The Nucleo-cytoplasmic Actin-binding Protein CapG Lacks a Nuclear Export Sequence Present in Structurally Related Proteins

Katrien Van Impe‡§, Veerle De Corte‡§, Ludwig Eichinger¶, Erik Bruyneel**, Marc Marcel**, Joël Vandekerckhove‡, and Jan Gettemans¶†¶†‡

From the ‡Department of Biochemistry, Flanders Interuniversity Institute for Biotechnology, Faculty of Medicine and Health Sciences, Ghent University, Rommelaere Institute, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium and Flanders Interuniversity Institute for Biotechnology, the Institut für Biochemie I, Medizinische Fakultät, Universität zu Köln, Joseph-Stelzmann-Strasse 52, 50931 Köln, Germany, and the **Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

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Despite thorough structure-function analyses, it remains unclear how CapG, a ubiquitous F-actin barbed end capping protein that controls actin microfilament turnover in cells, is able to reside in the nucleus and cytoplasm, whereas structurally related actin-binding proteins are predominantly cytoplasmic. Here we report the molecular basis for the different subcellular localization of CapG, severin, and fragminP. Green fluorescent protein-tagged fragminP and severin accumulate in the nucleus upon treatment of transfected cells with the CRM1 inhibitor leptomycin B. We identified a nuclear export signal in severin and fragminP, which is absent in CapG. Deletion of amino acids Met1–Leu27 resulted in nuclear accumulation of severin and fragminP. Tagging this sequence to CapG triggered nuclear export, whereas mutation of single leucine residues (Leu17, Leu21, and Leu27) in the export sequence inhibited nuclear export. Based on these findings, a nuclear export signal was identified in myopodin, a muscle-specific actin-binding protein, and the Bloom syndrome protein, a RecQ-like helicase. Deletion of the myopodin nuclear export sequence blocked invasion into collagen type I of C2C12 cells transiently overexpressing myopodin. Our findings explain regulated subcellular targeting of distinct classes of actin-binding proteins.

In eukaryotic cells, the nucleus and cytoplasm are separated by the nuclear envelope. Macromolecular traffic occurs via nuclear pore complexes, huge proteinaceous structures that span the double membrane that allow transport in essentially two modes: energy-independent passive diffusion and energy-dependent facilitated translocation (for reviews see Refs. 1 and 2). Passive diffusion is efficient for small molecules but becomes slow and inefficient as the size approaches a limit of 20–40 kDa. In contrast, facilitated translocation allows passage of macromolecules as large as several megadaltons and proceeds via specific nuclear transport receptors able to associate with components of the nuclear pore complex as well as cargo molecules that are translocated across the pore. Cargo molecules are recognized via import or export targeting signals, referred to as nuclear localization signals or nuclear export signals (NES), respectively (1–3).

There are several types of nuclear localization signals, which mediate nuclear import by direct binding to one or more importin receptors of the α or β type (reviewed in Ref. 3). In contrast, the pathways and details of signal-mediated nuclear export are less well defined. The best characterized nuclear export signal is the small, hydrophobic, leucine-rich NES, identified initially in the human immunodeficiency virus type 1 Rev protein (4) and the heat-stable inhibitor of cAMP-dependent protein kinase (PKI) (5). Structurally and functionally related export sequences have since been detected in many cellular and viral proteins (6, 7), including actin (8). Direct interaction with the export factor CRM1 (exportin 1) is essential for the export of proteins containing a leucine-rich NES (9).

An increasing number of actin-binding proteins has been reported to shuttle between nucleus and cytoplasm. Already in 1993, Onoda et al. (10) showed that CapG (Mbh1 or gCap39), a ubiquitous 39-kDa barbed end F-actin-binding protein particularly abundant in macrophages (11), is a nuclear and cytoplasmic protein. CapG does not contain a canonical nuclear localization signal, but it has been suggested that phosphorylation of CapG may be involved in controlling the subcellular localization of the protein (12). More recent data obtained from CapG null mice showed that CapG plays a role in phagocytosis and receptor membrane ruffling (13). Cofilin is a major actin depolymerizing protein, and its nuclear translocation is regulated by phosphorylation in some cells (14–17).

