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Cell Cycle Regulation of Menin Expression[1]

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Abstract

The multiple endocrine neoplasia type 1 gene product, menin, interacts with Jun D. The physiological role of menin in cell cycle control and the manner in which its inactivation contributes to tumorigenesis remain unknown. In the present study, the expression of menin was examined at various cell cycle stages in GH4C1 cells, a rat pituitary cell line. Cells synchronized at the G_1-S-phase boundary expressed menin at a lower level than G_0-G_1-synchronized cells. The expression of menin increased as the cells entered S phase, at which time Jun D expression also increased. In contrast, cells synchronized at the G_2-M phase expressed lower levels of menin. At G_0-G_1, G_1-S, and G_2-M phases of the cell cycle, menin was found predominantly in the nucleus. In summary, we show that in pituitary cells, menin is a nuclear protein whose expression is cell-cycle regulated. The data suggest that menin has an important role in cell growth regulation.

Introduction

MEN1[3] is an autosomal dominant disorder characterized by multiple endocrine tumors of parathyroids, pancreatic islets, and the anterior pituitary (1, 2). The MEN1 locus maps to chromosome 11q13 (3), and the *MEN1* gene itself was recently identified by positional cloning (4, 5). Over 200 independent germ-line and somatic mutations scattered throughout the protein coding region have been identified (Refs. 6 and 7 and references therein). Somatic mutations have been found to a variable extent in parathyroid adenoma, gastrinoma, insulinoma, lung carcinoid, and anterior pituitary tumor. Many of the mutations are clearly inactivating, leading to a truncated product. This would be consistent with menin acting as a tumor suppressor gene and a lack of menin caused by the loss of both alleles leading to tumor development. The human gene encodes a 610-amino acid protein with homology to no known protein nor any obvious conserved motifs that would provide clues to its function. Recently, two novel nuclear localization signal sequences have been identified at the COOH-terminal portion of the menin protein, which has been demonstrated to be primarily located in the nucleus of human embryonic kidney cells, mouse fibroblasts, and Chinese hamster ovary cells (8). Menin interacts with the activator protein 1 factor, Jun D, and represses Jun D-activated transcription (9), although the significance of this observation is unclear at present. However, the emerging knowledge of menin suggests that it functions in transcriptional regulation, DNA replication, or cell cycle control. Because the expression of several tumor suppressor genes is regulated during cell cycle progression (10, 11), the expression of menin may also be dependent on cell cycle stage.

In the present study, the subcellular localization of menin was examined in the rat pituitary GH4C1 cell line using a specific polyclonal menin antibody. In addition, we evaluated menin protein levels at various cell cycle stages in GH4C1 cells synchronized by serum starvation, mimosine, aphidicolin, or Colcemid.

Materials and Methods

Cell Lines and Antibodies. All cell lines were from the American Type Culture Collection (Manassas, VA). The anti-β-tubulin monoclonal antibody and the anti-FLAG M2 monoclonal antibody were from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada), and VWR Scientific (Bridgeport, NJ), respectively. The anti-TFIIH p89 antibody (S-19) and rabbit polyclonal anti-Jun D antibody (sc-74) were from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal Rb antibody 14001 A was from PharMingen (Mississauga, Ontario, Canada).

Menin Antibody and cDNA. A peptide, NH_2-REGRRRGPRRESKPC-COOH, was synthesized (by solid-phase chemistry at the Peptide Synthesis Facility of the Sheldon Biotechnology Centre of McGill University) corresponding to amino acids 476–489 of menin (this sequence is completely conserved between human and mouse) with an additional cysteine residue at the COOH terminus. The peptide was coupled through the cysteine residue to keyhole limpet hemocyanin, and a rabbit polyclonal antibody was raised by immunization with the conjugate. The antiserum was immunoaffinity-purified before use.

