Differential Binding of the Menin Tumor Suppressor Protein to JunD Isoforms

Oya Yazgan and Curt M. Pfarr
Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, and Southwest Cancer Center at Texas Tech University Medical Center, Lubbock, Texas 79430

Abstract

The role of the Jun family of proteins (c-Jun, JunB, and JunD) in oncogenesis has been extensively studied, but the distinct biological roles of each Jun protein is not known. For example, whereas c-Jun can transform primary cells in cooperation with an activated ras oncogene, JunD antagonizes ras-mediated transformation. We have discovered that two isoforms of the JunD transcription factor are ubiquitously expressed, resulting from use of an alternative translation start codon within the JunD mRNA. Here we report the first characterized functional difference between these JunD isoforms; only the full-length isoform of JunD binds to the Menin tumor suppressor protein. Furthermore, Menin suppresses transcriptional activity of the full-length but not the truncated isoform of JunD, which identifies the full-length JunD isoform as a functional target of Menin.

Introduction

The AP-1 transcription factor is composed of dimer combinations formed between the Jun and Fos transcription factors. AP-1 activity converts external signals generated by growth factors, cytokines, or genotoxic agents into stabilized, longer-lasting changes in the transcription of target genes (1, 2). The Jun family consists of three members: c-Jun, JunB, and JunD (3–6). Each of the Jun proteins exhibits different capacities to cooperate with other oncogenes in transformation assays. We have previously shown that whereas c-Jun efficiently transformed primary cells in cooperation with an activated ras oncogene, overexpressed JunD slowed fibroblast growth and antagonized transformation by ras (7). More recently, we have discovered that two distinct isoforms of JunD are generated by the use of two translational start sites within the single JunD mRNA. These two isoforms are identical except for 48 NH2-terminal amino acids in the longer JunD protein (referred to as JunD-FL). Both JunD-FL and the truncated isoform (ΔJunD) are expressed in all cell types and tissues thus far tested and are expressed at approximately the same stoichiometry.

Menin is an autosomal dominant disease characterized by a variety of tumors in endocrine tissues including the anterior pituitary, parathyroid, and pancreatic islets (8). The gene was identified by positional cloning in 1997 and is located at 11q13 (9–12). The encoded protein, named Menin, contains 610 amino acids and bears no discernable similarity with known sequences. Most of the identified sporadic and germ-line mutations result in truncations that likely inactivate the Menin protein; thus, Menin is considered a tumor suppressor protein. Loss or mutation of both MEN-1 alleles is thought to be etiological for familial MEN-1 tumor formation (13). Using the yeast two-hybrid interaction assay Menin was found to interact specifically with the JunD transcription factor, although the significance of this interaction is presently unknown (14). Agarwal et al. (14) demonstrated that JunD residues 8–70 were required for Jun-D-Menin interaction. This identified Menin-binding sequence overlaps the junction between JunD-FL and ΔJunD; thus, any possible difference in Menin binding between the two JunD isoforms was not revealed. Here we report that Menin binds only the JunD-FL isoform and that the binding site for Menin is entirely contained within the first 48 amino acids. Menin strongly suppresses the transcriptional transactivation activity of JunD-FL, whereas it does not affect the activity of the shorter JunD isoform (ΔJunD). We have tested the effects of two point mutations that commonly occur in familial MEN-1 tumors (A176P and A242V; Ref. 13). Either of these mutations resulted in lower binding of the mutant Menin protein to JunD-FL and less suppression of JunD-FL transcriptional activity.

An important regulatory mechanism for c-Jun and JunD function involves phosphorylation of their NH2-terminal transactivation domains by the JNKs (15). Because the docking domain within the JunD protein is immediately adjacent to the Menin interaction domain, we tested whether phosphorylation by JNK had any influence on Menin binding. We demonstrate that phosphorylation of the JNK target residues has no impact on Menin interaction.

These results suggest that JunD-FL is a functional target of the Menin tumor suppressor protein and that disruption of proper JunD-FL/Menin interaction is a component of the mechanism of tumorigenesis in MEN-1 disease.

Materials and Methods

Cell Culture. CHO cells were grown in Hams-F12 media (CellGro) supplemented with 5% fetal bovine serum (HyClone Laboratories, Inc.) and antibiotics under standard conditions.

