Menin-mediated Caspase 8 Expression in Suppressing Multiple Endocrine Neoplasia Type 1*

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Multiple endocrine neoplasia type 1 (MEN1), an inherited tumor syndrome linked to mutation of the MEN1 gene, which encodes a tumor suppressor, menin. We previously reported that menin up-regulates the caspase 8 expression and promotes TNF-α-induced apoptosis. However, it remains unclear how menin regulates caspase 8 expression and whether menin-mediated caspase 8 expression plays a role in repressing MEN1 development. Here we show that menin binds the 5′-untranslated region (5′-UTR) of the Caspase 8 locus in vitro and activates transcription of a reporter gene through the 5′-UTR. Menin directly binds the 5′-UTR in a sequence-independent manner in vivo. Moreover, Men1 ablation in cells reduces acetylation of histones H3 and H4 at the 5′-UTR of the caspase 8 locus bound by menin in vivo. Notably, the MEN1-derived menin point mutants lose their ability to bind the caspase 8 locus and fail to induce caspase 8 expression and TNF-α-mediated apoptosis. Consistent with these observations, the expression level of caspase 8 is markedly reduced in insulinomas from Men1+/− mice. Together, our results indicate that menin enhances the caspase 8 expression by binding the caspase 8 locus, and suggest that menin suppresses MEN1 tumorigenesis, at least in part, by up-regulating caspase 8 expression.

Multiple endocrine neoplasia type 1 (MEN1), an inherited tumor syndrome, is caused by mutation of the tumor suppressor gene, Men1 (1, 2), which encodes a protein of 610 amino acid residues, menin (3, 4). Due to a lack of conserved structural domains, the molecular basis for menin to act as a tumor suppressor is largely unknown. Mice with heterozygous Men1 inactivation develop a spectrum of endocrine tumors similar to that observed in patients with MEN1 syndrome (5–7). Homozygous Men1 disruption in mice leads to embryonic lethality on embryonic days 11.5–13.5 with a variety of developmental defects, including aberrant organogenesis of the multiple organs, such as the neural tube, the heart, and the liver (8). Men1+/− mice start to develop pancreatic insulinomas at approximately the sixth month after birth, and during the process, the remaining normal allele of Men1 is lost in the tumor, resulting in loss of heterozygosity of Men1 (5, 7).

Menin contains several nuclear localization signals in its C-terminal part (9, 10) and regulates expression of multiple genes, including Hoxc8, p27Kip1, p18Ink4c, telomerase, and IGFBP-2 (insulin-like growth factor-binding protein 2) (11–16). Menin interacts in vitro with multiple transcription factors, such as NFκB, Smad3, and JunD (17–19). It has been reported that menin physically binds to the loci of Hoxc8, p27Kip1, p18Ink4c, and telomerase (11, 12, 14, 15). Menin interacts with the histone methyltransferase complex containing mixed lineage leukemia (MLL) protein (11, 21) and promotes histone H3 lysine 4 (H3K4) trimethylation at the loci of genes, such as Hoxa9, p18Ink4c, and p27Kip1 (12, 15, 16, 22, 23). It has also been reported that menin, by interacting with histone deacetylases, suppresses the JunD-mediated transcription of a reporter gene, whereas tricostatin A, a histone deacetylase inhibitor, abrogates menin-mediated repression on gene transcription (24, 25). Thus, menin may regulate the gene expression by influencing the chromatin structure, including modifications of histones.

We previously demonstrated that caspase 8 expression was down-regulated in menin-null mouse embryonic fibroblasts (MEFs), whereas complementation of the MEFs with menin restored the high level of caspase 8 expression (26). Caspase 8 is a crucial component in the apoptosis pathway induced by death-related receptors (27, 28). Targeted caspase 8 disruption in mice leads to defects in apoptosis of lymphocytes as well as embryonic lethality (28). Because caspase 8 is reduced in several types of tumors (29–31), caspase 8 may play a role in suppressing tumorigenesis by potentiating death ligand-induced apoptosis. In agreement with this, caspase 8 expression is silenced due to DNA hypermethylation at the locus in neuroblastomas (29–31), leading to resistance of the tumor cells to death ligand-induced apoptosis (32–34).

