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Cell Cycle Regulation of Menin Expression

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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 G1-S-phase boundary expressed menin at a lower level than G0-G1-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 G2-M phase expressed lower levels of menin. At G0-G1, G1-S, and G2-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 is an autosomal dominant disorder characterized by multiple endocrine 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 localized 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 in mouse fibroblasts, and Chinese hamster ovary cells (8). Menin inter-

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 (Mississauga, Ontario, Canada).

Menin Antibody and cDNA. A peptide, NH2-REGRRRGPRRESKPC-

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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.

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

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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 mM NaCl, 1.5 mM MgCl2, 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 mM 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 G0-G1 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, G0-G1-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 G1-S-phase boundary (mimosine or aphidicolin) or G2-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 FACSscan (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. 1A). As shown in Fig. 1B, 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 TFIH p89 and β-tubulin as controls for nuclear and cytoplasmic fractions, respectively. As shown in Fig. 1C, 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 G0 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
aphidicolin are commonly used as potent and reversible late G1 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 G1-S-phase boundary, whereas Colcemid synchronizes cells released from serum starvation at the G2-M phase. As shown in Fig. 3, cells synchronized at the G1-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. 3A). Flow cytometry showed that 6–10 h after release from these G1-S-phase boundary-synchronizing agents, cells were entering into S phase (Fig. 3B). Consistent with this, a shift from the G0-G1 specific hypophosphorylated form of Rb to the hyperphosphorylated form that exists during cell cycle progression was observed in the cells treated with mimosine (Fig. 3C). 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 G1-S-phase boundary blockade. In contrast, cells synchronized at the G2-M phase expressed menin at a lower level compared to serum-starved cells. Similar findings were observed with another G2-M phase-synchronizing agent, nocodazole (data not shown).

**Subcellular Fractionation of Cells Synchronized at G0-G1, G1-S Phase, or G2-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 G0-G1, G1-S and G2-M. As shown in Fig. 4A, 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. 4B, 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. 4C). 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...
and harvested at the indicated times (h). C
blockade was released by culture in complete media for the indicated times (h). Nuclear
mimosine or 1 µg/ml Colcemid for 24 h. Nuclear (N), membrane (M), or cytoplasmic (C)
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)
were serum-starved for 24 h and cultured with complete media including 400 µM
mimosine or 1 µg/ml Colcemid for 24 h, and then the cell cycle
The precise subcellular localization of some proteins is dependent on
play some role at the G1-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 cyto-
plasm in dense cell cultures (G2-M) but is also found in the nucleus in
sparse cell cultures (G1-G2; Refs. 16 and 20). Thus, nuclear translo-
cation 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
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, me-
in 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 G2-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
posttranscriptionally modified forms of menin found in different loca-
tions 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 refrac-
tory 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 anti-
mitogenic, 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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References

1. Metz, D. C., Jensen, R. T., Bale, A. E., Skarnulis, M. C., Eastman, R. C., Nieman, L.,


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