Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1630–1634, February 1998
Cell Biology

Menin, the product of the *MEN1* gene, is a nuclear protein

SIRADANAHALLI C. GURU*, PAUL K. GOLDSMITH†, A. LEE BURNS†, STEPHEN J. MARX†, ALLEN M. SPIEGEL†, FRANCIS S. COLLINS*, AND SETTARA C. CHANDRASEKHARAPPA*‡

*Genetics and Molecular Biology Branch, National Human Genome Research Institute, and †Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Francis S. Collins, December 24, 1997

ABSTRACT **The *MEN1* gene, mutations in which are responsible for multiple endocrine neoplasia type 1 (MEN1), encodes a 610-amino acid protein, denoted menin. The amino acid sequence of this putative tumor suppressor offers no clue to the function or subcellular location of the protein. We report herein, based on immunofluorescence, Western blotting of subcellular fractions, and epitope tagging with enhanced green fluorescent protein, that menin is located primarily in the nucleus. Enhanced green fluorescent protein-tagged menin deletion constructs identify at least two independent nuclear localization signals (NLS), both located in the C-terminal fourth of the protein. Among the 68 known independent disease-associated mutations, none of the 22 missense and 3 in-frame deletions affect either of the putative NLS sequences. However, if expressed, none of the truncated menin proteins resulting from the 43 known frameshift/nonsense mutations would retain both the NLSs. The precise role(s) of menin in the nucleus remain to be understood.**

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder in which affected individuals variably develop tumors in the parathyroids, anterior pituitary, and enteropancreatic endocrine tissue (1). Recently, we identified the gene responsible for MEN1 (2), and germ-line mutations in this gene have been described for nearly all the fifty-nine MEN1 probands reported so far (3, 4). Also, somatic mutations in the *MEN1* gene have been identified in variable fractions of sporadic parathyroid adenomas, gastrinomas, insulinomas, lung carcinoids, and pituitary tumors (5–8). The nature of the mutations, which are consistent with a loss-of-function mechanism, the observation that the wild-type allele is consistently lost in tumors arising in patients with MEN1, and the observation that both alleles of the *MEN1* gene are often inactivated in sporadic tumors indicate that tumorigenesis is very likely due to loss of function of the *MEN1*-encoded protein menin. Thus the *MEN1* gene seems to be an excellent example of a classic tumor suppressor.

Analysis of the predicted menin amino acid sequence does not show homology to any known protein in the database, nor does it disclose any apparent sequence motifs, providing no clues as to the function of the protein. As a first step toward elucidation of the role of menin in tumorigenesis, we have designed experiments to identify its subcellular location and demonstrate herein that the majority of the protein resides in the nucleus. At least two independent nuclear localization signals (NLSs), both located in the C-terminal fourth of the protein, have been identified by deletion analysis.

MATERIALS AND METHODS

Generation of pcDNA3.1-Menin and EGFP-Menin Constructs. The isolation of the pCMV-Sport menin clone (A11) containing a full-length menin cDNA from a human leukocyte cDNA library has been described (2, 9). The coding region of menin from the A11 clone was amplified by PCR and cloned into the *Eco*RI site of pcDNA3.1(−) Myc-His (Invitrogen) in-frame with and upstream of myc-His epitope sequences to generate pcDNA3.1-menin construct. The same fragment was cloned into the enhanced green fluorescent protein (EGFP) expression vectors (CLONTECH), pEGFP-C2 (see Fig. 3*A*, EGFP1) or pEGFP-N2 to generate menin constructs tagged with EGFP at the N or C terminus, respectively. Each construct was characterized by restriction analysis and sequencing of the coding regions.

