The multiple endocrine neoplasia type 1 gene product, menin, inhibits the human prolactin promoter activity

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Abstract

Menin is a protein encoded by the gene mutated in multiple endocrine neoplasia type 1 (MEN1) characterized by multiple endocrine tumors of the parathyroid glands, pancreatic islets and the anterior pituitary, especially prolactinoma. In this study, we examined the effects of menin on human prolactin (hPRL) expression. In rat pituitary GH3 cells stably expressing menin, both PRL gene expression/secretion and thymidine incorporation into DNA were inhibited as compared with mock-transfected cells. The transcriptional activity of PRL promoter in GH3 cells co-transfected with menin was significantly decreased. A deletion mutation (569 delC), which we identified in a Japanese MEN1 family, was introduced into menin. When GH3 cells were transfected with a mutant menin expression vector, inhibition of hPRL promoter activity was partially reversed. These observations suggest that menin inhibits hPRL promoter activity and cell proliferation, raising the possibility that menin might play an important role in the tumorigenesis of prolactinoma.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by multiple endocrine tumors of the parathyroid glands, pancreatic islets and anterior pituitary (Shephherd 1991, Trump et al. 1996). Other endocrine tumors such as adrenal tumors and carcinoids, and non-endocrine tumors such as lipoma are seen less frequently. Pituitary tumors occur in 30% of MEN1 patients, with prolactinomas being the most common type (Shephherd 1991, Trump et al. 1996). The MEN1 gene, which represents a putative tumor-suppressor gene, has been mapped to chromosome 11q13 and was recently identified by positional cloning (Chandrasekharappa et al. 1997). Over 200 independent germline and somatic mutations scattered throughout the protein-coding region have been identified (Agarwal et al. 1997, Heppner et al. 1997, Lemmens et al. 1997, Bassett et al. 1998, Sato et al. 1998). The human MEN1 gene encodes a 610 amino acid protein termed menin. This protein has no homology to any known human or mammalian protein (Chandrasekharappa et al. 1997). Although the function of menin is not known, two recent studies provide important insights into its function. Studies by Guru et al. (1998) found two novel nuclear localization signal (NLS) sequences located at the C-terminal portion of the menin protein. Agarwal et al. (1999) showed that menin interacted with the activator protein 1 (AP1) factor, Jun D, but repressed Jun D-activated transcription.

Human prolactin (hPRL) gene expression and secretion are regulated by various hormones and growth factors such as dopamine, thyrotropin-releasing hormone and epidermal growth factor.
(Berwaer et al. 1991, 1993). These factors modulate different signaling pathways (cAMP, Ca$^{2+}$, protein kinase C, mitogen-activated protein kinases) that regulate hPRL gene transcription. These second messengers regulate hPRL gene activity via the proximal promoter region (Berwaer et al. 1991, 1993). Recent studies showed that transcription factor AP1 is involved in basal and okadaic acid (OA)-stimulated activity of the hPRL promoter (Caccavelli et al. 1998). In the present study, we have examined whether wild-type menin regulates hPRL promoter activity and cell proliferation. Additionally, we have also examined the actions of a mutant menin, isolated from a Japanese family with MEN1, and detailed its activity on the hPRL promoter.

**Materials and Methods**

**Northern blot analysis**

A single-step acid guanidinium thiocyanate–phenol–chloroform extraction technique was used to isolate total RNA from menin-transfected GH3 cells. Separation of the RNA samples, transfer to membrane and hybridization with the menin and PRL probes were described previously (Tamaki et al. 1996). Total RNA (2 µg) was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Life Science Co., Petersburg, FL, USA) and random primer (Takara Shuzo Co., Osaka, Japan). The cDNA product was amplified by PCR as previously described (Sato et al. 1998). The primers used for RT-PCR were 5’-GAG CTG TCC CTC TAT CCT CG-3’ (sense) and 5’-TGA CCT CAG CTG TCT GCT CC-3’ (antisense) and were designed to amplify the sequence between exons 2 and 3 of the MEN1 gene (Chandrasekharappa et al. 1997). Probes used in the hybridization were labeled with digoxigenin (Dig) (Boehringer Mannheim, Indianapolis, IN, USA) involving a nick translation methodology and then purified as described previously (Fujita et al. 1994). Blots were also probed with human β-actin to assess equal loading of samples (Fujita et al. 1994). After post-hybridization washes, the membranes were incubated with anti-Dig antibody (Boehringer Mannheim) followed by treatment with 3-(2'-spirooadamantane)-4-methoxy-(3’-phosphoryloxy) phenyl-1, 2-dioxetane (AMPPD) and then exposed to X-ray film for 15–45 min.

