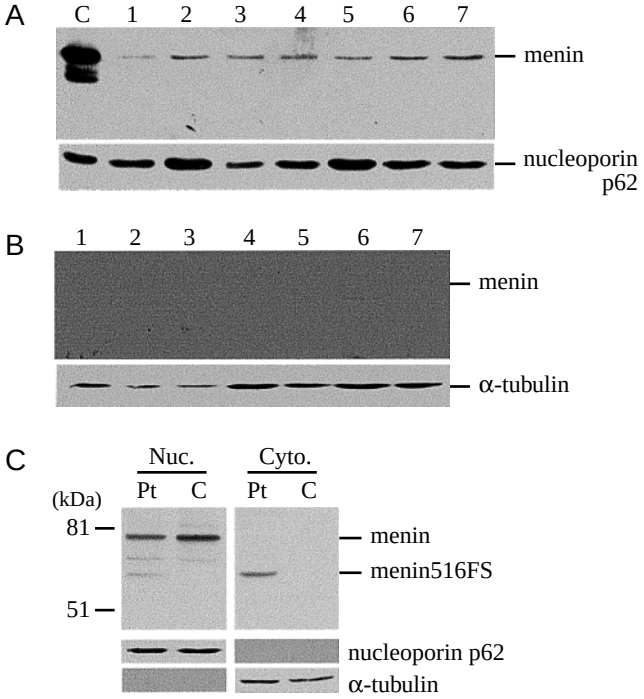

Fig. 4. A. Expression of menin in nuclear fraction of lymphocytes from patients with MEN 1. Lane C, COS transfected with pCMVsportMenin; lane 1, normal control; lane 2, MEN 1 with heterozygous *MEN1* gene mutation 359del4[7]; lane 3, MEN 1 with heterozygous *MEN1* gene mutation 621del9[7]; lanes 4–7, MEN 1 with no detectable *MEN1* gene mutations. Blots with HGK as a primary antibody are shown. Exposure, 5 min. After exposure, bound antibodies were stripped and the membrane was re-blotted with anti-nucleoporin p62 antibody to confirm proper sample preparation. Similar results were obtained using ARE. B. Expression of menin in the cytosolic fraction of lymphocytes from patients with MEN 1. Lane numbers correspond to those in A. Exposure, 15 min. After exposure, the membrane was re-blotted with anti-α-tubulin antibody. Similar results were obtained using ARE. C. Impaired nuclear localization of menin516FS. Nuclear (Nuc.) and cytosolic (Cyto.) fractions of lymphocytes from a MEN 1 patient with heterozygous *MEN1* gene mutation 516FS (1657insC) (Pt) and a normal control (C) were blotted. Note menin516FS in the cytosolic fraction. Primary antibody, ARE; exposure, 3 min. Similar results were obtained using HGK.

nize menin. A mutant menin, menin516FS, was recognized by both antibodies as efficiently as was the wild-type menin.

Expression of menin in various cell lines and mouse tissues Using these antibodies, we examined the expression of menin in various cultured cells. As shown in Fig. 3A, menin was detected in all cells examined as a single protein of 76 kDa. The expression was high in cells derived from neuroblastoma (NB19, NB69), anterior pituitary (AtT20, GH3), insulinoma (HIT) and pheochromocytoma (PC12), while its expression was low in cells derived from kidney (COS, 293), hepatoma (HepG2), leukemia (HL60) and thyroid (FRTL5). Expression of menin was also examined in mouse tissues. Both HGK and ARE detected high level expression of menin in brain (Fig. 3B). Menin was also abundantly expressed in lung and spleen, while its expression was low in heart and liver. In kidney, a more rapidly migrating band with the size of 66 kDa was detected by both antibodies. It is not known if this protein is the proteolytic product of menin or some other related protein. These results confirmed that menin is widely expressed in many cells and tissues.