Deletion constructs EGFP2–EGFP9 (see Fig. 3*A*) were generated by taking advantage of conveniently placed restriction sites in the construct EGFP1. Constructs EGFP2–EGFP5 were generated by digestion of the plasmid EGFP1 with enzymes *Acc*I, *Apa*I, *Kpn*I, and *Sma*I, respectively, to release fragments representing C-terminal menin deletions of various lengths, followed by recircularization of the remaining larger fragment. Similarly, the N-terminal deletion constructs EGFP7 and EGFP9, as well as the internal deletion construct EGFP8, were generated by using the enzymes, *Bgl*II, *Xho*I, and *Nae*I, respectively. EGFP6 was obtained as a result of a three-way ligation including two *Xho*I fragments, together representing the N-terminal 571 amino acids, to the vector EGFP-C2. The remaining three constructs, EGFP10–EGFP12, were generated starting from EGFP1 by an oligonucleotide-based site-directed mutagenesis method. EGFP10 and EGFP11 were mutants that contain engineered sequences to terminate menin at amino acids 587 and 603, respectively. EGFP12 is engineered to result in the deletion of 12 nucleotides, eliminating amino acids 588–591. The following forward and reverse primers were used to generate constructs EGFP10–EGFP12 by using the Quick Change site-directed mutagenesis kit (Stratagene): EGFP10F, GTCGCAAGTGCAGATGTAGAAGCAGAAAGTGTCC; EGFP10R, GGACACTTTCTGCTTCTACATCTGCACTTGCGAC; EGFP11F, GCACAGTCGCAAGTGCAGATGGTGTCCACCCCTAGTGAC; EGFP11R, GTCACTAGGGGTGGACACCATCTGCACTTGCGACTGTGC; EGFP12F, GACTACACTCTGTCTTTCCTCTAGCGGCAGCGCA; EGFP12R, GCGCTGCCGCTAGAGGAAAGACAGAGTGTAGTC.

Antibodies. Four polyclonal antibodies, KC27, SQV, AEA, and LEE, were generated in rabbits by immunization with synthetic peptides corresponding to menin residues 585–610 (VQMKKQKVSTPSDYTLSFLKRQRKGL), 583–610 (SQVQMKKQKVSTPSDYTLSFLKRQRKGL), 465–492 (AEAEEPWGEEAREGRRRGPRRESKPEEP), and 286–307 (LEELEPTPGRPDPLTLYHKGIA), respectively. Peptide conjugation, immunization, and antibody affinity purification

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/98/951630-5$0.00/0
PNAS is available online at http://www.pnas.org.

Abbreviations: EGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; DAPI, 4,6-diamidino-2-phenylindole.

‡To whom reprint requests should be addressed at: Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Building 49, Room 3E-13, 49 Convent Drive, Bethesda, MD 20892-4442. e-mail: chandra@nhgri.nih.gov.

on peptide-linked beads were as described (10). Mouse monoclonal antibodies specific for nuclear pore complexes (Babco, Richmond, CA) and tubulin (Oncogene Research Products) were used on immunoblots as markers for nuclear and cytoplasmic fractions, respectively. A monoclonal antibody for the myc epitope was obtained from Invitrogen.

Detection of Menin in Transiently Transfected Cells by Immunofluorescence and Green Fluorescence. Cells (HEK-293T, NIH 3T3, or CHO) were grown on coverslips in DMEM containing 10% fetal calf serum in a 60-mm plate to approximately 40% confluence. Transfection was carried out by using Lipofectamine (Life Technologies) with plasmid DNA (8–10 μg) expressing either full-length or truncated menin, as well as DNA from the respective vectors alone, pcDNA3.1 and EGFP-C2, for immunofluorescence and green fluorescence, respectively. Twenty-four hours after the transfection, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized by fluorescent microscopy, and the images were collected from a charge-coupled device camera.

For immunofluorescence, cells transfected with pcDNA 3.1-menin on the coverslip as above were washed three times with PBS, blocked with 5% BSA for 5 min, and washed again with PBS, followed by incubation for 2 hr at room temperature with affinity-purified menin antibody (KC27; 20 μg/ml) or myc antibody (1 μg/ml) and 5% BSA in PBS. After washing three times with PBS, coverslips were flooded with fluorescein isothiocyanate-tagged anti-rabbit/anti-mouse secondary antibodies (10 μg/ml) in 5% BSA and incubated for 30 min. After several washes with PBS, cells were stained with DAPI.

