Invasive breast cancer cells exhibit increased mobility of the actin-binding protein CapG

Malte Renz1,2, Beate Betz3, Dieter Niederacher2, Hans Georg Bender2 and Jörg Langowski1*

1Division of Biophysics of Macromolecules, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, TP3, D-69120 Heidelberg, Germany
2Department of Obstetrics and Gynecology, Heinrich-Heine-University, Düsseldorf, Moorstr. 5, D-40225 Düsseldorf, Germany

The CapG protein, a Gelsolin-related actin-binding protein, is expressed at higher levels in breast cancer, especially in metastasizing breast cancer, than in normal breast epithelium. Furthermore, it is known that an increased expression of the CapG protein triggers an increase in cell motility. According to in vitro experiments, it was supposed that it is the nuclear fraction of the protein, which causes the increase in cell motility. Here, we examined the dynamical distribution of the CapG protein within the living cell, i.e. the import of the CapG protein into the nucleus. The nuclear import kinetics of invasive, metastasizing breast cancer cells were compared to the import kinetics of non-neoplastic cells similar to normal breast epithelium. FRAP kinetics showed a highly significant increase in the recovery of photobleached CapG–eGFP in the cancer cells, so that a differentiation of invasive, metastasizing cells and non-invasive, non-metastasizing cells on the basis of transport processes of the CapG protein between the nucleus and the cytoplasm seems to be possible. Comprehension of the mobility and compartmentalization of the CapG protein in normal and in cancer cells in vivo could constitute a new basis to characterize the invasiveness and metastasizing potential of breast cancer.

Key words: live cell analysis; metastasizing breast cancer; nuclear import kinetics; FRAP

The CapG protein is an actin-binding protein of the Gelsolin family. The relatively small protein (39 kDa) is distributed throughout the cell, in the cytoplasm and in the nucleus. There is no typical nuclear localization signal (NLS), rather several regions of different subdomains are responsible for the nuclear import. A couple of positively charged amino acids within the PIP2-binding region of the CapG protein or the amino acids 134–147, respectively, are proposed as potential equivalents of the canonical NLS. In contrast to the other Gelsolin-related actin-binding proteins, the CapG protein lacks a nuclear export sequence, especially the decisive leucines5 (L17, L21, L27). Thus, it is the only member of this family accumulating in the nucleus.

The function of the CapG protein in the cytoplasm seems to be well known. It blocks the rapidly growing ends of actin filaments (capping), and is involved in the control of actin-based cell motility and membrane ruffling (phagocytosis) of nonmuscle cells. CapG was originally isolated from the cytoplasm of alveolar macrophages, these being the prototype of a motile and phagocytic cell. Knockout of the protein causes membrane ruffling and phagocytosis defects in macrophages and motility defects in neutrophil granulocytes and dendritic cells.

The function that CapG fulfills in the cell nucleus is largely unknown. There are very few experimental data and some hypotheses considering this context.

The sequence of the CapG–cDNA shows some overlap with the sequence of a group of DNA-binding proteins, the basic helix–loop–helix family, including the c-myc oncogene. Therefore, the CapG protein was called mbh-1, myc basic motif homolog-1. The actual binding of the protein to DNA could not be verified so far. A transactivation activity of the CapG protein was called into question, but a modulation of transcriptional activators was discussed.

Several studies have provided evidence that nuclear actin associates with three types of nuclear complexes involved in gene expression: chromatin remodeling complexes, ribonucleoprotein particles and the three RNA polymerases. CapG as a nuclear actin-binding protein could play a role in preventing the nuclear actin from polymerizing and keeping it in a monomeric globular or a short oligomeric form. Thus, it could have some indirect influence on the regulation of gene expression.

Phosphorylated CapG localized in the cell nucleus is described to impair the function of the nuclear PI 3-kinase of fibroblasts of rats and mice in terms of binding PIP2 (substrate-sequestration). The PI 3-kinase phosphorylates PIP2, PIP and PtdIns to create potential second messengers regulating directly or indirectly DNA synthesis.

Furthermore, an interaction with steroid receptors is possible. The founding member of the protein family, Gelsolin, modulates the transcriptional activity of PPAR-γ, and in response to stimulation by the ligand that of the androgen and glucocorticoid receptor; other members of the protein family are reported to interact with various steroid receptors.