However, it remains unclear how menin regulates caspase 8 expression and whether the menin-dependent caspase 8 expression is relevant to tumorigenesis in MEN1 syndrome. To address these questions, we have shown that menin specifically binds the 5′-UTR of the caspase 8 locus in vivo, and this menin-5′-UTR binding is correlated with an enhanced histone acetyl-
lation at the caspase 8 locus. The DNA fragment bound by menin in vivo also mediates menin-dependent transcriptional activation in vitro. Importantly, we have also shown that MEN1-derived menin point mutants not only lose their ability to bind the caspase 8 locus and induce caspase 8 expression but also fail to potentiate TNF-α-induced apoptosis. Moreover, caspase 8 expression is markedly decreased in islets or insulinomas in Men1+/− mice. These results suggest that menin suppresses MEN1 tumorigenesis, at least in part, through up-regulating caspase 8 expression.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—pMX-menin and pcDNA-menin were constructed by inserting PCR-amplified human menin cDNA (U93236) into the BamHI/NotI site of pMX-puro and pcDNA3 vectors, respectively, as previously described (13, 35). To generate L22R and A242V point mutations of menin, pMX-menin and pcDNA-menin were used as a template, and mutations were introduced to the template by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To express the GST-menin, the PCR-amplified cDNA was cloned into the BamHI/NotI site of pGEX-6p (GE Healthcare), and the protein was expressed in Escherichia coli strain BL21 (DE3) as a GST-tagged protein. pGEX-F1, -F2, and -F3 expressing the N-terminal part, the middle, and the C-terminal part of menin, respectively, were generated using pGEX-menin as a template and were expressed and purified as previously described (35). To construct pcas-Luc, genomic DNA covering −2935 to +623 of the caspase 8 locus was amplified from mouse genomic DNA and cloned into the KpnI and Smal site of the pGL3-basic vector (Promega, Madison, WI). Additionally, the mutant pcas-MLuc was constructed by cloning the PCR-amplified genomic DNA from −2935 to +40 of the caspase 8 locus, which lacked most of the 5′-UTR sequence, to the KpnI and Xhol site of the pGL3-basic vector.

**Cell Culture, Generation of Recombinant Retroviruses, and Complementation of Menin-null MEFs**—HEK 293 cells and E-NX cells, which were derived from 293 cells by transfecting the cells with individual cDNAs expressing retrovirus-packaging proteins (36) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and used for packaging recombinant retroviruses as previously described (37). MEF cell lines were generated from Men1ΔN3−8/− mice heterozygous for the Men1 locus (7, 37) and cultured in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), 1% minimal essential medium nonessential amino acid, and 1% L-glutamine. Menin-null MEFs complemented with menin or its mutants were established as previously described (26). Briefly, control retroviruses, menin, or its mutant-expressing retroviruses were used to infect Men1ΔN3−8/− MEFs. The resulting cells were selected with 2 µg/ml puromycin for 2 days.

**Transfection and Luciferase Assays**—E-NX cells were transfected using the calcium phosphate precipitation method. For the luciferase assay, 4 × 10⁵ cells were seeded per well in 6-well plates on day 0. On day 1, cells were transfected with reporter constructs, control pcDNA, pcDNA-menin, L22R, or A242V as indicated. pCMV-β-galactosidase expressing the LacZ gene (Clontech, Mountain View, CA) was cotransfected as an internal control to normalize the luciferase activity. After overnight incubation, cells were switched to a normal medium. On day 3, cells were harvested for luciferase (Promega, Madison, WI) and β-galactosidase assays (Clontech, Mountain View, CA), following the manufacturer’s instructions. Both luciferase and β-galactosidase activities were measured by a TR717 Microplate Luminometer (EG&G Berthold, Oak Ridge, TN).