For menin cDNA synthesis, the coding region of menin was amplified by reverse transcription-PCR of total RNA from human medullary thyroid carcinoma (TT) cells using 5′-ACCGCCCGCCGCCATGGGGCTGAAG-3′ as the forward primer, and 5′-CAGTAGTTCAGAGGCCTTTGCGCT-3′ as the reverse primer. A menin cDNA that encodes a FLAG epitope at the COOH terminus of menin was prepared as described above, except that the reverse primer was 5′-CAGTAGTTCAGAGCTTGTCGTCGTCGTCCTT-GTAGTCGAGGCCTTTGCGCTGCCGCTT-3′. The cDNAs were cloned into the pCRII vector (Invitrogen, San Diego, CA) according to the manufacturer's specifications. For mammalian cell transfection, the menin cDNA inserts were cloned into pcDNA3.1(+) (Invitrogen). The correctness of the constructs was verified by restriction enzyme analysis and nucleotide sequencing.

Cell Culture and Transfection. Rat pituitary tumor GH4C1 and African Green Monkey COS-7 cells were maintained in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotic-antimycotic (Life Technologies, Inc.) in a humidified atmosphere at 37°C with 5% CO_2. For transient transfection, cells were seeded at $2–5 \times 10^5$ cells/35-mm tissue culture dish and incubated for 24 h. Menin DNA was transfected by LipofectAMINE (Life Technologies, Inc.), and cells were harvested 48 h later.

Subcellular Fractionation. Cultures were trypsinized, and the cells were washed with PBS and collected by centrifugation (12). Cells were gently resuspended in 2 ml of buffer containing 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM EDTA, 0.5 mM PMSF, and one complete protease inhibitor mixture tablet (Roche, Laval, Quebec, Canada); allowed to swell for 10 min; processed by 20 strokes in a Dounce tissue homogenizer; and centrifuged at $2,000 \times g$ for 10 min to pellet crude nuclei. The supernatant from the low-speed spin was separated into cytoplasmic and membrane fractions by differential centrifugation at 35,000 rpm for 30 min in a SW50.1 rotor of a Beckman model L5-50 ultracentrifuge; the resulting cytoplasmic supernatant was adjusted to 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate and 0.1% SDS.

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[3] The abbreviations used are: MEN1, multiple endocrine neoplasia type 1; PMSF, phenylmethylsulfonyl fluoride; CDK, cyclin-dependent kinase; Rb, retinoblastoma gene product.

The membrane pellet was resuspended in 1 ml of radioimmunoprecipitation buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% NP40, 1% deoxycholate, 0.1% SDS, 0.5% aprotinin, and 0.5 mM PMSF], briefly sonicated and centrifuged at 10,000 × *g* for 10 min to pellet debris; nuclear pellets were obtained by centrifugation at 15,000 × *g* for 20 min at 4°C; resuspended in 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT; and again Dounce-homogenized. After a 20-min centrifugation at 15,000 × *g*, supernatants were dialyzed for 5 h against 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Protein content was determined by protein assay kit (Bio-Rad, Mississauga, Ontario, Canada), and samples were stored at −80°C.

Western Blotting. Cells were lysed in radioimmunoprecipitation buffer with 0.5 mM PMSF, complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatants were stored at −80°C. Protein quantitation was performed with a protein assay kit. Equal amounts of protein were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride (Trans-Blot, Bio-Rad). Blots were blocked with TBS [20 mM Tris-HCl (pH 7.5) and 137 mM NaCl] plus 0.1% Tween 20 containing 3% dried milk powder. The antigen-antibody complexes were visualized using the appropriate secondary antibodies (Bio-Rad), and the enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham Life Science, Inc., Oakville, Ontario, Canada).