Subcellular localization of mutant menin in lymphocytes from subjects with MEN 1 We next attempted to detect and localize mutant menin in lymphocytes from patients with MEN 1. Lymphocytes were isolated from peripheral blood and stimulated with phytohemagglutinin for 3 days in RPMI 1640 medium. Cells were collected and fractionated to obtain nuclear and cytosolic fractions. The results of immunoblotting using these fractions are shown in Fig. 4, A and B. In the normal control (lane 1), menin was detected only in the nuclear fraction as a 76 kDa single protein. One patient (lane 2) is affected with MEN 1 and carries heterozygous *MEN1* gene mutation 359del4.[7] This mutation causes a frame shift at amino acid 83, and thus neither HGK nor ARE recognizes the mutant protein. Although this patient expressed wild-type menin from only one allele, no quantitative reduction of menin protein was apparent. Another patient (lane 3) car-

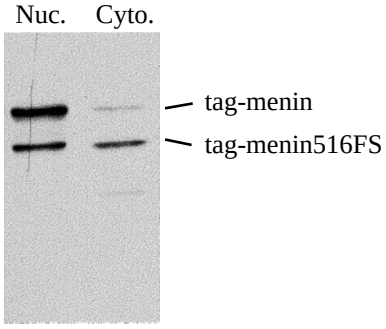

Fig. 5. Subcellular localization of wild-type and mutant tag-menin proteins. COS cells were transfected simultaneously with pcDNA/HisMenin and pcDNA/HisMenin516FS, and nuclear (Nuc.) and cytosolic (Cyto.) fractions were blotted. Primary antibody, HGK; exposure, 3 min.

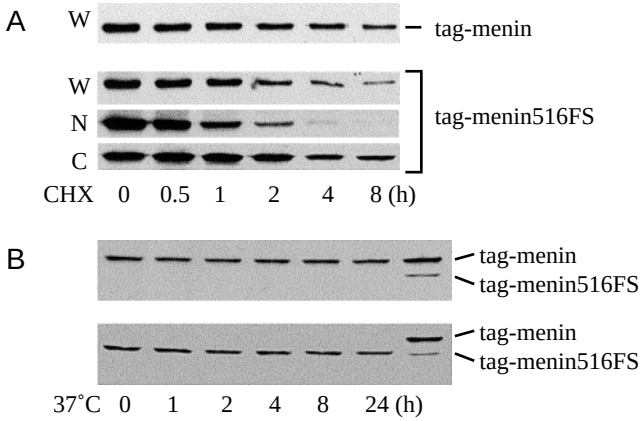

Fig. 6. A. Degradation of menin in the cell. COS cells were transfected with either pcDNA/HisMenin or pcDNA/HisMenin516FS. Forty-eight hours after transfection, 20 μg/ml cycloheximide (CHX) was added to the culture medium to halt protein synthesis in the cell. Cell extracts were prepared after the incubation of cells for an appropriate time. Primary antibody, HGK; exposure, 2–5 min. Essentially the same results were obtained in repeated experiments. W, whole cell extract; N, nuclear fraction; C, cytosolic fraction. B. Degradation of menin in cell extract. Whole cell extract was prepared from COS cells transfected with either pcDNA/HisMenin or pcDNA/HisMenin516FS, and incubated at 37°C for an appropriate time before gel electrophoresis and immunoblotting. No proteinase inhibitors were added during incubation. In the right-most lane, whole cell extract of COS cells transfected with both plasmids was loaded as a reference. Primary antibody, HGK; exposure, 2 min. Essentially the same results were obtained in repeated experiments.

