Menin, the Multiple Endocrine Neoplasia Type 1 Gene Product, Exhibits GTP-hydrolyzing Activity in the Presence of the Tumor Metastasis Suppressor nm23*

Hiroko Yaguchi‡, Naganari Ohkura‡, Toshihiko Tsukada, and Ken Yamaguchi

From the Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

*MEN1, the gene responsible for multiple endocrine neoplasia type 1, is a tumor suppressor gene that encodes a protein called menin, of unknown function with no homology to any known protein. Here we demonstrate that menin interacts with a putative tumor metastasis suppressor nm23H1/nucleoside diphosphate (NDP) kinase A in mammalian cells. Given the roles of nm23 as a multi-functional protein, we searched for the possible function of menin. Menin has no effect on the known activities of nm23; that is, nucleoside diphosphate kinase, protein kinase, or GTPase-activating protein for Ras-related GTPase Rad. However, we found that menin hydrolyzes GTP to GDP efficiently in the presence of nm23, whereas nm23 or menin alone shows little or no detectable GTPase activity. Furthermore, menin contains sequence motifs similar to those found in all known GTPases or GTP-binding proteins and shows low affinity but specific binding to GTP/GDP. These results suggest that menin is an atypical GTPase stimulated by nm23.

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by the combined occurrence of tumors of the parathyroid, pancreas, and pituitary that represents one of the familial cancer syndromes (1, 2). The responsible gene, MEN1, was localized to chromosome 11q13 (3) and identified by positional cloning (4). More than 400 independent germ line or somatic mutations distributed over the entire MEN1 coding region have been identified (5, 6). The majority are nonsense or frameshifts predicting a truncated product, but missense mutations have also been identified. The nature of the mutations, which are consistent with a loss-of-function mechanism, and the observation that the loss of both MEN1 alleles leads to tumor development support the prediction that MEN1 is a tumor suppressor gene.

Menin, the protein encoded by the MEN1 gene, contains 610 amino acids (4) and is highly conserved among human, mouse (98%) (7), and rat (97%) (8) and more distantly among zebrafish (75%) (9) and Drosophila (47%) (10), but there is no known homolog in the budding yeast. Menin RNA and protein are widely expressed in most human tissues analyzed (11), leaving the tumor development in endocrine tissues unaccounted for. Data base analysis of the menin protein sequence does not reveal homology to any other known proteins or any apparent conserved motifs, providing no clues as to its function. However, menin is found predominantly in the nucleus (12) and binds to transcription factors (13), suggesting its role related to transcriptional regulation. In addition, the expression of menin increases as GH4 cells entered S phase (14), suggesting that menin expression may be cell cycle-regulated. Even so, little is known about the biological role of menin in tumorigenesis or normal cellular functions.

We have recently identified a putative tumor metastasis suppressor nm23H1 as a menin-interacting protein in a yeast two-hybrid screen (15). Nm23, a putative metastasis suppressor, was originally identified by subtraction cloning in murine melanomas of differing metastatic potential (16). Transfection of nm23 cDNA into some tumor cell lines is associated with reduced metastatic potential (17) and cell motility (18). It is also thought to be involved in development, cellular proliferation, and differentiation (19). Nm23 belongs to a family of structurally and functionally conserved proteins of nucleoside diphosphate (NDP)1 kinases that are major suppliers of nucleoside triphosphates (NTPs), where ATP is the phosphate donor (20). In human tissue the two major isoforms are nm23H1 and nm23H2, which are identical to the NDP kinases A and B, respectively (16, 21). Until recently, NDP kinase activity was the only known function of nm23, but nm23 has been shown to be a multi-functional protein with other possible roles. It can also function as a protein kinase and undergo autophosphorylation on histidine and serine residues (20, 22, 23). Furthermore, nm23H1 has been shown to interact with Ras-related GTPase Rad and act as a GTPase-activating protein (GAP) for Rad, promoting the conversion of GTP associated with Rad to GDP (24).