Subcellular Fractionation, SDS/PAGE, and Western Blot Analysis of Untransfected and Transiently Transfected Cells. The pCMV-Sport menin clone (A11) containing a full-length menin cDNA isolated from a human leukocyte library (2, 9) was used for transfection. To create a vector-only control, the menin insert was removed by digestion with *Mlu*I and the vector was religated. Flasks (162 cm^2) were seeded with 20–30 million HEK-293T cells per flask in DMEM supplemented with 10% serum 1 day before transfection. Transfection was performed with 10 μg of plasmid DNA (vector with or without menin cDNA) per flask and the Superfect reagent from Qiagen (Santa Clarita, CA). The day after transfection, medium was replaced with DMEM without serum. Forty-eight hours after transfection, cells were harvested by washing with PBS and mechanical scraping from the flask. Cells were pelleted and kept on ice until fractionation. Fractionation of vector-only and menin-transfected HEK-293T cells into nuclear, membrane, and cytoplasm fractions was performed as described (11) with the following modifications: *N*-ethyl maleimide was eliminated from all buffers; aprotinin was set at 0.02%; 1 mM [4-(2-aminoethyl)-benzene sulfonylfluoride] (ICN) was substituted for 1 mM phenylmethylsulfonyl fluoride; and 2-mercaptoethanol was set at 5% (vol/vol). Immunoblot analysis of cell fractions on nitrocellulose membranes was performed after electrophoresis on 10% denaturing polyacrylamide gels (10), using the SQV (2 μg/ml), AEA (5 μg/ml), LEE (5 μg/ml), nuclear pore protein (1 μg/ml), and tubulin (5 μg/ml) antibodies. Protein concentrations of antibodies and cell fractions were determined by a dye binding assay (Bio-Rad).

RESULTS

Detection of Menin in the Nucleus of Cells Transiently Expressing Menin by Immunofluorescence. The complete coding region of menin was cloned into the mammalian expression vector pcDNA3.1, in-frame with the Myc-His epitope sequences, which were placed at the C terminus. HEK-293T (simian virus 40 tumor antigen-transformed human embryonic kidney cells) cells, transiently transfected with the pcDNA 3.1-menin construct, were analyzed by immunofluorescence with antibodies (KC27) raised against a peptide representing the C-terminal 26 amino acids of menin, as well as monoclonal antibodies for the myc epitope. Both antibodies detected fluorescence only in the nucleus (Fig. 1). Similar observations were also made with CHO (Chinese hamster ovary) cells transfected with the same plasmid (data not shown). These results show that menin is located primarily in the nucleus. The menin antibodies failed to detect endogenous menin in untransfected HEK-293T cells by immunofluorescence, presumably due to lower abundance of the protein in individual cells.

Detection of Menin by Western Blotting of Subcellular Fractions. The nuclear localization of menin was confirmed by subcellular fractionation and Western blot analysis. A similar antibody (SQV) raised against a synthetic peptide corresponding to the C-terminal 28 amino acids of menin reacted on immunoblots with full-length menin expressed in *Escherichia coli* (data not shown). SQV antibody was able to detect endogenous menin as an ~67-kDa band localized predominantly to the nuclear fraction with a smaller amount in the membrane fraction of HEK-293T cells (Fig. 2 *Top*). In addition to menin, SQV detects a more rapidly migrating band in the membrane and cytoplasmic fractions but not the nuclear fraction of vector-only transfected cells (Fig. 2 *Top*). We presume this band represents a protein cross-reacting with SQV because its appearance is blocked by the SQV peptide (data not shown), but the protein is not derived from menin itself because its abundance does not increase with menin transfection. In menin-transfected cells, a substantial increase in overall immunoreactivity compared with vector-only transfected cells was observed with SQV antibody. Menin was again predominantly localized in the nuclear fraction, but immunoreactivity was also detected in membrane and cytoplasmic fractions of menin-transfected cells (Fig. 2). In addition to the ~67-kDa menin band, bands of higher and lower mobility were seen with SQV in the nuclear fraction of menin-transfected cells. These appear to be menin-related because their appearance is dependent upon menin transfection. Their significance is uncertain, but the more rapidly migrating band could represent a proteolytic fragment of menin. Western blots with additional antibodies, AEA and LEE, raised against distinct peptides (residues 465–492 and 286–307, respectively) from the menin amino acid sequence confirmed the subcellular distribution of menin in vector-only and menin-transfected cells seen with SQV (data not shown). Because the Western blotting is sufficiently sensitive to detect endogenous menin in the nucleus as well, the nuclear localization observed by immunofluorescence in pcDNA3.1-menin transfected cells is not likely to be an artifact of overexpression.