Recent studies hint at the fact that the CapG fraction that is localized within the cell nucleus is crucial for cell motility, or rather for the increase in motility of benign cells:

The induced overexpression of the CapG–eGFP fusion protein in the cytoplasm and in the nucleus triggers an increase in cell motility in kidney cells of the dog and in human embryonic kidney cells, whereas overexpression of the protein only in the cytoplasm—by tagging a nuclear export sequence—does not alter the motility of these cells.

Endothelial cells exposed to unidirectional shear stress exhibit an increased migration ability and express more CapG: The actin-associated CapG in the cytoplasm doubles within 2 hr, whereas the CapG localized in the cell nucleus quadruples within 24 hr.

Beside these findings that CapG is involved in the control of cell motility of benign cells and that especially the nuclear fraction of the protein seems to be responsible for the increase in their ability to migrate, there is increasingly more evidence that CapG could be also decisive in several types of cancer. It seems to promote motility, invasiveness and metastasis. By screening of mRNA databases and analysis of the cDNA- (Cancer profiling arrays) and mRNA-levels (RT-PCR) of actual clinical specimen, i.e. matched pairs of normal and tumorous tissue, it was shown that CapG is overexpressed in breast cancer, in particular in metastasizing breast cancer (Niederacher, unpublished data). In ovarian and pancreatic cancer, in glioblastoma and in ocular melanoma an overexpression of the protein was described as well.

Regarding pancreatic cancer, the intensity of immunohistochemical staining of CapG within the cell nucleus appears to be correlated with tumor size. Further correlations between immunohisto-
chemical staining and established prognostic parameters could not be verified.22 A few lung- and stomach cancer cell lines and a melanoma cell line were reported, however, to be deficient in CapG, in contrast to the corresponding normal tissues.23

In summary, the involvement of CapG in cell motility and in particular in metastasis led us to investigate its dynamical distribution in the living cell. For this purpose, we constructed a CapG–eGFP fusion protein and used fluorescence recovery after photobleaching (FRAP) to assess the intracellular transport kinetics of this construct.

Material and methods

Cell culture

The MDA-MB-231 cell line, a human breast cancer cell line, was cultured in DMEM without phenol red and supplemented with 10% fetal bovine serum (FBS) and 1% glutamic acid.

The MCF-12A, a human non-neoplastic cell line similar to normal breast epithelium, was propagated in RPMI without phenol red and supplemented with 5% fetal horse serum, 20 ng/ml epidermal growth factor, 10 μg/ml insulin, 500 ng/ml hydrocortisone and 100 ng/ml cholera toxin.

Plasmids and transfection

For generating the vector pSV–CapG–eGFP, we amplified the coding sequence of the human CapG gene by PCR using the following primers: 5′-TG AGC TCA AGC TTC GTA GAA GGT GGT GT-3′ and 5′-GGGA CAG ATG AAC ACT GGA CAG ATG AAC C-3′. The amplified fragment was inserted into the EcoRI and SacII site of the pSV–eGFP vector (BD Biosciences Clontech, Heidelberg, Germany). The construct was sequenced.

MDA-MB-231 cells were transfected with the pSV–CapG–eGFP construct using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Final siRNA concentration with siRNA using HiPerFect (Qiagen, Hilden, Germany) accorded with the manufacturer’s instructions. Each experiment was done 3–4 times.

Rescue of CapG–eGFP knockdown

A rescue variant of CapG–eGFP was constructed containing silent mutations. CapG–eGFP–cDNA refractory to silencing was designed by introducing mutations in the third nucleotide position of 2 codons in each siRNA molecule that did not affect the amino acid sequence.24

The QuikChange Multi, a site-directed mutagenesis kit (Stratagene, La Jolla, CA), and following primers were used: 5′-TCG AGC TCA AGC TTC GTA GAG AAG GCT GGT GGT GT-3′ (silent mutations in positions 363 and 366 within the coding sequence of CapG); 5′-AAG GCC CAG GTG GAT ATT GTC ACT GAT GGG GAG-3′ (silent mutations in positions 363 and 633); 5′-CTG TAT AAG GTC TCT GAT GCC ACT GCA GAG ATG AAC CTG-3′ (silent mutations in positions 768 and 771).