**Plasmid constructs**

The protein-coding region (1833 bp) of the human menin cDNA (Chandrasekharappa et al. 1997) was amplified by PCR using primers that spanned the recognition sites of two restriction enzymes; NotI and BamHI (ATT AGG ATC CAT GGG GCT GAA GCC CGC CCA and ATG CGG CCG CTC AGA AGC CTT TGC GCT GCC respectively), as described previously (Namihira et al. 2000). After digestion with NotI and BamHI, the PCR products were purified and inserted into the plasmid vector, pcDNA3·1 (Invitrogen, Groningen, The Netherlands). The hPRL luciferase reporter gene (pHRL-LUC) was constructed using 940 bp of the hPRL promoter (−888/+52; +1 is defined as the start site of exon 1) (Truong et al. 1984) linked to the luciferase reporter gene (PGBV2; ToyoInk, Tokyo, Japan). The 940 bp fragment of the hPRL promoter was amplified by PCR using the primers, TAT GGT ACC GCT GGT GCG CAA GTG ACT GA and GAT CTC GAG CAA CGC AGT GAG TTG TCA CA. A point mutation (569 delC), identified in a Japanese MEN1 family (Namihira et al. 2000), was introduced into the menin sequence in the expression vector using site-directed mutagenesis (GeneEditor mutagenesis kit; Promega, Madison, WI, USA). All sequences of the synthetic mutant and PCR-amplified regions were confirmed by direct sequencing, as described previously (Yokote et al. 1998).

**Stable transfection and Western blotting analysis**

GH3 cells (Japan Health Sciences Foundation, Osaka, Japan) were cultured in Ham’s F10 medium (ICN Biomedicals, Inc., Aurora, OH, USA) supplemented with 15% fetal bovine serum and 2-5% horse serum. The menin expression vector (1·0 µg) was transfected into the cultured GH3 cells by the conventional cationic liposome transfection method (Lipofectamine; Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s instructions. The transfected cells were selected by addition of genetecine G418) (100 µg/ml) to the medium. Several clones were selected and checked for menin expression by Western blotting analysis. Nuclear extracts were prepared from the selected isolates for Western blot
analysis. Aliquots of 5 µg of each nuclear extract from menin- and mock-transfected cells were separated in a 10% SDS-PAGE. Mock-transfected cells were transfected with only empty vector (pcDNA3·1). Proteins were transferred onto PVDF membranes and the membranes were incubated with a 1:3000 dilution of anti-menin antiserum (Bhuiyan et al. 2000) followed by incubation in 1:3000 dilution of goat anti-guinea pig IgG coupled to horseradish peroxidase. Immunoreactive bands were visualized by the ECL system (Amersham, Tokyo, Japan) and exposed to Kodak XAR-5 film. The anti-menin antiserum was generated using a recombinant peptide (amino acid residues 443–535 of menin) and the homology of this peptide sequence is 87% between human and rat. This antibody recognized both human and rat menin. The preadsorption test of the antiserum showed complete disappearance of the 67 kDa band in Western blotting.

### PRL secretion assessed by reverse hemolytic plaque assay (RHPA) and proliferation assay

A clone of the stably transfected cells, showing the highest level of menin expression according to Western blot analysis, was subjected to the RHPA to assess PRL secretion, as described previously (Niimi et al. 1994). In brief, the cells (10⁶ cells/ml) were combined with protein-A-coupled erythrocytes and this mixture was infused into a poly-L-lysine-coated Cunningham slide chamber and incubated at 37 °C, in an atmosphere of 95% air/5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 1% non-essential amino acids for 24 h. Monkey anti-rat PRL serum (National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA (NIDDK)) diluted 1:150 was infused into the chambers and the mixture was incubated for 2 h. Plaque development was initiated by infusion of guinea pig complement and the reaction was terminated after 0-5 h by infusion of B-5 fixative. The cells were stained with 0-5% toluidine blue to facilitate observation of the hemolytic plaques. Two hundred cells/slide were counted and the percentage of plaque-forming PRL cells was calculated. Four different cell lines were examined in each group.