Here, we show that menin interacts with nm23 in mammalian cells, and the menin-nm23 interaction allowed us to find an unexpected activity of menin. Menin binds GTP with a low affinity and hydrolyzes GTP efficiently in the presence of nm23. Our data indicate that menin is an atypical GTPase stimulated by nm23.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293 and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin. HEK293 and COS-7 cells were transiently transfected with a calcium phosphate transfection kit (Edge BioSystems) and with Polyfect transfection reagents (Qiagen),

1 The abbreviations used are: NDP, nucleoside diphosphate; GAP, GTPase-activating protein; DTT, dithiothreitol.
respectively. The cells were used in experiments 30 h after transfection.

Co-immunoprecipitation Assay—The FLAG-tagged expression vector pCMV-Tag2 or Myc-tagged expression vector pCMV-Tag3 (Stratagene) was used to express tagged proteins in the cultured mammalian cells. HEK293 cells in 100-mm dishes at 4 × 10^6 cell/dish were co-transfected with pCMV-Tag2-FLAG expression constructs and 50 μg of pCMV-Tag3-Myc expression construct. The cells were lysed with 1.2 ml of low-stringent lysis buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.1% Nonidet P40, and one Complete™ protease inhibitor mixture tablet (Roche Molecular Biochemicals/25 ml of buffer) and sonicated briefly. Cell lysates were centrifuged at 15,000 × g for 5 min, and the supernatant was incubated with anti-Myc-agarose affinity (Sigma) or anti-FLAG affinity gel (Sigma) or anti-Myc antibody (Roche Molecular Biochemicals) as described (25).

NDP Kinase Assay—For the preparation of glutathione S-transferase (GST) fusion proteins, pEX2-5X-1 vectors were used (Amerham Biosciences). The fusion proteins were affinity-purified from the soluble fraction of cell extract with glutathione-Sepharose beads (Amerham Biosciences) according to the manufacturer’s instructions. NDP kinase activity was measured with [γ-32P]ATP as a substrate, and dCDP as an acceptor nucleotide. GST-nm23 (5 ng) was incubated with 1 μCi of [γ-32P]GTP (10 μCi) in 20 μl of assay buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol (DTT)) at room temperature for 20 min in the presence of 20 ng of either GST-menin or GST. At each time point, 1-μl aliquots were removed and directly spotted on the thin-layer chromatography (TLC) plate to resolve radioactive ATP and dCTP in 0.85 M KH2PO4 (pH 3.4). The labeled nucleotides were visualized by autoradiography and quantified with a bio-imaging analyzer (LAS2200, Fuji film).

In Vivo and in Vitro Phosphorylation—In vivo phosphorylation of menin, COS-7 cells plated at 5 × 10^5 cells/60-mm dish were transfected with 2 μg of FLAG-tagged menin expression construct. Metabolic labeling was carried out in phosphate-free Dulbeco’s modified Eagle’s medium containing 5% fetal calf serum and 1 mCi/ml of [32P]orthophosphate for 3 h. Immunoprecipitation with anti-FLAG monoclonal antibody was performed as described above. Immunoprecipitated protein was resolved by SDS-PAGE followed by autoradiography. For in vitro kinase assay, ~0.1 μg of GST-menin and 0.1 μg of GST-nm23 were incubated in a total volume of 20 μl of kinase buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 3 mM MnCl2, and 1 μl DTT) containing 10 μCi of [γ-32P]ATP at room temperature for 5 min. The reaction was terminated by the addition of an equal volume of 2× SDS sample buffer, the samples were boiled, and the reaction products were resolved by SDS-PAGE followed by autoradiography.