**Real Time RT-PCR Quantification, Northern Blotting, and Gel Shift Assay**—Exponentially growing MEF cells were seeded at 2 × 10⁵ cells/100-mm dish. After a 2-day culture, total RNA was isolated with a Qiagen RNeasy® minikit. Real time RT-PCR was performed using the ABI Prism 7900HT real time PCR system, with 5 µg of total RNA as a template. Taqman probes for caspase 8, Hoxc8, and GAPDH were purchased from Applied Biosystems (Mm00822247_ml, Mm00439369_ml, and Mm99999915_gl, Foster City, CA). Northern Blotting for identifying menin-induced genes was performed as previously described (35, 37). Briefly, total RNA was isolated from exponentially growing MEFs using the cesium chloride ultracentrifuge method. RNA (20 µg) was separated on an agarose gel and transferred to Hybond N+ membrane. The membranes were further incubated with the labeled caspase 8 probe or the GAPDH probe, followed by autoradiography. Gel shift assays were carried out as previously described with a double-stranded 5′-UTR of the caspase 8 gene (+208 to +623) (35, 38). The probe was radiolabeled with [γ-32P]ATP and T4 polynucleotide kinase (38).

**Cyclic Amplification and Selection of Target (CASTing)**—To amplify the potential menin-binding DNA sequence using CASTing (39), a pool of 60-mer nucleotides with 20-nucleotide random sequences in the middle part were synthesized, converted to double-stranded DNA, and labeled with [γ-32P]ATP. The labeled probe was incubated with each of the following proteins: GST-menin fusion protein, GST-F1 (amino acids 1–218), F2 (amino acids 219–395), F3 (amino acids 396–610), and Smad3, as described for the gel shift assay. The reactions were separated on a 4% native polyacrylamide gel (35). The gel, after completion of electrophoresis, was stained with coomassie blue, followed by autoradiography. The subregion of the gel with the dsDNA probe bound by menin protein or Smad3, as assessed by comparing the Coomassie-stained protein bands on the gel and the corresponding autoradiography, was excised and used to extract DNA. Using primers corresponding to the 5′-end and 3′-end sequences of the dsDNA, the purified DNA was further PCR-amplified and utilized as the DNA probe for the next round of gel shift assays with either menin or Smad3, which specifically binds to DNA (40). After four rounds of amplification, PCR-amplified dsDNA was subjected to incubation with 500 ng of GST-menin, GST-F1, F2, F3, or Smad3 in the presence of 1 µg of poly(di-l-c). The reaction was further separated on 4% native polyacrylamide gel and followed by autoradiography.

**Western Blotting and Detection of Apoptotic Cells**—MEFs were seeded at 2 × 10⁵ cells/100-mm dish on day 0. On day 2, cells were harvested and processed for Western blotting as previously described (35). Briefly, on day 0, MEFs (2 × 10⁵ cells)
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were seeded in a 100-mm dish. After 2 days of culture, cells were harvested. The whole cell lysate (50 μg of protein) was separated on a SDS-polyacrylamide gel and transferred to Hybond

CCTGTGGCCGAGTAC-3. Primers for the island were 5'-CCTGTGGCCGAGTAC-3. PCR or Taqman PCR. Primers for the 5'-UTR of caspase 8 locus in vivo were 5'-CATCCCCAGACACAGCAGAATTA-3'.

For analyzing TNF-α-induced apoptosis, MEFs were seeded at a density of 5 x 10^6 cells/well in 6-well dishes. On day 2, cells were treated with or without TNF-α (30 ng/ml)/actinomycin D (20 ng/ml). After 24 h of treatment, cells were collected for propidium iodide staining. The stained cells were subjected to fluorescence-activating cell scanning analysis (LSR cytometer; BD Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay—MEFs were seeded at 10^6 cells/100-mm dish on day 0, harvested, and analyzed on day 1 with a ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer’s instructions. Briefly, chromatin DNA was cross-linked to proteins by formaldehyde and sheared by pulsed ultrasonication. Sheared DNA-protein complex was incubated with the antibody or control IgG overnight. Antibodies used for ChIP were anti-menin (BL342; Bethyl Laboratory), anti-acetylated histone H3 antibody, and anti-acetylated histone H4 (Upstate Biotechnology). Antibody-precipitated DNA-protein complex was reverse cross-linked, followed by phenol/chloroform extraction, and the precipitated DNA was used as template for conventional PCR or Taqman PCR. Primers for the 5'-UTR Taqman PCR were 5'-CCCCAGCTGGAGTTGTGAC-3' and 5'-CACCCGAGCTGGAGTTGTGAC-3'. The Taqman probe was 5'-TCTCCGCTGGAGTTGTGAC-3'. Primers for the island were 5'-TGACGAGTGGCTTCTAGGACA-3' and 5'-GCGAGTGGCTTCTAGGACAC-3', and the Taqman probe was 5'-CGGAGTGGCTTCTAGGACAC-3'.