Flow Cytometric Analysis. Asynchronous GH4C1 cells were seeded at 5×10^5 cells/10-cm dish, cultured for 24 h in complete medium, and then synchronized at G_0-G_1 by culture in DMEM alone for 24 h. Cells were released back into the cell cycle by refeeding with DMEM supplemented with 20% fetal bovine serum. Alternatively, G_0-G_1-synchronized cells were refed with complete medium containing either 400 μM mimosine, 12 μM aphidicolin, or 1 μg/ml Colcemid and cultured for 24 h to synchronize at either the G_1-S-phase boundary (mimosine or aphidicolin) or G_2-M-phase (Colcemid). Synchronized cells were released back into the cell cycle by washing and refeeding with complete medium. For each time point, cells were harvested as described above, rinsed once with PBS, and stained with propidium iodide (13). The cell pellet was resuspended in 1 ml of 3.5 mM Tris, 7.5 mM propidium iodide (Calbiochem, La Jolla, CA), 0.1% NP40 (Sigma-Aldrich), 700 units/liter RNase (Roche), and 10 mM NaCl. After standing for 10 min on ice, the nuclei were analyzed in a FACScan (Becton Dickinson, Oxnard, CA). Calculation of the distribution in various phases of the cell cycle was performed with Cell Fit software (Becton Dickinson) using a sum of broadened rectangle fit.

Results

Identification of Menin Protein. A specific polyclonal antibody was raised against a peptide sequence overlapping nuclear localization sequence 1 of menin (Fig. 1*A*). As shown in Fig. 1*B*, by Western blot analysis this antibody detected endogenous menin as a 69-kDa species in rat pituitary GH4C1 and African Green Monkey COS-7 cells. Extracts of GH4C1 and COS-7 cells transiently transfected with menin cDNA demonstrated increased staining of the 69-kDa species. The band disappeared when antibody preabsorbed with the peptide against which it had been raised was used (data not shown). In COS-7 cells, transiently transfected with a FLAG-tagged menin cDNA, a 72-kDa species with FLAG immunoreactivity (representing the fusion protein of menin and the FLAG epitope) was detected (data not shown). By Western blotting, an endogenous 69-kDa menin species was detected in a variety of additional cell types and species. These included rat pituitary GH3, mouse pituitary corticotroph AtT-20, rat insulinoma RIN-5F, human lung carcinoma BEN, and human medullary thyroid carcinoma TT (data not shown).

Nuclear Localization of Menin in Pituitary Cells. Localization of menin in the nucleus was initially demonstrated in nonendocrine cells (8). We have examined the subcellular localization of menin by subcellular fractionation of rat pituitary GH4C1 cells followed by Western blotting with menin antibody, and with antibodies against

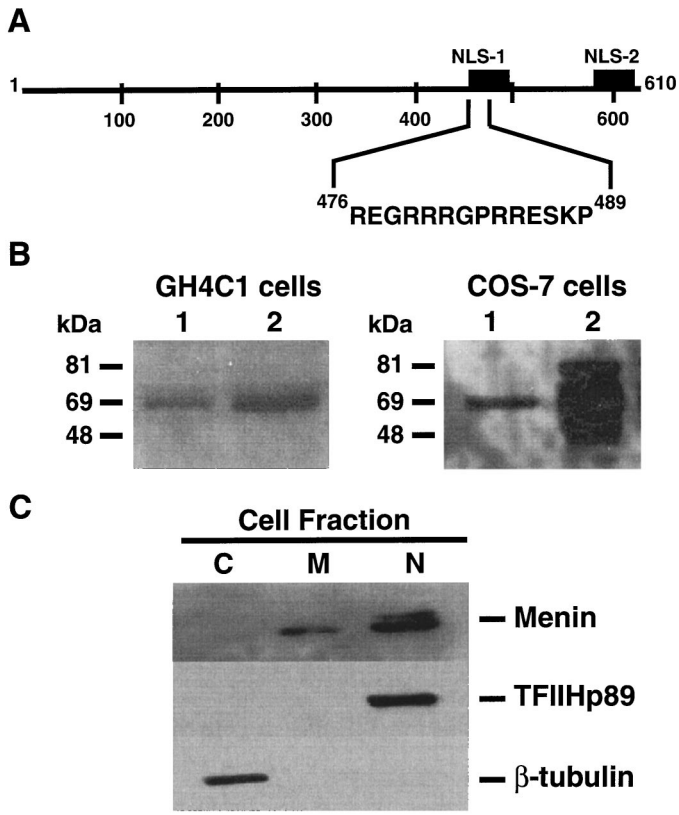
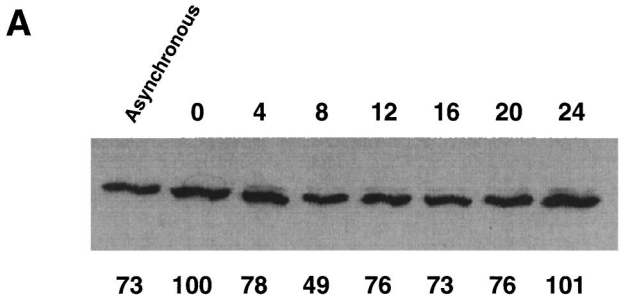