GAP Assay—The Rad-GAP activity was assessed based on the method of Zhu et al. (24). Briefly, 5 μg of GST-Rad bound to glutathione-Sepharose beads was loaded with 1 μCi of [γ-32P]GTP (Amerham Biosciences) in assay buffer A consisting of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 1 mg/ml bovine serum albumin containing the indicated concentrations of MgCl2 at room temperature. At given time points, aliquots of 8 μl were spotted in duplicate on BA 85 nitrocellulose filters (Schleicher & Schuell), and then the filters were washed 3 times for 5 min with 10 ml of cold washing buffer (50 mM Tris-HCl, pH 7.5, and 1 mM DTT) containing 1 mCi of [32P]GTP in an exchange buffer containing 5 mM MgCl2, as described above in the absence of various nucleotides at various concentrations. Incubation was continued at room temperature for 60 min, after which 8-μl aliquots were spotted on filters, the filters were washed 3 times with cold washing buffer containing 20 mM MgCl2, and the radioactivity was determined as described above.

Expression of Menin Mutants—Menin mutant constructs P12L, H139D, and P230L were generated by a PCR-based technique and cloned into pCMV-Tag2 expression vectors. All the constructs were confirmed by DNA sequencing. To study the expression levels, HEK293 cells were co-transfected with expression vectors of one of the FLAG-tagged menin variants (15 μg) and FLAG-luciferase (15 μg) as a cotransfection marker to monitor transfection efficiency; the total amount of transfected plasmid DNA was equalized by adding empty vector. Cell lysates were centrifuged at 15,000 × g for 5 min, and 2 × SDS-PAGE sample buffer was added to the supernatant. The samples were heated at 100 °C for 5 min, analyzed by SDS-PAGE, and blotted with anti-FLAG antibody as described above.

RESULTS

Menin Interacts with nm23 in Mammalian Cells—We previously identified nm23 as a potential menin binding partner in a yeast two-hybrid screening system and confirmed this association in vitro by GST pull-down assays (15). To further confirm this association, we performed a co-immunoprecipitation analysis in mammalian cells. HEK293 cells were co-transfected with plasmids expressing FLAG-menin or FLAG-luciferase and Myc-nm23 or Myc-luciferase, lysed with low-stringent buffer, and immunoprecipitated with anti-Myc or anti-FLAG antibodies. An anti-Myc monoclonal antibody co-immunoprecipitated FLAG-menin along with Myc-luciferase and Myc-nm23 or Myc-luciferase, lysed with low-stringent buffer, and immunoprecipitated with anti-Myc or anti-FLAG antibodies. An anti-Myc monoclonal antibody co-immunoprecipitated FLAG-menin along with Myc-nm23 (Fig. 1, lane 9) but did not co-immunoprecipitate FLAG-menin along with Myc-luciferase or FLAG-luciferase along with Myc-nm23 (lanes 4 and 5). In the reciprocal immunoprecipitation, an anti-FLAG monoclonal antibody co-immunoprecipitated Myc-nm23 along with FLAG-menin (Fig. 1, lane 9) but did not co-immunoprecipitate Myc-luciferase along with FLAG-menin or Myc-nm23 along with FLAG-luciferase (lanes 7 and 8). These results indicate that menin associates with nm23 in mammalian cells.

Menin Does Not Affect the NDP Kinase, Protein Kinase, or Rad-GAP Activity of nm23—To assess the functional implications of the binding of menin to nm23, we studied the effects of menin on the activities of nm23. Nm23 has been shown to be a multi-functional protein possessing a variety of enzymatic activities including NDP kinase, protein kinase, and GAP activities. First, to determine whether menin alters the NDP kinase activity of nm23, both proteins were expressed in bacteria as

At each time point, the reaction was stopped by the addition of 20 μl of elution buffer and heating at 65 °C for 5 min. The labeled nucleotides were resolved by TLC and analyzed as described above. For GDP hydrolysis assay of menin mutants, since the expression level of the mutants was lower than that of wild-type menin, cells were transfected with 30 μg of mutant plasmids or a smaller amount of wild-type plasmid (5 μg). The immunoprecipitates, each containing ~0.2 μg of protein, were used. The positions of GTP, GDP, and GMP on the plates were visualized under UV light by using unlabeled standards.
GST fusion proteins, purified, and subjected to a NDP kinase assay. As shown in Fig. 2A, GST-nm23 was able to transfer γ-phosphate from [γ-32P]ATP to dCDP, forming [γ-32P]dCTP. GST-menin had little effect on the NDP kinase activity of nm23, although there was a slight decrease in the activity. GST alone as a negative control had no effect on NDP kinase activity.