MDA-MB-231 cells stably expressing CapG–eGFP were first transfected with 5 nM siRNA and 48 hr later were transfected with the rescue construct using FuGene HD (Roche Diagnostics, Mannheim, Germany). 72 hr post siRNA transfection the invasion assay was performed.

Invasion assay

Invasion assays were performed in 24-well format cell culture inserts (BD Biosciences, Oxford, UK) with 8 μm pores and Matrigel matrix, a basement membrane preparation, according to the manufacturer’s instructions. Each experiment was done 3–4 times.

RT-PCR and Western blotting

Quantitative differential PCR was performed on an automated laser-activated fluorescent DNA sequencer (A.L.F., Pharmacia Biotech, Freiburg, Germany). Each PCR was done in a 50-μl volume containing 1.5 U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 200 μM dNTPs, 1 μM of GAPDH primer, 0.8 μM of CapG primer according to the manufacturer’s protocol. The primer sequences can be obtained on request. The housekeeping gene GAPDH was used as a reference.

SDS-PAGE was done using a Mini-Protean II system (Biorad, Hercules, CA) according to Laemmli.25 The proteins were transferred to a nitrocellulose membrane according to Kyhse-Andersen.26 Following antibodies were used according to the manufacturer’s protocols: anti-a-tubulin (Sigma–Aldrich, Schnell-dorf, Germany), goat-antimouse IgG-HRP and goat-antirabbit IgG–HRP (Santa Cruz Biotech, CA). Polyclonal anti-CapG antibodies were generated in rabbits by immunization with a carbohydrate-coupled CapG-peptide (6 injections, 200 μg each). After 56 days, antiserum was collected.

Epifluorescence microscopy

Live cells were examined using an Olympus CK40 epifluorescence microscope. Images were captured using an Optronis VX45 camera.

Confocal microscopy and FRAP

A laser-scanning confocal microscope (Nikon C1Si) with a 25-mW Argon ion laser was used to perform all photobleaching experiments using a 40 × 1.3 N.A. objective. The FRAP experiment was performed by exposing defined regions of cells to 100% laser intensity for 8 (MDA-MB-231) and 16 (MCF-12A) iterations. Images were taken with laser intensity set to 0.1% as indicated by the control software.

For quantitative analysis, regions were defined on the acquired images delineating the bleached nucleus, the cytoplasm and the extracellular space. By integrating over all pixels using the image acquisition software Imager (NIH, Bethesda), the fluorescence intensity was determined for each time point in each region. The determined mean gray values were background subtracted, corrected for acquisition photobleaching, laser intensity fluctuations and loss of fluorescence due to the bleaching event. The data were normalized to the initial fluorescence intensity. The following equation for the normalized fluorescence was applied:27

$$F(t) = \frac{[\text{Roi}(t) - \text{BG}(t)]}{[\text{Total}(t) - \text{BG}(t)]} \times \frac{[\text{Total}(0) - \text{BG}(0)]}{[\text{Roi}(0) - \text{BG}(0)]},$$

where Roi(t) denotes the fluorescence intensity at time t in the nucleus (region of interest), Total(t) denotes the fluorescence intensity in the cytoplasm at each time point and BG(t) the extracellular background fluorescence in each time point.

Results

Because the fraction of the CapG protein localized in the cell nucleus seems to be pivotal with respect to the increased cell motility, the studies were focused on the kinetics of CapG import from the cytoplasm into the cell nucleus. As model systems, a
highly invasive, metastasizing breast cancer cell line expressing CapG in very high amounts, the MDA-MB-231, and a non-malignant cell line similar to normal breast epithelium, the MCF-12A, were examined (Fig. 1a). It was attempted to characterize the kinetics of nuclear import in invasive and non-invasive breast epithelial cells beside the different expression levels.

**CapG knockdown reduces invasiveness**

The CapG protein was fused at the C-terminal with the auto-fluorescent protein eGFP. There are several reports that neither the localization nor the function of the protein is altered or diminished by this fusion.

