PATHOBIOLOGY IN FOCUS

Characterization of a group I Nme protein of *Capsaspora owczarzaki*—a close unicellular relative of animals

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Nucleoside diphosphate kinases are enzymes present in all domains of life. In animals, they are called Nme or Nm23 proteins, and are divided into group I and II. Human Nme1 was the first protein identified as a metastasis suppressor. Because of its medical importance, it has been extensively studied. In spite of the large research effort, the exact mechanism of metastasis suppression remains unclear. It is unknown which of the biochemical properties or biological functions are responsible for the antimetastatic role of the mammalian Nme1. Furthermore, it is not clear at which point in the evolution of life group I Nme proteins acquired the potential to suppress metastasis, a process that is usually associated with complex animals. In this study we performed a series of tests and assays on a group I Nme protein from filasterean *Capsaspora owczarzaki*, a close unicellular relative of animals. The aim was to compare the protein to the well-known human Nme1 and Nme2 homologs, as well as with the homolog from a simple animal—sponge (Porifera), in order to see how the proteins changed with the transition to multicellularity, and subsequently in the evolution of complex animals. We found that premetazoan-type protein is highly similar to the homologs from sponge and human, in terms of biochemical characteristics and potential biological functions. Like the human Nme1 and Nme2, it is able to diminish the migratory potential of human cancer cells in culture.

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The appearance of multicellularity is directly connected to the origin of cancer. Cancer can be viewed as a collapse of the cooperation within the multicellular organism and the proliferation of selfish cell lines.¹ Even some of the simplest ‘early-branching’ non-bilaterian animals, such as cnidarians, develop tumors.² Tumors have also been identified in other invertebrates, for example, flatworms, mollusks, and insects (reviewed earlier¹). Metastatic potential is the property that has the highest influence on the lethality and prognosis of cancer. Metastasis is a complex process. Cancer cells first need to detach from the primary site, pass through the basement membrane and the surrounding stroma, enter blood or lymphatic vessels, survive in the circulation, arrest and leave the vessel, and invade an unrelated tissue. Therein, the cells have to adapt to the new surroundings, multiply, and grow their own blood supply. Genes whose proper function inhibits metastasis are called metastasis suppressors. Different

(although overlapping) sets of metastasis suppressors are involved in each step of the metastatic process. One of the crucial events that occur during metastasis is the change in tumor cell adhesion properties. Precisely regulated cell adhesion is also one of the essential elements for the evolution of complex multicellularity. Some metastasis suppressors are directly or indirectly involved in cell–cell or cell–ECM adhesion, and others modulate the function of adhesion molecules (Četković et al, this issue). Therefore, the same molecular interactions are crucial for both, the origin of multicellular organization and the metastatic processes, and they include genes that are in mammals recognized as metastasis suppressors.

The human Nme1 is the first known and the most studied metastasis suppressor.³ Nme (nucleoside diphosphate kinases—NDPKs, Nm23 proteins) is a family of evolutionary conserved proteins present in all domains of life.⁴ Their

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canonical role is the maintenance of NTP pool in the cell by phosphorylation of NDPs, although many other functions of this protein have been described. In animals, Nme proteins are divided in two distinct groups. Non-bilaterian animals have one or two group I Nme proteins, which have further diversified after the appearance of vertebrates.⁵ Group II contains members have more ancient origins and homologs present in unicellular eukaryotes. Ten Nme proteins have been identified in human: Nme1–4 belong to group I; and Nme5–9 to group II. Human Nme10, a.k.a. XRP2, has only a partial NDPK domain and does not belong to either group.⁶

Decreased expression of Nme1 has been linked to metastatic phenotype of several tumor types. Human Nme2 is highly homologous to human Nme1, but has different pI value.⁷ These two proteins form enzymatically active hetero- or homohexamer in any possible combination of subunits (6Nme1, 5Nme1+1Nme2,... 6Nme2). Besides diphosphate kinase activity, Nme1 and Nme2 also act as histidine kinases,^{8,9} 3'–5' exonucleases,¹⁰ and transcriptional regulators.¹¹ As expected for an enzyme with basic and diverse functions, mammalian Nme1 is involved in many cellular processes such as proliferation,¹² differentiation,^{13,14} development,^{15,16} apoptosis,^{17,18} adhesion, migration,¹⁹ and vesicular trafficking.²⁰ In spite of the large effort put in the research on Nme proteins, the mechanism of metastasis suppression is still unknown. Nme1 has been linked with several steps in the metastatic cascade,³ but without clear evidence for the mechanism of its action.

