JunD-Menin Interaction Regulates c-Jun-mediated AP-1 Transactivation

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Abstract. The gene responsible for multiple endocrine neoplasia type 1, MEN1, encodes the 610-amino acid-protein, menin. Although menin has been reported to bind AP-1 transcription factor JunD and suppress its transcriptional activity, little is known about its molecular mechanisms and physiological role. To better understand the function of menin and its significance in tumorigenesis, we investigated the effect of wild-type and mutant menin proteins on AP-1 transactivation. In COS cells, wild-type menin suppressed JunD-mediated transactivation in a dose-dependent manner, while it augmented c-Jun-mediated transactivation also in a dose-dependent manner. These effects were lost or reduced in all menin mutants examined. Electrophoretic mobility shift assay using AP-1 binding elements as a probe revealed that menin does not affect binding of c-Jun to DNA. Coexpression of menin mutants did not affect the function of wild-type menin. Coexpression of JunD amino-terminal fragment abolished menin-mediated enhancement of c-Jun transactivation, suggesting that Menin-JunD interaction may negatively regulate the enhancing effect of menin on c-Jun-mediated transactivation in COS cells.

Key words: MEN1 gene, Multiple endocrine neoplasia type 1, Endocrine tumor, Tumor suppressor, Signal transduction

MULTIPLE endocrine neoplasia type 1 (MEN1) is a hereditary disease characterized by hyperplastic and neoplastic disorder of endocrine organs such as parathyroid, anterior pituitary and gastroenteropancreatic endocrine tissues [1]. The responsible gene (MEN1) has been mapped to 11q13 [2] and later identified by positional cloning [3, 4]. Most subjects with MEN1 harbor a germline MEN1 gene mutation, and somatic mutations of the MEN1 gene have been identified in sporadic endocrine tumors to some extent [1]. Mutation of the MEN1 gene along with the loss of the unaffected allele in the tumor of patients indicates that this gene functions as a tumor suppressor and the loss of both alleles leads to tumor development [5]. The physiological function of the MEN1 gene product, menin, is still largely unknown. Menin is a 610 amino-acid (611 amino-acid in mouse) nuclear protein [6] and is ubiquitously expressed in many cultured cells and various tissues including non-endocrine organs [7–9]. Organ-specific tumor formation in MEN1 thus suggests involvement of other factors or organ-specific posttranslational modification of menin. Moreover, intense expression of the MEN1 transcript in early stage of fetal embryogenesis and in actively proliferating cells suggests its significant roles in cell growth and differentiation [7, 10, 11]. To date, several cellular proteins have been shown to interact with menin, although the exact physiological roles of those interaction remain largely elusive [12–21].

Activator protein-1 (AP-1) is a sequence-specific transcription factor composed of members of the Jun
(c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families [22]. These proteins associate to form a variety of dimers that bind to a promoter element referred to as the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE). Variations in core AP-1 sequences, compositional changes and posttranslational modifications in AP-1 components enable functional versatility of AP-1. Menin has been shown to bind JunD and suppress its transcriptional activation through the histone deacetylase-dependent mechanism, although the critical domain of menin to bind JunD has yet to be established [12, 13]. AP-1 plays key roles in cell proliferation, differentiation and transformation, and aberrant AP-1 function is often observed in cancer cells [23–26]. Regulation of AP-1 activity thus could be the critical function of menin as a tumor suppressor. In the present study, we examined the functional interaction between AP-1 transcription factors and menin. Menin inhibited JunD-mediated AP-1 transactivation while it enhanced c-Jun-mediated AP-1 transactivation in COS cells. These effects did not accompany the change of DNA binding or phosphorylation status of Jun proteins. Interestingly, the effect on c-Jun function was abolished when the amino-terminal fragment of JunD was coexpressed, implying that JunD regulates c-Jun function by controlling the influence of menin on c-Jun.