FIGURE 1. Menin specifically binds to the caspase 8 locus in vivo. Menin-null or menin-expressing MEFs were seeded at 1 x 10^6 cells/100-mm dish on day 0. On day 1, cells were processed to cross-link the DNA-protein complexes, which were incubated with anti-menin antibody or rabbit IgG control. On day 2, DNA-protein complexes were precipitated with protein A-agarose beads, followed by reverse cross-linking. Purified DNA was used for PCR templates with 0.1% input as positive controls. A pair of primers covering the 5'-UTR, +208 to +623, from the transcription start site (TSS) were used for PCR amplification.

RESULTS

Menin Binds to the 5'-UTR of Caspase 8 Locus in Vivo—We have previously shown that menin increases caspase 8 expression specifically (26). We sought to determine whether menin binds the caspase 8 locus in vivo and, if it does, whether the binding is DNA sequence-specific or not. Thus, we performed a ChIP assay in menin-null MEFs complemented with vector or menin to address these questions. Three pairs of primers used for ChIP target three distinct regions of the caspase 8 locus, including 5'-UTR, 3'-UTR, and a region containing a ~80-bp fragment that was highly conserved between human and murine genomic DNA with 78% identity at the 5' end of the caspase 8 locus, which was named as the “island.” The ChIP assay indicates that anti-menin antibody, but not the control IgG, pulled down the 5'-UTR fragment in menin-expressing cells (Fig. 1, lanes 5 and 6, middle), whereas anti-menin antibody failed to pull down the DNA fragment in vector cells (Fig. 1, lane 3). In contrast, menin failed to pull down the island and 3'-UTR regions in either menin-expressing or menin-null cells (Fig. 1, lanes 3 and 6, top and bottom). These results demonstrate that menin specifically associated with the 5'-UTR region of the caspase 8 locus in vivo.

We have previously shown that menin binds dsDNA in vitro (35). However, it remains unknown whether menin binds the 5'-UTR of caspase 8 in vitro and, if it does, whether menin binds it in a DNA sequence-dependent manner. To address this question, we amplified the 5'-UTR and end-labeled it with 32P. The labeled probe was used to perform a gel shift assay to determine whether the probe binds the C-terminus of menin (GST-menin fusion protein (GST-F3), amino acids 396–610) (Fig. 2A), which was previously shown to bind dsDNA (35). Our results clearly show that GST-F3 binds the 5'-UTR DNA fragment (Fig. 2A, lanes 3 and 4). We further tested whether the menin-DNA binding is affected by nonspecific dsDNA. As shown in Fig. 2A, even the lowest amount of poly(dI-dC) (0.125 μg), a homopolymer of nonspecific dsDNA, markedly blocked GST-F3 binding to the 5'-UTR DNA fragment (Fig. 2A, lanes 5–8), suggesting that C-terminal menin (residues 396–610) binds the 5'-UTR in a sequence-independent manner.

However, we could not rule out the possibility that menin might prefer to bind some DNA sequences with a relatively high affinity. To address this question, we applied CASTing
Menin Stimulates Caspase 8 Transcription Activity through Its Binding Sites—Menin binds the 5′-UTR region specifically in vivo; however, it is unclear if menin activates caspase 8 transcription through the menin-bound fragment. To address this question, we cloned the 5′ part of the caspase 8 gene containing the 5′-UTR to upstream of a luciferase reporter gene (Fig. 3A, pcas-Luc). A luciferase reporter without a menin binding site was used as a control (Fig. 3A, pcasM-Luc). The resulting constructs were cotransfected into cells with or without menin cDNA, followed by luciferase and β-galactosidase assays. The results indicate that menin increased expression of the luciferase reporter driven by the menin-binding sequence by 5-fold, whereas removal of the menin-binding sequence abolished menin-mediated induction of reporter expression (pcasM-Luc) (Fig. 3B). These results suggest that menin can activate reporter expression through its binding sequence, 5′-UTR, further indicating that menin regulates caspase 8 transcription by binding the caspase 8 locus. It is conceivable that additional cellular factors must be required for menin-dependent expression of caspase 8, since menin by itself does not bind DNA specifically.