EGFP-Tagged Menin Deletion Constructs Identify Two NLSs in Menin. EGFP-tagged constructs expressing full-length and truncated versions of menin were constructed. The extent of the menin coding sequence represented and the location of the green fluorescence observed in the transfected cells for each deletion construct is shown in Fig. 3. Stability of the protein product was confirmed by the presence of strong fluorescence in transfected, but not sham-transfected, cells for each construct. HEK-293T cells, transfected with constructs expressing full-length menin tagged with EGFP at either the N terminus (EGFP1) (Fig. 3) or the C terminus (data not shown) were found to generate green fluorescence only in the nucleus. All the C-terminal deletion constructs expressing various lengths of the menin coding region up to amino acid 476 (EGFP2–EGFP5) were found to display green fluorescence in the cytoplasm, indicating that no NLS is present in the first 476 amino acids. However, a construct extending up to amino acid 571 (EGFP6) targets the protein to the nucleus, suggesting that a NLS must be present in the additional 95 amino acids (NLS-1). Analysis of this sequence identified a stretch of 19 amino acids (from positions 479 to 497, RRRG-

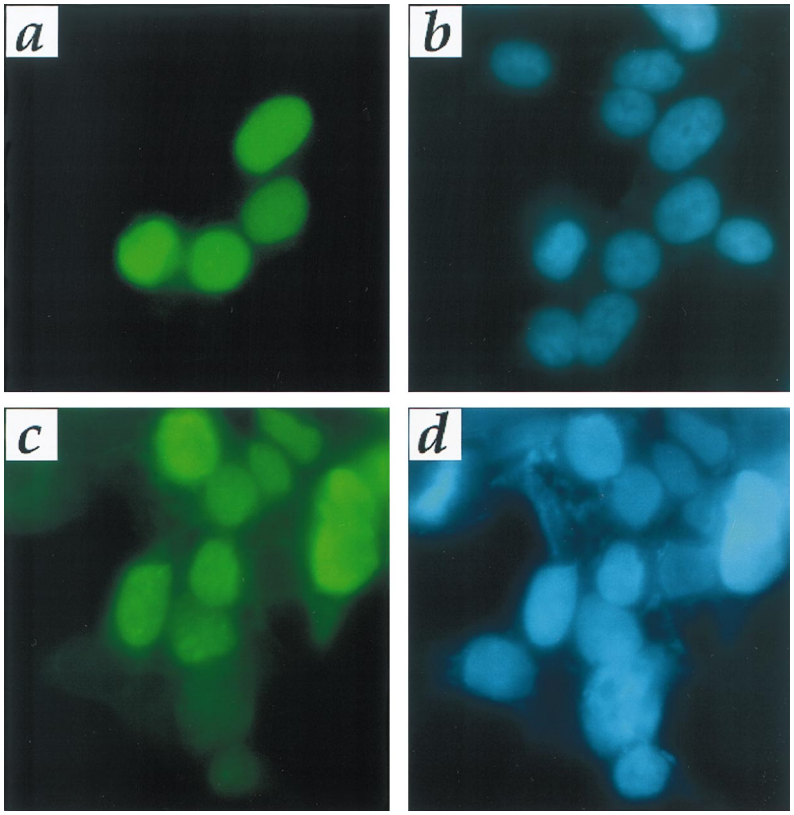

FIG. 1. Immunofluorescence of menin-transfected HEK-293T cells with menin and myc epitope antibodies. Twenty-four hours after transfection with pcDNA3.1-menin, cells were processed for immunofluorescence with menin antibody (KC27) or with myc antibody followed by fluorescein isothiocyanate-conjugated secondary antibody detection. Immunofluorescence pattern with menin antibody (*a*) and the DAPI staining (*b*) showing the nuclei from the same cells. Immunofluorescence with myc antibody (*c*) and the DAPI staining (*d*) showing the nuclei from the same cells. Note that not all cells are positive, because this is a transient transfection. Endogenous levels of menin are not detectable above background.