**Materials and Methods**

**Plasmids**

Expression vector for wild-type human menin, pcDNA/HisMenin, was generated as previously described [11]. Expression vectors for following mutants were also generated: G42D, W183S, V184E and 516FS. 516FS mutant contains a single base insertion (1657insC) in exon 10 which causes a frame shift at amino acid codon 516 and early termination [27]. Expression vectors for these menin mutants, pcDNA/HisMenin42D, pcDNA/HisMenin183S, pcDNA/HisMenin184E and pcDNA/HisMenin516FS, respectively, were created from pcDNA/HisMenin by the insertion or substitution of nucleotide at the corresponding position. To obtain GST-menin fusion protein, a coding region of the MEN1 cDNA was subcloned in frame into pGEX6P-2 (Pharmacia, Uppsala, Sweden) to generate pGEX-Menin. A reporter plasmid pAP1-Luc was purchased from Stratagene (La Jolla, CA). Expression vectors for mouse c-Jun and mouse JunD (pcDNA3.1 mouse c-Jun, pcDNA3.1 mouse JunD and GST-JD1) were kindly provided by Drs. S. C. Chandrasekharappa (NHGRI) and S. K. Agarwal (NIDDK). Expression vector for truncation mutant of JunD, pcDNA3.1 mouse JunD(1–196), was generated by partial digestion of pcDNA3.1 mouse JunD with Apa I followed by self-ligation. The expected translation product of this construct is a 197 amino-acid protein that contains 1–196 of JunD and valine at its carboxy-terminal. Nucleotide sequence of this plasmid was confirmed by dideoxy sequencing.

**GST pull-down assay**

[35S]Methionine-labeled menin was synthesized by TNT T7-coupled Reticulocyte Lysate System (Promega) using the above described pcDNA/HisMenin constructs as templates. GST-JunD fusion protein or GST (approximately 5 μg) was attached to GST-sepharose beads (Pharmacia) and incubated with in vitro-translated menin for 2 h at 4°C. Mixtures were then centrifuged, washed five times in binding buffer, and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Radio-labelled signals were visualized and quantified with a Bioimaging Analyzer System BAS1500 (Fuji Photo Film, Tokyo, Japan). Pull-down assay using [35S]methionine-labeled Jun proteins and GST-menin was also performed with the same procedure.

**Luciferase assay**

COS cells were cultured in Dulbecco’s modified eagle medium (Gibco, Rockville, MD) supplemented with 7% fetal bovine serum in 24-well plates. At 60–70% confluence, cells were transfected by the calcium phosphate precipitation method with 100–150 ng of either pcDNA3.1 mouse JunD or pcDNA3.1 mouse c-Jun, 100 ng of pAP1-Luc and various amounts of menin-expression vectors. Total amount of plasmids in each well was corrected by empty vector, pcDNA3.1/HisC (Invitrogen, Carlsbad, CA). Cells were harvested 48 h after transfection and luciferase activity was measured by Luciferase Assay System (Promega, Madison, WI). Efficiency of transfection in each well was corrected with β-galactosidase activity. All transfection studies were repeated more than four times in duplicate.
Electrophoretic mobility shift assay

Cell extracts were prepared as described before [28]. Double stranded oligonucleotides containing wild-type or mutant TRE sequence were prepared. Nucleotide sequences of these are as follows: TRE-W (wild-type), 5'-cgcttgaTgACTCagcggaa-3'; TRE-M (mutant), 5'-cgcttgaTgACTTggcggaa-3'. Capital letters denote consensus TRE sequence. In mutant TRE, 2 nucleotides in TRE were substituted as underlined. Double-stranded oligonucleotides were end-labeled and 10 fmol of oligonucleotide was incubated at room temperature for 20 min with nuclear extracts from COS cells. Reaction mixture contained 20 mM Tric-Cl (pH 7.6), 100 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol, 50 ng/µg 10⁹ poly (dI-dC) and 5% glycerol. Protein-DNA complexes were separated from protein-free DNA by non-denaturating gel electrophoresis in 5% polyacrylamide gels. Radiolabelled signals were visualized with a BAS1500.

Western blotting

Experimental procedure for western blotting of menin has been described previously [9]. In the present study, 2,000 × diluted polyclonal anti-menin antibody HGK, which recognizes amino acids 199–212 of menin, was used.

