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Isolation and characterization of human CapG expressed and post-translationally modified in *Pichia pastoris*

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ABSTRACT

CapG is an actin-binding protein, which is overexpressed in a variety of tumors, i.e. breast, ovarian, pancreatic and lung carcinoma. We successfully expressed human CapG in the wild type strain X-33 of the methylotrophic yeast *Pichia pastoris* (*P. pastoris*), which does not express endogenous CapG, in order to characterize this protein in more detail. After mechanical cell lysis, debris was centrifuged and the soluble protein was precipitated with ammonium sulfate. This protein pellet was dialyzed and used for CapG purification. Ca^{2+}-dependent exposure of hydrophobic sites allowed single step and selective elution from a Phenyl Sepharose™ matrix. 3.5 mg CapG/10 g wet biomass were isolated and showed a Ca^{2+}-sensitive and dose-dependent capping activity of actin in a fluorometric assay. In *P. pastoris*, CapG is located at actin patches, actin cables and arranges along the budding neck. The proliferation rate and morphology of the yeast cells are not influenced by the interaction of CapG with actin. The modification pattern of human CapG from *P. pastoris* and human carcinoma cells is highly similar. We validated most of the known post-translational modifications and found three new phosphorylation and nine new acetylation sites by mass spectrometry. The N-terminus is acetylated or truncated. Truncated CapG is not phosphorylated at the residues S10, T212 and S337. First mutagenesis experiments indicate an N-terminal acetylation dependent C-terminal phosphorylation.

1. Introduction

CapG (macrophage-capping protein, gelsolin-like) is an actin-binding protein (ABP). It caps the fast growing end of actin filaments in a Ca^{2+}- and phosphatidylinositol 4,5-bisphosphate-dependent (PIP_2) manner [1,2] and promotes restructuring of the actin cytoskeleton [2,3]. CapG was isolated from rabbit alveolar macrophages [1]. As a result, the protein was called CapG, which is an abbreviation for macrophage-capping protein gelsolin-like. The name refers to its origin, function and similarity to gelsolin, another ABP. CapG is necessary for membrane ruffling in macrophages and it is overexpressed in various tumors, such as in breast, ovarian, pancreatic and lung carcinoma [4–7]. Its expression relates to tumor progression and metastasis [8–10], most likely due to changes of the expression profile of tumor related proteins such as STAT4, ANK3 and IGF2 [11]. CapG dependent differential expression was measured by microarray analysis in case of CapG overexpression and repression by siRNA, respectively [11]. STAT4 is a transcription factor regulating cell growth, differentiation und survival. IGF2 is a growth hormone important for mitogenesis. ANK3 belongs to the

Abbreviations used: CapG, macrophage-capping protein, gelsolin-like; ABP, actin-binding protein; 2DE, two-dimensional electrophoresis; PTM, post-translational modification; iP, isoelectric point; SNP, single nucleotide polymorphism; p.a., pro analysis; LB, Luria-Bertani Medium; YPD, Yeast Extract Peptone Dextrose Medium; Tris, tris(hydroxymethyl)aminomethane hydrochloride; MD, Minimal Dextrose Medium; YNB, Yeast Nitrogen Base; BMMY, Buffered Methanol-complex Medium; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; CCD, charge-coupled device; PBS(T), phosphate buffered saline (TWEEN® 20); Na_3VO_4, sodium orthovanadate; NaF, sodium fluoride; PFA, paraformaldehyde; NaN_3, sodium azide; ASB-14, amidosulfobetaine-14; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate; IAA, iodoacetamide; DTT, dithiothreitol.

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family of ankyrin proteins, which link surface receptors to the actin cytoskeleton. Ankyrins are important for cell motility and proliferation among others [11]. Due to the stated facts CapG has been described as a potential oncogene [4].

CapG was expressed in *Escherichia coli (E. coli)* to study its molecular structure [12,13]. Purification of CapG was performed by DEAE ion exchange chromatography. Other purification protocols used Phenyl Sepharose™ matrix [3], CM-Sepharose, MONO S or MONO Q [14] and obtained a purity of about 99%. The protein has a molecular mass of 38 kDa and is composed of three subunits, which are connected via two linkers [12]. The recombinant protein showed a Ca^{2+}- and PIP_2-sensitive activity [12–14]. It binds Ca^{2+} in the first and third segment and PIP_2 at the beginning of the second segment [12,15]. If CapG binds two Ca^{2+} ions, it changes its conformation [12]. Subunit 1 moves away from the subunits 2 and 3 and exposes the actin-binding site in the first segment [12,14]. On the contrary the interaction with PIP_2 prevents binding of CapG to actin [2]. Ca^{2+} depletion leads to Ca^{2+} dissociation from CapG, which subsequently changes its structure into a closed conformation [1]. Any truncation of the N-terminus resulted in a loss of capping activity and in poorly soluble protein mutants [14]. Truncation of the last segment of CapG reduced the capping activity by 50%, but did not change its Ca^{2+}- and PIP_2-sensitivity. The loss of the second Ca^{2+}-binding site also reduced the capping activity of CapG evidently [14].