There is evidence in favor of a role for actin in nuclear processes, ranging from chromatin remodelling (18) to nuclear export (19). The presence of actin-binding proteins in the nucleus suggests that actin may not merely be present in the nucleus but that its polymerization is controlled as well. Alternatively, nuclear actin-binding proteins may display functions distinct from controlling actin polymerization. For instance, supervillin, an F-actin bundling protein, contains nuclear localization signals (20) and associates with the androgen receptor membrane ruffling.
tor, modulating its transcriptional activity (21). Zyxin, a component of focal adhesions, is involved in controlling mitosis through association with h-warts/LATS1, a serine/threonine kinase and constituent of the mitotic apparatus (22).

CapG is structurally related to Dictyostelium discoideum severin and Physarum polycephalum fragminP (FrgP). These proteins are characterized by repeats of 125–150-amino acid segments. In CapG, severin, and FrgP, the subdomains are organized in triplicate, whereas zyxin contains six repeats. CapG displays 54% similarity with severin (23) and FrgP (24). In contrast to CapG, however, they have not been reported to shuttle between nucleus and cytoplasm (25, 26). In addition to binding the fast growing end of actin filaments, severin and fragminP display F-actin severing activity in the presence of calcium, enabling these proteins to modulate cell morphology and cell motility processes.

We show here that severin and FrgP, but not CapG, contain Rev-like NESs at their N termini that control nucleo-cytoplasmic trafficking of these proteins. Mutation of single leucine residues in the export sequence abrogated export activity. As such, these findings may provide a molecular basis for the observed nuclear and cytoplasmic localization of CapG as compared with the predominantly cytoplasmic localization of severin and FrgP. Myopodin, a muscle-specific actin bundling protein, was recently shown to shuttle between nucleus and cytoplasm in a developmentally regulated manner (27). Delineation of a nuclear export sequence in severin and fragminP enabled us to identify a nuclear export sequence in myopodin, as well as in the Bloom syndrome protein.

EXPERIMENTAL PROCEDURES

Reagents—Texas Red-X-phalloidin and Alexa 488-conjugated goat anti-mouse IgG conjugate were obtained from Molecular Probes (Eugene, OR). Restriction enzymes were from New England Biolabs, Inc. (Beverly, MA). Platinum Taq HIFI polymerase, pcDNA3.1 V5-His-TOPO, pCR®/T7 CT-TOPO, and anti-V5 antibody were purchased from Invitrogen. Pf6 Turbo polymerase and Quikchange site-directed mutagenesis kit were from Stratagene (La Jolla, CA). pEGFP-N1 was purchased from Clontech (BD Biosciences, Palo Alto, CA). Leptomycin B and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma. DEAE-cellulose (DE-52) was purchased from Whatman (Maidstone, UK). Molecular mass markers for SDS-PAGE were from Bio-Rad. Phenylmethylsulfonyl fluoride was from Serva (Heidelberg, Germany). Other protease inhibitors were from Roche Applied Sciences.

Cell Culture and Transfection—MDCK-AZ (28), C2C12, and HEK293T cells were maintained at 37 °C in a humidified 10% CO2 incubator and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg of streptomycin (Invitrogen). HEK293T cells seeded on rat tail collagen-coated cover slips were transfected with cDNA constructs using calcium phosphate. MDCR-AZ and C2C12 cells were transfected with LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. The cells were grown for 24–72 h before processing for microscopy. For quantitation of experimental data, 200 cells were counted and scored for GFP localization (cytoplasmic only, or nuclear and cytoplasmic). Mean values and standard deviations were calculated.

dDNA Cloning—Total RNA was isolated from C2C12 myoblasts (generously provided by Dr. J. C. Adams, Lerner Research Institute, Cleveland, OH) with the RNeasy kit (Qiagen), and mRNA was reverse transcribed into first strand cDNA. For PCR, 50-μl reactions were set up containing 2.5 units of Taq polymerase, 2 μl of template cDNA, and 0.8 μM each of primer. The number of cycles was performed to obtain full-length myopodin: 5′-cgattgctcagatcggcagcccagc-3′ and 5′-cgggtgctccgtctctccacaaagacggttcc-3′. For myopodin lacking the NES, the primer 5′-cgattgctcagatcggcagcccagc-3′ was used. cDNAs were cloned into pcDNA3.1/V5-His (Invitrogen). All of the constructs were verified by sequencing.

