CROATICA CHEMICA ACTA
CCACAA **78** (3) 343–348 (2005)

ISSN-0011-1643
CCA-3018
Original Scientific Paper

Identification and Analysis of cDNAs Encoding Two Nucleoside Diphosphate Kinases (NDPK/Nm23) from the Marine Sponge *Suberites domuncula**

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RECEIVED NOVEMBER 26, 2004; REVISED FEBRUARY 14, 2005; ACCEPTED MARCH 15, 2005

Suberites domuncula is a member of the most ancient and simplest extant phylum of multicellular animals – sponges (Porifera). A database of *S. domuncula* expressed sequence tags (ESTs) was recently constructed by random cDNA sequencing. Two NDPK/Nm23 proteins from the sponge *Suberites domuncula* are reported here. Sponge proteins were named Nm23-SD1 and Nm23-SD6, because they display the highest sequence similarity with human Nm23-H1 and -H6 proteins. Overall sequence conservation of Nm23-SD1 with human Nm23-H1 is very high – 79 % (71 % identical amino acids). Nm23-SD6 possesses an insertion at the C-terminus and displays 55 % overall homology (40 % identical amino acids) with human Nm23-H6. Secondary structure predictions for both sponge and human Nm23 protein pairs are almost identical. *S. domuncula* Nm23 proteins display high similarity to homologues from mammals/humans, higher than to *e.g.* NDPK/Nm23 proteins from *Drosophila* or other invertebrates. Sponge Nm23 proteins are more similar to mammalian/human Nm23 proteins than most known Nm23 proteins of invertebrates.

Keywords
antimetastatic proteins
nucleoside diphosphate kinases
sponge cDNA library
Porifera

INTRODUCTION

Nucleoside diphosphate kinases (NDPK) are enzymes conserved throughout evolution and present in all three domains of life: Bacteria, Archaea and Eukarya. Basic function of NDPKs is phosphorylation of the nucleoside diphosphates via a »ping pong« mechanism.[1] However, NDPKs are known to play important roles in a variety of regulatory processes associated with cell proliferation, development and differentiation. In eukaryotes, these proteins most probably modulate transmembrane signaling pathways.[2] NDPKs in higher animals are encoded by several genes, called *nm23* genes. Eight *nm23* genes have been identified in human genome.[3] Their products, Nm23 proteins, are involved in many important cellular processes, including the control of oncogenic transformation and metastatic potential of tumor cells. *nm23-H1* gene was the first to be recognized as a metastasis suppressor

* Dedicated to Professor Željko Kućan on the occasion of his 70th birthday. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology, $HDBMB_{2004}$, Bjelolasica, Croatia, September 30 – October 2, 2004.

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gene by Steeg *et al*.[4] and Nm23-H1 protein is the most studied member of Nm23 proteins. Reduced expression of *nm23-H1* is linked with increased metastatic potential of several human malignant tumors: breast carcinomas,[5] hepatoma[6] and gastric carcinoma,[7] while the overexpression of *nm23-H1* inhibits the metastatic phenotype.[8,9] Nucleoside diphosphate kinase activity is not relevant to metastasis suppression[10] and other biochemical activities of the Nm23-H1 protein, *i.e.,* histidine-dependent protein kinase activity[11] and recently discovered 3'–5' exonuclease activity[12] have been proposed to underlie metastasis suppression. In addition, Nm23 proteins also interact with DNA.[13] Both Nm23-H1 and -H2 repress transcription of the platelet-derived growth factor A gene.[14]

A relatively new member of human NDPKs is Nm23-H6, localized in mitochondria, with a suggested function in cell growth and cell cycle progression.[15] Despite extensive work, the biochemical mechanism of action of Nm23 proteins in suppressing invasive phenotype in some mammalian cancers is still unknown. However, increasing evidences suggest that the *nm23* genes are involved in the control of normal development and differentiation in all living organisms.[16] The *awd* gene in *Drosophila melanogaster*, an *nm23* homologue from invertebrates, was the first *NDPK* gene correlated with proper differentiation of the tissues of epithelial origin.[17]

Until now, nothing was known about *nm23* genes/Nm23 proteins in the basal, most simple metazoan phylum Porifera, and besides *Caenorhabditis elegans* and *D. melanogaster* Nm23 homologues, only three other NDPK/Nm23 proteins were known from lower Metazoa. We report here the primary structure and *in silico* analysis of two Nm23 proteins from the marine sponge *S. domuncula*, which display high conservation of primary and predicted secondary structures with their mammalian/human homologues.