The conformation of CapG and its interaction with potential binding partners such as Ca^{2+}, PIP_2 and actin might be influenced by post-translational modifications (PTMs). Tyrosine phosphorylation might regulate Ca^{2+}-sensitivity as shown for supervillin [16]. But it also may increase PIP_2 affinity and hence decrease actin-binding as shown for gelsolin [17]. In the case of villin phosphorylation is required for its nuclear localization [18]. Supervillin, villin, gelsolin and CapG belong to the gelsolin family of ABPs, whose members share a similar structure and a Ca^{2+}- and PIP_2-dependent binding activity of actin [19]. A PIP_2-dependent increase of tyrosine phosphorylation *in vitro* has been also demonstrated for CapG [20]. It is supposed that phosphorylated CapG is located mainly to the nucleus than to the cytoplasm. But this observation has not been validated since. The significance of PTMs for CapG is not known and the characterization of most PTMs of CapG is based on overall proteome or phosphoproteome analyses in the context of methodical development e.g. for the enrichment of PTMs [21]. These results include acetylation, phosphorylation, ubiquitination and succinylation. However, they still have to be validated by a focused mass spectrometry analysis. So far, the only low throughput analysis of PTMs of human CapG was performed with human CapG from leukocytes [22], which is phosphorylated at position S337 and truncated and acetylated at its N-terminus, respectively.

Our aim was therefore the characterization of an easy to use expression system for human CapG, which is comparable to human cells and which provides sufficient amounts of CapG to analyze its PTMs. Hence, we needed a eukaryotic expression system and decided on yeast cells. We chose the methylotrophic yeast *Pichia pastoris* (*P. pastoris*), because the integration of the exogenous gene into the host genome is as simple as in *Saccharomyces cerevisiae* (*S. cerevisiae*). In comparison to *S. cerevisiae* the expression of recombinant proteins in *P. pastoris* is controlled by a tightly regulated and methanol activated and dextrose inactivated promoter [23]. Therefore, the expression of heterologous proteins is nearly repressed during biomass production. Additional benefits of *P. pastoris* are the cultivation to a high cell density, because of a very low ethanol production [24,25] and an expression efficiency of recombinant proteins that reaches up to 1% of the total protein. The possibility to cultivate *P. pastoris* to high cell densities and the use of a tightly regulated promoter makes this yeast a favorable candidate for a high scale protein production. The expression of human CapG in *P. pastoris* and its purification yielded high amounts of pure CapG. We subsequently controlled the similarity between heterologous and human CapG regarding its capping activity, localization and modification pattern after two-dimensional electrophoresis prior to mass spectrometry analysis of heterologous CapG.

2. Material and methods

2.1. Chemicals

Restriction and ligation enzymes were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The yeast expression vector pPICZ B and wild type strain X-33 were part of the EasySelect™ Pichia Expression Kit Invitrogen (Carlsbad, CA, USA). *Escherichia coli* (*E. coli*) strains TOP 10F′ and DH5α were taken from Invitrogen (Carlsbad, CA, USA) and NEB (Ipswich, MA, USA), respectively. Herring sperm DNA was obtained from Promega (Madison, WI, USA). Q5® Hot Start High-Fidelity DNA Polymerase was used from NEB (Ipswich, MA, USA) and nucleosides for PCR from Qiagen (Hilden, Germany). We used an acrylamide/bisacrylamide mixture (1:39.5) from Carl Roth (Karlsruhe, Germany) for SDS-PAGE. All other chemicals were obtained from AppliChem (Chicago, IL, USA), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), SERVA Electrophoresis (Heidelberg, Germany), Sigma-Aldrich (St. Louis, MO, USA) and VWR (Radnor, PA, USA) except where noted otherwise.

2.2. Oligonucleotides

We used the sequencing primers AOX1 5′ 5′–GACTGGTTCCAATTGACAAGC–3′ and AOX1 3′ 5′–GCAAATGGCATTCTGACATCC–3′, which were part of the EasySelect™ Pichia Expression Kit (Invitrogen, CA, USA), at an annealing temperature of 57.9 °C to control cloning success. The primer pair CapG-EcoRI_for 5′–GAATTCAAAGAATTCATGTACACAGCCATTCCCCAG–3′ and CapG_XhoI_rev 5′–GCAATTTTTCAAGGACTGGAAATGACTCGAGTTTAAGTTC–3′ was used for cDNA amplification and includes the consensus sequence for Eco RI and Xho I, respectively. The primers were used at an annealing temperature of 68.3 °C and 68.4 °C, respectively. For N-terminal truncation the primer CapGNT2_trunc_f 5′–GAATTCAAAGAATTCATGACAGCCATTCCCCAGAGT–3′ and CapGNT6_trunc_f 5′–GAATTCAAAGAATTCATGCAGAGTGGCT–3′ were used together with CapG_XhoI_rev at an annealing temperature of 68.3 °C. The primers were purchased from Eurofins Genomics (Ebersberg, Germany).

2.3. Plasmids, vectors, strains

CapG 335R cDNA was obtained from Dr. Nicole Seier and integrated into the vector pPICZ B. CapG 335H mRNA was isolated from the cell line T3M4, transcribed into cDNA and also integrated into the vector pPICZ B. We used *E. coli* TOP 10F′ and DH5α strains for plasmid amplification and the prototroph wild type strain X-33 of *Pichia pastoris* (*P. pastoris*) for protein expression.

2.4. Restriction of vector and template

Vector and template restriction were performed in two steps using each enzyme separately (Eco RI, Xho I) at a concentration of 10 U with a maximum of 2 μg of DNA in a reaction volume of 50 μl according to the manufacturer instructions [26,27]. Restriction