Menin Enhances Acetylation of Histones in the Caspase 8 Locus—It has previously been reported that menin interacts with MLL complex, and the menin-MLL complex methylates histone H3 lysine 4 (H3K4) (11, 21). Thus, we attempted to test if MLL associates with caspase 8 locus, especially in the region bound by menin. The ChIP assay failed to detect any significant binding of MLL to the 5′-UTR and other regions of the caspase 8 locus (data not shown). Using antibodies against di- or trimethylated lysine 4 of histone 3, we did not observe any enrichment of the modified histones around the caspase 8 locus in the presence of menin. These data suggest that menin may regulate
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A

B

FIGURE 4. Menin promotes the histone acetylation at the 5′-UTR of the caspase 8 locus. Menin-null MEFs complemented with vector or menin were used for ChIP assays as described under “Experimental Procedures.” The cross-linked DNA-protein complexes were extracted, and the purified DNA was used for real-time PCR with Taqman probes detecting the 5′-UTR or the island sequence. Association of acetylated histone H3 (Ac H3) and histone H4 (Ac H4) to the caspase 8 5′-UTR (A) or the island sequence (B) was expressed as the percentage of input (relative occupancy). The results were representative of three independent experiments.

expression of caspase 8 in a H3K4 methylation-independent manner.

Thus, we turned our attention to the potential impact of menin on histone acetylation, which also might regulate gene expression in the caspase 8 locus. To this end, we performed ChIP assay using anti-acetylated H3 or H4 antibodies to detect the histone acetylation on the 5′-UTR and the island in menin-null cells complemented with either menin or vector. The results indicate that the level of acetylated H4 at the 5′-UTR increased about 4.5-fold in menin-expressing cells as compared with that in menin-null cells (Fig. 4A). Notably, the amount of acetylated H3 in the 5′-UTR region was 38 times higher than that of the menin-null cells (Fig. 4A). In contrast, although a high level of acetylated histone H4 was detected in the island region (Fig. 4B, 40% of the input) in menin-expressing cells, loss of menin only slightly reduced the level of acetylated H4 (Fig. 4B, 35% of the input). Furthermore, the amount of acetylated H3 in the island was barely detectable in both menin-expressing and menin-null cells (Fig. 4B). Together, these results demonstrate that menin facilitates acetylation of both histones H3 and H4 at the menin-binding 5′-UTR region but not in the island without the menin binding site.

Treating menin-null MEFs with vaproic acid, a histone deacetylase inhibitor similar to tricostatin A (41), markedly increased expression of Hoxc8, another known menin target gene.3 However, in the present study, increasing concentrations of valproic acid did not increase the expression of caspase 8, suggesting that menin-related acetylation of histones H3 and H4 surrounding the 5′-UTR may not be the rate-limiting factor for menin-mediated induction of caspase 8 expression.

Men1-derived Point Mutants Lose Their Ability to Induce Caspase 8 Expression and Sensitivity to TNF-α-induced Apoptosis—Menin is mutated in MEN1 patients, and Men1+/− mice have a spectrum of tumors similar to that of MEN1 patients (7). Thus, we wondered if menin-dependent caspase 8 expression contributes to the repression of MEN1 tumorigenesis. We determined whether MEN1-derived menin mutations compromise the ability of menin to induce caspase 8 expression and TNF-α-mediated apoptosis. Two menin point mutants, L22R and A242V, were tested for their ability to promote caspase 8 expression. We performed the luciferase assay using the reporter driven by the caspase 8 5′-UTR, pcas-Luc, in cotransfection with either control, wild type menin or one of the mutants, L22R or A242V. Western blotting confirmed that menin, L22R, and A242V were all expressed at a similar level in the transfected cells. Wild type menin induced the luciferase expression by 7.5-fold, whereas the L22R and A242V mutants failed to stimulate expression of the luciferase reporter gene (Fig. 5A).