![Figure 1](image-url)

**Figure 1** – (a) RT-PCR data of CapG mRNA expression levels relative to the housekeeping gene GAPDH in MCF-12A and in MDA-MB-231. MCF-12A: 0.89 ± 0.28; MDA-MB-231: 1.63 ± 0.18. (b) Western blotting: Using 5 nM siRNA, 3 days post-transfection more than 90% of CapG expression was inhibited in MDA-MB-231 cells. (c) siRNA knockdown (2.5 nM) reduces amount of CapG–eGFP as assessed by epifluorescence live cell microscopy: MDA-MB-231 stably expressing CapG–eGFP untreated and 4 days post-transfection (×400 magnification). (d) siRNA knockdown reduces invasiveness of MDA-MB-231 stably expressing CapG–eGFP as assessed by the Matrigel invasion assay. Bars represent invasiveness index of each treatment, expressed as a value relative to the number of cells translocating in the untreated control. Error bars represent standard errors. Each experiment was done 3 times. Control: untreated MDA-MB-231 stably expressing CapG–eGFP; 2.5 nM: siRNA molecules at end concentration of 2.5 nM each in a total of 2 ml medium reduce invasiveness to 70.6% ± 2.2%; 5 nM: siRNA molecules at end concentration of 5 nM each in a total of 2 ml medium reduce invasiveness to 92.7% ± 2.3%. (e) Rescue of CapG knockdown noninvasive phenotype in MDA-MB-231 stably expressing CapG–eGFP as assessed by the Matrigel invasion assay. Bars represent invasiveness index of each treatment, expressed as a value relative to the number of cells translocating in the untreated control. Error bars represent standard errors. Each experiment was done 4 times. Control: untreated MDA-MB-231 stably expressing CapG–eGFP; 5 nM: siRNA molecules at end concentration of 5 nM each in a total of 2 ml medium reduce invasiveness to 97.5% ± 7.2%; rescue: supertransfection of siRNA treated MDA-MB-231 with a rescue variant of CapG–eGFP restores invasiveness to 32.0% ± 7.2%.

The localization of the fluorescent fusion protein in fixed cells is consistent with the localization of the nonmodified protein as revealed by immunostaining. Both the transient overexpression of the CapG–eGFP fusion protein and the transient overexpression of the native CapG protein trigger an increase in cell motility. On the other hand, the knockdown using siRNA in carcinoma cells constitutively overexpressing endogenous CapG impairs cell motility.

In our model cell lines, we observe a distribution of the CapG–eGFP fusion protein in the living cells, which is concurrent with the reported distribution in fixed and immunostained cells. The invasiveness of the MDA-MB-231 expressing CapG–eGFP is...
comparable with the invasiveness of the native MDA-MB-231 cells, whereas the knockdown reduces their invasiveness significantly as assessed by the Matrigel invasion assay (Fig. 1d). An increase in the amount of siRNA (from 2.5 to 5 nM) shows an increase in knockdown effectivity. Using 5 nM siRNA, more than 90% of CapG expression was inhibited in MDA-MB-231 cells.

siRNA transfection had no effect on α-tubulin expression levels (Figs. 1b and 1c). Corroborating CapG knockdown data has been published recently. As expected the non-neoplastic cells, the MCF-12A, expressing the CapG–eGFP are not invasive in the applied assay. These observations can be taken as evidence that the assumed precondition of our model system considering invasive, metastasizing cancer cells and non-invasive normal cells holds even if the cells under study are transfected with the CapG–eGFP fusion protein.

**Rescue of knockdown noninvasive phenotype**

We attempted to rescue CapG depleted cells by expressing CapG–eGFP refractory to the siRNA. Successful rescue would not only provide a stringent test for the specificity and reversibility of the knockdown noninvasive phenotype but it would also test whether CapG tagged with fluorescent protein could efficiently substitute for endogenous capping activity. In the presence of inhibitory siRNA, the MDA-MB-231 cells were supertransfected with the CapG–eGFP construct refractory to siRNA. The supertransfected MDA-MB-231 exhibits a distinct increase in invasiveness. 32% ± 7.2% of the supertransfected cells were invasive compared with only 2.5% ± 1.3% of the cells treated with siRNA only (Fig. 1e).