Statistical analysis

All statistical analyses were performed using the Statview program for t test analysis.

Results

Binding of menin to Jun proteins

Menin has been shown to bind JunD and repress JunD-mediated transactivation, but its critical domain to bind JunD has yet to be established. By deletion analyses, Agarwal et al. reported that three regions of menin, amino acid codons 1–40, 139–242 and 322–448 are important for binding [12]. Meanwhile, Gobl et al. suggested the importance of carboxyterminus of menin for JunD binding [13]. Effect of menin on c-Jun-mediated AP-1 transactivation is also controversial. Enhancement of c-Jun-mediated AP-1 transactivation by menin has been reported despite its inability to bind c-Jun [12], while the other report mentioned the lack of the effect of menin on c-Jun function [13]. To address these issues, we generated a number of expression vectors for menin mutants and examined the effect of these mutants on the function of AP-1 transactivation. G42D, W183S, and 516FS have been identified in patients with MEN1, and V184E has been found in a kindred with familial isolated hyperparathyroidism. G42D mutation locates at the flanking region of the first JunD binding domain, while W183S and V184E mutations locate in the center of second JunD binding domain proposed by Agarwal et al. [12]. 516FS mutant lacks carboxyterminus of menin, which Gobl et al. reported to be essential for JunD binding [13].

We initially examined binding of these mutants to JunD by GST pull-down assay. As shown in Fig. 1A, [³⁵S]methionine-labeled wild-type menin bound GST-JunD fusion protein. One mutant, G42D, bound GST-JunD as efficiently as wild-type menin. Binding of W183S, V184E and 516FS to GST-JunD was significantly reduced compared to that of wild-type menin (p<0.05). Quantitative analysis revealed that relative binding of G42D, W183S, V184E and 516FS to GST-JunD was 115%, 25%, 35% and 45% of wild-type menin, respectively. Our results show that the carboxyterminus of menin may be important but not essential for JunD binding.

Despite enhancement of c-Jun-mediated transactivation, menin has been reported not to bind c-Jun [12]. To confirm this, we performed a pull-down assay using GST-menin and [³⁵S]methionine-labeled Jun proteins (Fig. 1B). In vitro-translated wild-type JunD bound GST-menin fusion protein, and a truncation mutant of JunD, JunD (1–196), which retains its menin-binding domain (amino acids 1–70) but lacks both the dimerization domain and DNA binding domain, also bound GST-menin as efficiently as wild-type JunD. On the other hand, in vitro-translated c-Jun did not bind GST-menin.

Effect of menin on AP-1 transactivation

To investigate whether binding of menin to JunD correlates with its suppressive effect on JunD-mediated transactivation, we next examined the effect of wild-type and mutant menin proteins on JunD-mediated AP-1 transactivation. In agreement with previous
reports [12, 13], wild-type menin suppressed JunD-mediated transactivation in a dose dependent manner (Fig. 2A). When COS cells were transfected with equal amount of expression vectors for mouse JunD and wild-type menin (150 ng each per well), JunD-mediated transactivation through TRE was suppressed to about 50% of control. Transactivation was further suppressed to less than 30% of control when the amount of expression vector for wild-type menin was increased to 300 ng. Expression of JunD was not affected by the coexpression of increasing amount of menin (data not shown). On the other hand, three missense mutants showed impaired suppressive effects and a frame-shift mutant 516FS showed no apparent effect on JunD-mediated transactivation even with four-fold excess amount (600 ng) of expression vector. These results indicate that binding of menin to JunD is not sufficient to suppress JunD-mediated transactivation. Although nuclear localization of 516FS is partly impaired, about 50% quantity of protein compared to wild-type menin was identified in the nucleus [9].

The effect of menin on c-Jun-mediated transactivation was also investigated. As shown in Fig. 2B, wild-type menin augmented c-Jun-mediated AP-1 transactivation in a dose dependent manner. Transactivation was enhanced to about 400% and 500% of control with 300 ng and 600 ng of expression vector for wild-type menin, respectively. All four mutants did not show any significant positive effect on c-Jun function. These results corroborate that in COS cells menin negatively regulates JunD-mediated transactivation while it positively regulates c-Jun-mediated transactivation despite its inability to bind c-Jun.