We further determined if L22R and A242V also fail to bind the caspase 8 locus in vivo, using a quantitative ChIP assay. To this end, menin-null MEFs were transduced with vector retroviruses or retroviruses expressing either wild type menin, L22R, or A242V. The resulting cell lines were used for a ChIP assay. The results indicate that wild type menin binds the 5′-UTR of caspase 8 locus, as expected (Fig. 5B), although L22R and A242V fail to bind the 5′-UTR (p = 0.02 and 0.03, respectively, as compared with the wild type menin). It is noteworthy that both wild type and menin mutants fail to bind the island sequence, strongly suggesting the specificity of menin binding to the caspase 8 locus (Fig. 5B). Together, these results demonstrate that menin associates with the 5′-UTR and activates caspase 8 expression, whereas the menin mutants lose the ability to associate with the 5′-UTR and fail to activate the luciferase reporter expression. It is possible that the mutants failed to be recruited or stabilized to the caspase 8 locus, since the steady level of the mutant proteins is similar to that of the wild type menin (Fig. 5B, bottom).

To further test if MEN1-derived menin point mutants, L22R and A242V, lose their ability to induce caspase 8 expression, we examined the effect of the two mutants on the endogenous caspase 8 gene expression. Thus, we analyzed the mRNA and protein levels of caspase 8 in vector cells as well as cells expressing either wild type menin or one of the mutants. Northern blotting shows that complementation of the menin-null cells with wild type menin markedly increased the caspase 8 mRNA level (Fig. 5C, lane 2). In contrast, point mutants, L22R and A242V, completely lost their ability to promote caspase 8 expression at the mRNA level (Fig. 5C, lanes 3 and 4). Consistent with the Northern blotting results, Western blotting also shows that only wild type menin promoted expression of caspase 8 at the protein level (Fig. 5D, lane 2), and the two menin point mutants failed to do so. As a control, wild type along with the two menin point mutants were all expressed at a similar protein level (Fig. 5D, lanes 2–4, middle). Together, these results demonstrate that menin binds to the 5′-UTR region in vivo and stimulates caspase 8 expression, whereas MEN1-derived point mutants lose the ability to associate with the 5′-UTR and thus fail to induce gene expression.

The response of menin-null MEFs or MEFs expressing wild type menin or mutant L22R or A242V to TNF-α-induced apoptosis was also examined. Cells were treated with TNF-α and followed by staining with propidium iodide and flow cytometry analysis. The results indicate that wild type menin potentiated TNF-α-induced apoptosis by 28% (Fig. 6), as previously reported (26). In contrast, A242V failed to potentiate TNF-α-induced apoptosis (12%; Fig. 6). Notably, TNF-α-in-

3 P. La, unpublished data.
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MEN1-derived menin point mutants failed to promote TNF-α-induced apoptosis. These findings strongly suggest that menin may control the threshold of TNF-α or other death ligand-induced apoptosis through up-regulating caspase 8 expression, leading to repressing MEN1 tumorigenesis.

The Reduction of Caspase 8 Expression in Mouse MEN1 Insulinomas—To further test if menin-mediated caspase 8 expression contributes to menin suppressing MEN1 tumorigenesis, we monitored the caspase 8 expression in Men1+/− mice that develop a tumor syndrome similar to human MEN1 tumor syndrome. It has been reported that ~62% Men1+/− mice develop hyperplasia but not adenoma in pancreatic islets at an early age (5, 42). At an age of 9.5 months, most Men1+/− mice develop insulinomas in which the normal allele of Men1 is lost (loss of heterozygosity) (7).

To determine the caspase 8 expression during MEN1 tumorigenesis, pancreatic islets or insulinomas were isolated from age-matched Men1+/+ or Men1+/− mice. The isolated islets or insulinomas were used to prepare RNA and proteins for evaluating caspase 8 expression using RT-PCR and Western blotting. At an age of 4.5 months, islets from both Men1+/+ and Men1+/− mice expressed a similar level of caspase 8 (Fig. 7A, lanes 2 and 4, top). From the age of 6.5 months to 9.5 months, however, the caspase 8 mRNA level was progressively reduced in islets/insulinomas in Men1+/− mice, as compared with that in wild type mice (Fig. 7A, lanes 2 and 4, middle and bottom). These results are consistent with the notion that when insulinomas developed after 6 months of age and loss of heterozygosity occurred, caspase 8 expression was markedly reduced due to loss of Men1. In agreement with this explanation, the caspase 8 protein level was also markedly reduced in islets/insulinomas from Men1+/− mice at age of 9.5 months, as compared with that from Men1+/+ mice (Fig. 7B). However, due to the technical restrain, the ideal demonstration of menin loss of heterozygosity and reduced caspase 8 expression in the same islet cells from Men1+/− mice remains to be done. Together, these results suggest that caspase 8 may play a crucial role in menin-mediated suppression of MEN1 tumorigenesis, since loss of Men1 in aged Men1+/− mice was precisely correlated with the reduction of caspase 8 expression.

