Nocturnin in the demosponge *Suberites domuncula*: a potential circadian clock protein controlling glycogenin synthesis in sponges

*Werner E. G. MÜLLER*,†, Xiaohong WANG*,†, Vlad A. GREBENJUK*, Michael KORZHEV*, Matthias WIENS*, Ute SCHLOSSMACHER* and Heinz C. SCHRÖDER*

**INTRODUCTION**

Sponges are filter feeders that consume a large amount of energy to allow a controlled filtration of water through their aquiferous canal systems. It has been shown that primmorphs, three-dimensional cell aggregates prepared from the demosponge *Suberites domuncula* and cultured in vitro, change their morphology depending on the light supply. Upon exposure to light, primmorphs show a faster and stronger increase in DNA, protein and glycogen content compared with primmorphs that remain in the dark. The sponge genome contains nocturnin, a light/dark-controlled clock gene, the protein of which shares a high sequence similarity with the related molecule of higher metazoans. The sponge nocturnin protein was found showing a poly(A)-specific 3′-exoribonuclease activity. In addition, the cDNA of the glycogenin gene was identified for subsequent expression studies. Antibodies against nocturnin were raised and used in parallel with the cDNA to determine the regional expression of nocturnin in intact sponge specimens; the highest expression of nocturnin was seen in the epithelial layer around the aquiferous canals. Quantitative PCR analyses revealed that primmorphs after transfer from light to dark show a 10-fold increased expression in the nocturnin gene. In contrast, the expression level of glycogenin decreases in the dark by 3–4-fold. Exposure of primmorphs to light causes a decrease in nocturnin transcripts and a concurrent increase in glycogenin transcripts. It was concluded that sponges are provided with the molecular circadian clock protein nocturnin that is highly expressed in the dark where it controls the stability of a key metabolic enzyme, glycogenin.

Key words: circadian clock, glycogenin, light/dark response, nocturnin, sponges, *Suberites domuncula*.

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BMAL1</td>
<td>Brain and muscle ARNT (aryl hydrocarbon receptor nuclear translocator)-like 1</td>
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<tr>
<td>CRY</td>
<td>Cryptochrome</td>
</tr>
<tr>
<td>Cy5</td>
<td>Indodicarbocyanine</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>hnRNA</td>
<td>Heterogeneous nuclear RNA</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>Per</td>
<td>Period</td>
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<tr>
<td>qRT–PCR</td>
<td>Quantitative real-time reverse transcription PCR</td>
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<tr>
<td>Ror</td>
<td>Retinoic acid-related orphan receptor</td>
</tr>
<tr>
<td>SDGAPDH</td>
<td>Suberites domuncula glycerol 3-phosphate dehydrogenase</td>
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<td>SDGYG</td>
<td>S. domuncula glycogenin</td>
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<tr>
<td>SDNOC</td>
<td>S. domuncula heterogeneous nuclear RNA</td>
</tr>
<tr>
<td>REV-ERBα</td>
<td>Retinoic acid-related orphan receptor</td>
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1 To whom correspondence should be addressed (email wmueller@uni-mainz.de). The nucleotide sequence data reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number HE663082 and HE663083 for nocturnin and for glycogenin respectively.

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Sponges and primmorphs

Specimens of the marine sponge *S. domuncula* (Porifera, Demospongiae, Hadromerida) were sampled in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 17°C for more than 12 months [21].

Prammorphs, three-dimensionally growing cell aggregates *in vitro*, were prepared from single cells, obtained from *S. domuncula*, as described previously [22]. They were cultivated for up to 10 days in natural seawater (Sigma) supplemented with organic nutrients (0.2 % RPMI 1640 medium; Gibco). After 1 day in culture, the cells form 100–200 μm aggregates which increased in size during the following 5 days. Unless otherwise stated, 3-day-old aggregates were used. Where indicated, the primmorphs remained in complete darkness or had been illuminated with 30 lux, using a 15-watt Cool White fluorescent light (Westinghouse Electric) and kept in a temperature-controlled incubator at 16°C.

In one series of experiments, intact animals were exposed to 200 lux by a white fluorescent light penetrating through the 5–10 cm of water in front of the animals. Prior to analysis by immunohistology, those animals remained under continuous light exposure or in complete dark for 3 days.

Identification and cloning of the poriferan nocturnin and glycogenin cDNAs

The two cDNA sequences [SDNOC (*S. domuncula nocturnin*)] and [SDGYG (*S. domuncula glycogenin*)] were isolated. Details are given in the Supplementary Online data (at http://www.BiochemJ.org/ij/bj448/bj4480233add.htm).

Preparation of recombinant nocturnin and antibodies

The recombinant nocturnin (rNOC_SUBDO) was prepared in *Escherichia coli* cells as described in the Supplementary Online data. This protein sample was used for raising polyclonal antibodies in rabbits.

SDS/PAGE and Western blot analysis

Details of the SDS/PAGE and for Western blot analyses are given in the Supplementary Online data.

qRT–PCR (quantitative real-time reverse transcription PCR)

The absolute steady-state expression level of *SDNOC*, as well as of *SDGYG*, was determined by qRT–PCR. From single cell suspensions, where prammorphs were formed, RNA was isolated, as described with the respective experiments in the Results section.