Fig. 1. Identification of the menin protein. *A*, a rabbit polyclonal antibody was raised against the peptide shown. Total cell lysates (50 μg) were separated by SDS-PAGE, Western blotted, and probed with the anti-menin polyclonal antibody. *B*, *left panel*, GH4C1 cells transfected with (*Lane 1*) empty vector and (*Lane 2*) menin cDNA. *B*, *right panel*, COS-7 cells transfected with (*Lane 1*) empty vector and (*Lane 2*) menin cDNA. *C*, subcellular localization of menin. Nuclear (*N*), membrane (*M*), or cytoplasmic (*C*) fractions of GH4C1 cells were separated by SDS-PAGE (20 μg protein/lane) and immunoblotted. As controls for the fractionation procedure, antibodies against TFIIHp89 and β-tubulin were used for nuclear and cytoplasmic proteins, respectively.

TFIIH p89 and β-tubulin as controls for nuclear and cytoplasmic fractions, respectively. As shown in Fig. 1*C*, menin was predominantly found in the nuclear fraction, with a lesser amount found in the membrane fraction, and no staining was seen in the cytoplasmic fraction. This indicates that menin is located mainly in the nucleus in endocrine GH4C1 cells.

Cell Cycle Regulation of Menin. The expression of some cell cycle regulators such as cyclins, cyclin-dependent kinase (CDK), or CDK inhibitors, changes according to the progression of cell cycle. We therefore examined the expression of menin at different cell cycle stages. We first examined the expression of menin throughout the cell cycle in GH4C1 cells that had been synchronized in G_0 by serum starvation. Cells were released back into the cell cycle by refeeding with 20% serum and analyzed at various times after serum stimulation. The cell cycle profile and menin expression were monitored by flow cytometry of propidium iodide-stained cells and Western blotting, respectively. As shown in Fig. 2, menin was expressed after serum starvation, but at 4−8 h after serum stimulation, menin expression transiently decreased. At this time, the flow cytometry profile indicated that cells had not yet entered S phase. Then, from 12 h onward, as the cells were starting to progress into S phase, menin expression increased. Thus, the induction of menin correlated with the entry of the cells into S phase.

Treatment of Cells with Cell Cycle-synchronizing Agents. We next used mimosine, aphidicolin, and Colcemid for further analysis of regulation of menin expression during the cell cycle. Mimosine and



B

	Asynchronous	0	4	8	12	16	20	24
G_0/G_1	48	71	67	70	55	56	49	54
S	37	18	20	17	24	27	40	26
G_2/M	16	11	13	13	21	17	11	20

Fig. 2. Cell cycle regulation of menin. *A*, total cell lysates (30 μg) from unsynchronized GH4C1 cells (*Asynchronous*) or from cells serum-stimulated for 0, 4, 8, 12, 16, 20, and 24 h (*Lanes 0, 4, 8, 12, 16, 20,* and *24*) after serum starvation for 24 h were separated by SDS-PAGE and immunoblotted with anti-menin antibody. Results of densitometric analysis are indicated *below* the immunoblot. *B*, flow cytometry of propidium iodide-stained cells.