To further evaluate the role of menin as a tumor suppressor in pancreatic islets, we determined menin expression in normal islets from wild type Men1+/+ mice or in insulinoma cells from Men1+/− mutant mice, using immunohistochemistry staining. Menin was readily detectable in the nucleus of normal islet cells duced apoptosis was even repressed in cells expressing L22R (2%; Fig. 6), and the reason for this is unclear. It is possible that the mutant menin inhibits one of the components in the TNF-α pathway, including TNF-α receptors. Together, these results indicate that menin up-regulates caspase 8 expression and potentiates TNF-α-induced apoptosis, and the...
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**DISCUSSION**

We and others have previously shown that ectopic expression of menin causes apoptosis in vitro (26, 43, 44). Consistent with the role of menin in apoptosis, menin also induces expression of caspase 8, a proapoptotic protease (27). However, it was poorly understood how menin induces caspase 8 expression and whether menin-mediated caspase 8 expression is crucial for suppressing MEN1 tumorigenesis. Our current findings demonstrate that menin specifically binds the caspase 8 locus in vivo and stimulates transcription of the reporter gene driven by the menin-binding DNA fragment from the caspase 8 locus. Importantly, Men1-derived menin point mutants lose their ability to induce caspase 8 expression and TNF-α-induced apoptosis. Caspase 8 expression is markedly decreased in islets or insulinomas from Men1+/− mice. Thus, menin-dependent caspase 8 expression may sensitize death ligand-induced apoptosis in normal islets, and the dysregulation of caspase 8 expression caused by MEN1 mutations may in part contribute to the development of MEN1 syndrome.

*Menin Specifically Binds the Caspase 8 Locus in Vivo to Regulate Its Expression*—Menin regulates transcription of a number of endogenous genes, including hTERT, Hoxc8, p27Kip1, p18Ink4c, and IGFBP-2 (11–15, 45). Menin has also been shown to bind the loci of most of these genes in vivo, as demonstrated by ChIP assays. We have demonstrated that menin specifically binds to the 5′-UTR of the caspase 8 locus, and the menin-bound sequence also mediates menin-induced transcription of the reporter gene. Our previous studies have shown that menin binds dsDNA (35). We have now further demonstrated that menin binds dsDNA in a sequence-independent manner, as shown by a CASTing assay (Fig. 2B). Since menin specifically binds to the 5′-UTR of the caspase 8 gene in vivo (Fig. 1) but binds dsDNA nonspecifically in vitro, these results suggest that menin may be recruited directly or indirectly by sequence-specific DNA binding transcription factors or coregulators to the caspase 8 locus. In agreement with the reasoning, comparison of the DNA sequences from these menin-bound fragments from caspase 8, p18Ink4c, p27Kip1, and Hoxa9 did not show significant homology (data not shown).

Consistent with this notion, a recent report shows that menin interacts with ligand-activated estrogen receptor to stimulate transcription of an endogenous gene (46). Thus, it is likely that menin interacts with a transcription factor or a coregulator and then targets the caspase 8 locus to modulate caspase 8 transcription. We have previously shown that two patches of positively charged residues in the C terminus of menin mediate binding to dsDNA, and mutations of these residues reduce caspase 8 expression in cells (10, 35). It is likely that menin-DNA interaction, albeit nonspecific, is crucial for stabilizing the transcriptional machinery at the caspase 8 locus. Since mutations in mutants L22R and A242V reside in the N-terminal and middle parts of menin, outside of the DNA-binding C terminus, these mutants may not directly affect the ability of menin to bind DNA. Nevertheless, we cannot rule out
the possibility that the mutations can indirectly affect the DNA binding.