In animals, cell adhesion is achieved through several classes of proteins, including integrins and cadherins. There is evidence suggesting that Nme1 affects cell adhesion in metastasis.^{19,21} The mechanisms probably include modulation of cadherins²² and integrins.²³

Because of large research efforts, we now have considerable knowledge of Nme1 properties in complex animals such as mammals. In an earlier study, we demonstrated that the earliest-branching simple animals—sponges (Porifera) possess an Nme1 homolog, NmeGp1Sd, with biochemical properties very similar to the mammalian homologs. Moreover, we found that the sponge Nme1 (NmeGp1Sd) is recognized by human cells as their own protein, and is able to diminish migratory (metastatic) potential of cancer cells.⁷ So far the work on Nme1 in unicellular eukaryotes has been very limited with no data available on the properties of Nme homologs in close unicellular relatives of animals. Herein, we present a study of a group I Nme protein from *Capsaspora owczarzaki*—a close unicellular relative of animals. The aim was to compare the properties of 'ancestral-type' protein before the emergence of multicellularity, with the homologs from simple non-bilaterian animals and vertebrates. We were especially interested in investigating whether the premetazoan version of the group I Nme has properties that would make it a metastasis suppressor.

There are three major lineages of unicellular eukaryotes closely related to animals: Ichtiostorea; Filisterea; and

Choanoflagellata.²⁴ *C. owczarzaki* is one of only two known species in the phylum Filisterea. It was identified as an ameoboid endosymbiont of a pulmonate snail *Biomphalaria glabrata*.^{25,26} In laboratory conditions, *C. owczarzaki* has a life cycle consisting of three stages—filopodial (adherent), aggregative (pseudomulticellular), and cystic. Recently sequenced *C. owczarzaki* genome²⁷ encodes many proteins with functions related multicellularity, previously thought to be metazoan innovations, such as a rich repertoire of transcription factors²⁸ and tyrosine kinases,²⁹ as well as proteins directly involved in adhesion. *C. owczarzaki* possesses cadherins and all components of the integrin-mediated adhesion and signaling complex, including four integrin- α and four integrin- β homologs.³⁰ Many of the adhesion-related proteins are more highly expressed in aggregative stage, indicating their potential role in adhesion between aggregated *Capsaspora* cells.³¹ It has been shown that some conserved *C. owczarzaki* proteins can substitute the function of their homologs in arthropods³² and vertebrates.³³

We showed that *C. owczarzaki* has one typical group I Nme protein named NmeGp1Co and that the properties of this premetazoan protein are strikingly similar to the human Nme1 and Nme2. NmeGp1Co can diminish the migratory potential of human cancer cells, which strongly suggests that the antimetastatic properties of group I Nme proteins were present before the appearance of animals, and therefore before the origin of cancer in the animal lineage.

MATERIALS AND METHODS

Sequence Analyses

Homology searches and sequence retrievals were done using BLAST (NCBI, NIH, Bethesda, MD, USA: <http://www.ncbi.nlm.nih.gov>). Protein domains and sites were analyzed by Prosite (<http://prosite.expasy.org/>). Multiple sequence alignments were constructed by clustalX (<http://www.clustal.org/>).

Statistical Analyses

RNAseq data are available from Figshare (https://figshare.com/articles/RNAseq_data_-_Seb_-_Pedr_s_et_al_2013_/4585447). We analyzed gene expression using previously described methodology.³¹ Briefly, we used four different methods (DESeq, EdgeR, CuffDiff, and NOISeq) to identify differences in gene expression. Only if a gene was identified by at least three out of four methods, it was considered to be significantly differentially expressed. We used Kruskal–Wallis test with *post hoc* test for pairwise comparison of subgroups according to Conover³⁴ to check whether different Nme proteins have significantly diminished the migratory potential of human cancer cells.

Plasmid Constructions

The PCR-generated cDNA fragment was cloned into pGEM vector using forward primer 5'-ATGTCGACCGAGCGTAC-3' and the reverse primer 5'-TTAGTTCTCGTAGACCTGGG-3'. The cDNA for NmeGp1Co was recloned into pET15b using

PCR into *Nde*I (5'-GTCTAGCATATGTCGACCGAGCGTAC-3') and *Bam*HI (5'-CTAGACGGATCCTTAGTTCTCGTACAGCTGGGGGCGG-3') restriction sites of pET15b expression vector (Novagen) downstream from the thrombin cleavage site. For colocalization assay, the full-length coding sequence *NmeGp1Co* was cloned in frame into pEGFPC1 expression vector (Clontech, USA). The fragment of 456 bp was amplified using 5'-GTCTAGCTCGAGTAATGTCGACCGAGCGTAC-3' and 5'-CTAGACGAATTCCTTATTAGTTCGCTAGACCTGGGGGCGG-3' primers. The PCR product was digested with *Xho*I and *Eco*RI, and ligated with the *Xho*I/*Eco*RI sites of pEGFPC1 expression vector. The pEGFP-N1-Nme1 was a kind donation of Dr Marie-Lise Lacombe, INSERM UMRS_938, Paris, France. *NmeGp1Co* cDNA was amplified using a forward primer containing the FLAG sequence (F: 5'-GTCTAGGGATCCACGAGATGGACTACAAGGACGACGACGATAAGATGTCGACCGAGCGTAC-3') and the reverse primer (R: 5'-CTAGACGAATTCCTTATTAGTTCGCTAGACCTGGGGGCGG-3'). The PCR product was cloned into the *Bam*HI/*Eco*RI sites of eukaryotic expression vector pcDNA3 (Invitrogen).