**Effect of menin on DNA binding of c-Jun**

To elucidate how menin augments c-Jun mediated transactivation, we examined whether menin affects the binding of c-Jun to DNA by electrophoretic mobility shift assay (Fig. 3A). Nuclear extracts from COS cells transfected with pcDNA3.1 mouse JunD produced an intense retarded signal (lane 1). This signal disappeared by the addition of 250-fold excess of specific competitor (unlabeled oligonucleotide containing TRE) (lane 2), but not by the non-specific competitor (unlabeled oligonucleotide containing mutated TRE) (lane 3). Addition of menin-rich extract in the reaction did not reduce, rather it augmented JunD binding to DNA (lane 5). This augmentation was, however, also observed when untransfected COS nuclear extract was added (lane 4). Expression of menin in Menin-NE was confirmed by Western blotting using polyclonal anti-menin antibody [9] (Fig. 3B). The same experiment was performed for c-Jun and the binding of c-Jun to
TRE was not affected by menin (Fig. 3A, lanes 7–11). Increase of signal intensity was seen with COS nuclear extract expressing menin (lane 11), but the similar degree of enhancement was also seen with untransfected COS nuclear extract (lane 10). These results indicate that suppression of JunD function and augmentation of c-Jun function by menin is not mediated by changes in DNA binding of those Jun proteins.
Lack of a dominant negative effect by menin mutants

In some tumor suppressor gene products, the mutant protein exerts an inhibitory effect on the function of wild-type protein (dominant negative effect). If binding of menin to JunD is essential to exert a regulatory function on AP-1 activity, coexpression of menin mutants, especially G42D, would inhibit the function of wild-type menin since G42D binds JunD but does not suppress JunD-mediated transactivation as efficiently as wild-type menin. To determine whether menin mutants possess a dominant negative effect on the function of wild-type menin, we next performed the coexpression study, the results of which are shown in Fig. 4. Coexpression of equal- or four-fold excess amount of menin mutants did not affect the inhibitory effect of wild-type menin on JunD-mediated transactivation (Fig. 4A). Menin mutants did not affect the augmentation of c-Jun function by wild-type menin, either (Fig. 4B). These results demonstrated the lack of a dominant negative effect by menin mutants and also imply that menin may not form a stable complex with JunD at least under this experimental condition.

Abrogation of the function of menin by JunD N-terminal fragment

Finally, we examined whether menin-JunD interaction could affect the c-Jun function. Since c-Jun and JunD function through the same response element, simple coexpression study is not suitable to investigate the effect of JunD on the functional interaction between c-Jun and menin. Therefore, we performed coexpression study using JunD(1–196), which binds menin as efficiently as wild-type JunD (Fig. 1B), but it neither induce target gene expression nor dimerize with other AP-1 proteins due to its lack of both DNA binding domain and dimerization domain. When coexpressed with c-Jun, JunD(1–196) suppressed c-Jun-mediated AP-1 transactivation only when a large amount of plasmid was used for transfection (Fig. 5A). When coexpressed with menin, JunD(1–196) effectively abrogated positive effect of menin on c-Jun-mediated transactivation in a dose dependent manner. Menin-mediated enhancement was totally attenuated with 150 ng of JunD(1–196) expression vector. When W183S mutant, which poorly binds JunD (Fig. 1A), was coexpressed instead of wild-type menin, the suppressive effect by JunD(1–196) was marginal. Similarly, JunD(1–196) attenuated a suppressive effect of menin on JunD-mediated transactivation (Fig. 5B). JunD(1–196) itself induced no luciferase activity over the background level (data not shown).