The details of the qRT–PCR procedure to determine the expression level of *SDNOC* were described previously [13,23]. All reactions were run with an initial denaturation at 95°C for 10 min, followed by 40 cycles each at 95°C for 20 s, then 60°C for 20 s and 72°C for 35 s. The following primers were used for amplification of *SDNOC*: forward, 5′-GCCTTGTCTCCTGCTC-3′ (nt 413–432) and reverse, 5′-AGACTCTTAGTCTTCCAAACC-3′ (nt 535–516); PCR product size is 123 bp. As a reference housekeeping gene, we used *SDGAPDH* (*S. domuncula* glyceral 3-phosphate dehydrogenase; GenBank®, accession number AM902265.1) with the following primers: forward, 5′-TCCACCAGGGCAGTGATG-3′ (nt

**MATERIALS AND METHODS**

**Chemicals, materials and enzymes**

The sources of the materials used in the present study were described previously [11].
816–837) and reverse, 5′-AGTGAGTGCTCCTCGGAGTC-3′ (nt 945–924); fragment size was 130 bp. After qRT–PCR, the threshold position was set to 50.0 RFU (relative fluorescence units) above PCR-subtracted baseline for all runs. Mean Ct values and efficiencies were calculated by the iCycler software. Relative expression level was calculated by $E_{\text{GAPDH}}^{C_{\text{GAPDH}}} / E_{\text{NOC}}^{C_{\text{NOC}}}$, where $E$ is the PCR efficiency and $C_t$ is the threshold cycle.

The quantitative steady-state expression level of $SDGYG$ was determined in parallel assays to those of $nocturnin$ expression. For this series of experiments the following primers were used: forward, 5′-GGCAACATCATAGTGGCAGAGG-3′ (nt 732–755) and reverse, 5′-ATCACTCTGTCCAGATCTCG-3′ (nt 868–847); PCR product size of 137 bp. The expression level was calculated by $E_{\text{GAPDH}}^{C_{\text{GAPDH}}} / E_{\text{VYG}}^{C_{\text{VYG}}}$.

Immunohistology

Tissue slices were prepared as described previously [24]. Slices of 8 μm thickness were fixed in paraformaldehyde. The specimens were incubated with the primary antibodies, the rabbit anti-nocturnin serum (PoAb-rNOCSUBDO), diluted 1:1500 in blocking solution and incubated while shaking at 4°C overnight. As controls, both pre-immune serum and PoAb-rNOCSUBDO antibodies, adsorbed with recombinant nocturnin, were used. Unbound antibodies were removed by washing four times with PBS prior to the incubation with fluorescence labelled [with Cy5 (indodicarbocyanine)] secondary antibodies (1:3000 dilution). Parallel slices were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma). The slices were inspected with an Olympus AHTB3 microscope under immunofluorescence light at an excitation light wavelengths suitable for either Cy5-stained structures or for DAPI.

Determination of the glycogen level

The microdetermination method, performed as described by Templeton [25], was used to quantify the concentration of glycogen in the primmorphs. In brief, the anthrone reagent (catalogue number 10740, Sigma; for determination of carbohydrates) was used, which was applied in the assay after washing the sample in distilled water and alcohol and subsequent drying as described previously [25]. Glycogen was extracted and precipitated from hydrolysates with 80% ethanol and treated with ethanol to remove low-molecular-mass carbohydrates. The amount of glycogen in the samples was determined using the glycogen from Mytilus edulis (G1767, Sigma) as a standard. Optical density was determined against a water blank at 630 nm in a spectrophotometer. Protein determinations were performed in homogenates prior to centrifugation.

Deadenylase assay

In order to prove that nocturnin has enzymatic activity as a 3′-exoribonuclease, the full-length nocturnin protein (rNOCPAE_SUBDO) was prepared recombinantly (details are given in the Supplementary Online data). The 37.5 kDa protein obtained was assayed for deadenylase activity using the poly(A) tail of a partial glycogenin cDNA. A part of the $SDGYG$ cDNA comprising the ORF (open reading frame) nucleotide sequence (from nt 465–575) was cloned into the pSP64 poly(A) Vector system (Promega). This vector has a stretch of 30 dA:dT residues allowing the synthesis of a RNA containing a 30-residues-long poly(A) tail [26]. To generate the synthetic poly(A)$^+$ mRNA substrate, the glycogenin cDNA (nt 465–575) was digested with HindIII and SacI enzymes (New England Biolabs) and ligated into the vector, digested with the same restriction enzymes. The pSP64[poly(A)$^+$] construct was transcribed using SP6 polymerase and the transcribed poly(A)$^+$ mRNA was purified by phenol/chloroform extraction, precipitated and then resuspended in diethylpyrocarbonate-treated water [27]. The mRNA concentration was measured at $A_{260}$ and the transcript size was determined by gel electrophoresis followed by staining with SYBR Green II (Molecular Probes) [28,29]. RNA size markers (peqGOLD, PEQLAB) were used.

The deadenylase assay was performed as described previously [16,17,30] and contained, in a total reaction volume of 80 μl, the following components: 100 mM Tris/HCl (pH 8.7), 2 mM MgCl₂, 0.2 mM dithiothreitol, 0.02% Nonidet P40, 0.3% spermidine and 10 pg/ml BSA, as well as 10 μg of poly(A)$^+$ glycogenin mRNA. The enzyme reaction was started with either 3 μg of recombinant rNOCPAE_SUBDO or 1 μg of poly(A)$^+$ specific 3′-exoribonuclease, purified from the calf thymus [17]. After incubation at 37°C, aliquots of 20 μl were size-separated, stained with SYBR Green II and the bands were visualized with a short-wave ultraviolet transilluminator [29].

Northern blot analysis

RNA was isolated from primmorphs, essentially as described previously [31]. RNA was extracted from liquid-nitrogen-pulverized tissue with Trizol® (Invitrogen). The concentration and the purity of the RNA were determined osimetrically using the absorbance at 260 nm and the $A_{260}/A_{280}$ ratio respectively.