The pRSET A-Nme2 was kindly donated by Dr Shantanu Chowdhury, Institute of Genomics and Integrative Biology, New Delhi, India.

Protein Expression and Purification

NmeGp1Co and *Nme2* were overproduced in *Escherichia coli* strain BL21 tagged with six histidine residues at the N terminus and purified to homogeneity from bacterial lysates using nickel-affinity chromatography. *E. coli* strain BL21 harboring the plasmid constructs were grown to 0.85 at OD₆₀₀ and induced with 0.8 mM IPTG for 2.5 h at 37 °C. Cells were incubated 30 min on ice in lysis buffer (25 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole and 1 mg/ml lysozyme) and sonicated 8 × 30 s (50% of full power). After centrifugation (12 000 r.p.m.) for 20 min at 4 °C, the supernatant was applied onto nickel-charged agarose column (Qiagen). Histidine-tagged proteins bound to nickel-affinity resins were eluted with 200 mM imidazole. *NmeGp1Co* and *Nme2* proteins were applied to a PD-10 desalting gel filtration column (Sephadex, GE Healthcare) equilibrated with 25 ml of Nme buffer³⁵ (20 mM HEPES at pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, and 0.1 M KCl) and then eluted with 3.5 ml of Nme buffer.

Protein Crosslinking with Glutaraldehyde

Oligomerization of the recombinant protein *NmeGp1Co* was performed in Nme buffer. Reactions contained 14 µg of purified *NmeGp1Co* protein. In first reaction glutaraldehyde was added to final amount of 0.04%. Reaction was incubated at 25 °C for 15 min. Second reaction proceeded with 1.6% glutaraldehyde for 40 min at 37 °C. The reaction product was subjected to 12% SDS-PAGE and visualized by protein staining with Coomassie brilliant blue.

Gel Filtration Chromatography

Recombinant human *Nme2* and *NmeGp1Co* were loaded onto Bio-Sil SEC 250 gel filtration columns (300 mm × 7.8 mm) and eluted with Nme buffer³⁵ at a flow rate of 0.5 ml/min (BioLogic Duo Flow, BIO-RAD). Peak fractions were used for NDPK assay.

DNA-Binding Assay

The DNA-binding activity of the *NmeGp1Co* protein was assayed *in vitro* as described.³⁶ Reactions contained 20 ng of single-stranded circular DNA (ssDNA) from bacteriophage ϕ X174 (NEB). The amount of purified *NmeGp1Co* protein is indicated (Figure 4). The reaction mixture was incubated in 20 µl of buffer containing 50 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 30 µg/ml BSA. After 30 min of incubation at 37 °C products were subjected to 0.8% agarose gel and stained with SYBR gold (Invitrogen).

NDPK Assay

NDPK activity was measured using a coupled pyruvate kinase–lactate dehydrogenase assay.^{35,37} Reaction kinetics was followed by connecting the turnover of ATP to the turnover of NADH to NAD. NADH concentrations were measured by spectrophotometry. A volume of 500 µl of reaction mixtures were incubated in quartz cuvettes at room temperature in the presence of ATP as phosphate donor and dTDP as phosphate acceptor. The final concentrations were as follows: 50 mM Tris-HCl (pH 7.4); 75 mM KCl; 5 mM MgCl₂; 1 mM phosphoenolpyruvate; 0.1 mg/ml NADH; 1 mM ATP; 0.2 mM dTDP; 2 U/ml of pyruvate kinase; 2.5 U/ml of lactate dehydrogenase; and 1 mg/ml bovine serum albumin. All reagents and enzymes were purchased from Sigma. Reactions were initiated by the addition of 50 ng of *NmeGp1Co* enzyme and activity was monitored in an Ultrospec[®] 2100 *pro* (Amersham Pharmacia Biotech, USA) measuring the decrease in absorbance at 340 nm. The control reactions omitting enzyme produced minor rates. Human *Nme2* was used as control. All reactions were done in triplicate.