Discussion

Our present study revealed that 1) menin suppressed
JunD-mediated AP-1 transactivation while it augmented c-Jun-mediated transactivation in COS cells, 2) suppression of JunD function by menin mutants did not correlate with the efficiency of each mutant to bind JunD, 3) menin did not affect binding of JunD and c-Jun to DNA, 4) menin mutants did not have a dominant negative effect on the function of wild-type menin, and 5) the amino-terminal fragment of JunD abrogated the effect of menin on c-Jun-mediated transactivation. The lack of a correlation between suppression of JunD function by menin and its binding to JunD, and an augmentation of c-Jun function by menin agree with a previous report by Agarwal et al. [12]. It is of interest that effect of menin on c-Jun function is highly cell-type specific as menin suppressed c-Jun-mediated transactivation in CHO cells [28]. AP-1 function is controlled by the composition of dimers, posttranslational modification of AP-1 proteins and interaction with other nuclear regulatory proteins [22, 29]. We have recently demonstrated that menin does not influence DNA binding and phosphorylation of c-Jun and JunD [28].

Considering that menin affected c-Jun function without binding it, the most likely mechanism by which menin affects AP-1 function is the regulation of interaction between AP-1 complex and other nuclear regulatory protein(s) that is essential for appropriate AP-1 function. For example, menin could release activator protein(s) from JunD and that activator could be supplied to c-Jun. Overexpression of JunD amino-terminal fragment would squelch menin and that will attenuate menin-mediated augmentation of c-Jun activity. The lack of such effect for W183S supports this speculation. A protein which binds carboxy-terminal region of JunD (thus does not bind JunD(1–196)) or a
protein which binds only DNA-bound JunD could be a candidate for such a factor. Similarly, menin could recruit repressor protein(s) to JunD, which accompanies with the release of the repressor from c-Jun. Despite its high JunD binding activity, G42D mutant showed only reduced effects on AP-1 function compared to those of wild-type. G42D may be unable to regulate interaction between JunD and cofactor(s) or induce the conformational change of JunD that is necessary for interaction with other proteins. There are a number of proteins that bind JunD and regulate its transcriptional activity [30–32]. Further elucidation is required to evaluate the above hypothesis. It is to be noted that Kim et al. recently reported that menin recruits mSin3A/histone deacetylase complex to suppress JunD-mediated transactivation [33]. The effect of menin mutants we examined herein on JunD-corepressor interaction is of our current interest.

c-Jun regulates the transcription of target genes through formation of homodimers or heterodimers with Fos family members. In addition to transcriptional regulation, c-Jun also plays a crucial role in cellular proliferation, differentiation and apoptosis [22, 34, 35]. c-Jun has been reported to be essential for Ras-induced transformation and tumorigenesis [36], which is in clear contrast to the function of JunD that antagonizes Ras-induced transformation [37]. Antagonistic effect of JunD on c-Jun function may, at least in part, be exerted through JunD-menin interaction.

Considering menin mutants are able to bind JunD but are unable to control JunD-mediated transactivation effectively, it is reasonable to assume that the coexpression of mutant proteins would disturb the function of wild-type menin. However, we did not observe a dominant negative effect by menin mutants on the function of wild-type menin. The reason why all mutants especially G42D did not affect the function of wild-type menin is not clear. Other nuclear proteins could control the binding of menin to JunD, and the actual binding of G42D in vivo may not be as good as wild-type menin. Alternatively, menin may not form a stable complex with JunD and once other nuclear proteins are released from (or recruited to) JunD, menin might be released from JunD. Although elucidation of the molecular mechanism awaits further investigation, lack of a dominant negative effect in our study well agrees with clinical observations that in tumors in MEN1 patients or sporadic tumors with MEN1 gene involvement, inactivation of both alleles are constantly observed [38].

In summary, we demonstrated, albeit indirectly, that menin regulates interaction of AP-1 and other regulatory proteins. Especially, c-Jun function could be regulated by the formation of a JunD-menin complex. Collection of data on a functional study involving a large number of mutants, identification of key proteins for functional interaction between AP-1 and menin, and elucidation of tissue specific function of menin will facilitate our understanding on the molecular function of menin as well as its domain structure, and the mechanism of tumor suppression by menin.

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References

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