ries a heterozygous *MEN1* gene mutation, 621del9.[7] This mutation causes deletion of three amino acids, 171 to 173, but the epitopes recognized by HGK and ARE are preserved in the mutant protein. Mutant menin in this patient would not be distinguished from wild-type menin in terms of its molecular size. In this patient, menin was observed only in the nuclear fraction, indicating that the mutant menin is also localized in the nucleus. This is in agreement with the observation by Guru *et al.*, that the two nuclear localization signals of menin are located in the carboxyl-terminal fourth of the molecule.[16] Subjects in lanes 4 to 7 were clinically diagnosed as MEN 1 but no mutations were identified in the coding region of the *MEN1* gene. Patients in lanes 4 and 6 are familial cases while patients in lanes 5 and 7 are sporadic. Because mutation search by PCR followed by sequencing is unable to detect the deletion of the entire exon in an affected allele, we speculated that these patients may have a large deletion of the *MEN1* gene, as has previously been reported,[19] and if the deletion involves only exons encoding the carboxyl terminus of the menin, a truncated menin could be detected by immunoblotting. In these subjects, however, menin was only observed in nuclear fraction and proteins with altered molecular size were not seen either in nuclear or cytosolic fractions.

The 1657insC (or 1650insC) is a relatively frequent mutation in patients with MEN 1, regardless of ethnic origin.[4, 7, 8] This mutation causes a frame shift at amino acid 516 and the mutant menin terminates at amino acid 530 (Fig. 1, see pcDNA/HisMenin516FS). When we examined the localization of this mutant protein using lymphocytes from the patient with this mutation, the rapidly migrating protein corresponding to menin516FS was observed both in the nuclear and cytosolic fractions (Fig. 4C). The signal of menin516 in the nucleus was consistently weaker than that of wild-type menin. This result suggests that nuclear localization of the menin516FS is partly impaired, even though it retains one of the two nuclear localization signals, NLS-1 (Fig. 1).[16] To confirm this, we transfected COS cells with pcDNA/HisMenin and pcDNA/HisMenin516FS simultaneously, and cell fractions were subjected to immunoblotting. As shown in Fig. 5, wild-type menin (tag-menin) was predominantly localized in the nucleus while a significant amount of tag-menin516FS remained in the cytoplasm. This result implies that both NLS-1 and NLS-2 may be necessary for efficient nuclear localization of menin.

Stability of wild-type and mutant menin As described above, we recognized that signals of menin516FS were always weaker than those of wild-type menin (See Fig. 2A, lane 5 and Fig. 4C). Since HGK and ARE recognize both wild-type and mutant menins (Fig. 2), we reasoned that menin516FS may be unstable compared to the wild-type. We therefore examined the stability of wild-type and mutant menin. After transfection of cells with either pcDNA/HisMenin or pcDNA/HisMenin516FS, protein

synthesis was inhibited by 20 μg/ml cycloheximide (CHX) and remaining immunoreactivity to HGK was chased. The results are shown in Fig. 6A. Under this condition, tag-menin gradually disappeared and its half-life was approximately 10 h. On the other hand, menin516FS disappeared significantly faster than the wild-type menin. Since tag-menin516FS is expressed in both nucleus and cytoplasm (Fig. 5), we reasoned that rapid degradation of the menin516FS may occur in the cytoplasm. However, immunoblotting using cell fraction revealed that is not the case. In the nucleus, tag-menin516FS rapidly disappeared with an estimated half-life of 1.2 h, while in the cytoplasm, tag-menin516FS was apparently stable. Its half-life in the cytoplasm was approximately 6 h. We wished to know if this rapid disappearance of menin516FS in the nucleus is due to active degradation or merely reflects the instability of the aberrant protein. To examine this, the stability of the wild-type and mutant menin was examined *in vitro* using cell extracts. Whole cell extract of COS cells transfected with either pcDNA/HisMenin or pcDNA/HisMenin516FS was subjected to immunoblotting after incubation at 37°C for a suitable time. No proteinase inhibitors were added during incubation. As shown in Fig. 6B, both tag-menin and tag-menin516FS were stable under this condition; more than 70% of immunoreactivity of these proteins remained even after 24 h. These results imply that the short half-life of 516FS is due to degradation in the cell rather than to fragility of the protein.