Plasmid Cleavage Assays

Cloning of pUC19 plasmid containing 57-bp NHE (nuclease hypersensitive element) of the *c-myc* promoter was described previously.⁷ Recombinant plasmid pUC19-NHE (50 ng) and empty pUC19 was incubated with *NmeGp1Co* protein as indicated (Figure 4). The reaction was assembled in 50 mM KCl, 10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 30 µg/ml BSA, and incubated 30 min at 37 °C. The reaction was terminated with 1% SDS, 10 mM EDTA, and proteinase K treatment (200 µg/ml) for 30 min at 55 °C. Topoisomerase I (Gibco BRL) and *Nme2* proteins were used as controls and were processed in the same conditions. Samples were analyzed in 0.8% agarose gel in TAE buffer and then stained with SYBR gold (Invitrogen).

Cell Culture

Human HeLa cells (ATCC) and MDA-MB-231T (donated by Dr Patricia S. Steeg, Center for Cancer Research, National Cancer Institute, USA; for reference, Palmieri *et al*³⁸) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified chamber with 5% CO₂ at 37 °C.

Transient Transfections and Laser-Scanning Confocal Microscopy

Twenty-four hours before transfection 3×10^4 HeLa cells were seeded on circular coverslips placed in 24-well dishes to obtain 60% confluence. Each well was transfected with 1 µg of plasmid DNA: pyRedC1-Nme1; pyRedC1-Nme2; or pEGFPC1-NmeGp1Co or cotransfected with of pyRedC1-Nme1 and pEGFPC1-NmeGp1Co and/or pyRedC1-Nme2 and pEGFPC1-NmeGp1Co (0.5 µg of each construct). The transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Forty-eight hours post transfection the cells were washed with phosphate-buffered saline (PBS, pH 7.5), fixed in 4% formaldehyde, and mounted in mounting medium (DAKO) supplemented with 1 µg/ml 4, 6-diamino-2-phenylindole (DAPI; Sigma) for nuclear staining.

Fluorescent images were obtained by Leica TCS SP2 AOBS laser-scanning confocal microscope equipped with HCX PL APO λ-Blue 63×1.4 objective. GFP was excited by 488 nm laser line, DsRed using 543 nm, and DAPI using 405 nm laser line. The digital images were assembled using Adobe Photoshop software.

Preparation of Stably Transfected MDA-MB-231T Clones

Stably transfected cell lines were prepared as previously described.³⁹ In brief, MDA-MB-231T cells were transfected using Lipofectamine reagent (Invitrogen) with pcDNA3FLAG-Nme1, pcDNA3MYC-Nme2, pcDNA3FLAG-NmeGp1Co, and pcDNA3 as control. Post transfection the cells were resuspended and incubated in DMEM supplemented with geneticin (Sigma) until development of resistant colonies. Positive clones were screened by western blotting, propagated, and frozen until further usage.

Immunoprecipitation and Western Blotting

For detection of stably transfected MDA-MB-231T (control), two selected clones expressing the Capsospora variant of the NmeGp1 protein (Co2 and Co9) were seeded in 100 mm Petri dishes, washed two times in PBS, collected in lysis buffer (20 mM Tris, pH 7.6–8, 150 mM NaCl, 1% Triton, and 1 mM EDTA) supplemented with protease inhibitors and degraded by sonication. The immunoprecipitation was done with either anti-Nme1 antibody (Calbiochem) using Dynabeads, or with anti-FLAG M2 affinity gel (Sigma) according to the

manufacturer's instructions. Immunoprecipitation with IgG (Sigma) was used as a negative control.

The samples were loaded on SDS-PAGE and electrotransferred to an Immobilon-PSQ membrane (Milipore). The membranes were incubated with anti-FLAG M2 antibody (Sigma) of affinity-purified polyclonal anti-Nme1 antibody for detection of complex formation. Protein bands were visualized using Western Lightning Plus-ECL (PerkinElmer). The images were acquired by Alliance 4.7 (Uvitec) and assembled in Adobe Photoshop.

Migration Assay

For migration assay 3×10^5 cells were seeded on 60 mm Petri dishes in DMEM supplemented with 10% FBS. After 24 h the cells were starved in serum-free medium for another 24 h. Further, the cells were centrifuged and washed with DMEM supplemented with 0.1% BSA. A total of 2×10^4 cells were placed in to the upper chamber of Cell Culture Inserts (Beckton-Dickinson) and allowed to settle down for 20 min. DMEM supplemented with 1% FBS served as a chemoattractant and was added to the lower chamber. The cells were allowed to migrate for 5 h, after which the medium was removed, non-migratory cells were detached with cotton swabs while membranes with migrated cells were washed twice in PBS, and fixed in 4% formaldehyde for 15 min at room temperature. The cells were stained with 0.1% crystal violet, cut out from the inserts, mounted in mounting medium (DAKO), analyzed by light microscopy, and photographed. The cells from four representative images of every sample were assembled in Adobe Photoshop and counted. The experiments were performed three times.