Aliquots of total RNA (5 μg) were electrophoresed through 1% formaldehyde/agarose gel and blotted on to Hybond-N+ nylon membranes following the manufacturer’s instructions (Amersham) as described previously [32]. Hybridization was performed with a 0.37 kb part of the $SDGYG$ cDNA, spanning the region nt 13–376 within the coding region. The probe was labelled with the PCR DIG (digoxigenin) probe synthesis kit according to the manufacturer’s instructions (Roche). After washing, DIG-labelled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase; 1:10000 dilution) and visualized by a chemiluminescence technique using CDP-Star, according to the instructions of the manufacturer (Roche) and as described previously [32]. The ssRNA (single-stranded RNA) ladder from New England BioLabs was used as a marker for the size determination.

The poly(A) tails of the mRNAs were digested with RNaseH as described previously [33]. In brief, 50 μg of RNA was denatured by heating and immediately chilling on ice. Then the samples were incubated at 25°C for 10 min either in the absence or in the presence of oligo(dT)$_{12-18}$ (Invitrogen). The hybrid RNA:oligo(dT) samples were incubated (37°C for 30 min) with 3 units of RNase H (Invitrogen) per 50 μl assay. Those digested RNA samples were then used for Northern blot analysis.

Additional methods

For the quantification of protein, the Bradford method (Roti-Quant solution, Roth) [34] was used. DNA content was measured as described previously [35]. In order to determine the dry mass, primmorph samples were dried at 105°C and then weighed on a microbalance (Sartorius Expert LE26P). Six parallel determinations were performed.
Figure 1 Specimens of S. domuncula and primmorphs, the three-dimensional aggregates obtained from them

(A) Two specimens of S. domuncula are shown, a red and a yellow colour-type. On to the yellow specimen a grazing gastropod/mollusc Bittium sp. (indicated by arrowheads) is seen. The animals harbour shells which are used by hermit crabs (cr). Primmorphs formed in the light from single cells after 2 (B) or 4 (C) days in culture. In contrast, three-dimensional aggregates, kept in the dark form only comparably small aggregates after 2 days (D) which grow slowly to larger cell clumps after an incubation period of 4 days (E).

RESULTS

S. domuncula and its primmorphs

Three different colour types of S. domuncula are found in nature [11], primarily as red specimens (Figure 1A), secondarily as yellow ones (Figure 1A) and finally as whitish/magenta ones. The specimens live in association with the hermit crab Pagurites oculatus (Decapoda, Paguridea; Figure 1A) which resides inside of shells of the mollusc Trunculariopsis trunculus (Gastropoda, Muricidae). For the experiments described in the present study, the common red specimens were used. On the surface of both colour types, gastropods/molluscs Bittium sp. (Mollusca, Gastropoda, Cerithiidae, Bittium) occasionally graze.

Effect of light on morphology and DNA, protein and glycogen content in primmorphs

If exposed to the light, the small aggregates, formed during the first 2 days, re-aggregate to larger units with sizes of around 1 mm at day 4 (Figures 1B and 1C). In contrast, those three-dimensional cell aggregates which were left in the dark showed a significantly retarded re-aggregation; only small aggregates are seen at day 2 and the primmorphs at day 4 show a lobated morphology and have not yet started to form round-shaped units (Figures 1D and 1E).

The DNA and protein content in growing primmorphs differs in dependence on the light conditions used for cultivating them. During continuous illumination of the primmorphs, 1 day after the onset of the re-aggregation process the DNA and protein content was determined as 0.95 and 125 μg/mg of dry mass respectively. During the following 4 days, the mass increased to 2.9 and 211 μg/mg of dry mass of DNA and protein respectively (Figure 2). When kept in the dark, the primmorphs show a similar increase in DNA content during the incubation period of 0–4 days; however, the content of protein in the primmorphs is significantly lower, with a value of 105 μg/mg of dry mass at day 0 and 142 μg/mg of dry mass at day 4.

The content of glycogen showed similar kinetics. Under light exposure, the glycogen content in the primmorphs increased significantly from 28 μg/mg of dry mass to 52 and 67 μg/mg of dry mass at days 3 and 4 respectively. No significant increase was seen in the primmorphs kept in the dark; values around 15–22 μg/mg of dry mass are measured during the incubation period of 0–4 days (Figure 3).

Sequence analysis of sponge nocturnin

The complete SDNOC cDNA was isolated (Supplementary Figure S1 at http://www.BiochemJ.org/bj/448/bj4480233add.htm). The
Figure 2  DNA and protein content in primmorphs grown in the light or dark

At 1 day after the onset of the re-aggregation process, the growing primmorphs were exposed to light or dark as described in the Materials and methods section. At zero time up to a total incubation period of 4 days, the primmorph samples were taken and used for determination of DNA or protein; the values are correlated with the amount of dry mass used for the analyses. Results are means ± S.D. from ten separate experiments. *P < 0.05.

Figure 3 Alteration of glycogen content in the primmorphs during light exposure or dark maintenance from 0–4 days

Results are means ± S.D. from 10 separate experiments. *P < 0.05.

size of the deduced protein (NOC_SUBDO), comprising 314 aa, is 34836 Da. As shown in the Supplementary Online data, the sponge nocturnal mRNA had a complete size of 1.2 kb. The deduced protein comprises the domain that is characteristic for the superfamily of exonuclease-endonuclease-phosphatases. In addition, a phylogenetic analysis revealed a similarity/homology score of 50% with the corresponding cnidarian protein, that the phosphatases. In addition, a phylogenetic analysis revealed characteristic for the superfamily of exonuclease-endonuclease-1.2 kb. The deduced protein comprises the domain that is

Recombinant SDNOC and antibodies raised against it

A part of the cDNA comprising the ORF for nocturnal (NOC_SUBDO) was expressed in E. coli cells. After purification by affinity chromatography, the 30 kDa protein had a purity of >95% (see Supplementary Online data). This protein sample (rNOC_SUBDO) was used to raise polyclonal antibodies (PoAb-rNOCSUBDO). The serum recognized the 30-kDa recombinant protein after size separation by SDS/PAGE and Western blotting (Supplementary Figure S2, lane d at http://www.BiochemJ.org/bj/448/bj4480233add.htm). This protein did not cross-react with a pre-immune serum (Supplementary Figure S2, lane e).