Expression Vector Construction. GST fusion proteins were prepared using the pGEX4T-3 vector (Pharmacia). Insert DNA encoding the NH2-terminal fragments of c-Jun (amino acids 1–123), JunD (amino acids 1–149), JunD (amino acids 1–115) and ΔJunD (amino acids 49–149) with BamHI and EcoRI restriction sites at their 5′ and 3′ ends, respectively, were generated using PCR from cDNA templates. The inserts were gel-purified, digested, and ligated into pGEX4T-3 cut with the same enzymes. Expression vectors for the GAL4-fusion proteins were constructed using the pGal4–0 plasmid (16). Insert DNA encoding the same NH2-terminal fragments indicated above were prepared by PCR with BamHI and XhoI restriction sites at their 5′ and 3′ ends, respectively. The inserts were gel-purified, digested, and ligated into pGAL4–0 cut with the same enzymes. Plasmid DNA stocks used for transformations were prepared using a standard cesium chloride density gradient procedure. The myc-tagged Menin expression vector (pcDNA3.1-Mein-Myc) was kindly provided Dr. S. Chandrasekharappa (Bethesda, MD). The A176P and A242V point mutations in Menin were introduced using a PCR-based site-directed mutagenesis protocol (Quick Change, Stratagene). All of the constructs were verified by direct sequencing using an ABI automated sequencer.

Binding and Kinase Assays. GST-fusion proteins were inductively expressed in Escherichia coli and purified by a standard protocol using glutathione-coated agarose beads (Sigma). GST-fusion proteins were eluted from the beads with excess glutathione and dialyzed in 50 mM Tris (pH 7.5) and 20.

Received 9/12/00; accepted 12/4/00.
mM MgCl₂. Menin protein was prepared using a coupled in vitro transcription/translation system (TNT, Promega), that was supplemented with [³⁵S]methionine and [³⁵S]cysteine (Trans-label, ICN). Binding assays were performed using approximately 4 μg of GST-fusion proteins, 5 μl of Menin TNT-lysate, and 20 μl of 50% glutathione-agarose beads in binding buffer [50 mM Tris (pH 8.0); 0.01% NP-40; 5 mM MgCl₂; 10% glycerol; 10 mM NaCl; and 4 μg/ml each aprotonin, leupeptin, and pepstatin A] at 4°C for 3 h. After centrifugation, the beads were washed three times in binding buffer and boiled in SDS-sample buffer, and the complexed proteins were resolved on 12% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany) by electoblotting and the membranes dried down. For the kinase/GST-pull-down dual assay, the kinase reaction was performed first in a total volume of 40 μl using 4 μg of each fusion protein, 1 μl of TNT-lysate programmed with a cDNA for JNK(β2) (kindly provided by Dr. R. Davis) in the presence of 50 μM [γ-³²P]ATP (10 Ci/mmol) in kinase buffer [50 mM Tris (pH 7.5), 20 mM MgCl₂, 20 mM β-glycerophosphate, 2 mM DTT, and 0.1 mM sodium orthovanadate] at room temperature for 1 h. ³³S-labeled Menin protein and 20 μl of 50% glutathione-agarose in binding buffer were then added and the mixture further incubated for 4 h at room temperature. The beads were then harvested by centrifugation. The protein complexes were analyzed as described above. The ³⁵S and ³²P signals were detected using a PhosphorImager instrument (Molecular Dynamics).

Transfection Assays. CHO cells were plated at a density of 45,000 cells/well in 24-well plates, 24 h before transfection. The reporter plasmid Gₛ₆E₇-Luc (0.3 μg) along with 2 ng of the indicated expression vector (pGAL4-Jun), encoding each of the GAL4-Jun fusion proteins, and the indicated amount (0, 5, or 10 ng) of expression vector (pcDNA3.1-Menin-Myc) for the wild-type or mutant Menin proteins were transfected into the cells using Transfast reagent (Promega) in serum-free media according to the manufacturer’s instructions. At 24 h posttransfection, cells were harvested by incubation in Passive Lysis Buffer (Promega) for 30 min at room temperature with agitation. The luciferase activity in the extracts was measured in a Turner Designs luminometer using the Luciferase Assay System (Promega).

Western Blot Analysis. Transfected CHO cells were rinsed with cold PBS, harvested, and boiled in SDS-sample buffer for 5 min. Equal amounts of total protein from whole-cell extracts were fractionated on a 12% denaturing SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Non-specific interactions were blocked by preincubation of the membranes with 5% nonfat dry milk powder in PBS supplemented with 0.1% Tween 20 for 1 h. GAL4-Jun fusion proteins were detected using an α-GAL4 mouse monoclonal antibody directed against the GAL4 DNA binding domain (SC-510, Santa Cruz Biotechnology). Myc-tagged Menin proteins were detected using an α-myc mouse monoclonal antibody (Invitrogen). Horseradish peroxidase-conjugated donkey antimouse antibody (Jackson Labs) was used for the secondary antibody. The blots were developed using an enhanced chemiluminescence system (Pierce).