PRRESKPEEPPPPKK). Four basic amino acid residues out of a stretch of 6 residues can sometimes function as a single basic type NLS signal (12–15), and the region between positions 479 and 485 (RRRGPRR) would fit this. Alternatively, two strings of 2 or 3 basic residues (Arg or Lys) separated by a stretch of 10–12 amino acids have been shown to function in some proteins as a bipartite basic type NLS (13, 15). It is possible that the sequence from positions 484 to 497 (*RR*ESKPEEPPPP*KK*, where the basic residues mentioned are italic) within this stretch of 19 amino acids could function in this manner.

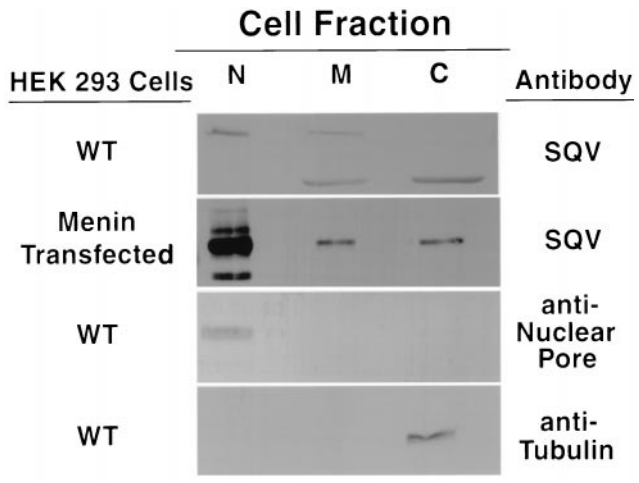

FIG. 2. Immunoblot of representative nuclear (N), membrane (M), and cytoplasmic (C) fractions of HEK-293T cells transfected with vector only (WT) or with menin. Fifty micrograms of protein was loaded for each fraction for the menin blot in WT cells, 5 μg of protein was used for the menin blot in menin-transfected cells, 25 μg of protein is present in each lane for the nuclear pore protein blot, and 12.5 μg of protein was used per lane for the tubulin blot. The blots shown for tubulin and for nuclear pore protein were from WT cells, but blots of fractions from menin-transfected cells gave comparable results.

Analysis of N-terminal deletion constructs identified another NLS (NLS-2) within the C-terminal 39 amino acids of menin, as can be seen from the results of construct EGFP9 (Fig. 3). The sequence from positions 588 to 608 (*KKQK*VSTPSDYTLSFL*KRQRK*) in this region could provide a bipartite NLS and/or the sequence from positions 604 to 608 (KRQRK) could function as a single basic type NLS. Three additional constructs that eliminate NLS-2 completely (EGFP10) or delete either component of this bipartite signal (EGFP11 and EGFP12) still targeted menin to the nucleus, presumably because they all still carried NLS-1.

DISCUSSION

Immunofluorescence, Western blot analysis of subcellular fractions, and epitope tagging with EGFP indicate that menin is primarily located in the nucleus. Many proteins that localize to the nucleus contain a polybasic motif, the NLS, which is necessary for proper nuclear targeting (12–15). Although no precise match to the consensus NLS sequence is present in menin, we hypothesized that such signal(s) might exist. Studies with EGFP-tagged menin deletion constructs indicate that at least two functionally independent NLSs exist in the menin protein. There are several prior examples of proteins carrying multiple functional NLSs. For instance, SRY and SOX9, members of the family of high-mobility group domain transcription factors, each contain two independent NLSs (16).