cDNA clones encoding FrgP, CapG, or severin were cloned into pEGFP-N1. FrgP was subcloned into CapG-pEGFP-N1 as a KpnI/AgeI fragment. CapG-FrgP chimera were created by a unique procedure using partially complementary primers in a PCR with CapG-FrgP-pEGFP-N1 as template. In the example of swapping the CapG S3 subdomain for FrgP S3, the forward primer contains 21 nucleotides at the end of the S2 domain of CapG and 24 nucleotides at the beginning of the S3 domain of FrgP. The reverse primers contains 45 nucleotides at the end of the S2 domain of CapG, including the complementary nucleotides of the forward primer. After the addition of Pf6 Turbo polymerase and temperature cycling (18 cycles), the nonmutated, parent cDNA was digested with DpnI. Following transformation, XL1-Red E. coli competent cells were used to amplify the DNA as a consequence of the 21 complementary nucleotides. Using CapG-pEGFP-N1 as a template, the coding sequence for the FrgP NES was fused N-terminally to CapG in a similar manner. The forward primer encodes amino acids 18–27 of the NES sequence, a Kozak sequence, an extra ATG codon, and 24 nucleotides of the N terminus of CapG, starting with the second codon, omitting the start codon of CapG, to prevent incorrect initiation of translation. The reverse primer contains the complementary nucleotides of the NES sequence and 24 nucleotides starting from the HindIII cloning site in the pEGFP-N1 vector. Additional sequences were fused to CapG in separate PCRs. N-terminal tagging of the myopodin or Bloom NES to CapG-EGFP was performed according to the same strategy. Mutations in the NES region of CapG construct were generated using the Quikchange site-directed mutagenesis kit (Stratagene).

Recombinant Protein Expression and Purification—The full-length CapG sequence was cloned into the pLT1073 expression vector (29) and transformed into Escherichia coli MC1061 that already contained the pSCM26 plasmid (30) and harbors the T7 polymerase gene (see also Ref. 24). The cells were grown in 500 ml of LB medium containing 100 μg/ml ampicillin. The cultures were grown to an A600 of 0.6 at 37 °C. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-galactoside for 4 h at 30 °C. The cells were collected by centrifugation at 5,000 rpm for 15 min and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) by lysozyme (0.1 mg/ml, 40 min at room temperature) and sonication. Soluble CapG was further purified by ion-exchange chromatography on a DEAE and monoQ column. Full-length CapG DNA was also amplified by PCR, whereby a sequence encoding a Myc tag was introduced N-terminally. This construct was cloned into the pCR T7 CT-TOPO vector, resulting in a fusion protein with a C-terminal V5 and His8 tag. Expression of this fusion protein was achieved as described above, and purification was accomplished by ion-exchange chromatography on a DEAE column.

Fluorescence Microscopy and Immunocytochemistry—The cells were viewed directly for EGFP fluorescence after fixation with 3% paraformaldehyde in phosphate-buffered saline (Invitrogen) for 20 min at room temperature and staining with DAPI. For myopodin staining, the cells were fixed, permeabilized in 0.1% Triton X-100, washed in phosphate-buffered saline, incubated with anti-V5 antibody for 1 h at 37 °C, and followed several washes in phosphate-buffered saline incubated with Alexa 488-conjugated goat anti-mouse antibody for 30 min at room temperature. Microscopic images were captured with a Zeiss Axioplan epifluorescence microscope (×40 objective) equipped with an Axiocam cooled CCD camera and processed using ZEISS software (Zeiss).

Capping of Actin Filaments—The experiments were performed essentially as described earlier (31, 32). Briefly, preassembled unlabeled actin filaments (final concentration, 1 μM) were used as “nuclei.” They were subsequently mixed with 3 μM G-actin (25% pyrene-labeled) in the absence or presence of untreated recombinant CapG or Myc-V5-HisCapo. The capped G-actin filaments were observed using a phase contrast microscope controlled by a computer program. Invaginated and superficial cells were counted in 12 fields of 0.157 mm2. The invasion index is the percentage of cells invading the gel over the total number of cells.
Nuclear-Cytoplasmic Shuttling of Actin-binding Proteins