Sequence analysis of SDGYG

The ORF of the SDGYG cDNA encodes a protein (GLYG_SUBDO) of a calculated size of 37023 Da; (Supplementary Figure S3 at http://www.BiochemJ.org/bj/448/bj4480233add.htm). In this sequence the characteristic domains for the glycogenins, the N-terminal β-α-β Rossmann-like fold as well as the glycosyl transferase family 8 segment, are present.

Expression of nocturnin in the tissue of the animals

The regional expression of nocturnin within the sponge tissue was analysed by immunofluorescence. Intact animals were kept for 3 days in either dark or under continuous light. Tissue slices within the surface region of a dark-kept animal were prepared and reacted with the anti-nocturnin antibodies (PoAb-rNOCSUBDO). The immunofluorescence image convincingly shows that the cells around aquiferous canals (the endopinacoderm layer) are brightly stained (Figures 4A and 4C). The cell arrangement around the canals was visualized by DAPI staining (Figures 4B and 4D). In contrast, when the slices of the light-maintained animals were reacted with PoAb-rNOCSUBDO almost no staining was seen (Figures 4E and 4G). Two control series were performed to demonstrate that the immunofluorescence signals are specific. First, when slices from a dark-kept animal were reacted with pre-immune serum, almost no staining is seen (Figure 4I). The corresponding DAPI-stained slice shows that a region around a canal was selected for sectioning (Figure 4J). Secondly, when adsorbed PoAb-rNOCSUBDO antibodies had been applied for the immune reaction, again no immunofluorescent signals were seen on the sections obtained from a dark-kept animal (Figure 4K); the corresponding DAPI image is shown in Figure 4(L).

Expression levels of nocturnin and glycogenin in primmorphs in either the light or dark

A quantitative determination of the steady-state expression level both for the SDNOC and the SDGYG was performed by using qRT–PCR. Primmorphs cultivated for 3 days under light exposure showed an expression level for SDNOC of 2.7 × 10⁻³, with respect to the steady-state expression of the SDGAPDH gene (Table 1). However, if those three-dimensional aggregates were kept for the same period of time in dark, the expression level significantly increased to 48.2 × 10⁻³. Contrasting day/night expression was also measured for SDGYG. The amount of transcripts is considerably higher in light-exposed primmorphs with 5.2 × 10⁻¹, compared with the dark-maintained primmorphs with 1.2 × 10⁻¹ (Table 1).

The time-dependent induction of SDNOC mRNA transcript formation during dark onset was determined in primmorphs that had been kept for 3 days under continuous light. After onset of complete darkness, the SDNOC transcript level increased significantly from 3.2 × 10⁻¹ to 8.3 × 10⁻³, even after 3 h, and reached a maximum value of 49.0 × 10⁻³ after a 12-h incubation.
Figure 4  Expression of nocturnin in tissue of *S. domuncula* as analysed by immunohistology and applying antibodies against nocturnin.

(A–D) An animal kept in the dark was sectioned through a tissue region of the sponge approximately 0.5–1 mm below its surface. (A) The slice was reacted with antibodies against nocturnin (PoAb-rNOCSUBDO) and the immunoreactions were monitored with a labelled antibody. (B) A parallel slice was inspected at 490 nm to localize DAPI staining. Canals (ca) are marked. (C and D) A section through a similar region was inspected at higher magnification.

(E–H) An animal exposed to the light for 3 days was analysed by immunofluorescence (E). (F) The same region was likewise analysed for DAPI staining, to highlight the canal (ca). (G and H) A similar region at higher magnification. (I–J) Control analysis from an animal kept in the dark and analysed for immunofluorescence, using pre-immune serum (I) and in parallel by DAPI staining (J). (K and L) In a further control, adsorbed antibodies, PoAb-rNOCSUBDO, were used. (K) No staining reaction is seen; parallel DAPI image (L). The tissue area around a canal (ca) is marked.

Figure 5  Reverse kinetics of the increase/decrease of the abundance of the nocturnin compared with glycogenin transcripts after transfer of primmorphs from light to dark.

Primmorphs were kept for 3 days in the light and then transferred to the dark. Samples of primmorphs were taken immediately after the onset of darkness and during the following 24-h incubation period. RNA was then extracted and the expression levels for the two genes were quantified using qRT–PCR. Each data point represents the mRNA level of the respective expressed gene normalized to the amount of GAPDH transcripts and results are means ± S.D. (five experiments per time point).

Table 1  Expression levels of the *SDNOC* as well as the *SDGYG* genes in primmorphs dependent on light exposure.

Primmorphs were prepared from cell suspension and maintained for 3 days under light exposure (Light), or under complete darkness (Dark). RNA was then extracted and the expression levels of nocturnin as well as GYG were quantified through qRT–PCR. Each data point represents the mRNA level of the respective expressed gene normalized to the amount of SDGAPDH transcripts, as means ± S.D. (five experiments per time point).