RESULTS

C. owczarzaki has One Canonical and One Atypical Group I Nme Gene

Genomic and transcriptomic data show that *C. owczarzaki* has two group I Nme genes. Both proteins have a standard architecture of the group, ie, only one NDPK domain (marked in Figure 1a) and no other protein domains. One of them encodes a 152-amino-acid-long typical group I Nme protein with a conserved NDPK active site. Protein sequence is deposited in NCBI's GenBank under accession number XP_004365544. According to the accepted nomenclature, the protein encoded by this gene is named NmeGp1Co. The other gene encodes a 243-amino-acid-long protein. Its sequence is deposited in GenBank with accession number XP_004349949. Significantly bigger size compared to other NmeGp1 homologs is due to the addition of ~90 amino-acid residues at the N terminus. The N terminus has no homology with NDPKs or with any other protein in the NCBI's GenBank, and contains no recognizable domains. Although the remaining part of the protein shows high homology with other group I Nme proteins, it does not have a conserved NDPK active site (boxed in Figure 1a), and therefore probably has no NDPK activity. As this homolog clearly belongs to

Figure 1 (a) Alignment of human, sponge, and *C. owczarzaki* group I Nme proteins. Amino acids crucial for hexamer formation are marked with *. NDPK active site is boxed and NDPK domain is marked by a gray arrow. (b) Expression levels of *C. owczarzaki* group I Nme proteins. ↑ NmeGp1Co is significantly more expressed in the filopodial stage compared to the cystic stage, ↓ ↓ NmeGp1Co-like is significantly less expressed in the cystic stage compared to the other two stages.

group I Nme proteins, but also has unique characteristics, we named it NmeGp1Co-like.

We checked the position of both genes in the genome of *C. owczarzaki* and found that they are not tandem duplicates. We also found that the synteny is not conserved between *C. owczarzaki*, sponge *Amphimedon queenslandica* and human. The phylogenetic trees of group I Nme proteins that we constructed in an attempt gain insight in their evolutionary history at the origin of animals were uninformative, with little congruence and almost no statistical support for the branches of interest. Therefore, we aligned the NDPK domains of the two *C. owczarzaki* proteins, sponge NmeGp1Sd, and human Nme1 and Nme2 proteins, and checked the percentage of conserved (identical or similar) amino-acid residues (Table 1). In general, NDPK domains are relatively conserved, with a notable exception of the NmeGp1Co-like protein,

Table 1 Percentage of identical/similar amino-acid residues in NDPK domains of human, sponge, and *C.owczarzaki* group I Nme proteins

NDPK domain	Nme2-human	NmeGp1Co	NmGp1Sd	NmeGp1Co-like
Nme1-human	90/96%	74/81%	73/83%	59/77%
Nme2-human		75/83%	73/86%	63/79%
NmeGp1Co			66/83%	57/76%
NmGp1Sd				57/76%

which is the most diverged compared to all the other analyzed proteins.

We checked the expression levels of the two proteins in different stages of the *C. owczarzaki* life cycle (Figure 1b)

using the transcriptomics data analyzed earlier.³¹ NmeGp1Co is significantly more expressed in the filopodial stage compared to the cystic stage, while NmeGp1Co-like is significantly less expressed in the cystic stage compared to the other two stages.

According to its sequence, especially due to the conserved NDPK active site, we concluded that NmeGp1Co is a true NDPK with other typical functions of group I Nme proteins in the cell. Therefore, we focused our work on this Nme homolog.

Biochemical Characteristics of the NmeGp1Co Protein

In order to test the biochemical properties of NmeGp1Co, the protein was produced in *E. coli* and purified. The NDPK assay^{35,37} was used to determine NmeGp1Co enzymatic activity. The determined *C. owczarzaki* NmeGp1Co kinase activity of 254.37 U/mg was within the previously reported ranges for the human protein.^{10,40}

Various oligomeric structures, such as hexamers, tetramers, dimers, and monomers, were found after crosslinking the NmeGp1Co protein with glutaraldehyde. Recombinant NmeGp1Co is predominantly in the hexameric form when incubated in 1.6% glutaraldehyde for 40 min at 37 °C (Figure 2a). The hexameric form was expected due to the conserved primary protein sequence elements, KPN loop (residues 93–112 in NmeGp1Co), and residues Lys 30⁴¹ and Ser 119,⁴² known to be crucial for hexamer formation (Figure 1). The structure of *C. owczarzaki* NmeGp1Co enzyme was determined by gel filtration (Figure 2b). The previously characterized human Nme2⁴³ was used as marker. Overlapping gel filtration peaks confirm that NmeGp1Co protein has a hexameric structure like the human Nme2.