RESULTS AND DISCUSSION

CapG-EGFP, Severin-EGFP, and fragminP-EGFP Localize Differentially in Transfected Cells—The molecular mass of native CapG (39 kDa) is compatible with passive transport of the protein to the nucleus (2). Despite their similar size, severin and FrgP are predominantly cytoplasmic, and these proteins are not observed in the nucleus under normal growth conditions. We cloned CapG upstream of EGFP, resulting in a fusion protein with a predicted molecular mass of 67 kDa. Transient expression of CapG-EGFP in HEK293T cells showed a distribution identical to wild type CapG (10); fluorescence was detected in the cytoplasm as well as in the nucleus (Fig. 1A). By contrast, FrgP-EGFP and severin-EGFP were present in the cytoplasm under these conditions. In a limited number of cells, very weak nuclear staining could be observed (Fig. 1, B–D). Western blots on crude extracts of transfected HEK293T cells showed a band corresponding with the expected molecular mass of the fusion proteins (not shown). Because passive diffusion of bovine serum albumin, a protein with similar mass (68 kDa), is extremely slow (2), we considered it unlikely that CapG-EGFP (67 kDa) translocates to the nucleus by passive diffusion.

Leptomycin B Induces Nuclear Accumulation of fragminP—We further examined nuclear trafficking of these proteins by generating a CapG-FrgP-EGFP fusion polypeptide. We expected that CapG-FrgP-EGFP would display a nuclear and cytoplasmic localization similar to those of CapG-EGFP. Surprisingly, however, the CapG-FrgP-EGFP fusion protein was present in the cytoplasm following transfection in HEK293T or MDCK cells (Fig. 2, A and B). This may be due to inhibition of interaction between CapG and a putative nuclear import factor by FrgP in the large fusion protein. Alternatively, FrgP may contain a nuclear export sequence that prevents nuclear accumulation of the CapG-FrgP-EGFP fusion protein. The second possibility was further investigated using leptomycin B (LMB), a drug that interferes with the nuclear export factor CRM1 (35). When MDCK cells transiently expressing CapG-FrgP-EGFP were incubated with LMB, we observed nuclear enrichment of the fusion protein (Fig. 2C). Similarly, FrgP-EGFP (Fig. 2, D and E) accumulated in the nucleus upon incubation of transfected MDCK cells with LMB. These findings suggest that FrgP contains a nuclear export sequence. If CapG should lack such a regulatory sequence, it could explain why this actin-binding protein is constitutively present in the nucleus and cytoplasm under normal growth conditions. This was further investigated by transfection studies using CapG-FrgP chimerical proteins (domain swapping).

The N-terminal Region of Severin and fragminP Constitutes a Nuclear Export Sequence That Is Absent in CapG—To identify the putative regulatory sequences in severin, FrgP, and CapG controlling their subcellular localization, we generated CapG-FrgP chimerical proteins whereby CapG subdomains were exchanged for structurally similar FrgP subdomains (see “Experimental Procedures”). Because FrgP is cloned downstream of CapG, hybrid proteins always start with the CapG methionine residue (Table I). We predicted that when a nuclear export sequence in FrgP is deleted, the hybrid protein would be nuclear. Surprisingly, all of the chimeras showed a distribution identical to CapG-EGFP, displaying cytoplasmic as well as nuclear distribution. Even the chimeras that contained 95% of the FrgP sequence and barely 5% of the CapG sequence (CapG M1-L27/FrgP Q52-D371) showed nuclear localization (Fig. 3A).

Aligning the N-terminal regions of CapG, severin, and FrgP revealed several conserved hydrophobic residues in severin and FrgP, a hallmark of nuclear export sequences (36). This stretch of amino acids is absent in CapG (Fig. 3B). This sequence resembles the Rev-type export signal (37, 38), but the spacing between the hydrophobic amino acids is different from the Rev NES signal.

We tested the functionality of this region by deleting FrgP amino acids Met1–Leu27 in CapG-FrgP-EGFP, FrgP-EGFP, and the corresponding 27 amino acids in severin-EGFP and analyzed the distribution of these truncated fusion proteins following transfection in HEK293T cells. Interestingly, fluorescence was detected in the nucleus as well as in the cytoplasm (Fig. 3, C–E), similar to CapG-EGFP. This result shows that the N-terminal region of severin and FrgP constitutes a functional nuclear export sequence.