EXPERIMENTAL

S. domuncula *Database of Expressed Sequence Tags*

Construction of *S. domuncula* cDNA library was described earlier.[18] Random sequencing of sponge cDNA clones was performed at MWG – The Genomic Company, Germany.

Database of *S. domuncula* expressed sequence tags (ESTs) contains over 11000 individual sequences and can be searched via Internet (http://spongebase.genoserv.de).

Sequence Analysis

DNA and protein sequences were analyzed and stored using the Lasergene sequence analysis software (DNA-Star, Madison, WI). Homology searches and sequences retrieval were done via Internet server BLAST (NCBI, NIH, Bethesda, MD, USA: http://www.ncbi.nlm.nih.gov). Multiple sequences alignments (MSA) and constructions of the phylogenetic tree from the MSA were performed with the CLUSTAL X program.[19] Programs GeneDoc[20] and TreeView, version 1.6.6.,[21] were used for graphic presentation of the results. Predictions of proteins' secondary structures were performed using »PROF – Secondary Structure Prediction System program«[22] at the University of Wales, Aberystwyth Computational Biology Group Server (http://www.aber.ac.uk/~phiwww/prof/).

RESULTS

Initial homology searches of *S. domuncula* ESTs database were performed by TBLASTN using human Nm23-H1A protein (NP_937818) and resulted in 6 positives. According to detailed homology analysis of 6 positive cDNAs by BLASTX, 4 cDNAs encoded an identical protein with the highest homology with human Nm23-H1B (NP_000260) and 2 cDNAs encoded an identical protein with the highest homology with human Nm23-H6 protein (O75414). Sponge homologues of human Nm23 proteins were therefore named Nm23-SD1 and Nm23-SD6.

S. domuncula cDNAs encoding Nm23-SD1 and Nm23-SD6 proteins are 541 and 787 nucleotides (nt) long, respectively, and are deposited under accession numbers AY764255 and AY764256. Open reading frame (ORF) for Nm23-SD1 starts with ATG at position 19 and stops with TAA at position 472. ORF for Nm23-SD6 starts with ATG at position 68 and stops with TGA at position 674. Putative Nm23-SD1 protein is 151 aa long (Figure 1a), with calculated *M* of 17138 Da. Nm23-SD6 is 202 aa long (Figure 1b), with *M* of 23268 Da.

Nm23-H1B Homologue from S. domuncula

Nm23-SD1 and human Nm23-H1B share 71 % identical amino acids (79 % overall sequence similarity) and differ in size by only 1 aa (Figure 1a). Longer form of human Nm23-H1 protein is called Nm23-H1A and has additional 25 aa at the N-terminus. We carefully inspected *S. domuncula* EST database and could not find any evidence that this sponge also possesses the longer form of Nm23-H1. Figure 1a shows the alignment of Nm23-SD1 and human Nm23-H1B proteins. In addition to the high overall conservation in the primary structure between two proteins, all important aa (required for different functions of NDPK/Nm23 proteins) are perfectly conserved in sponge protein and are marked in Figure 1a. Consensus for nucleoside diphosphate kinase active site N-x(2)-H-[GA]-S-D-[GSA]-[LIVMPKNE] is also well conserved (boxed in Figure 1a). The only difference between human and sponge NDPK active sites is the isoleucine to cysteine change (I→C) at nonconserved position of the site. Cysteine at this position is often found in Nm23