**Comparison of CapG mobility in normal and in breast cancer cells**

One possibility to determine the mobility of fluorescent proteins within the living cell is the technique of FRAP. Exposed to recurrent cycles of excitation and emission, fluorescent molecules lose their ability to fluoresce, they are bleached. By bleaching the fluorescent molecules in a distinct region of a cell with high laser intensity and by detecting afterwards the redistribution of
fluorescence intensity of the whole cell, it is possible to characterize the mobility of the fluorophores.

To perform FRAP experiments a region exposed to high laser intensity was chosen so that the cell nucleus was bleached homogeneously (Fig. 2). After the bleaching the increase in fluorescence intensity corresponding to the uptake of the CapG–eGFP fusion protein into the nucleus was monitored over time by taking a picture of the cell every 10 sec, in total 80 pictures in 13 min. Potential photobleaching reversibility was improbable due to the long observation period—reversible dark states due to triplet sequestration occur on a timescale of micro- to milliseconds. Furthermore, we used a constant acquisition frequency in order not to alter the fraction of potential light-dependent reversible dark states of the GFP protein.

Even by examining the microscopic pictures of the cells just bleached and at the end of the detection period by eye, the difference between the MDA-MB-231 and the MCF-12A is evident. The highly invasive, metastasizing breast cancer cells regain nearly all the initial fluorescence intensity within the detection period, the non-neoplastic cells on the contrary exhibit only a slow increase in fluorescence intensity. Analyzing the pictures, subtracting the background, correcting for laser fluctuations and acquisition photobleaching and normalizing to the initial fluorescence intensity before the bleaching event—as described in detail in the Material and methods section—results in the graphs in Figures 3 and 4.

The normalized fluorescence intensity $F(t)$ was fit with a single-exponential function: $F(t) = 1 - [a - b(1 - e^{-\lambda t})]$, where $a$ is the fraction of initial fluorescence intensity that was bleached, $b$ is the fraction of bleached fluorescence intensity recovered after time $t$ and $\lambda$ is the recovery rate.

For the averaged recovery curve of the non-neoplastic cells (MCF-12A) we obtained $a = 0.770 \pm 0.001; b = 0.533 \pm 0.046; \lambda = (0.576 \pm 0.064) \times 10^{-3}$ sec$^{-1}$, whereas the highly invasive, metastasizing breast cancer cells (MDA-MB-231) yielded $a = 0.783 \pm 0.001; b = 0.757 \pm 0.006; \lambda = (1.373 \pm 0.020) \times 10^{-3}$ sec$^{-1}$.

Kinetic parameters distinguish normal and breast cancer cells

The recovery time $\tau$ is the reciprocal of the recovery rate $\lambda$ and is directly related to the characteristic time for transporting the fluorescent CapG–eGFP fusion protein from the cytoplasm into the cell nucleus. It is $\tau = 1.736 \pm 193$ sec for the non-neoplastic MCF-12A cells similar to normal breast epithelium and $\tau = 730 \pm 11$ sec for the invasive, highly metastasizing MDA-MB-231 breast cancer cells.

Another characteristic kinetic criterion can be derived from the fraction of initial fluorescence bleached, $a$, and the fraction of bleached fluorescence recovered, $b$, namely the immobilized or extremely slowly exchanging fraction of the CapG–eGFP fusion protein within the nucleus, $I_1 = 1 - b$. Its value is $30.8\% \pm 6\%$ for the CapG–eGFP fusion protein within the nucleus of the non-neoplastic cells (MCF-12A), and only $3.3\% \pm 1\%$ for the CapG–eGFP fusion protein within the nucleus of the highly invasive metastasizing cells (MDA-MB-231).