<table>
<thead>
<tr>
<th>Gene expression (mRNA/mRNA_GAPDH) ± S.D.</th>
<th>SDNOC</th>
<th>SDGYG</th>
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<tr>
<td>Light</td>
<td>2.7 ± 0.3 × 10^{-3}</td>
<td>5.2 ± 0.6 × 10^{-1}</td>
</tr>
<tr>
<td>Dark</td>
<td>48.2 ± 5.1 × 10^{-3}</td>
<td>1.2 ± 0.1 × 10^{-1}</td>
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period (Figure 5). The decay kinetics of *SDGYG* expression proceeds in the opposite direction, starting with 475 × 10^{-3} transcripts, with respect to the *SDGAPDH* level, and reached a minimum after the 12–24 h incubation period with 153 × 10^{-3} and 138 × 10^{-3} transcripts respectively.

Similarly fast is the decay/synthesis kinetics of the SDNOC and SDGYG transcripts in primmorphs kept in the dark after onset of light exposure (Figure 6). The primmorphs were cultured in the dark during the first 3 days after starting aggregation and they were then exposed to light. Starting with the high transcript level for SDNOC of 41.7 × 10^{-3} with respect to the SDGAPDH transcripts, the decay starts after 3 h and significantly dropped to 29.6 × 10^{-3}, after just a 3-h dark period, to almost the lowest level that is reached after 6–24 h of light exposure of approximately 10 × 10^{-3}. The increase of the SDGYG transcript level becomes, again, significant even after a 3-h exposure to light (193 × 10^{-3}) and reaches maximal levels after 12–24 h of 487 × 10^{-3} and 541 × 10^{-3} respectively.
Figure 6   Dark/light reciprocal expression studies of the nocturnin and the glycogenin gene; quantification through qRT–PCR

The primmorphs remained in the dark for 3 days before being exposed to light; time 0, onset of light exposure. Samples were taken after 0–24 h.

Figure 7   Nocturnin-mediated deadenylation of the synthetic poly(A)-containing mRNA substrate

The poly(A)-glycogenin mRNA was prepared using the pSP64 poly(A) Vector system and assayed with the full-length recombinant nocturnin protein (rNOCPAE) or the native poly(A)-specific 3'-exoribonuclease (PAE), purified from the calf thymus, for deadenylation activity as described in the Materials and methods section. The assays were run for 0, 5, 10 or 30 min, as indicated. After the incubation (37°C), aliquots were taken and size separated by gel electrophoresis followed by SYBR Green II staining. The arrows indicate poly(A)' and poly(A)− sizes; they had been estimated by comparison with the 100 nt and 200 nt size markers.

Synthetic SDGYG mRNA as a target for the deadenylase SDNOC

The SDGYG mRNA was synthesized using the pSP64 poly(A) Vector system. The poly(A)+ mRNA contained a 30-nt long poly(A) stretch and a 100-nt long partial SDGYG mRNA section. This mRNA was incubated with the full-length recombinant nocturnin protein from S. domuncula (rNOCPAE_SUBDO) or with the native poly(A)-specific 3'-exoribonuclease, purified from the calf thymus [17]. The incubation was performed at 37°C. After a 10-min incubation period, the poly(A)+ mRNA already became partially deadenylated, if assayed with the recombinant nocturnin (Figure 7, left-hand panel). After an extended incubation period for 30 min, the poly(A)+ mRNA added to the assay underwent complete deadenylation (Figure 7, left-hand panel), as did the native poly(A)-specific 3'-exoribonuclease (Figure 7, right-hand panel). This deadenylation of the poly(A)+ mRNA was prevented if the assays were supplemented with 10 mM EDTA, an inhibitor of the nuclease activity [17] (results not shown).

Northern blot analysis of SDGYG mRNA from light- and dark-exposed primmorphs

The size of the mRNA encoding SDGYG was determined by Northern blot hybridization using the labelled SDGYG cDNA as a probe. The experiments revealed that the mRNA for SDGYG, isolated from primmorphs exposed to light had a larger size, compared with the transcripts isolated from primmorphs kept in the dark (Figure 8A). Correlating with the markers that run in parallel, the SDGYG mRNA existing in primmorphs kept for 96 h under light was determined to be approximately 1280-nt long (Figure 8A, lane a), whereas the SDGYG transcripts from the primmorphs that had been subsequently exposed for 3 h to the dark were determined to be about 1180-nt long (Figure 8A, lane b). After exposure of the primmorphs to the dark for longer than 8 h, no clear band could be detected any more under the conditions tested.

The length of the poly(A) tail of the SDGYG mRNA was further estimated by digesting this segment with RNase H after a prehybridization step with oligo(dT). Using this approach, it could be visualized by Northern blot analysis that the transcripts, which were obtained from the primmorphs kept in the light and not hybridized with oligo(dT), had an apparent length of 1280 nt (Figure 8B, lanes a and b), whereas those transcripts which had been hybridized with oligo(dT) and subsequently exposed to RNase H, in order to remove selectively their poly(A) tail, had a size of approximately 1120 nt (Figure 8B, lanes c and d).

DISCUSSION

In the last decade the molecular basis of the core oscillatory mechanisms had been elaborated, as outlined in the Introduction section and a previous review [36]. In animals, the major ‘clock genes’ have been determined and found to code for the transcription factors BMAL1 and CLOCK; these gene products have the characteristics to act as regulatory proteins that negatively regulate their own expressions after a distinct time delay. This limb comprises a series of clock-controlled genes/gene products, e.g. Per, Cry, Ror (retinoic acid related orphan receptor)-α and nocturnin. The Rev-Erb-α gene product reduces the expression of Bmal1 via interaction with the ROR-response elements. This system is depicted in Figure 9. The phase precision of the oscillating circadian rhythm is under the control of variations in light intensities [37], and is likely to be adjusted by those. Surely the sponges do not have light perception systems, known from the opsin-based visual systems of protostomians or deuterostomians [14].