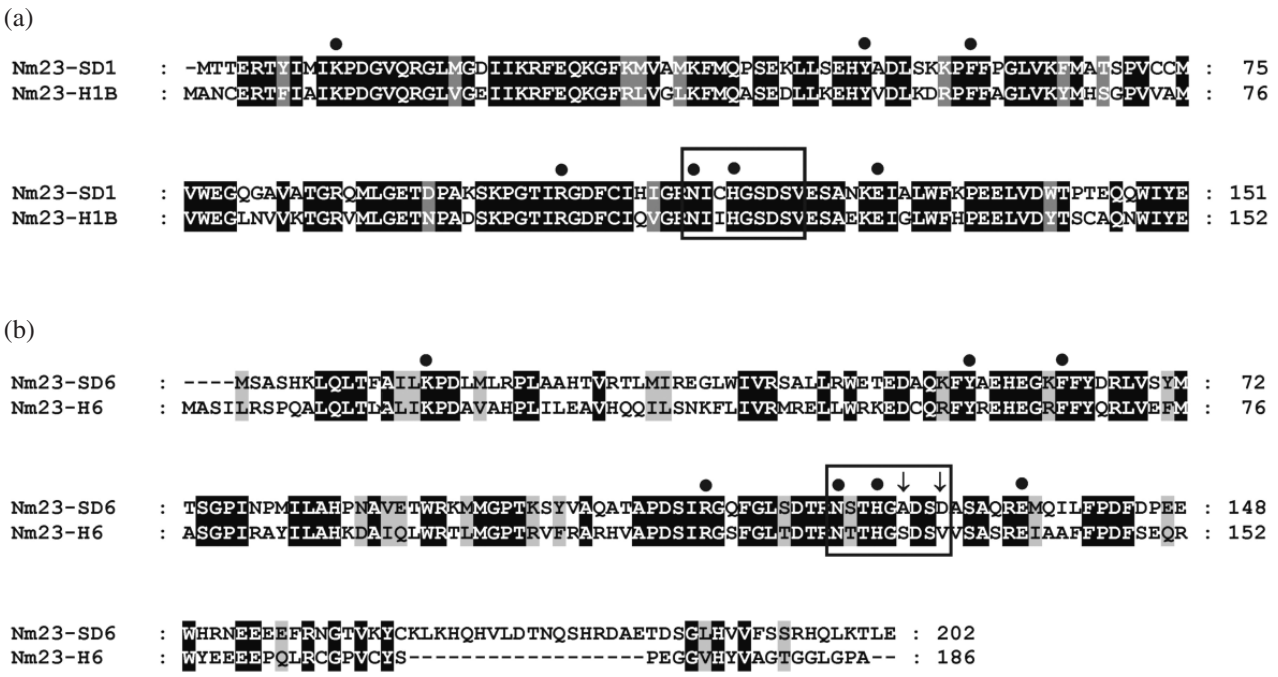

Figure 1. Alignment of amino acid sequences of Nm23 proteins from *S. domuncula* and humans: a) Nm23-H1B (NP_000260) and Nm23-SD1 (AY764255) proteins and b) Nm23-H6 (O75414) and Nm23-SD6 (AY764256) proteins. Identical aa are shown in white on black and similar ones in white on gray. Different aa are shown in black on white. NDPK active sites are boxed. Residues involved in nucleotide binding and catalysis[27] are marked with •. Residues in Nm23-SD6 protein differing from NDPK active site consensus sequence are marked with arrows.

proteins from fungi and is also present in *C. elegans* NDPK protein (NP_492761).

We analyzed sequence conservation between the known Nm23-H1 homologues from invertebrates, as well as the degree of their homology with human (mammalian) Nm23-H1B protein. The results are presented in Table I. Nm23-SD1 displays the highest homology with human Nm23-H1B protein. Only Nm23-H1B homologue (Awd protein; P08879) from *D. melanogaster* shows slightly higher sequence conservation with human Nm23-H1B.

Prediction of secondary structures for NM23-SD1 and human Nm23-H1B was performed according to Ouali *et al.*[22] and the results are presented in Figure 2a. Almost identical results were obtained for both proteins, although their primary structures differ by more than 20 %.

Unrooted phylogenetic tree of selected NDPK/Nm23-H1 proteins from different eukaryotic organisms is shown in Figure 3 to illustrate the close position of sponge protein with regard to the vertebrate branch.

TABLE I. Percentage of identical amino acids and overall sequence similarity (in parentheses) between Nm23 proteins from humans and their homologues from invertebrates[(a)]

	Organism	*Homo sapiens* (Mammals)	*Suberites domuncula* (Porifera)	*Drosophila melanogaster* (Insects)	*Caenorhabdits elegans* (Nematodes)	*Hydra vulgaris* (Cnidaria)
Nm23-H1B and homologues	*H. sapiens*	100 %	71 % (79 %)	76 % (86 %)	66 % (79 %)	61 % (80 %)
	S. domuncula	–	100 %	67 % (79 %)	58 % (73 %)	64 % (80 %)
	D. melanogaster	–	–	100 %	64 % (76 %)	62 % (77 %)
	C. elegans	–	–	–	100 %	59 % (77 %)
	H. vulgaris	–	–	–	–	100 %
Nm23-H6 and homologues	*H. sapiens*	100 %	40 % (55 %)	35 % (51 %)	Not found	Not found
	S. domuncula	–	100 %	20 % (48 %)		
	D. melanogaster	–	–	100 %		

[(a)] The following sequences were used for comparison – a) Nm23-H1: *Homo sapiens* (NP_000260), *Suberites domuncula* (AY764256), *Drosophila melanogaster* (P08879), *Caenorhabditis elegans (*NP_492761) and *Hydra vulgaris* (AAK51137); b) Nm23-H6: *Homo sapiens* (O75414), *Suberites domuncula* (AY764255) and *Drosophila melanogaster* (AAF64469).