Miscellaneous—The protein concentrations were determined by the method of Bradford (34) using bovine serum albumin as a standard.
alone is not sufficient for nuclear export. This finding also demonstrates that modification of the CapG N terminus per se does not interfere with nuclear trafficking. Furthermore, a CapG fusion protein containing an N-terminal Myc tag and a C-terminal V5 epitope in addition to a His6 tag was found to cap the barbed ends of actin filaments as efficiently as the untagged recombinant protein (Fig. 4). Therefore, N-terminal attachment of amino acids does not interfere with the F-actin capping activity of the actin-binding protein. However, appendage of FrgP amino acids Leu17–Leu21, containing three leucine residues, promoted export of CapG-EGFP in 22% of the cells (Fig. 5, A and D). FrgP M1-L27-CapG-EGFP, containing the full stretch of the 27 N-terminal amino acids of FrgP, promoted cytoplasmic localization of the fusion protein in nearly 100% of the cells (Fig. 5, B and D), demonstrating that the FrgP N-terminal region represents a functional nuclear export sequence able to export CapG from the nucleus. Transfection of this construct into MDCK cells also resulted in a cytoplasmic localization (Fig. 5C) in the absence of LMB. However, the addition of LMB to the cells resulted in nuclear enrichment of FrgP NES-tagged CapG-EGFP (Fig. 5C).

The FrgP nuclear export sequence contains two isoleucines and three leucine residues (Fig. 3A). In severin, Ile14 is substituted by a Val residue. To examine the contribution of these hydrophobic residues in directing nuclear export, we generated single and double point mutants in the CapG-EGFP cDNA construct that harbors the FrgP nuclear export sequence (FrgP M1-L27-CapG-EGFP). Single mutants in which either Ile9, Ile14, or Val25 were substituted by an alanine residue did not significantly prevent nuclear export of FrgP M1-L27-CapG-EGFP (I9A, I14A, or V25A, 99.5% cytoplasmic). Expression of the I9A/I14A double mutant yielded similar results (Fig. 5, B and E). These results suggest that both isoleucine residues as well as Val25 do not play a significant role in export activity of the fragminP NES.

### Table 1: CapG-FrgP-EGFP hybrid proteins

| CapG sequence | FrgP sequence | Location |
|---------------|---------------|----------|
| CapG(M1–A244) | FrgP(K273–D371) | nucl/cyt (100%) |
| CapG(M1–F125) | FrgP(H157–D371) | nucl/cyt (100%) |
| CapG(M1–P92) | FrgP(V123–D371) | nucl/cyt (100%) |
| CapG(M1–L61) | FrgP(P92–D371) | nucl/cyt (100%) |
| CapG(M1–Q39) | FrgP(G64–D371) | nucl/cyt (100%) |
| CapG(M1–L27) | FrgP(Q52–D371) | nucl/cyt (100%) |

* Contains the first two domains of CapG (S1 and S2) and the third domain of FrgP (S3).
* Contains the first domain of CapG (S1) and the second and third domains of FrgP (S2 and S3).
* nucl, nucleus; cyt, cytoplasm.

Three Leucine Residues in the Severin/FrgP NES Are Indispensable for Directing Nuclear Export—To ascertain whether the FrgP/severin nuclear export sequence is able to export an otherwise nuclear protein, we cloned the FrgP export sequence upstream of CapG-EGFP. Appendage of amino acids Gly18–Leu27 of FrgP to the N terminus of CapG-EGFP followed by transfection in HEK293T cells did not affect the localization of the fusion protein (not shown), suggesting that this region of myopodin is sensitive to LMB, despite the absence of a classical NES (27). Sequence alignment between myopodin and FrgP/severin showed conservation of the three leucine resi-
dyes, which, incidentally, are also located at the N terminus of myopodin (Fig. 6A). To examine the role of this myopodin region as a potential nuclear export sequence, we tagged this sequence onto CapG-EGFP following the same strategy as for FrgP and analyzed its distribution in transfected HEK293T cells. Surprisingly, fusion of myopodin amino acids Leu14–Leu24 to CapG-EGFP yielded no change in the subcellular localization (Fig. 6B), in contrast to FrgP L17-L27-CapG-EGFP (see above). However, the myopodin Pro3–Val13 segment on its own exported CapG-EGFP in 10% of the cells (Fig. 6C). Finally, myopodin P3-L24-CapG-EGFP gave rise to cytoplasmic localization of the fusion protein in 16% of the cells (Fig. 6, D and F). This finding shows that the N-terminal region of myopodin displays weaker nuclear export activity as compared with the severin/FrgP NES. Furthermore, despite the similarity between FrgP and myopodin N-terminal regions at the primary structure level, both export sequences are probably different because the myopodin region containing the leucine-rich stretch displays no export activity, in contrast to the corresponding region in fragminP. Finally, our data do not exclude the presence of other regions in myopodin involved in controlling the subcellular distribution of this actin bundling protein.