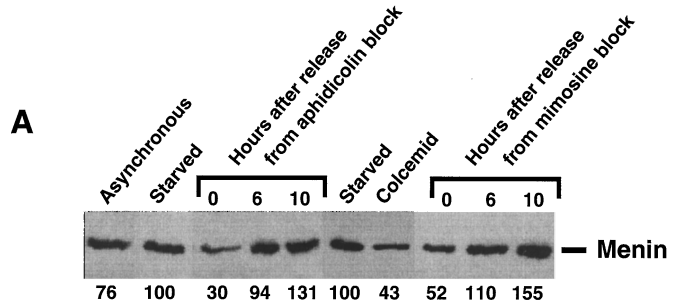
aphidicolin are commonly used as potent and reversible late G_1 and S-phase blockers of the cell cycle. Colcemid is a microtubule inhibitor and M-phase blocker. Mimosine and aphidicolin can synchronize cells released from serum starvation at the G_1-S-phase boundary, whereas Colcemid synchronizes cells released from serum starvation at the G_2-M phase. As shown in Fig. 3, cells synchronized at the G_1-S-phase boundary expressed menin at a lower level than in serum-starved cells. Six or 10 h after release from mimosine or aphidicolin, the expression of menin increased (Fig. 3*A*). Flow cytometry showed that 6–10 h after release from these G_1-S-phase boundary-synchronizing agents, cells were entering into S phase (Fig. 3*B*). Consistent with this, a shift from the G_0-G_1 specific hypophosphorylated form of Rb to the hyperphosphorylated form that exists during cell cycle progression was observed in the cells treated with mimosine (Fig. 3*C*). Ten h after release from mimosine (corresponding to S phase), the amount of the hyperphosphorylated form increased. Thus, the expression of menin increased when the cells were entering into S phase after release from G_1-S-phase boundary blockade. In contrast, cells synchronized at the G_2-M phase expressed menin at a lower level compared to serum-starved cells. Similar findings were observed with another G_2-M phase-synchronizing agent, nocodazole (data not shown).

Subcellular Fractionation of Cells Synchronized at G_0-G_1, G_1-S Phase, or G_2-M Phase. The subcellular localization of some tumor suppressors or other cell growth-regulating factors has been documented to change depending upon the cell cycle stage (14, 15). We therefore examined the subcellular localization of menin at G_0-G_1, G_1-S and G_2-M. As shown in Fig. 4*A*, the relative amount of menin in each fraction was similar in serum-starved, mimosine-treated, and Colcemid-treated cells. This indicates that menin exists mainly in the nucleus throughout the cell cycle in nondividing cells.

Jun D Expression at Different Cell Cycle Stages. The transcription factor Jun D is a direct menin-interacting partner, and menin inhibits Jun D-activated transcription (9). Therefore, we investigated Jun D expression at different cell cycle stages by Western blotting. As shown in Fig. 4*B*, Jun D expression was low after serum starvation, and it increased as cells entered into the progression phase. Next, we examined the effect of various cell-synchronizing agents on Jun D expression (Fig. 4*C*). The cells treated with mimosine expressed Jun D at a higher level compared to serum-starved cells, and 10 h after release from mimosine, Jun D expression increased further. Jun D expression also increased with Colcemid treatment, but to a lesser extent compared to the response 10 h after release from mimosine.

Discussion

A polyclonal antibody raised against a peptide sequence overlapping nuclear localization signal 1 of menin recognized a 69-kDa protein in rat pituitary GH4C1 cells by Western blotting. This likely represents endogenous menin for the following reasons. First, in cells transiently transfected with menin cDNA, the amount of the 69-kDa protein detected was increased. Second, the protein was not detected when antibody preadsorbed with the immunizing peptide was used. And, third, in cells transiently transfected with a FLAG-tagged menin cDNA, a similarly-sized species with FLAG immunoreactivity was detected. By Western blotting, we detected an endogenous 69-kDa menin species in a variety of cell types and species. Thus, menin has



B

	Asynchronous	Starved	Hours after release from aphidicolin block 0	6	10	Starved	Colcemid	Hours after release from mimosine block 0	6	10
G_0/G_1	60	68	62	52	45	71	12	64	31	11
S	21	20	25	39	46	16	18	14	50	57
G_2/M	19	10	13	8	10	13	70	23	19	32