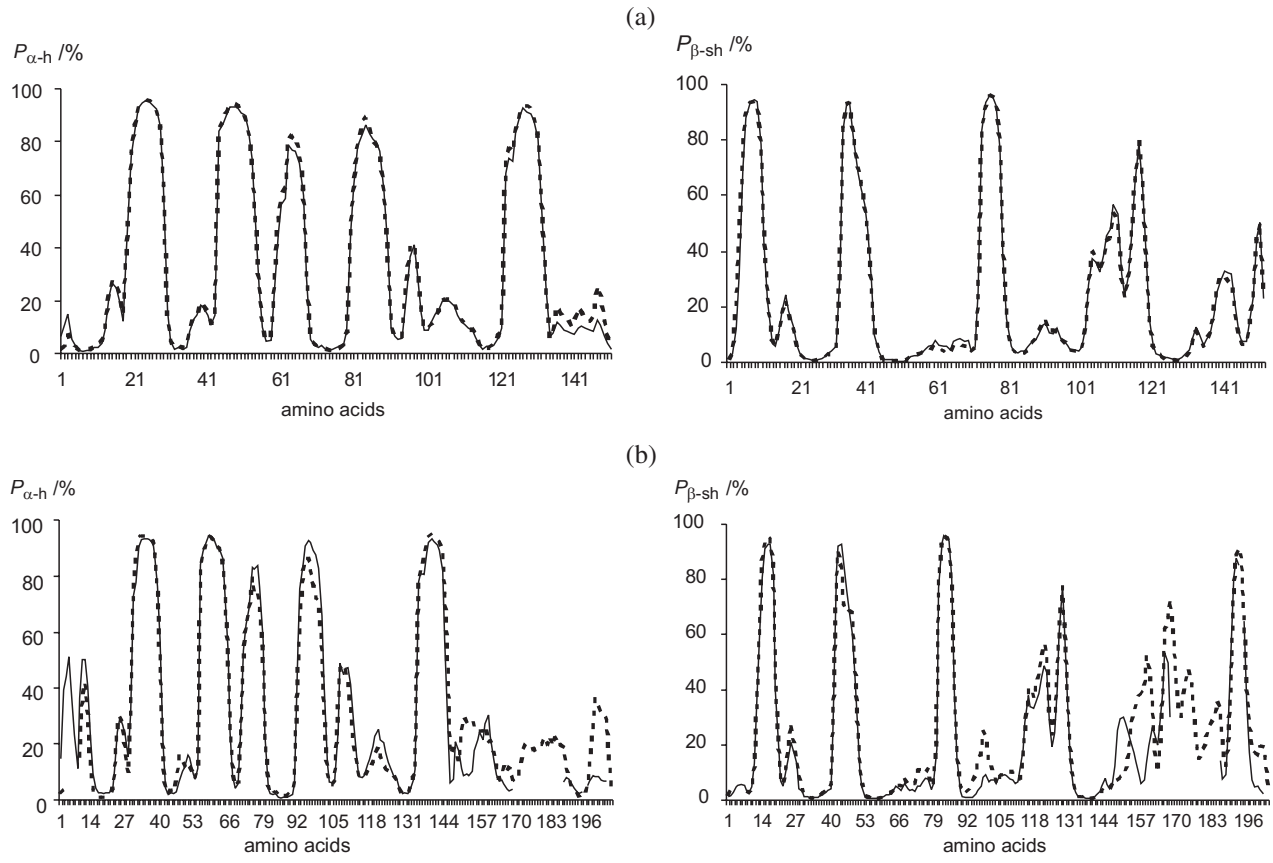

Figure 2. Predictions of secondary structures of Nm23 proteins from humans and *S. domuncula*: a) Nm23-H1B and Nm23-SD1 b) Nm23-H6 and Nm23-SD6. Probability profiles for human proteins are shown with full lines and for *S. domuncula* proteins with dotted lines. $P_{\beta\text{-sh}}$ – probability for extended β-sheets; $P_{\alpha\text{-h}}$ – probability for α helices.