To analyze whether this immobile component is independent of the recovery time, i.e. whether $\tau$ and $I_1$ are two independent parameters characterizing normal and cancerous cells, the MCF-12A and MDA-MB-231 cells examined were divided into two groups of equal size according to their recovery rate: one group of relatively fast recovering cells and one of slowly recovering cells (Fig. 5). The average bleaching curves of two sub-groups, namely the relatively slowly recovering neoplastic cells and the relatively fast recovering non-neoplastic cells were compared with regard to their recovery time and their immobile component. As expected, the recovery times of these 2 groups approach each other, MCF-12A $\tau_{\text{fast subgroup}} = 1.227 \pm 81$ sec and MDA-MB-231 $\tau_{\text{slow subgroup}} = 1.134 \pm 71$ sec, whereas the immobile component appears to be stably differing: $I_1(\text{MCF-12A fast subgroup}) = 28.2\% \pm 3.5\%$ and $I_1(\text{MDA-MB-231 slow subgroup}) = 1.4\% \pm 4\%$. We conclude that the immobilization status, related to the binding properties of the CapG–eGFP fusion protein within the cell nucleus does not correlate to the recovery time of the observed cells, but is an independent characterizing parameter of benign and cancer cells. Thus, the non-neoplastic cells similar to normal breast epithelium and the invasive, highly metastasizing breast cancer cells differ distinctly both in the import kinetics of the CapG–eGFP fusion protein from the cytoplasm into the cell nucleus and in the immobilized fraction within the nucleus. The CapG–eGFP fusion protein seems to be transported into the cell nucleus of cancerous cells more rapidly and appears to be far less immobilized in this compartment compared to the corresponding benign cells.

Discussion

In our study, we present an in vivo comparison of the dynamical distribution of the CapG protein in normal and breast cancer cells. The chosen model cell lines exhibit differences in the expression level of the Gelsolin-related actin-binding protein CapG and in their motility capacity. We could demonstrate that the C-terminal fusion of the CapG protein does not alter its localization nor the
invasiveness of the MDA-MB-231 cells, whereas the knockdown of the CapG–eGFP fusion protein reduces their invasiveness significantly as assessed in Matrigel invasion assay. Expressing CapG–eGFP refractory to the siRNA restores the invasiveness of the MDA-MB-231, a stringent test to corroborate specificity and reversibility of CapG knockdown and the functionality of the CapG–eGFP construct.

Because there is increasing evidence that the nuclear fraction of the CapG protein determines the increase in cell motility, we focused our kinetic studies on the import of CapG into the nucleus. Applying a FRAP-based kinetic analysis it could be shown for the first time that invasive, highly metastasizing and non-neoplastic breast epithelium cells exhibit highly significant differences in the import kinetics and even in the binding characteristics of a protein within the cell nucleus. The live cell analysis revealed that the CapG protein is transported much faster into the nucleus of the breast cancer cell line and is actually far less immobilized in the nucleus of the cancer cells than in the normal breast epithelium cell line.

The studied model system suggests that there exist kinetic properties of cancerous and normal cells distinguishing them beyond the mere differences in expression level. Studying living cells is the very precondition of such a kinetic analysis. Live cell examinations of dynamic properties of proteins also seem to be an interesting way to broaden tumor diagnostic capabilities, and could complement the set of established prognostic parameters. FRAP-based analysis of CapG nuclear import kinetics might not only help in understanding the function of this protein, e.g. in binding nuclear actin and determining microfilament dynamics, but is also a possible method to estimate the invasiveness and metastasizing potential of cancerous cells on a cell-by-cell basis. Such assays may even be implemented in a high-throughput arrangement, e.g. using cell microarrays. Further investigations will clarify whether neoplastic and non-neoplastic cells can show differences in the kinetics even at equal CapG expression levels, and whether the invasiveness and metastasizing potential of a single breast epithelium cell can be predicted by analyzing the kinetics. A generalization of our observation on a larger number of cell lines will be the subject of a later study.

Understanding of the mobility and compartamentalization of the CapG protein in normal and in cancer cells in vitro can help to gain an insight into its function in the context of cell motility, invasiveness and metastasis and could constitute a new basis to characterize the invasiveness and metastasizing potential of breast cancer.

Acknowledgements

The authors thank Dr. Waldemar Waldeck for very helpful advice and discussions and Ms. Gabriele Müller for expert technical assistance. The microscopic work was conducted at the Nikon Imaging Center of Heidelberg University; they also thank Dr. Ulrike Engel for help with the microscopic techniques. Malte Renz is a recipient of a doctoral fellowship by the German National Merit Foundation.