Proliferation assay was performed using a clone of stably transfected cells. The cells were exposed to 1 µCi/well [methyl-³H]thymidine (Moravek Biochemicals, Brea, CA, USA) for 6 h. The cells were harvested, and the radioactivity incorporated into DNA was quantified by a scintillation counter. The experiment comparing the proliferation rates of menin-overexpressing GH3 cells and mock cells was performed by seeding 2 × 10⁵/well in six-well plates containing regular medium with G418 on day 0. At 24-h intervals for a total of 5 days, the medium was aspirated, and the cells were rinsed twice with cold PBS and incubated in 1 ml 0-25% trypsin–1 mM EDTA for approximately 5 min. Cell suspensions were counted by hemocytometer. Each time point represents the number of cells ± S.E.M. of four different replicates. Each experiment was repeated a minimum of three times.

### Transient transfection and luciferase assay

Wild-type menin or mutant menin expression vector was co-transfected with phPRL-LUC into GH3 cells for luciferase reporter assay as described above. Aliquots of 1 µg phPRL-LUC and 0-1, 0-5 or 1-0 µg wild-type menin expression vector or 1 µg mutant menin expression plasmid were used in this experiment. The final DNA concentration was adjusted using empty vector DNA. The efficiency of transfection was monitored using co-transfection of β-galactosidase (β-Gal) expression vector (pTKβ; Clontech, Palo Alto, CA, USA). Cells were harvested 48 h after transfection. The luciferase activity was measured using a luciferase assay kit (ToyoInk). Preparation of the cell lysates and reporter assays was performed according to the manufacturer’s instructions. To control the variability of transfection, assay results were normalized by β-Gal expression in each sample.

### Statistical analysis

The data were analyzed by means of an unpaired Student’s t-test with one-way ANOVA. The data are given as means ± S.E.M. and significance was taken as P<0-05.

### Results

In MEN1 patients, prolactinoma is the most common type of pituitary tumor. To evaluate the
effect of menin on PRL expression, we created GH3 cells that overexpressed the protein menin. That these cells expressed the protein was demonstrated using Western blot analysis of extracts from cells stably transfected with the menin expression vector. Results (Fig. 1A) showed that use of an antibody directed against menin revealed a single band with an estimated molecular mass of 67 kDa. That expression was not limited to a single clone; we assessed several separate isolates and found that they also expressed menin. Whereas stably transfected cells expressed high levels of menin, the mock-transfected cells containing only pcDNA3.1 had very low levels of menin arising from endogenous expression (lane 1). The electrophoretic mobility of menin in mock- and stably transfected cells was the same (lane 2).

To assess expression of menin and the effect of this protein on PRL mRNA expression, Northern blot analysis was performed. Total RNA extracted from stably transfected cells that expressed menin was probed for its content of menin, PRL and β-actin mRNA. Results (Fig. 1B) revealed signals that matched the expected size of the sequences of interest in both mock- and stably transfected cells. Consistent with the Western blot studies above, the abundance of menin mRNA was higher in the stably transfected compared with that in control cells. In contrast, the exact opposite was observed for PRL mRNA, being lower in stably transfected compared with control cells. As expected, β-actin mRNA was the same in both cell types. This observation suggests that overexpression of menin decreases expression of the PRL gene.

Next we examined whether menin-induced down-regulation of PRL expression correlates with secretion of PRL from these cells. Therefore, we employed RHPA to determine the secretion of PRL from transfected cells. The percentage of plaque-forming PRL cells was one-third of that in control cells. This parameter is significantly ($P<0.01$) lower in the menin-transfected cells than in mock transfectants (Fig. 2).

Figure 1 (A) Western blotting analysis of menin in GH3 cells transfected with menin expression vector or mock vector. Lane 1, mock-transfected cells (pcDNA3.1); lane 2, menin-transfected cells. Proteins were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with specific antibodies (Bhuiyan et al. 2000). Western blot analysis revealed a specific band of menin protein of approximately 67 kDa, as indicated by the arrow. The clone showing highest level of menin expression (lane 2) was used for RHPA studies. Expression of the nuclear protein TFIID served as the internal control and was similar among all samples. The preadsorption test of the menin antiserum showed complete disappearance of the 67 kDa band. (B) Northern blot analysis of PRL mRNA expression. Total RNA was isolated from GH3 cells and Northern blot analysis was performed as described in the Materials and methods section. The RNA was probed for its content of menin, PRL and β-actin mRNA. Lane 1, menin-overexpressing GH3 cells; lane 2, mock-transfected GH3 cells. An identical experiment independently performed gave similar results.
Previous reports suggesting that menin affects cell proliferation raise the possibility that it may act as a tumor-suppressor gene. This idea prompted us to ask whether menin affects the proliferation of GH3 cells. To test this idea we measured the ability of menin-expressing cells to proliferate by assessing cell numbers on plastic dishes after 5 days of growth. In the same cells we also measured \[^{3}H\]thymidine uptake. Results (Fig. 3A) showed that the number of cells was significantly less (4.2 \times 10^4) in menin-expressing transfectants than in controls (7.6 \times 10^4). Not surprisingly (Fig. 3B) \[^{3}H\]thymidine uptake was significantly lower in menin-expressing cells compared with that of mock-transfected GH3 cells.