It is well established that sponges undergo rhythmic contraction resulting in a reduction of their body volume by up to 70%, as shown for the species Tethya wilhelma [38]. Interesting to note is that the contraction phases are significantly longer during the night than during the day, indicating that the sponges are provided with a nervous-like signal transmission system (e.g. [39]). However, in these animals, neurons are absent indicating that a nervous system, known from higher animal phyla, does not exist (e.g. [40]) even though neuronal receptors had been identified, e.g. the metabotropic glutamate/GABA (γ-aminobutyric acid)-like receptor [41] and also neurotransmitters, e.g. GABA [42], had been found. As a potential compensation for the lack of a neuron-based transmission system in sponges, we proposed (see the Introduction section) a neuron-like wiring circuit composed of a light-generating luciferase, that is coupled to the glass fibre network of the spicules and finally of a photon-detecting Cry.

Although the clock-controlled genes act on the expression of the central core clock molecules Bmal1 and Clock either negatively (Per and Cry) or positively (Rorα), nocturnin remains in the cytoplasm and controls the half-life and stability of key mRNA species involved in the adjustment of the homoeostasis of the intermediary metabolism, especially of the fatty acid and
had originally been described as a 3′-exonuclease by us [18,19]. This enzyme controls the network by acting positively on the expression of the downstream gene targets, primarily Per, Cry, Ror and Rev-Erb. It is assumed that the light/dark cycle in general and the luciferase light-generating system, here in sponges, adjusts the fidelity of the clock oscillation. The gene products PER and CRY, the major negative limb, inhibit the activity of BMAL1 and CLOCK.

A further clock controlled gene product is the nuclear receptor Rev-Erb. It is assumed that the light/dark cycle in general and the luciferase light-generating system, here in sponges, adjusts the fidelity of the clock oscillation. The gene products PER and CRY, the major negative limb, inhibit the activity of BMAL1 and CLOCK. A further clock controlled gene product is the nuclear receptor Rev-Erb that likewise negatively affects the expression of Bmal1 via interaction with the Rev-Erb/ROR responsive elements (RORE). In addition, the expression of the clock controlled gene Noc (nocturnin) is positively controlled by BMAL1 and CLOCK. The protein product, termed nocturnin, is a deadenylase that had originally been described as a 3′-exonuclease [17,18]. This enzyme controls the decay kinetics via specific removal of the poly(A) tail of the mRNAs. It is assumed that nocturnin removes (especially in the dark/night) key regulatory proteins of the fatty acid and glycogen metabolism [6].

**Figure 8** Northern blot analysis of glycogenin mRNA isolated from primmorphs that had been incubated for the indicated periods of time (in h) either in the light or dark

(A) RNA was isolated from primmorphs exposed for 96 h to light (lane a, 2 μg of RNA per lane) or for 96 h in the light and subsequently for 3 h in the dark (lane b, 5.5 μg of RNA per lane). After size separation and blot transfer the membranes were hybridized with a DIG-labelled SDGYS cDNA probe; hybridization was detected by using the CDP/chemiluminescence procedure. (B) mRNA samples, isolated from specimens exposed for 96 h to light, remained untreated or had been hybridized to oligo(dT), as indicated. Then the transcripts were incubated with RNAse H, as outlined in the Materials and methods section. Subsequently, the samples were analysed by Northern blotting (4.5 μg of RNA per lane).

**Figure 9** Scheme presenting the major molecules contributing to the core molecular clock machinery

The transcription factor BMAL1 and CLOCK constitute the positive limbs of the core circadian network by acting positively on the expression of the downstream gene targets, primarily Per, Cry, Ror and Rev-Erb. It is assumed that the light/dark cycle in general and the luciferase light-generating system, here in sponges, adjusts the fidelity of the clock oscillation. The gene products PER and CRY, the major negative limb, inhibit the activity of BMAL1 and CLOCK. A further clock controlled gene product is the nuclear receptor Rev-Erb that likewise negatively affects the expression of Bmal1 via interaction with the Rev-Erb/ROR responsive elements (RORE). In addition, the expression of the clock controlled gene Noc (nocturnin) is positively controlled by BMAL1 and CLOCK. The protein product, termed nocturnin, is a deadenylase that had originally been described as a 3′-exonuclease by us [18,19]. This enzyme controls the decay kinetics via specific removal of the poly(A) tail of the mRNAs. It is assumed that nocturnin removes (especially in the dark/night) key regulatory proteins of the fatty acid and glycogen metabolism.

The quantitative steady-state expression of both nocturnin and glycogenin was determined by qRT–PCR, as a function of light/dark exposure. It was seen that after changing the primmorphs from the light-exposed state to the dark, a strong up-regulation of the nocturnin expression results; half-maximal levels are found after 3–6 h onset of the dark. A similar induction kinetics had been determined also for nocturnin in the mammalian system [6]. Glycogenin gene expression was described to be induced by special cells, the grey cells [43], which are localized below the epithelial pinacoderm layer.

After the two cDNAs for nocturnin and glycogenin had been cloned, the deduced proteins were characterized. The nocturnin polypeptide shows the characteristic exonuclease-endonuclease-phosphatase domain [44], whereas glycogenin comprises the glycosyl transferase family 8 domain [45,46]. Subsequently, the nocturnin protein was recombinantly expressed in E. coli cells and then used to raise polyclonal antibodies. With these tools in hand, the expression/synthesis level of nocturnin was determined.