Next, we examined the possibility that menin is phosphorylated by nm23. There are 28 putative phosphorylation sites in menin (5), but it has not yet been confirmed if menin is phosphorylated. To analyze the phosphorylation of menin, COS-7 cells transfected with plasmid expressing FLAG-tagged menin were metabolically labeled with [32P]orthophosphate and immunoprecipitated with anti-FLAG antibody. We found that FLAG-menin was phosphorylated (Fig. 2B, lane 2). Then, to determine whether nm23 can phosphorylate menin, GST-menin and GST-nm23 were co-incubated in the presence of [γ-32P]ATP for 5 min at room temperature. The phosphorylation of GST-menin was not detected on the addition of GST-nm23 (lane 3). In addition, autophosphorylation of nm23 (22) was not altered by the co-incubation with menin (lane 3).

Nm23H1 was also shown to act as a specific GAP of the Ras-related GTPase Rad (24). Therefore, we next examined whether menin affected the Rad-GAP activity of nm23. GST-Rad-Sepharose was loaded with [α-32P]GTP and incubated with or without GST-nm23 in the presence of GST-menin or GST. In the absence of nm23, we could not observe any detectable GTPase activity of Rad within 20 min (Fig. 2C). In contrast, as shown by Zhu et al. (24), in the presence of GST-nm23 there was a marked increase in the rate of GTP hydrolysis. However, the Rad-GAP activity of nm23 was not altered by the co-incubation with GST-menin.

Co-incubation of Menin with nm23 Efficiently Hydrolyzes GTP—As shown above, menin did not affect the Rad-GAP activity of nm23, which could promote the hydrolysis of GTP bound to Rad; however, menin-nm23 interaction might be involved in Rad-independent GTP hydrolysis. To test this hypothesis, GST-menin (0.5 μg) and GST-nm23 (0.5 ng) alone or together were incubated with [α-32P]GTP. GST-menin alone did not show GTP-hydrolyzing activity, and GST-nm23 hydrolyzed only a small amount of GTP to GDP (Fig. 3). Intriguingly, when both nm23 and menin were present, a much greater amount of GTP was converted to GDP, reaching 20% by 5 min and 45% by 20 min. This finding suggests one possibility in which menin may enhance the catalytic activity of nm23 as a NDP kinase that transfers the terminal phosphate from any NTP to any NDP. However, the observation in this assay that GST-Rad with undetectable intrinsic GTPase activity displayed high GTPase activity in the presence of Rad-GAP nm23 (Fig. 3, lower panel) suggests an alternative possibility that menin, like Rad, may be a GTPase stimulated by nm23.

Menin Contains Several Sequence Motifs Found in All GTPases—Based on the possibility that menin is a GTPase like Rad, we examined if menin has a structure similar to that of known GTPases or GTP-binding proteins including the Ras-
related small GTPases and the α subunits of heterotrimeric G proteins. Although data base searching has not shown any homology to previously known proteins including GTPases, we found that menin contains within its N-terminal region several sequence motifs similar to those found in all known GTPases (G1 to G4) (27). Fig. 4 shows the consensus sequences for these motifs, which are known to be involved in GTP binding and GTP hydrolysis, and the corresponding amino acids in menin. The predicted G1 region (consensus GXXXXGK(S/T)), the phosphate binding motif, begins at position 2 in the amino acid sequence of menin, but the highly conserved second glycine is replaced by glutamine (GLKAAQKT). The G2 region, a conserved threonine, which is usually conserved within each GTPase family but not between different subfamilies, cannot unambiguously be identified in the sequence of menin, but a candidate threonine is found at positions 56 and 62. The DXXG Mg2+ binding motif (G3) and the (N/T/K)XD guanine binding motif (G4) are found at positions 70–73 and 150–153, respectively. In addition, a motif similar to the sequence G5 (consensus E(A/C/S/T)SA(K/L)) conserved in the small GTPases (27), was also found in a non-canonical form (IASAK) at positions 306–310.