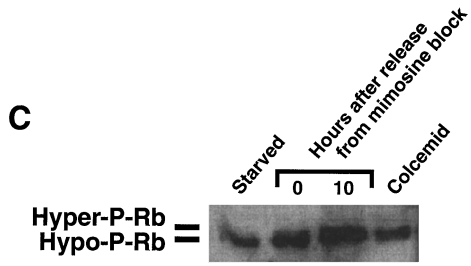


Fig. 3. Cell cycle synchronization and menin protein expression. *A*, GH4C1 cells were serum-starved for 24 h; cultured with complete media including 12 μM aphidicolin, 400 μM mimosine, or 1 μg/ml Colcemid for 24 h; and then released from cell cycle blockade by culture in complete media for the indicated times (h). Total cell lysates (30 μg) were examined with anti-menin antibody by Western blotting. Results of densitometric analysis are indicated *below* the immunoblot. *B*, flow cytometry of propidium iodide-stained cells. *C*, Western blot of nuclear extracts (20 μg) probed with anti-Rb antibody.

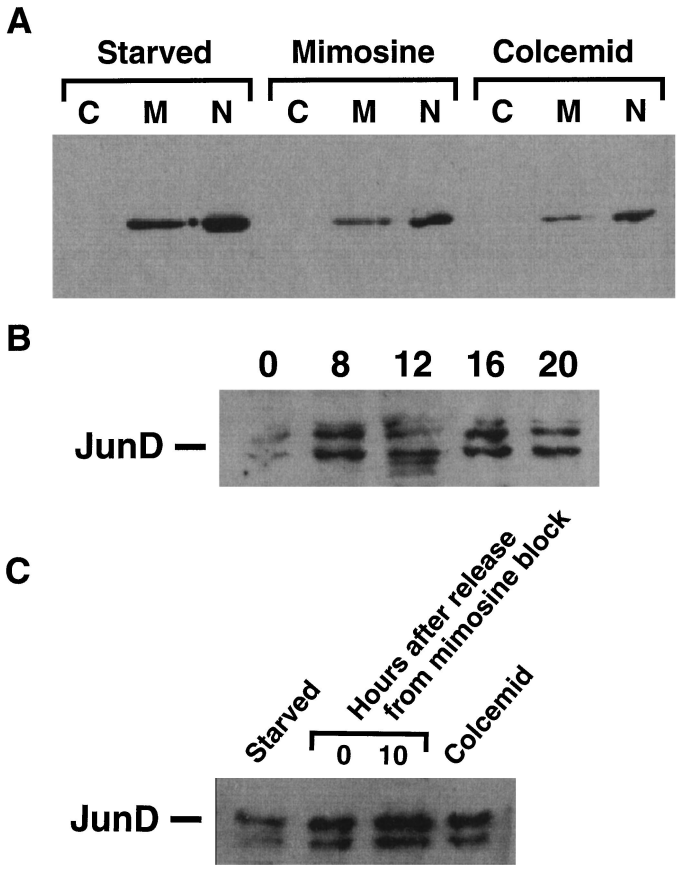


Fig. 4. *A*, subcellular localization of menin at different cell cycle stages. GH4C1 cells were serum-starved for 24 h and cultured with complete media including 400 μM mimosine or 1 μg/ml Colcemid for 24 h. Nuclear (*N*), membrane (*M*), or cytoplasmic (*C*) fractions of GH4C1 cells were separated by SDS-PAGE (20 μg protein/lane) and immunoblotted with anti-menin antibody. Jun D expression is shown at different cell cycle stages. GH4C1 cells were serum-starved for 24 h. *B*, cells were then stimulated with serum and harvested at the indicated times (h). *C*, cells were cultured with complete media including 400 μM mimosine or 1 μg/ml Colcemid for 24 h, and then the cell cycle blockade was released by culture in complete media for the indicated times (h). Nuclear extracts were prepared, 20-μg aliquots were separated by SDS-PAGE, and Western blotting was carried out with anti-Jun D antibody.

a wide tissue distribution, is present in endocrine and nonendocrine cells, and is conserved as a similarly sized protein across rodent, simian, and human species.