NmeGp1Co protein binds nonspecifically to sscDNA. Binding activity was observed with concentration of 100 ng, manifested by DNA band retardation (Figure 3). Human

Nme1 did not display the ability to bind sscDNA, while human Nme2 binds sscDNA.⁷

Nme2 is involved in DNA structural changes necessary for the activity of the *c-myc* promoter. It binds to the NHE sequence of the *c-myc* promoter cloned into pUC19, which yields mostly nicked circular plasmid.⁴⁴ Our experiments showed that the NmeGp1Co does not have DNA topoisomerase-like activity, contrary to the human Nme2. To test this activity, NmeGp1Co protein was incubated with supercoiled pUC19 plasmid containing the 57-bp *c-myc* NHE sequence. Topoisomerase I and Nme2 proteins were used as controls. Control topoisomerase I cleaved negatively supercoiled plasmid DNA. Human Nme2 specifically cleaved NHE sequence cloned into pUC19 unlike NmeGp1Co, which did not display this activity (Figure 4a). However, the experiment showed that NmeGp1Co nonspecifically binds double-stranded circular DNA (Figure 4b), unlike Nme2, which does not display this activity.

NmeGp1Co Behaves like Human Nme1/Nme2 in the Human Cell

Along with studying biochemical properties of NmeGp1Co, we wanted to determine whether the human cell recognizes the *Capsaspora* protein as its own. In order to check the colocalization of NmeGp1Co with human Nme1 and Nme2, we co-expressed the fluorescently labeled proteins in human

Figure 2 Oligomerization of NmeGp1Co. **(a)** Crosslinking NmeGp1Co with glutaraldehyde. In the first reaction glutaraldehyde was added to a final amount of 0.04%. Reaction was incubated at 25 °C for 15 min. The second reaction was performed with 1.6% glutaraldehyde for 40 min at 37 °C. **(b)** Gel filtration chromatography. The blue line represents human Nme2 while the green line represents NmeGp1Co protein. A full color version of this figure is available at the *Laboratory Investigation* journal online.

Figure 3 DNA-binding activity of the NmeGp1Co protein. Reactions contained 20 ng of single-stranded circular DNA from bacteriophage ϕ X174 (NEB). The amount of purified NmeGp1Co protein is indicated.

Figure 4 Plasmid cleavage assays. (a) Recombinant plasmid pUC19-NHE and (b) empty pUC19 was incubated with NmeGp1Co protein as indicated. Topoisomerase I and Nme2 proteins were used as controls and were processed in the same conditions.

HeLa cell line. The colocalization experiments (Figure 5) show that both human Nme proteins colocalize with the *C. owczarzaki* homolog. The colocalization is seen in the cytoplasm and in the nucleus.

Next, we wanted to test whether NmeGp1Co forms complexes with the human Nme1 protein in live human cells. For this purpose several MDA-MB-231T clones stably expressing FLAGNmeGp1Co were formed. Two clones were chosen for further analysis (Co2 and Co9). To test the possible interactions of NmeGp1Co and human Nme1 protein, FLAG or Nme1 was immunoprecipitated from cell lysates of FLAGNmeGp1Co-expressing clones, and with anti-FLAG M2 affinity gel. The results of the western blot analysis with anti-Nme1 and anti-FLAG antibody reveal that FLAG/NmeGp1Co forms complexes with the endogenous Nme1 (Figure 6). In the precipitate of human protein, the *Capsaspora* protein was co-precipitated and vice versa. Therefore, we showed that the human Nme1 recognizes the NmeGp1Co homolog as a partner for complex formation.

Finally, we checked whether NmeGp1Co can influence the migration of human cancer cells. We tested the migration potential of MDA-MB-231T cells overexpressing the FLAG/NmeGp1Co (clone Co2), and compared them with the human MDA-MB-231T cells overexpressing Nme1 and Nme2 that are known to diminish migratory potential of cancer cells.⁴⁵ We found that that NmeGp1Co-expressing clone, as well as human Nme1- and Nme2-overexpressing clones have substantially diminished the migratory potential of MDA-MB-231T, and same result was obtained in three independent experiments (Figure 7). According to Kruskal–Wallis test, this result was statistically significant ($P=0.009$). On the contrary, the differences among clones overexpressing Nme1, Nme2, and NmeGp1Co were not statistically significant. Therefore, NmeGp1Co has the ability to suppress the motility of the tested cells.

DISCUSSION

The main goal of this study was to characterize a group I Nme protein from *C. owczarzaki*, a close unicellular relative of animals, in order to better understand the evolution of Nme proteins and their functions, especially at the origin of animals. Comparison with the previous study of a group I Nme protein from a sponge, a simple non-bilaterian animal,⁷ and research on mammalian (mostly human) group I Nme proteins provide a better insight into changes that occurred in transition to multicellularity and subsequently in the animal lineage.