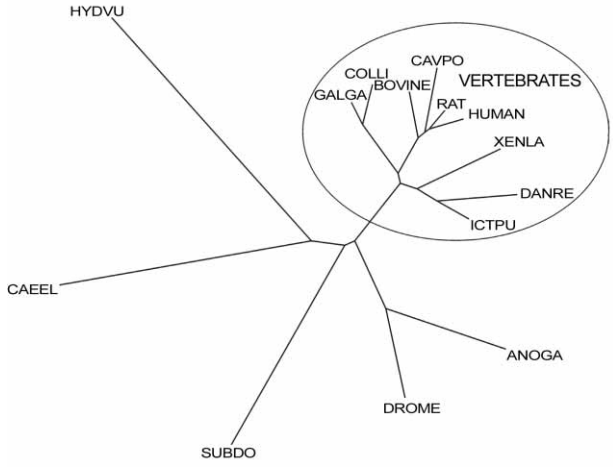

Figure 3. Unrooted phylogenetic tree based on Nm23-H1 homologues from different eukaryotes. Vertebrate branch is shown in the circle. The following sequences were used: ICTPU – *Ictalurus punctatus* (AAG14350), DANRE – *Danio rerio* (AAF20910), XENLA – *Xenopus laevis* (CAA66474), HUMAN – *Homo sapiens* (NP_000260), RAT – *Rattus norvegicus* (NP_612557), CAVPO – *Cavia porcellus* (AAK00527), BOVINE – *Bos taurus* (P52175), COLLI – *Columba livia* (Q90380), GALGA – *Gallus gallus* (NP_990378), HYDVU – *Hydra vulgaris* (AAK51137), CAEEL – *Caenorhabditis elegans* (NP_492761), SUBDO – *Suberites domuncula* (AY764256), DROME – *Drosophila melanogaster* (P08879) and ANOGA – *Anopheles gambiae* (XP_308641).

Nm23-H6 Homologue from S. domuncula

Nm23-SD6 and human Nm23-H6 (O75414) have 40 % identical amino acids (55 % overall similarity) and differ considerably in size: sponge protein is 202 aa long, due to an insertion of 18 aa close to the C-terminus, while human H6 protein is only 186 aa long (Figure 1b). Mammalian Nm23-H6 proteins have longer C-termini than Nm23-H1 proteins and vary in size from 186 aa to 189 aa (in mouse; NP_061227). They are also less conserved than H1 proteins and the overall degree of sequence conservation between human NM23-H6 and mouse Nm23-M6 is only 91 %. Besides that from vertebrates, Nm23-H6 homologue was only described from *D. melanogaster* (AAF64469) and is the product of *nmdyn-d6* gene.[23] *C. elegans* genome does not encode putative H6 homologue, nor it was until now found in any other lower metazoan organism. Of the two known invertebrate H6 homologues, sponge Nm23-SD6 is closer to Nm23-H6, because *D. melanogaster* protein shares only 35 % identical amino acids (51 % sequence similarity) with human H6 protein. In addition, *nmdyn-d6* gene product is only 151 aa long and has a short C-terminus. *S. domuncula* and *Drosophila* homologues of human Nm23-H6 protein are not much related – they share only 20 % identical amino acids (48 % overall homology); see Table I.

Prediction of secondary structures for Nm23-SD6 and human Nm23-H6 are shown in Figure 2b. Predicted secondary structures are highly similar for the first 140 aa in both proteins and the differences are found close to the C-termini, as a result of the above mentioned insertion in sponge protein. However, in both proteins aa at the very C-ends form extended beta sheets with highly similar predicted probabilities.

DISCUSSION

S. domuncula encodes at least two NDPK/Nm23 proteins, homologues of human Nm23-H1 and -H6 proteins. Interestingly, 2 cDNAs encoding Nm23-SD1 protein differ from other 2 cDNAs in nucleotide sequence at 3 silent positions, indicating the presence of 2 alleles (genes?) for Nm23-SD1 in the sponge genome. cDNAs encoding homologues of other human Nm23 proteins are not present in the *S. domuncula* ESTs database, which does not exclude the possibility of their presence in the sponge genome/proteome. The inspected database contains full or partial sequences of about 3700 different transcripts/proteins and is far from being complete.