Henderson and Eleftheriou (37) previously predicted a putative Rev-like NES in the Bloom syndrome protein (Lys1146–Asp1163). However, this region was unable to export EGFP from the nucleus, suggesting that it is not a functional NES. Based on our data we examined the Bloom sequence for a potential NES. An 11-amino acid region in the center of the Bloom DEAD box domain (Ile749–Ile759) contains one leucine and two isoleucine residues, spaced at identical intervals as in the FrgP NES (Fig. 6A). Although the corresponding myopodin segment (Leu14–Leu24) showed no significant export activity (Fig. 6B), the Bloom amino acid sequence Ile749–Ile759 was able to export CapG-EGFP from the nucleus with an efficiency comparable with that of myopodin Pro3–Leu24; in 20% of the cells, CapG-EGFP was exported from the nucleus (Fig. 6A, D–F). Secondary structure predictions did not reveal particular features that are common to the export signals of Bloom, FrgP, or myopodin, apart from the hydrophobic residues. The presence of this NES in an ATP-dependent helicase is not fully clear at present but
opens up new avenues for future research. Of note, the Bloom NES and the surrounding residues are conserved in a number of other helicases.

The NES of Myopodin Modulates Collagen Invasiveness—The partial effect of the myopodin NES on nuclear exclusion of CapG-EGFP, as compared with the efficiency of the FrgP/severin NES, is not unexpected. Indeed, as reported earlier (37–38), different NES sequences do not export EGFP from the nucleus with the same efficiency. These differences in relative NES strength are physiologically relevant in that some shuttling proteins require more rapid export from the nucleus than others. Other regions in the protein may also affect the subcellular localization, as illustrated in the case of myopodin. When amino acids Met1–Leu64 of myopodin were deleted, no signifi-
cant enrichment in the nucleus was observed as compared with the wild type protein following overexpression in differentiating C2C12 muscle cells (Fig. 7A). In both cases, co-localization with F-actin was observed as reported previously by Weins et al. (27), most likely because of the actin-binding region in myopodin (amino acids 410–563). Therefore, we performed collagen type I invasion experiments as an independent method to explore a potential role for the myopodin NES. Whereas HEK293T or C2C12 cells did not invade a collagen matrix (Fig. 7B, lanes 1, 2, 5, and 6), cells expressing full-length myopodin invaded into collagen type I (Fig. 7B, lanes 3 and 7). Significantly, the myopodin deletion mutant lacking Met1–Leu24 was unable to induce collagen invasion (Fig. 7B, lanes 4 and 8). This result argues in favor of a regulatory role of the myopodin NES in mediating collagen invasion, although its exact mode of action in this mechanism is not clear at present. Possibly, deletion of the NES perturbs the natural ability of myopodin to shuttle between nucleus and cytoplasm in a subtle manner, causing the mutant to reside longer in one cellular compartment. This hypothesis requires analysis of putative myopodin interaction partners that are involved in promoting invasion by the actin-binding protein.

In summary, we have shown that the occurrence of a nuclear export signal can account for the differential localization in cells of several representatives of a subfamily of gelsolin-related actin-binding proteins. The observations that CapG lacks such a NES and that it does not translocate to the nucleus in a passive manner may suggest that it plays an active role in that cell compartment. Possibly, deletion of the NES perturbs the natural ability of myopodin to shuttle between nucleus and cytoplasm in a subtle manner, causing the mutant to reside longer in one cellular compartment. This hypothesis requires analysis of putative myopodin interaction partners that are involved in promoting invasion by the actin-binding protein.

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Nuclear-Cytoplasmic Shuttling of Actin-binding Proteins

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