Since menin interacts with H3K4 methyltransferases (11, 21) and affects the H3K4 methylation at the Hoxa9 locus (22, 23), we analyzed the MLL-related H3K4 methylation at the caspase 8 gene. The ChIP assay did not show significant impact of menin on the H3K4 modification. This is consistent with a recent report that a substantial number of menin target genes may not be coregulated by MLL (47). Instead, we found that menin enhances H3 and H4 acetylation specifically at caspase 8 5′-UTR, which is bound by menin. However, menin does not affect the status of histone H3 and H4 acetylation at the island that is further upstream of the 5′-UTR, and menin does not bind the island sequence in vivo. These results suggest that menin may bind to the 5′-UTR and affect the acetylation of the histones surrounding the menin-binding region, resulting in activation of caspase 8 transcription.

It is unclear how menin regulates acetylation of the histones, which is usually correlated with transcriptional activation (48). It is likely that menin directly or indirectly affects the activity of histone acetyltransferases, but not histone deacetylases, at the caspase 8 locus, since histone deacetylase inhibitor, valproic acid, did not affect caspase 8 expression.3 On the other hand, it is also possible that the impact of menin on histone acetylation is not a rate-limiting factor in regulating caspase 8 transcription.

**Menin May Suppress MEN1 Tumorigenesis through Up-regulating Caspase 8 and Potentiating Death Ligand-induced Apoptosis**—Caspase 8 plays a pivotal role in apoptosis induced by death ligands, including TNF-α (49). Expression of caspase 8 is silenced by hypermethylation of DNA in the caspase 8 locus in neuroblastosomas. Re-expression of caspase 8 sensitizes the tumor cells to death ligand-induced apoptosis (32–34), suggesting a potential role for caspase 8 in suppressing the development of neuroblastosomas. We have previously shown that menin enhances caspase 8 expression, caspase 8 enzymatic activity, and apoptosis in MEFs upon treatment with TNF-α (26). However, it remains unclear whether menin-mediated caspase 8 expression contributes to suppressing MEN1 tumorigenesis.

Thus, crucial questions remain as to whether menin mutations derived from MEN1 patients affect caspase 8 expression and sensitivity to TNF-α-induced apoptosis. Our current findings indicate that the tested missense point mutants of menin not only fail to up-regulate caspase 8 mRNA and protein but also cause menin to lose its ability to potentiate TNF-α-induced apoptosis. Consistent with these results, the menin mutants, as compared with the wild type menin, also failed to bind the caspase 8 locus in vivo and lost their ability to up-regulate reporter expression in vitro.

We observed that caspase 8 expression in MEN1 insulinomas was progressively decreased, following the trend of the development of insulinomas in Men1+/− mice. These results indicate that accompanying the loss of heterozygosity of Men1, which takes place in most of the insulinomas (7), caspase 8 expression also decreases. This suggests that loss of menin expression in MEN1 insulinomas leads to a reduction of caspase 8 expression. These observations enforce the notion that menin-dependent caspase 8 expression plays a crucial role in repressing MEN1 tumorigenesis. Supporting this conclusion, caspase 8 is frequently silenced in neuroblastosomas and ectopic expression of caspase 8 in the tumor cells render the cells sensitive to death ligand-induced apoptosis (29, 32–34).

Thus, our results are consistent with a model in which menin normally binds to the caspase 8 locus, alters chromatin structure, and increases caspase 8 expression. The up-regulated level of caspase 8 may potentiate death ligand-mediated apoptosis in endocrine cells, resulting in suppression of MEN1 tumorigenesis. Since menin is also actively involved in repressing cell proliferation through up-regulating p16ink4a and p27kip1 and repressing CDK2 and Cdc7/ASK activity (12, 15, 16, 50) and genome stability (37, 51), menin-dependent caspase 8 expression may coordinate with other menin functions in suppressing MEN1 tumorigenesis.

As targeted disruption of the caspase 8 locus in mice resulted in embryonic lethality, cardiac deformations, the neural tube defects, and deficiency in proliferation of T lymphocytes (20, 28), it remains unclear whether the nonapoptotic function of caspase 8 is also involved in suppressing MEN1-related tumorigenesis. Investigation of the precise role of caspase 8 in MEN1 development using conditional caspase 8 knock out mice is currently under way.

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