**Menin Binds GTP**—The presence of similar motifs to those found in all known GTPases suggests that menin could similarly exhibit GTP binding activity as well as GTP-hydrolyzing activity. To test this, we examined whether menin directly bound GTP by filter binding assay. Although GST alone did not bind [γ-32P]GTP, GST-menin bound GTP in a time-dependent manner, reaching a maximum by 20 min (Fig. 5A). However, the relative binding affinity of GST-menin was much lower than that of GST-Rad (Fig. 5, A and B).

We next examined whether the binding of menin to GTP is affected by the concentration of Mg2+, as observed with many GTPases (28, 29). As shown in Fig. 5C, in the absence of Mg2+, menin bound GTP only poorly. However, the binding activity was increased in the presence of Mg2+, and maximum activation was obtained at 10 mM Mg2+, indicating that, like many GTPases, menin requires Mg2+ for GTP binding. GST alone had no activity to bind the guanine nucleotide at any concentration of Mg2+ from 0–20 mM (data not shown).

To further characterize the binding of GTP, we carried out competition experiments with a number of nucleotides and determined whether menin is a specific guanine nucleotide-binding protein. Binding of [γ-32P]GTP was almost completely blocked by GTP and GDP at higher concentrations than 0.01 mM, whereas GMP did not compete (Fig. 5D). ATP, UTP, and CTP also competed to some extent at high concentrations, but weakly when compared with GTP and GDP.

**Menin Immunoprecipitated from HEK293 Cells Hydrolyzes GTP**—We found that menin expressed in bacteria as a GST fusion protein hydrolyzed GTP in the presence of nm23 and bound GTP. To further confirm this, we investigated whether menin expressed in mammalian cells exhibited GTP-hydrolyzing activity. We immunoprecipitated FLAG-tagged proteins from transiently transfected HEK293 cells lysed with a low-stringent buffer and then subjected the immunoprecipitates to a GTP hydrolysis assay. As shown in Fig. 6, A and B, left panels, immunoprecipitates from empty vector-transfected cells showed no activity, and immunoprecipitates from Rad-transfected cells hydrolyzed a small amount of GTP to GDP. However, immunoprecipitates from menin-transfected cells showed strong GTP-hydrolyzing activity and converted a large amount of GTP to GDP. Unexpectedly, menin also produced a small amount of GMP, whereas Rad or empty vector did not (Fig. 6, A, left panel, and C).

We found that menin immunoprecipitates hydrolyzed GTP to both GDP and GMP; however, we have not determined whether the detected activity is derived from menin itself or menin protein complexed with associated proteins. To examine whether menin itself can hydrolyze GTP, we further purified FLAG-menin by immunoprecipitation using a stringent lysis buffer with anionic detergent to free the menin from most, if not all, interacting proteins. Under the stringent conditions, no association between FLAG-menin and Myc-nm23 was detected (data not shown), whereas this association was confirmed under the low-stringent conditions (Fig. 1). Even when purified under stringent conditions, menin immunoprecipitates were able to convert GTP to GDP (Fig. 6, A and B, right panels), although the level of conversion was lower than that shown by menin purified under the low-stringent conditions (Fig. 6B), and GMP was not detected (Fig. 6A, right panel). There was no...
increased GTP hydrolysis by Rad immunoprecipitated under low-stringent conditions when compared with Rad immunoprecipitated under stringent conditions, probably due to any detectable association between FLAG-Rad and Myc-nm23 under low-stringent conditions in this assay (data not shown). In addition, as observed with GST-FLAG and Myc-nm23, the menin immunoprecipitates bound GTP with a 10-fold lower affinity than Rad immunoprecipitates, whereas empty vector did not (data not shown). From these results, we confirmed that menin expressed in mammalian cells bound and hydrolyzed GTP.