Results

Two Isoforms of JunD Are Generated through Translational Regulation. The human, rodent, and avian junD genes contain no introns and generate a single mRNA species (6, 17, 18). Two isoforms of JunD are generated from this single mRNA through the use of two translation start sites (19). The longer isoform, JunD-FL, contains 484 amino acids in the mouse and rat and 348 amino acids in human. The shorter JunD isoform (∆JunD) is truncated by 48 NH₂-terminal amino acids in the rodent and 53 residues in human. JunD is ubiquitously expressed as determined by immunoblotting analyses from a wide range of cell lines and animal tissues. 3 A representative JunD Western blot using extract from mouse thymus is shown in Fig. 1A. No functional differences between JunD-FL and ∆JunD have yet been reported.

Menin Binds Only to the JunD-FL Isoform. Because JunD was identified in a yeast two-hybrid screen as a Menin binding protein, we tested whether Menin would preferentially interact with either JunD isoform (14). We prepared fusion proteins between GST and portions of the NH₂-termini of JunD-FL, ∆JunD, or c-Jun (Fig. 1B). These fusion proteins were expressed in bacteria, purified, and used in pull-down assays. GST-Jun fusion proteins were incubated with Menin protein that was produced by in vitro transcription/translation. Menin protein was radiolabeled by incorporation of [³⁵S]methionine and [³⁵S]cysteine. Fig. 2A illustrates a typical binding assay. Menin efficiently binds to GST-JunD-FL and copellets with GST-JunD-FL when harvested using glutathione-agarose beads (Fig. 2A, Lanes 4 and 5). However, ∆JunD displays no measurable binding to Menin above the background detected when GST alone was used as a control (Fig. 2A, Lanes 6 and 8). Furthermore, GST-Jun also exhibits no measurable binding to Menin in this assay. A GST fusion containing the first 48 NH₂-terminal amino acids of JunD-FL (the residues truncated in ∆JunD) also bound Menin strongly (Fig. 2A, Lane 1). As can be seen comparing Lanes 1 and Lane 4 in Fig. 2, most, if not all, of the Menin binding activity within the JunD molecule is fully contained within the first 48 amino acids.

Menin Binding Is Unaffected by JunD Phosphorylation. The JNKs interact with both JunD-FL and ∆JunD via a JNK docking domain positioned between residues 49 and 73 (numbered with respect to JunD-FL). Both JunD-FL and ∆JunD are phosphorylated by JNK on three residues adjacent to the docking domain (serine-90,
c-Jun and placed in a mammalian expression vector (Fig. 1C). These vectors were tested by Western blot analysis for their ability to produce the correct size fusion proteins after transfection and expression in CHO cells as shown in Fig. 3B. These expression vectors were cotransfected into CHO cells along with a GAL4-luciferase reporter vector (G7-E1b-Luc). Gal4-JunD-FL displayed consistently higher transactivation capacity than either Gal4-JunD or Gal4-c-Jun (Fig. 3A). When cotransfected with an expression vector for wild-type Menin, transactivation of JunD-FL was suppressed in a dose-responsive manner, with activity decreased 5-fold with 10 ng of Menin expression vector. Transactivation by either ΔJunD or c-Jun was unaffected by coexpression of Menin. Menin protein was monitored by Western blot analysis using an anti-myc antibody to detect the myc epitope-tagged Menin protein (Fig. 3B).

Mutations in Menin Disrupt Both JunD Binding and Transcriptional Suppression. Two Menin point mutations were tested for their effect on JunD interaction (Fig. 1D). Both of these mutations (A176P and A242V) are found in both sporadic and familial tumors (13). As shown in Fig. 2A, Lanes 2 and 3, these mutations resulted in lower levels of Menin binding to JunD in GST-pull-down assays. In

serine-100, and threonine-117). Phosphorylation of JunD-FL and ΔJunD by JNK significantly increases their transcriptional activity in transfection reporter assays, which suggests that JNKs are important regulators of JunD function*. Therefore, we tested whether phosphorylation by JNK affected the interaction between JunD and Menin. GST-biding assays were performed after incubation of GST-Jun and Menin in the absence or presence of in vitro transcribed/translated JNK along with [γ-32P]ATP. Under these reaction conditions, phosphorylation of the GST-JunD protein is nearly complete (Fig. 2B and data not shown). Again, JunD-FL bound 35S-labeled Menin (Fig. 2B, Lane 1), but this binding was unaffected by phosphorylation of the GST-JunD-FL (Fig. 2B, Lane 3). Although ΔJunD was similarly phosphorylated under these conditions, it showed no measurable binding to Menin (Fig. 2B, Lane 7).