Evolution and diversification of group I Nme proteins at the dawn of the animals is difficult to reconstruct. *C. owczarzaki*, a close relative of animals analyzed herein, has two group I Nme genes. The same is true for different unicellular eukaryotes.⁴⁶ We checked the available genomes of Choanoflagellates—the closest unicellular relatives of animals, and found that they have only one group I Nme gene. Among the early-branching metazoan lineages the presence of NmeGp1 genes varies. Demosponges and placozoans have one NmeGp1 homolog, while cnidarians and ctenophores have two.⁴⁷ Reconstructing the state in the last common ancestor of animals with any certainty is problematic for two reasons. First, the deep phylogeny of animals is unresolved. Currently, there are two competing theories placing either sponges⁴⁸ or ctenophores⁴⁹ as the earliest-branching lineage. Second, the sequences of Nme genes and proteins often do not contain sufficient phylogenetic signal to reconstruct their evolutionary history at the base of the animal tree. However, upon examining the available evidence it seems more likely that the ancestor of animals had only one group I Nme gene that subsequently went through diversification in different animal lineages. Lineage-specific duplications have already been found in animals such as lancelet *Branchiostoma floridae* and sea squirt *Ciona intestinalis*,⁵ sea anemone,⁴ and some sponges.⁷ *C. owczarzaki* also has two group I Nme proteins that are not tandem duplicates. NmeGp1Co is a typical highly conserved member of the group. Our results show that it is a true NDPK similar to the human protein, and that it probably has all standard roles crucial for the survival of the cell. On the other hand, NmeGp1Co-like protein has some unusual characteristics: a long insertion at the N terminus; no NDPK active site; and much less-conserved NDPK domain. This points out to an ancient, probably lineage-specific duplication. NmeGp1Co kept the original essential function and remains under strict evolutionary constraint, while NmeGp1Co-like was able to accumulate more mutations. The function of the NmeGp1Co-like protein is currently unknown.

In laboratory culture, *C. owczarzaki* has a life cycle consisting of three stages: filopodial; aggregative; and cystic. Each stage has a distinct pattern of gene expression.³¹ NmeGp1Co is significantly more expressed in the filopodial stage. This is the most proliferative and metabolically active stage, so it is not surprising that an enzyme with basic

Figure 5 Subcellular localization of NmeGp1Co and human Nme1 and Nme2. (a, b) HeLa cells transiently transfected with pyRedC1-Nme1 (red fluorescence) and pEGFPC1-NmeGp1Co (green fluorescence). The signal is visible mainly in the cytoplasm, but can be seen in the nucleus as well. (c, d) HeLa cells transiently transfected with pyRedC1-Nme2 (red fluorescence) and pEGFPC1-NmeGp1Co (green fluorescence). The signal is visible mainly in the cytoplasm, but can be seen in the nucleus as well. In all cases (a–d) the colocalization signal is visible in the cytoplasm, but can be seen in the nucleus as well. The colocalization signal of the cytoplasmic ‘granum-like’ structures is visible, but incomplete. Bar = 10 μ m. A full color version of this figure is available at the *Laboratory Investigation* journal online.

metabolic functions is highly expressed in filopodial cells. On the other hand, NmeGp1Co-like protein is significantly less expressed in the cystic stage, while the levels of expression are similar in the filopodial and aggregative stage. The function of this protein is unknown, but it is highly unlikely that it has a kinase activity due to the mutation in the NDPK active site, and is probably not involved in basic cellular metabolism.

Biochemical characterization of the *C. owczarzaki* NmeGp1Co protein shows that the NmeGp1Co possesses

some biochemical characteristics typical for human Nme1 and others typical for human Nme2 protein. NmeGp1Co has properties highly similar to the sponge NmeGp1Sd homolog. As predicted from its primary sequence, recombinant NmeGp1Co appears to be predominantly in the hexameric form like human Nme1/Nme2⁵⁰ and NmeGp1Sd.⁷ We showed that NmeGp1Co is a NDPK whose activity is within the previously reported ranges for the human protein^{10,40} and the sponge NmeGp1Sd protein.⁷

Figure 6 Nme1/NmeGp1Co complex formation analysis. (a) Input control: cell lysates from control K (MDA-MB-231T cells stably transfected with pcDNA3 vector), Co2, and Co9 pcDNA3FLAG/NmeGp1Co construct tested with anti-FLAG antibody and anti-Nme1 antibody. (b) Immunoprecipitation: MDA-MB-231T cells stably transfected with pcDNA3 vector (K) and FLAG/NmeGp1Co (Co2 and Co9) were immunoprecipitated with anti-FLAG M2 affinity gel and immunoblotted with anti-FLAG and anti-Nme1 antibody. FLAG/NmeGp1Co produces heteromers with exogenous (FLAG/NmeGp1Co, upper band) and endogenous (lower band) Nme1. (c) Immunoprecipitation: FLAG/NmeGp1Co (Co2 and Co9) were immunoprecipitated with mouse anti-IgG (negative control) and anti-Nme1 antibody and immunoblotted with anti-FLAG and anti-Nme1 antibody. Endogenous Nme1 produces complexes with exogenous (FLAG/NmeGp1Co produces heteromers with exogenous (FLAG/NmeGp1Co, upper band) and endogenous (lower band) Nme1.