Reduced Expression Levels of Menin Mutants Identified in MEN1 Patients—To examine whether the GTP-hydrolyzing activity of menin is involved in the pathogenesis of MEN1 tumors, we constructed FLAG-tagged menin mutants with missense mutations identified in MEN1 patients, P12L, H139D, and P320L, and tried to perform a GTP hydrolysis assay using the immunoprecipitates of these mutants. Immunoprecipitates of these mutants still retained the ability to hydrolyze GTP (Fig. 7A), but we had difficulty in comparing the GTPase activity because the expression of these mutants was marked decreased compared with that of wild-type menin in transiently transfected cells. As shown in Fig. 7B, the immunoprecipitates of mutants P12L, H139D, and P320L each contained only a very small amount of protein. To confirm the decreased expression level of mutants, HEK293 cells were transfected with FLAG-tagged menin or mutants and FLAG-tagged luciferase as a co-transfection marker to monitor transfection efficiency, and cell lysates were blotted with anti-FLAG antibody. As shown in Fig. 7C, unlike wild-type menin, mutants P12L, H139D, and P320L were expressed at extremely low levels, although FLAG-luciferase was expressed well in all transfections.

DISCUSSION

GTP-hydrolyzing and Binding Activity of Menin—In the present study, we have shown that menin interacts with nm23 in mammalian cells. The binding to nm23 has allowed us to uncover the potential activity of menin. We have demonstrated by using GST fusion proteins that menin shows no detectable GTP-hydrolyzing activity, and nm23 hydrolyzed GTP only poorly but that co-incubation of menin with nm23 results in a much greater conversion of GTP to GDP. It may be possible that the observed GTP hydrolysis is attributable to the enhancement by menin of the NDP kinase activity of nm23, which transfers the terminal phosphate from any NTP to any NDP. However, the observation that menin has little effect on the NDP kinase activity of nm23 and the fact that Ras-related GTPase Rad exhibits only a low intrinsic GTPase activity but can be greatly stimulated by nm23 as a Rad-specific GAP (24) gives rise to an alternative hypothesis, that menin, like Rad, may be a GTPase stimulated by nm23. This hypothesis is consistent with the observation that menin protein immunoprecipitated under stringent conditions is able to hydrolyze GTP to on the filters was quantified with a bio-imaging analyzer. The lower panel shows an autoradiogram of the radioactivity remaining on the filters. Results shown in B and D are means from two separate experiments.
GDP, probably because of the intrinsic GTPase activity of menin, which is detectable in protein expressed in more native forms in mammalian cells, unlike bacterially expressed GST-menin. However, we have not ruled out the possibility that unidentified menin-associated proteins still bind menin even under stringent conditions and promote the GTP-hydrolyzing activity of menin. The hypothesis that menin is a GTPase is also consistent with the GTP binding of menin. Competition experiments with a number of nucleotides demonstrating that GTP and GDP are the strongest competitors with \( \alpha^32P \text{GTP} \) to bind menin indicates that menin specifically binds GDP as well as GTP. Moreover, the observed Mg\(^{2+}\)-dependent binding of GTP suggests that menin binds GTP as a complex with Mg\(^{2+}\), like many GTPases (28). These binding properties are shared by most GTPases. Considering that menin binds GTP with a much lower affinity than Rad GTPase, the lack of an effect of menin on the Rad-GAP activity of nm23 may be explained by the notion that Rad as competitor completely blocked the binding of menin to GTP. An alternative possible explanation is that menin and Rad share the same binding site on nm23, and therefore, menin and Rad are mutually exclusive.

**FIG. 6.** Menin immunoprecipitates exhibit GTP-hydrolyzing activity. HEK293 cells transfected with FLAG-menin expression vector were lysed with low-stringent or stringent buffer and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were then incubated with [\( \alpha^32P \text{GTP} \). The reaction was stopped by the addition of elution buffer and boiling for 5 min. The labeled nucleotides were resolved by TLC and quantified with a bio-imaging analyzer. Empty vector pCMV-tag2 and FLAG-Rad expression vector were used as negative and positive controls, respectively. A, a representative TLC profile at 20 min of incubation. B, percent conversion of GTP to GDP quantified with a bio-imaging analyzer. C, percent GDP hydrolyzed per total guanine nucleotide (GMP + GDP + GTP). Low stringent and stringent indicate lysis conditions used in immunoprecipitation. Data shown are representative of three experiments.