Menin was initially shown to be a nuclear protein by studies of nonendocrine cells (8). Here we have demonstrated that menin is predominantly nuclear in rat pituitary GH4C1 cells. Thus, menin could function in transcriptional regulation, cell cycle progression, DNA repair, or DNA replication in endocrine cells. The present study has examined the expression level of menin throughout the cell cycle and, by comparison with other tumor suppressors or cell growth regulators, this may provide clues as to the type of functions menin subserves.

Tumor suppressors BRCA-1 and BRCA-2 are poorly expressed in quiescent cells (10, 11, 16). By contrast, as shown in the present study, menin is relatively well-expressed in quiescent cells at G_0-G_1. The cdk inhibitors, p21 and p27, are also well-expressed in quiescent cells (17, 18), and therefore, potentially, menin may function like these CDK inhibitors. As shown here, the levels of menin transiently decrease as the GH4C1 cells enter the cycle and then increase again as the cells enter S phase from the G_1-S-phase boundary onward. It is at this same stage that the expression of tumor suppressors such as BRCA-1, BRCA-2, and p53 increases (10, 16, 19). Thus, menin may play some role at the G_1-S-phase checkpoint, analogous to BRCA-1, BRCA-2, and p53.

The precise subcellular localization of some proteins is dependent on cell cycle stage and/or cell density. For example, the von Hippel-Lindau tumor suppressor gene is found predominantly in the cytoplasm in dense cell cultures (G_2-M) but is also found in the nucleus in sparse cell cultures (G_0-G_1; Refs. 16 and 20). Thus, nuclear translocation is often closely linked to the cell cycle. In the present study, by using subcellular fractionation of synchronized cell populations, we did not find any evidence for the subcellular localization of menin changing to any major extent with it being predominantly nuclear throughout the cell cycle. In support of this, it was recently reported that in a pituitary tumor cell line derived from a MEN1 patient and in HEK293 cells transfected with an epitope-tagged menin cDNA, menin was found by immunofluorescence to be nuclear in nondividing cells (21). However, immediately after cell division, some staining was observed in the cytoplasm. In the present study, in GH4C1 cells synchronized in G_2-M using two different blocking agents, Colcemid and nocodazole, we did not find significant amounts of menin in the cytoplasm. The apparent difference in findings may relate to the different methodologies used. Additionally, it may be that there are posttranslationally modified forms of menin found in different locations in the cell, and that some antibodies selectively recognize these modified proteins. This remains to be determined.

Menin has been identified as an interacting partner of the activator protein 1 transcription factor Jun D and represses Jun D-activated transcription (9). Several studies suggest that Jun D has antimitogenic activity, in contrast to other Jun and Fos family members (22, 23). Because menin is a putative tumor suppressor gene, its repressive effect on Jun D-mediated transcriptional activation is paradoxical, and the significance of menin and Jun D binding remains unknown. The expression of Jun D is generally constitutive and is relatively refractory to growth factor stimulation (24). The relative expression of Jun D at different cell cycle phases is dependent on the cell type. In fully differentiated postmitotic cells, Jun D mRNA is generally higher than that of either c-Jun or Jun B (25), suggesting that Jun D protein may have a unique role in resting cells. In the present study, Jun D expression was demonstrated to be higher in proliferating cells than in resting GH4C1 tumor cells. This is compatible with the Jun D expression patterns in NIH3T3 cells and osteoblastic-like osteosarcoma cells (26, 27). In the GH4C1 cells, Jun D expression paralleled that of menin, and when the cells entered S phase, the amounts of both proteins increased. Considering that both Jun D and menin are antimitogenic, it is possible that their mutual binding suppresses the growth-inhibitory action of each molecule. However, whereas menin is a regulator of Jun D action, Jun D may not be the main mediator of menin action.

In summary, we have demonstrated that menin is predominantly located in the nucleus, and its expression is regulated during cell cycle progression in a rat anterior pituitary tumor cell-line. Additional studies will be required to determine the factors regulating alterations of menin during the cell cycle and to ascertain its precise role in modulating cell cycle kinetics.

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