Figure 7 Migration assay. MDA-MB-231T cell transfected with empty vector pcDNA3 (KO), pcDNA3FLAG/Nme1, pcDNA3FLAG/Nme2, and pcDNA3FLAG/NmeGp1Co were tested for migration potential in Boyden chambers. The cells were stained with crystal violet, while the images were recorded with light microscope. The cells were counted on four representative images. The results were presented as relative numbers of migrated cells compared to KO (empty vector transfectants; $\pm$ s.d.).

Regarding its affinity for DNA binding, NmeGp1Co has a unique combination of properties, although it shares most of them with the sponge and human proteins. NmeGp1Co binds nonspecifically to ssDNA like NmeGp1Sd and human Nme2, but unlike Nme1. Furthermore, NmeGp1Co is not able to cleave *c-myc* NHE sequence, same as sponge NmeGp1Sd and human Nme1 protein.⁷ In contrast, human Nme2 is able to cleave the *c-myc* NHE sequence. This property is linked to the structural changes of the promoter and activation of the *c-myc* proto-oncogene.³⁵ Nme1 and Nme2 appeared by *cis*-duplication in amniotes.⁵ It seems that after the duplication event, Nme2 developed this unique regulatory feature. Finally, NmeGp1Co nonspecifically binds

to double-stranded circular DNA unlike any of the proteins we tested.

In addition to testing the biochemical properties of NmeGp1Co, we wanted to check how it behaves in live mammalian cells. The localization of fluorescently labeled NmeGp1Co in the mammalian cell is mostly in the cytoplasm, but also in the nucleus. The granular structures visible in the cytoplasm have already been described accompanying both homologous and heterologous over-expression of Nme proteins.^{7,51} It is still not clear whether the granules are part of the cell (eg, ribosomes of the rough endoplasmic reticulum) or an artifact of the experiment. NmeGp1Co does not colocalize completely with the human Nme1 and Nme2 homologs while colocalization of the Nme1, Nme2, and sponge NmeGp1Sd was complete.⁷ This discrepancy could be due to the subtle differences in some of the properties that were not included in our testing. *C. owczarzaki* protein is expressed in a cell of a distantly related species and is possibly not finding or recognizing all its usual interaction partners and thus not performing all biological functions and biochemical activities. The disparity may also be due to the fact that different human cell lines were used in the experiments: HEp-2 cells for the sponge homolog and HeLa cells for the *C. owczarzaki* protein. In spite of only partial colocalization, we found that NmeGp1Co forms complexes with native human Nme1, same as the sponge NmeGp1Sd.⁷ Finally, we showed that NmeGp1Sd diminishes the migration potential of human cancer cells in culture, same as Nme1/Nme2 and NmeGp1Sd. While it is clear that Nme1 in humans is a metastasis suppressor, the mechanism of suppression is still not understood. Here we show that whichever property or function of Nme1 is responsible for the metastasis suppression, it was present in ancestral-type group I Nme protein before the origin of animal and the diversification of Nme proteins. It is, therefore, likely that the metastasis suppression property of Nme1 is linked to some of its standard functions in the cell, possibly ones that still haven't been described. This function is probably equally important for unicellular eukaryotes as it is for the animals. However, in the multicellular context, at least in mammals, Nme1 has been co-opted to also function as a metastasis suppressor. Many cancer- and disease-related genes have ancient origins, before the appearance of animals.^{52,53} The same is true for metastasis suppressors (Četković et al, this issue). Therefore, their functions and mechanisms of action should be viewed in a wider context that takes into account homologs from simple unicellular organisms and also vast interaction networks within and between the cells of highly complex animals.

To conclude, this study showed that NmeGp1Co, a group I Nme protein from filasterean *C. owczarzaki*, a close unicellular relative of animals, has properties strikingly similar to the human Nme1 and Nme2 proteins in terms of primary sequence, biochemical characteristics, and functions in the cell. The protein did not change significantly in the transition to multicellularity. It is recognized as a native protein by

human cancer cells and it diminishes their migratory potential. This means that even before the appearance of animals, the ancestral group I Nme homolog was a versatile protein with characteristics and functions similar to the human Nme1.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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