Menin Suppresses Transcriptional Activity of the JunD-FL Isoform. To test the effect of Menin on JunD transcriptional activity, transient transfections were performed using a GAL4 transcription reporter system. GAL4 fusions were prepared by replacing the DNA binding domain of GAL4 and NH2-terminal fragments of JunD or

---


---

Fig. 2. In vitro binding assays between JunD and Menin. A, GST-pull-down binding assays using GST-Jun proteins and in vitro transcribed and translated 35S-labeled Menin protein. The GST fusion proteins contained fragments of Jun corresponding to JunD-FL (1–79, 1–115, and 1–149), or to ΔJunD (1–79), or to c-Jun (1–79). GST protein alone was used as a negative control. After incubation, the GST protein complexes were harvested, separated by SDS-PAGE, transferred to nitrocellulose membranes, and the 35S signal detected. Bottom panel, the labeled Menin signal remaining in the supernatant. B, combination GST-pull-down and JNK kinase assay. GST fusion proteins were first phosphorylated by mixing GST-JunD (1–115), corresponding to JunD-FL, or GST-JunD (1–115), corresponding to ΔJunD, with [γ-32P]ATP alone; [γ-32P]ATP plus rabbit reticulocyte lysate (RRL) programmed with JNK; or with [γ-32P]ATP plus unprogrammed RRL. This kinase reaction was incubated for 1 h, at which time 35S-labeled Menin protein was added. After incubation, the complexes were harvested and analyzed as in A. The bottom radiolabeled bands result from 35S incorporation into the GST fusion proteins and the top band from the 35S-labeled Menin protein. S, supernatant; P, pellet.
the case of the A242V mutation, the amount of Menin that copelleted with GST-JunD was similar to that in the GST control, indicating that this residue is critical for Menin binding (Fig. 2A, Lanes 3 and 8). These mutant isoforms of Menin were also tested in cotransfection transciptional assays. Both mutations had a similar impact. Each mutant Menin protein resulted in a lower level of inhibition of JunD-FL transcriptional activity than that seen with the wild-type Menin (Fig. 3A). The mutants exhibited ~50% of the inhibitory activity of the wild-type Menin (Fig. 3A, top bar graph). No effect of either mutation was detected on the transcriptional activity of ΔJunD or c-Jun.

Discussion

Whereas the three Jun family members (c-Jun, JunB, and JunD) have strong similarities at the primary sequence and structural levels, they exhibit distinct biological properties (1, 7, 20). For example, both c-Jun and JunB are considered immediate-early genes, based on their rapid and strong induction by growth factors and superinduction of mRNA levels in the presence of cycloheximide, whereas JunD is relatively refractory to such stimuli (6, 21, 22). The Jun proteins also differ markedly in their capacity to transform cells, whereas c-Jun can transform immortalized cells as a single gene and can cooperate strongly with activated alleles of ras in transformation assays, JunD overexpression slows the proliferation of fibroblasts, and JunD antagonizes transformation by activated ras (7, 23). These transformation assays were performed before the two distinct isoforms of JunD were identified; thus, it is presently unclear what differences exist between JunD-FL and ΔJunD in their ability to slow fibroblast growth or to antagonize ras-induced transformation.

The positional cloning of the gene responsible for multiple endocrine neoplasia (MEN-1) and its characterization as a classical tumor suppressor has focused attention on the encoded protein (10). The Menin protein is 610 amino acids in length and has no identified similarities with database sequences except for two putative nuclear localization signals (Fig. 1D; Ref. 24). Using the yeast two-hybrid interaction system with Menin as the bait, JunD was identified as a Menin-interacting protein (14). Here we have demonstrated that Menin binds directly to the full-length isoform of JunD (JunD-FL) but not to the truncated JunD isoform (ΔJunD). Furthermore, the Menin binding domain of JunD is contained within the first 48 amino acids, precisely the same residues that are truncated in ΔJunD. We have demonstrated that this differential binding has pronounced functional effects. Menin suppresses transcriptional activity mediated by a GAL4-JunD-FL fusion molecule in a dose-dependent manner, but activity of GAL4-ΔJunD is unaffected. Supporting the hypothesis that JunD-Menin interaction is functionally relevant, two naturally occurring Menin mutations that were engineered into our Menin expression vector resulted in lower suppression of JunD-FL transcriptional activity, although having no measurable effect on ΔJunD activity. Consistent with these observations, these same Menin mutations resulted in lower levels of binding to GST-JunD-FL fusion proteins in our in vitro assays.