DISCUSSION

Here we report the development of two polyclonal antibodies against menin. These antibodies specifically recognized menin expressed in cells or translated *in vitro*. Development of antibodies against menin has been reported by others, but the expression of menin has only been demonstrated in a few tissues.[15, 16, 18] Huang *et al.* raised an antibody against the same epitope as that of HGK (#622),[18] but it did not recognize *in vitro*-translated menin. ARE probably recognizes the same epitope as the AEA reported by Guru *et al.*,[16] M1C2 by Stewart *et al.*,[15] and #625 by Huang *et al*.[18]

We have observed wide-spread expression of menin in cultured cell lines and mouse tissues by western blotting. These results are in accordance with the report by Stewart *et al.* on *Men1* mRNA.[15] They showed, by northern blotting, high-level expression of mouse *Men1* mRNA in brain, liver and testis. In our present study, abundant expression of menin in brain was also observed. On the other hand, expression of menin in the liver was very low (Fig. 3B). The reason for this discrepancy between mRNA and protein levels is not clear, but *in situ* hybridization by Stewart *et al.* suggested that *Men1* mRNA is less abundant in liver.[15]

Wide-spread expression of menin raises the question of how mutation of the *MEN1* gene causes tissue-specific lesions. Agarwal *et al.* reported that menin directly interacts with the AP1 transcription factor JunD and represses its function.[17] Since JunD functions as a negative regulator of cell growth and antagonizes *ras*-induced transactivation,[20–22] the relationship between impaired repression of JunD function by mutation of the *MEN1* gene and tumorigenesis in endocrine organs remains unclear. Furthermore, despite wide-spread expression of menin, its involvement in non-endocrine tumors is not known. There might be functional interactions between menin and nuclear protein(s) other than JunD which are expressed in target organs of MEN 1. Alternatively, menin-associated protein(s) may be expressed widely in most organs, but not in endocrine organs. Our antibodies should be useful to search for such proteins.

Guru *et al.* identified two nuclear localization signals in menin (Fig. 1)[16] and indicated that one of these signals should be sufficient to translocate the protein to the nucleus. Our results show that the nuclear localization of menin516FS, which lacks NLS-2 due to frame shift mutation, is partly impaired. The presence of both NLS-1 and NLS-2 may be necessary for the efficient nuclear translocation of menin. Alternatively, aberrant amino acid residues 517–530 (PEASWDCRWHSPRP) of menin516FS may somehow inhibit the nuclear localization of the protein. A further analysis will be necessary to resolve this issue. The significance of cytoplasmic retention of the mutant menin for cell physiology is uncertain. Many frame-shift or nonsense mutations identified in patients with MEN 1 will produce truncated proteins with impaired nuclear localization.[2–9] However, no apparent genotype-phenotype correlation has been observed in MEN 1 patients.[23]

We have demonstrated that the wild-type menin is stable, while the frame-shift mutant menin516FS is relatively unstable in the nucleus. This is probably due to active degradation of the mutant menin rather than its instability *per se*, because menin516FS was as stable as the wild-type *in vitro* (Fig. 6B). An important domain that stabilizes the protein in the nucleus may exist in the carboxyl terminus of menin, and mutant menin may be digested by active proteolysis in the nucleus. It is also possible that the mutant is transported intact into the cytoplasm and degraded there. However, we could not detect any proteolytic lower-molecular-weight fragments by immunoblotting. It is unclear how the mutant protein disappears from the cell.

MEN1 is a tumor-suppressor gene and Agarwal *et al.* recently showed that menin plays a role in transcriptional regulation.[17] Many tumor suppressor gene products located in the nucleus are involved in cell cycle control, transcriptional regulation and DNA replication and

repair.[24–30] Cellular stresses such as DNA damage dramatically change the expression of those proteins by modulating mRNA expression or by affecting the stability of the protein.[31–33] The effect of DNA damage by irradiation, ultraviolet and chemicals on the expression and posttranslational modification of menin is one of our current interests.[34, 35] Our antibodies should be useful for such studies.

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