By application of whole-mount in situ hybridization (cDNA: results not shown) and immunohistology (polyclonal antibodies; see above), the highest level of nocturnin was identified in the cortex region of the sponge. In this region, the expression of luciferase [12] and Cry [13], the two elements of the light generation and harvesting system in sponge, had also been identified. Nocturnin is abundantly present in the aquiferous canal-lining cells of the dark-kept animals, the endo-pinacocytes, as identified by immunohistology. Those cell layers had been implicated to act as a water diffusion barrier and also as phagocytizing cells.

Moreover, glycogen metabolism [6] (Figure 9). Nocturnin is a circadian deadenylase that had originally been described as a 3′-exonuclease from the calf thymus [17,18]. As a function for this enzyme, a selective hydrolysis of the poly(A) tail of mRNAs had been proposed [16], allowing a control of the post-transcriptional net polyadenylation of hnRNA (heterogeneous nuclear RNA), especially during the DNA synthesis phase. These data had been confirmed and extended for the hnRNA of nocturnin by nuclear run-on analyses [7]. For the present study, the steady-level of the transcripts for glycogenin had been used. This enzyme is the crucial regulator of glycogen synthesis through acting both as a glycosyltransferase and as a primer [20].

To define the model, the response of *S. domuncula* and primmorphs derived from this species via aggregation of single cells to aggregates containing proliferating and differentiating three-dimensional clumps [22] was determined as a function of the dark and light. It is shown that primmorphs exposed to light develop into compact three-dimensional aggregates, whereas those that remained in the dark form irregular and small aggregates. If the primmorphs were kept in the dark only a slight increase in the protein and DNA contents were measured, whereas in the presence of light those aggregates vigorously formed those biopolymers. Next, and as expected, the light-exposed primmorphs heavily deposited glycogen in contrast with the dark-maintained aggregates. In sponges, glycogen is produced by special cells, the grey cells [43], which are localized below the epithelial pinacoderm layer.

The quantitative steady-state expression of both nocturnin and glycogenin was determined by qRT–PCR, as a function of light/dark exposure. It was seen that after changing the primmorphs from the light-exposed state to the dark, a strong up-regulation of the nocturnin expression results; half-maximal levels are found after 3–6 h onset of the dark. A similar induction kinetics had been determined also for nocturnin in the mammalian system [6]. Glycogenin gene expression was described to be induced...
faster after starvation for 30–60 min [47]. In the sponge system, this enzyme reaches its 50% maximal expression level after about 6–12 h of light exposure.

In order to verify that the sponge nocturnin also possesses a poly(A)-specific 3'-exoribonuclease activity, the cDNA was used to prepare a complete nocturnin in *E. coli* cells recombinantly. As described for the vertebrate nocturnins [15,30], *SDNOC* also possesses a specific exoribonuclease activity. In our approach, the poly(A)+ glycogenin mRNA was incubated with the recombinant sponge nocturnin and the native poly(A)-specific 3'-exoribonuclease, purified from the calf thymus as described previously [17]. Along with the data gathered from vertebrate nocturnins (summarized in [5]) and the results of the present study, it must be concluded that the previously described poly(A)-specific 3'-exoribonuclease, discovered by us [17] is identical with the nocturnin proteins.

Furthermore, based on previous studies from our group and others [48,49], it was established that the length of the poly(A) tail of mRNAs is positively correlated with the half-life of the respective transcripts [50]. Therefore we determined the overall size of the transcript encoding glycogenin isolated from light-exposed primmorphs in comparison with the size of the transcripts from primmorphs kept, after light exposure, in the dark. It was found that the transcripts from the primmorphs under light exposure were about 100 nt longer than those isolated from the primmorphs kept in the dark. A distinct transcript band reflecting glycogenin could be identified after 3 h in the dark, whereas a longer incubation period resulted in a drastic decay of the glycogenin-specific transcripts. The conclusion that the reduction of the transcript size is due to a removal of the poly(A) tail of the glycogenin mRNA was supported by RNase H-digestion experiments, following a previously described protocol [51].

These digestion experiments revealed that the size of the transcripts from light-exposed animals is reduced by about 160 nt, after hybridization with oligo(dT) and RNase H treatment. The two sets of experiments strongly suggest that also in the intact cell system the size of the transcripts for glycogenin undergo a shortening of about 100–160 nt, which should be attributed to the removal of the poly(A) tail of the glycogenin transcripts. In future experiments we will use qRT-PCR under different pulse-chase conditions to determine the half-life of glycogenin mRNA isolated from the primmorphs kept in the dark in the absence and in the presence of cycloheximide, following a strategy described previously [52]. We expect from those experiments a clarification about the effect of the inhibition of protein (nocturnin) synthesis on the rapid decay of the glycogenin transcripts. Those experiments are now in progress in our group. However, until now it has not been possible to knockout the nocturnin gene *in vivo* in the sponge system as described for mice [53], since this technique could not be established in these animals.

To conclude, the experimental results obtained in the present study show that in sponges the expression of nocturnin is up-regulated during the dark phase and undergoes decay during light exposure. The expression level of glycogenin is inversely correlated with nocturnin. On the basis of the existing data, it is reasonable to accept that the exonuclease nocturnin is, perhaps directly, involved in the dark-induced decay of the glycogenin transcripts via the deadenylation of mRNAs. The nocturnin-based and light-controlled expression system of glycogenin is economically meaningful. The sponges, as filter-feeders, filtrate huge amounts of water, 0.002–0.84 cm3 per second per cubic centimetre of sponge tissue, through their aquiferous canal system to extract edible micro-organisms and, by that, meet their extremely high energy demands. It is interesting that sponges are using the 'high energy' phosphate, the arginine-phosphate instead of creatine-phosphate used in mammalian systems, as the phosphoryl group donor to synthesize ATP via an enzymatic reaction catalysed by arginine kinase [54]. In this context, the first molecular approach is outlined to understand the dark/light response at metabolic level.