In addition to the anti-proliferative actions of menin, recent studies also suggest that it is a nuclear protein. The nuclear localization of menin points to the possibility that it may affect transcriptional activity of selected genes. To test this idea, we analyzed the effect of menin on PRL promoter activity. Therefore, GH3 cells were co-transfected with either a vector that overexpressed menin or an empty vector together with the reporter, phPRL-LUC. Luciferase activity was determined in the absence of any chemical or receptor-derived stimulation of the transfected cells. The luciferase activity was significantly inhibited by...
co-transfection with wild-type menin in a dose-dependent manner (Fig. 4). At maximal doses of menin expression vector of 1 µg, luciferase activity was noted at 37% of control. We have previously identified a point mutation (569 delC) in menin isolated from a Japanese patient with MEN1, who had a prolactinoma (Sato et al. 1998). This mutation caused truncation of menin (from amino acid 183 to 610). The ability of the mutant menin to inhibit PRL promoter activity was significantly (*P*<0·01) weaker than that of wild-type menin (Fig. 4).

**Discussion**

MEN1Burrin, a familial cancer syndrome with prolactinomas, carcinoid tumors of the lung and thymus, and hyperparathyroidism has been described in four large kindreds from the Burin peninsula of Newfoundland (Farid et al. 1980). A nonsense mutation in the MEN1 gene is believed to be responsible for this disease in the affected members of the MEN1Burrin families, thus providing convincing evidence of a common founder (Olufemi et al. 1998). Also, prolactinoma is the most common type of MEN1-related pituitary tumor, suggesting that the PRL gene may be a target for the actions of menin in the pituitary. This prior information points to the possibility that the PRL gene may be a target of menin activity. In addition, OA, a tumor-promoting agent that acts by inhibiting protein phosphatases, stimulates activity of the hPRL at the proximal promoter. Caccavelli et al. (1998) demonstrated that a member of the AP1 family, containing Jun D and c-fos, binds to the proximal element P1 within the hPRL promoter and AP1 is involved in both basal and OA-stimulated expression of the hPRL gene. Menin was recently identified as a partner that interacts with AP1 factor Jun D. This interaction represses Jun D-activated transcription (Agarwal et al. 1999). Based on the clinical and basic observations summarized here, we postulated that menin may affect activity of the hPRL promoter. Consistent with the hypothesis, the results of our studies show that menin inhibits hPRL promoter activity in rat pituitary GH3 cells.

Although our results showed that menin negatively regulated the hPRL promoter activity, the role of Jun D in this regulation still remains undefined because this is not a topic of the current study. Further support for the inhibitory effects of menin comes from results with the mutant menin derived from an MEN1 patient with a prolactinoma (Sato et al. 1998). Perhaps the defective function of the mutant menin in this patient underlies the increased expression of PRL. Consistent with this idea, the ability of mutant menin to attenuate hPRL promoter activity was less than that of wild-type menin. Despite the fact that truncated menin was only 183 amino acids in length, it still impaired hPRL promoter activity. These observations suggest that the N-terminal 183 residues retained, in part, the ability of wild-type menin to inhibit hPRL promoter activity despite the loss of the NLS by the mutant.

Previous studies have shown how menin mutations affect its cellular functions. Guru et al. (1998) reported that nuclear transport of menin is disturbed by some frame-shift and nonsense mutations due to the lack of NLS sequences in the C-terminal region. This information, added to the results of our studies, suggests that the blunted inhibitory activity of the mutant menin on the hPRL promoter may be attributed to disturbed nuclear transport of the mutant menin.

Although we demonstrated the inhibitory effects of menin on hPRL promoter activity, the role of
Menin inhibits PRL gene expression

H Namihira and others

Menin regulates the expression of PRL in vitro studies require further work to elucidate the role of menin in the tumorigenesis of prolactinoma in MEN1.

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