Nm23-SD1 and human Nm23-H1B display high overall similarity in the primary structure as well as in predicted secondary structures. Positions of secondary structure elements (α-helices and β-sheets) in two predictions correlate very well with the previously determined general pattern for the Nm23 protein family.[24] The same is also true of Nm23-SD6 protein. All highly conserved aa involved in different biological functions of Nm23 proteins are present in the sponge Nm23-SD1. This is a strong indication that sponge protein is the true homologue of human Nm23-H1 and might therefore function in both sponge and mammalian cells in an identical way, *i.e.,* it could potentially be able to substitute human Nm23-H1 protein. It would therefore be interesting to study *in vitro* the ability of the sponge Nm23-SD1 to suppress metastatic potential of human cancer cells.

Nm23-SD6 and human Nm23-H6 are less conserved in their primary structure than sponge/human H1 proteins. Interestingly, their predicted secondary structures are highly similar. This is particularly evident in the poorly conserved N-termini of H6 proteins, where only 15 out of 50 aa are conserved (Figure 1b). However, the predicted secondary structures show an almost perfect overlap of structure elements with high probability (β-sheet, α-helix, β-sheet; see Figure 2b). In order to corroborate the similarity in the sponge and human homologues secondary structures, we also applied the PSIPRED prediction method.[25] This method again predicted almost identical secondary structures for both protein pairs (data not shown), although there are minor differences between predictions obtained with different methods.

Nm23-SD6 is more similar to human H6 that the putative H6 from *Drosophila*, the only other known H6 homologue from invertebrates. Most of the highly conserved aa are present at predicted positions in Nm23-SD6. Surprisingly, Nm23-SD6 does not have the consensus NDPK active site, characteristic of all NDPK proteins. Two conserved positions in the active site are occupied by non-permissive aa (Figure 1b), which could lead to aberrant NDPK function of this protein in sponge cells. One of the »non-permissive« changes in sponge protein includes serine to alanine (S→A) substitution in the NDPK active site consensus sequence (Figure 1b). Human Nm23-H1 protein with identical mutation was normally active; the mutation influenced neither the phosphorylation level nor the tumor suppression activity[26] despite mutations of this serine to some other aa. On the other hand, human Nm23-H5, involved in spermatogenesis, with as many as three non-permissive mutations in the NDPK active site is lacking NDPK activity *in vitro*.[27] It was already mentioned in the Introduction that NDPK activity is not required for many other biological activities of Nm23 proteins. Unfortunately, we know little about the Nm23-H6 biological role(s). Human Nm23-H6 was first described in 1999[15] and is poorly investigated: the protein is localized in mitochondria and is involved in cytokinesis. Because the gene for Nm23-SD6 is expressed in sponges (2 identical cDNA sequences were obtained by random sequencing), we have reasons to speculate that the gene product is present in *S. domuncula* and is biologically active, although not necessarily as a NDPK. What is/are the function(s) of Nm23-SD6 and Nm23-SD1 proteins in sponges still remains to be investigated.

Acknowledgement. – This work was supported by the grant to V.G. from the Croatian Ministry of Science, Education and Sports (MZT-P0098072) and to W.E.G.M. from the Deutsche Forschungsgemeinschaft (Mü/14-3).

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SAŽETAK

Dvije nukleozid-difosfat kinaze (NDPK/Nm23) iz morske spužve *Suberites domuncula*

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Suberites domuncula je pripadnik najstarije i najjednostavnije postojeće skupine (koljena) višestaničnih životinja – spužvi (Porifera). Nasumičnim sekvenciranjem cDNA *S. domuncula* nedavno je konstruirana baza EST-ova (expressed sequence tags). U ovom radu opisana su dva NDPK/Nm23 proteina iz spužve *Suberites domuncula.* Spužvini proteini nazvani su Nm23-SD1 i Nm23-SD6 jer su po aminokiselinskim sljedovima najsličniji ljudskim Nm23-H1 i -H6 proteinima. Ukupna sačuvanost sekvence između Nm23-SD1 i Nm23-H1 vrlo je visoka i iznosi 79 % (71 % je identična). Nm23-SD6 ima inserciju na C-kraju i pokazuje ukupnu homologiju od 55 % (40 % identičnosti) s ljudskim Nm23-H6. Predikcija sekundarne strukture pokazuje da su oba proteinska para gotovo jednaka. Nm23 proteini iz spužve *S. domuncula* pokazuju visoku sličnost s homolozima iz sisavaca/čovjeka, višu nego s npr. NDPK/Nm23 proteinima iz vinske mušice ili drugih beskralješnjaka. Osim toga, sličniji su proteinima iz sisavaca/čovjeka od većine poznatih Nm23 proteina iz beskralješnjaka.