The Jun proteins have a highly modular structure with the dimerization, DNA-binding, and nuclear localization signal domains located within the COOH-terminus, and the transactivation domain located within the NH2-terminal one-third of the molecule (1). In addition, a discrete JNK docking domain is present in both JunD isoforms (Fig. 1A). JNK binds both JunD-FL and ΔJunD and activates transcription of both isoforms by phosphorylating target residues located adjacent to the docking domain. Phosphorylation of these sites did not affect the binding of Menin to JunD-FL in our binding assays, suggesting that JNK-mediated phosphorylation does not modulate Menin binding. However, these assays used in vitro translated and transcribed JNK and Menin with the GST-JunD substrate in large excess. Thus, whereas the kinase reactions were allowed to run to completion, thereby phosphorylating most of the available target residues, the possibility of direct steric interference between Menin and JNK was not tested. Because Menin has a molecular mass of 68 kDa and JNK isoforms have masses of 45 or 54 kDa, the possibility of steric interference certainly exists, considering that their respective binding domains within JunD are immediately adjacent.

In addition to Menin, several other nuclear proteins have been shown to interact with the NH2-terminal region of JunD, including JAB-1 (25) and CBP (26). The precise binding domains for these proteins have not yet been mapped, nor have any complexes composed of JunD and several of these binding proteins been described. Thus, although the manner in which Menin suppresses the transcriptional activity of JunD-FL is presently unknown, a reasonable conjecture is that Menin functions by regulating the binding of JunD-interacting proteins or by participating in protein complexes positioned on the NH2-terminal of JunD.

These data together suggest that the full-length isoform of JunD is a relevant physiological target for the Menin tumor suppressor protein. Moreover, disruption of proper JunD-FL-Menin interaction by mutational inactivation or deletion of Menin may underlie at least part of the mechanism leading to tumor development in MEN-1 disease. Interestingly, the JunD-FL isoform has higher basal transcriptional activity than the ΔJunD isoform, which suggests that JunD-FL activity must be more tightly modulated for growth regulation. Because we have previously shown that constitutive expression of both isoforms of JunD had growth-inhibitory effects in mouse fibroblasts, an important experimental question is whether this effect is predominantly attributable to the ΔJunD isoform.

A working model for tumorigenesis resulting from loss of Menin activity is presented in Fig. 4. Although it is not known whether JunD-FL and ΔJunD regulate unique subsets of target genes, there is presently no evidence to suggest that they possess different dimerization properties. Therefore, both isoforms would be expected to form...
dimers with other Jun and Fos proteins, thereby contributing to the total AP-1-dependent regulation of target genes. Some of these genes are likely to be critical for proper regulation of cell proliferation (Fig. 4A). Our data suggest that JunD-FL would be capable of exerting a stronger transcriptional activation response than would ΔJunD. Although wild-type Menin would inhibit JunD-FL, the strength of this inhibition in any given cell type or tissue is unknown; i.e., important parameters such as the stoichiometry of Menin and JunD-FL or the regulation of Menin-JunD binding within the nucleus have not yet been determined. When Menin activity is absent or attenuated, as is the case for MEN-1 disease, the transcriptional inhibition of JunD-FL would be lifted resulting in an altered AP-1 activity. This in turn would lead to an alteration of AP-1 target gene expression leading, through unknown mechanisms, to tumorigenesis. Although JunD is the only Menin-interacting protein identified to date, undoubtedly additional interactions will be uncovered that also play a role in MEN-1 disease.

Acknowledgments

We thank Drs. S. C. Chandrasekharappa (NHGRI, NIH, Bethesda, MD) and R. Davis (University of Massachusetts Medical School, Worcester, MA) for providing the Menin and JNK expression vectors, respectively. We also thank Drs. Simon Williams and Martine Coue for helpful discussions and critical reading of the manuscript.

References

Differential Binding of the Menin Tumor Suppressor Protein to JunD Isoforms

Oya Yazgan and Curt M. Pfarr

Cancer Res 2001;61:916-920.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/3/916

Cited articles
This article cites 26 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/3/916.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/61/3/916.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.