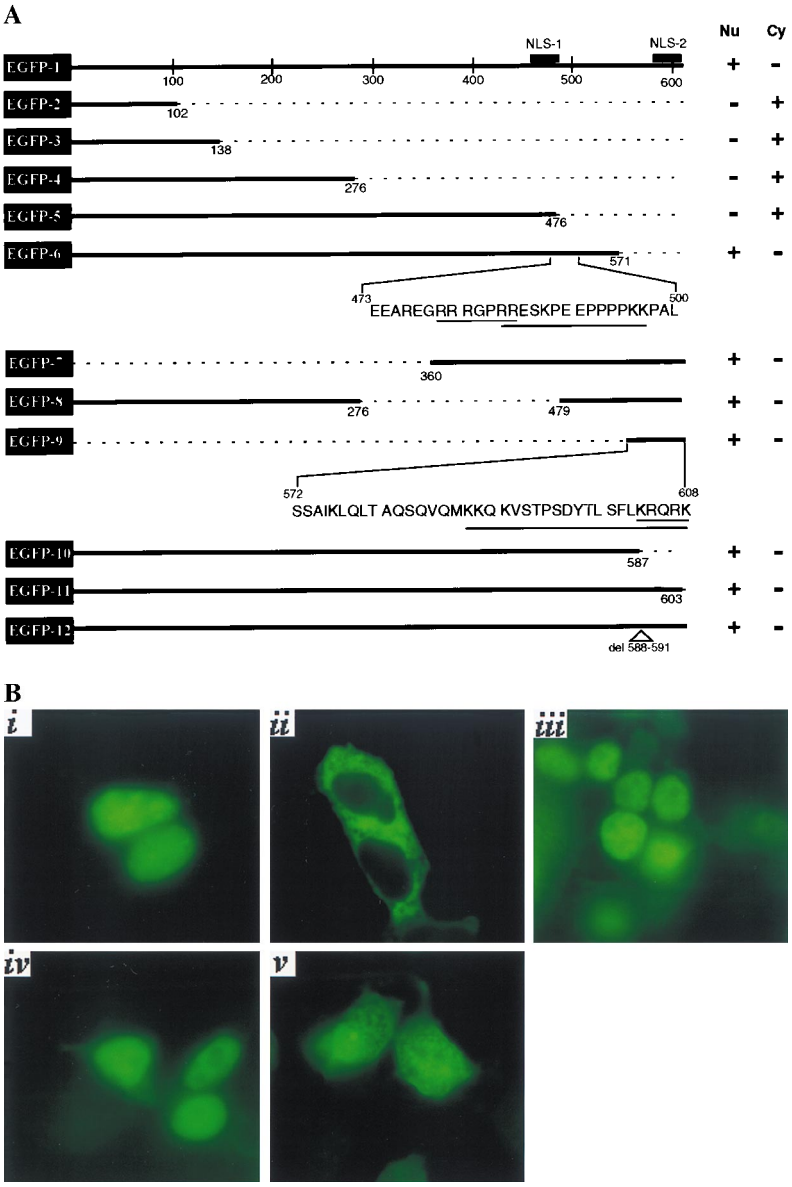

FIG. 3. (*A*) Schematic of deletion constructs of menin fused to EGFP and localization of green fluorescence observed in the transfected cells. All constructs (EGFP1–EGFP12) were generated with EGFP (boxed) at the N terminus of the menin coding region. The extent of the menin coding region retained in each construct is represented by a solid thick line and the deleted region is represented by a dotted thin line. The position of the starting and the terminating amino acid in each deletion construct is also indicated, except for amino acids 1 and 610. The presence (+) or absence (−) of green fluorescence observed in the nucleus (Nu) or cytoplasm (Cy) for each construct is shown. The sequence around the two putative independent NLSs mapped in this study (NLS-1 and NLS-2) are shown for constructs EGFP6 and EGFP9, respectively. Underlined sequences represent potential single basic type and bipartite sequences in both NLS-1 and NLS-2. (*B*) Representative examples of green fluorescence in HEK-293T cells transfected with EGFP-menin fusion constructs. The presence of green fluorescence in the nucleus or cytoplasm of cells transfected with EGFP1 (*i*), EGFP5 (*ii*), EGFP6 (*iii*), and EGFP9 (*iv*) is shown. The diffuse pattern of fluorescence observed in cells transfected with EGFP vector alone (*v*) is also shown

Both NLS-1 (amino acids 479–497) and NLS-2 (amino acids 588–608) are present in the C-terminal fourth of menin. However, none of the known 22 missense and 3 in-frame germ-line or somatic MEN1 single amino acid deletion mutations fall within either of the putative NLS-1 and NLS-2 sequences (3–8). Thirty-nine of the 43 known frameshift/nonsense mutations would result in a truncated menin lacking both NLS-1 and NLS-2 and, therefore, should be retained in the cytoplasm. The truncated proteins from the four most distal mutations should retain the NLS-1 and, if expressed, would be predicted to localize to the nucleus. Because there are no discernible phenotypic variations associated with these four MEN1 mutations (3, 5, 7), it is unclear whether there is any functional significance to whether a truncated menin protein is located in the nucleus or cytoplasm.