**FIG. 7.** Decreased expression levels of menin missense mutants. A, HEK293 cells transfected with 5 \( \mu \)g of FLAG-tagged menin or 30 \( \mu \)g of mutant expression vector were lysed with low-stringent buffer, and the immunoprecipitates were subjected to a GTP hydrolysis assay. For preparation of equal amounts of protein, a smaller amount of wild-type plasmid was transfected. The total amount of transfected plasmid DNA was equalized by adding empty vector. A representative TLC profile at 20 min of incubation is shown. B, HEK293 cells were transfected with 30 \( \mu \)g of FLAG-tagged menin or mutant expression vector. Cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were resolved by SDS-PAGE. The proteins were visualized on the gel by GelCode Blue Stain reagent (Pierce) and blotted with anti-FLAG antibody. C, HEK293 cells were co-transfected with expression vectors of one of the FLAG-tagged menin variants (15 \( \mu \)g) and FLAG-luciferase (Luc, 15 \( \mu \)g) as a co-transfection marker to monitor transfection efficiency. The total amount of transfected plasmid DNA was equalized by adding empty vector pCMV-tag2. Cell lysates were blotted with anti-FLAG antibody.
on binding to nm23. Moreover, we find it interesting that menin contains several sequence motifs similar to those found in all GTPases (27), which are known to be involved in GTP binding and GTP hydrolysis.

The GTPase superfamily comprises at least three subfamilies, that of the small GTPases (e.g. Ras, Rad), that of the heterotrimeric G-proteins (e.g. transducin), and that of the GTPases involved in protein synthesis (e.g. elongation factor Tu), which are involved in an array of cellular functions including cell growth, differentiation, membrane trafficking, and nuclear transport (27, 30, 31). The ability to hydrolyze and bind GTP and the presence of similar sequence motifs to those commonly found in GTPases suggest that the functions of menin partially overlap with those of members of the GTPase superfamily.

However, menin is distinct from most members of the GTPase superfamily in that it is relatively large (68 kDa) and lacks a canonical form of the G1 motif or any space before the motif. Moreover, outside the individual predicted motifs there is no significant homology to any known GTPase. These features indicate that menin is not a typical GTPase. Rather, its relatively large protein size and the N-terminal placement of its GTPase motifs are reminiscent of some atypical GTPases with multidomains. For example, dynamin GTPase (100 kDa), which mediates vesicle trafficking, is a multidomain protein whose N-terminal third contains the GTPase domain (32, 33). Dynamin has been referred to as a “non-classical” GTPase, to distinguish it from the classical GTPases that act as regulatory switches, because dynamin uses its GTPase activity to drive vesiculation (32–34). Interestingly, the Drosophila homologue of nm23 (Awd) has been shown to be required for dynamin-dependent synaptic vesicle recycling (35). Another example is p190 Rho-GAP, which is a multidomain protein that also contains GTPase motifs at its N terminus and binds GTP but lacks intrinsic GTPase activity (36, 37). We note that menin contains a threonine residue instead of asparagine in the conserved (N/T)KXD GTP binding motif, which is shared by only a small number of GTPases including dynamin and p190 (27, 33, 36).

If menin functions as a classical GTPase, one might suggest for example, like Ras, menin serves to regulate cell growth or differentiation by acting as a molecular switch between active GTP-bound and inactive GDP-bound states, where nm23 acts as a GAP for Rad but not for other Ras-related GTPases such as Ras, Rho, Ran, and Rac (24). This specificity is consistent with the lack of any detectable sequence homology of nm23 with other GTPases. In addition, nm23H1 consisting of a four-stranded antiparallel β-sheet and surrounding α-helices (38) shows no tertiary structural similarity to those of GTPases for Ras, Rho, or Ran subfamilies, which are mostly helical (39). Nor does it contain any arginine residues that are good candidates for arginine finger, which is often critical for the Ras-GAP and Rho-GAP activities (39, 40). These observations suggest that nm23 can act on menin or Rad by a distinct catalytic mechanism of GAP action.