It is possible that some truncated mutant proteins present in MEN1 patients are unstable and degraded. However, the clear signal either in the nucleus or cytoplasm from the cells expected to express truncated EGFP-tagged proteins suggest that truncated versions of menin are partially stable, at least in this cell culture system.

The nuclear localization of menin suggests a variety of possible functions, such as serving as a component in transcriptional regulation, DNA replication, or cell cycle control. Certainly there are many other tumor suppressor genes whose normal protein products are found in the nucleus. But the precise role played by menin in the regulation of endocrine cell growth control will require additional investigation.

We thank Regina Collins for expert assistance with transfection and cell culture. We thank Drs. Neeraj Adya and Kevin Brown for their

assistance with immunofluorescence and Dr. Jeff Trent for reviewing the manuscript.

1. Metz, D. C., Jensen, R. T., Bale, A. E., Skarulis, M. C., Eastman, R. C., Nieman, L., Norton, J. A., Friedman, E., Larsson, C., Amorossi, A. *et al.* (1994) in *The Parathyroids*, eds. Bilezekian, J. P., Levine, M. A. & Marcus, R. (Raven, New York), pp. 591–646.
2. Chandrasekharappa, S. C., Guru, S. C., Manickam, P., Olufemi, S. E., Collins, F. S., Emmert-Buck, M. R., Debelenko, L. V., Zhuang, Z., Lubensky, I. A., Liotta, L. A., *et al*. (1997) *Science* **276,** 404–407.
3. Agarwal, S. K., Kester, M. B., Debelenko, L. V., Heppner, C., Emmert-Buck, M. R., Skarulis, M. C., Doppman, J. L., Kim, Y. S., Lubensky, I. A., Zhuang, Z., *et al*. (1997) *Hum. Mol. Genet.* **6,** 1169–1175.
4. The European Consortium on MEN1. (1997) *Hum. Mol. Genet.* **6,** 1177–1183.
5. Heppner, C., Kester, M. B., Agarwal, S. K., Debelenko, L. V., Emmert-Buck, M. R., Guru, S. C., Manickam, P., Olufemi, S. E., Skarulis, M. C., Doppman, J. L., *et al*. (1997) *Nat. Genet.* **16,** 375–378.
6. Zhuang, Z., Vortmeyer, A. O., Pack, S., Huang, S., Pham, T. A., Wang, C., Park, W. S., Agarwal, S. K., Debelenko, L. V., Kester, M., *et al*. (1997) *Cancer Res.* **57,** 4682–4686.
7. Debelenko, L. V., Brambilla, E., Agarwal, S. K., Swalwell, J. I., Kester, M. B., Lubensky, I. A., Zhuang, Z., Guru, S. C., Manickam, P., Olufemi, S. E., *et al*. (1997) *Hum. Mol. Genet.* **6,** 2285–2290.
8. Zhuang, Z., Ezzat, S. Z., Vortmeyer, A. O., Weil, R., Oldfield, E. H., Park, W. S., Pack, S., Huang, S., Agarwal, S. K., Guru, S. C., *et al*. (1997) *Cancer Res*. **57,** 5446–5451.
9. Guru, S. C., Agarwal, S. K., Manickam, P., Olufemi, S.-E., Crabtree, J., Weisemann, J. M., Kester, M. B., Kim, Y. S., Emmert-Buck, M. R., Liotta, L. A. *et al.* (1997) *Genome Res.* **7,** 725–735.
10. Goldsmith, P. K., Gierschik, P., Milligan, G., Unson, C. G., Vinitsky, R., Malech, H. & Spiegel, A. M. (1987) *J. Biol. Chem.* **262,** 14683–14688.
11. Abrams, H. D., Rohrschneider, L. R. & Eisenman, R. N. (1982) *Cell* **29,** 427–439.
12. Boulikas, T. (1994) *J. Cell. Biochem.* **55,** 32–58.
13. Yoneda, Y. (1997) *J. Biochem.* **121,** 811–817.
14. Garcia-Bustos, J., Heitman. J. & Hall, M. N. (1991) *Biochem. Biophys. Acta* **1071,** 83–101.
15. Dingwall, C. & Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481.
16. Sudbeck, P. & Scherer, G. (1997) *J. Biol. Chem.* **272,** 27848–27852.