Potential Role of Menin in Tumorigenesis—In recent years, several proteins have been shown to interact with menin, providing clues to the potential functions of menin (13, 41–43). It has been shown that interaction with JunD induces a repression of JunD-mediated transactivation on an AP-1 binding site (13) and that menin is involved in the TGF-β-signaling pathway through its interaction with Smad3 (41), which interacts with JunD. Recently, menin has also been shown to interact with NF-κB and repress NF-κB-mediated transactivation (42). These findings provide some evidence that menin may participate in transcriptional regulation through its association with transcription factors; however, the molecular mechanism for tumor suppression by menin remains elusive. We do not know whether the GTP-hydrolyzing activity of menin plays a key role in transcriptional regulation, but it is an interesting possibility. Menin has been said to be a nuclear protein, but it is also found in cytosolic and membrane fractions (12, 14, 44). Given the facts that most Ras-related proteins are membrane-associated and specific membrane localization is both essential and central to their biological activity and that nm23 proteins are also found in cytosol or membrane fractions (45), it is feasible that menin localized to the membrane or cytoplasm would have distinct roles from that as a transcriptional regulator in the nucleus.

We find it interesting that GMP is also produced by menin immunoprecipitates from mammalian cells. The observation that the complex of GST-menin and GST-nm23 hydrolyzed GTP to GDP but not to GMP suggests that, among the menin immunoprecipitates, there would be unidentified interacting proteins other than nm23, with unique enzymatic activity to convert GTP to GMP, such as hGBP1 (human interferon-induced guanylate-binding protein) (46), or with GDPase activity such as the PCHP oncogene product (47). It would be interesting to investigate the possible interactions of menin and nm23 with the proteins with such activities.

Loss or mutations of both MEN1 alleles are considered to be an important step in tumor formation. More than 70% of the germ line or somatic MEN1 mutations identified in patients with MEN1 are nonsense and frameshifts, predicting a truncation or absence of menin, but missense mutations have also been identified (5, 6). If menin activity to hydrolyze GTP is involved in tumorigenesis, menin missense mutations identified in patients with MEN1 may lead to decreased GTP-hydrolyzing activity. We have so far found no experimental confirmation of this prediction. Rather, we observed the decreased expression levels of menin mutants as commented previously (13), which leads us to surmise that at least some identified missense mutations may decrease the half-life of the affected menin protein, thereby resulting in a reduced steady-state level and, consequently, loss of function. Consistent with this hypothesis is the fact that identified mutations are scattered throughout the MEN1-coding region with no apparent mutational hot spots (5). Among these mutations, there may also be missense mutations affecting functionally critical amino acid residues like those involved in the catalytic mechanism, but such mutations are considered to be rare in most genetic diseases (48). Furthermore, the instability of missense or short in-frame deletion mutants has been shown in some genetic diseases, and these mutations are often situated in regions of the proteins that are not part of the active site or of interaction areas or binding sites (48, 49). Therefore, we can postulate that loss of GTP hydrolysis and/or transcriptional regulation by menin resulting from the truncation or instability of MEN1 products could lead to MEN1 tumor formation, although there is no direct evidence to support this assumption. There are several members of the GTPase superfamily whose loss is implicated in the development of tumors; for example, NOEY2, a Ras-related, imprinted tumor suppressor gene in ovarian and breast cancers, functions as a negative regulator of cell growth (50), and a recently identified Ras-related gene, RERG, is im-
plicated as a growth-inhibitory gene in breast cancer (51). The molecular mechanism for growth suppression by such GTPases may provide clues as to the role of menin in tumorigenesis.

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