**AUTHOR CONTRIBUTION**

Werner Müller, Heinz Schröder and Xiaohong Wang designed the experiments; Vlad Grebenjuk, Michael Kozhhev and Ute Schloßmacher performed the experiments; and Werner Müller, Heinz Schröder, Xiaohong Wang and Matthias Wiens wrote and/or revised the paper.

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**REFERENCES**


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SUPPLEMENTARY ONLINE DATA

Nocturnin in the demosponge *Suberites domuncula*: a potential circadian clock protein controlling glycogenin synthesis in sponges

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MATERIALS AND METHODS

Identification and cloning of the poriferan nocturnin and glycogenin cDNAs

Several ESTs (expressed sequence tags) had been found in the *S. domuncula* EST database (SpongeBase https://octavia.vk.medizin.uni-mainz.de/login.cgi) encoding parts of the sponge nocturnin and glycogenin cDNAs.

*S. domuncula* nocturnin

The cDNA encoding the putative nocturnin polypeptide was completely sequenced by application of the 3′- and 5′-racing technique using the CapFishing Full-length cDNA Premix kit (Seegene). The sequence was identified and termed putative nocturnin. The complete nucleotide clones of the *SDNOC* comprises 1066 bp, excluding the poly(A) stretches; the ORF ranges from nt 74–76 to nt 1012–1014. The products were subcloned in the pGEM-T vector (Promega) and sequenced.

*S. domuncula* glycogenin

The partial sponge cDNA for glycogenin was also identified in the SpongeBase and completed by 3′- and 5′-RACE (rapid amplification of cDNA ends). The complete clone of *SDGYG* is 1242 nt long; the ORF spans from nt 25–27 to nt 1037–1039. The deduced polypeptide was termed GLYG_SUBDO.

Sequence analyses

Homology searches were conducted through servers at the European Bioinformatics Institute and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments were run with ClustalW version 1.6 [1]. Phylogenetic trees were constructed on the basis of protein sequence alignments applying the Neighbor-Joining method to distance matrices with the Dayhoff PAM matrix model [2]. The degree of support for internal branches was further assessed by bootstrapping (Phylip; http://evolution.genetics.washington.edu/phylip.html).

Preparation of recombinant nocturnin

The Gateway System from Invitrogen was used to facilitate subcloning. Two kinds of recombinant nocturnin proteins were prepared; a partial protein sequence of nocturnin (termed rNOC_SUBDO) and the full-length poly(A)-specific 3′-exoribonuclease enzyme (rNOCPAE_SUBDO). The partial protein sequence was used for raising antibodies, whereas the complete recombinant protein was assayed for deadenylase activity.

Partial protein sequence

The major part of the cDNA/protein was amplified by standard PCR as described previously [3]. In short, the cDNA segment of *SDNOC*, spanning nt 137–985 coding for the enzyme part (aa 22–304), was amplified with a combination of forward primer, 5′-GGGGACAGT TTGTGCAAAA AGCAGGCTTACTGAAA-GTGTCCTCAATGGAAT-3′ and reverse primer, 5′-GGGGACACCTTGTGACAAGAAAGCCTGGTACTAAGAACC AAGCTC-3′ (underlined is the attB recombination site). The attB PCR product [913 nt (852 nt for the nocturnin gene and 61 nt for the attb recombination site)] was purified from the gel using NucleoSpin Extract II (Macherey-Nagel) according to the instructions of the manufacturer. The BP recombination reaction was performed with the obtained attB PCR product (including the nocturnin cDNA fragment) and using the pDONR 221 entry vector (Invitrogen). This construct was used to transform TOP10 cells (Invitrogen); positive clones were used for subsequent LR recombination reaction with the attR-containing pDEST 17 destination vector (Invitrogen). Positive expression clones were transformed into competent *E. coli* BL21 AI One Shot cells (Invitrogen). The cells were incubated in LB (Luria–Bertani) medium with 50 μg/ml of carbenicillin (Roth) at 37°C. After reaching a turbidity of 0.6–0.8 (D600), L-arabinosine was added at a final concentration of 0.2% and the cells were incubated for 12 h. The cell pellet was collected by centrifugation and analysed by SDS/PAGE (12% gel). Two sequential metal-ion-affinity chromatography steps were performed to obtain the (almost) pure sample. The recombinant nocturnin had a purity of >95%, as checked by SDS/PAGE. Refolding of the protein was induced with a 0.7 M L-arginine buffer (containing 5.0 mM EDTA, 0.1% CHAPS, 10 mM glutathione and 1.0 mM glutathione disulfide, pH 7.2). The expected molecular mass of the recombinant nocturnin is 34.1 kDa (rNOC_SUBDO).

Complete protein sequence

The complete nocturnin protein was obtained in the same way with the exception that the complete ORF of *SDNOC* was ampli...
Figure S1  The S. domuncula nocturnin (NOC_SUBDO), deduced from its cDNA (SDNOC)

(A) The deduced protein was aligned with the nocturnin sequence from human (NOC_HOMO, NCBI reference sequence NP_036250.2) and the cnidarian N. vectensis (NOC_NEMATO; NCBI reference sequence XP_001626780.1). Residues conserved (identical or similar with respect to their physicochemical properties) in all three sequences are shown in white on black; those which share similarity to at least two residues are shown in white on grey. The exonuclease-endonuclease-phosphatase (EEP) domain is shown. (B) These three proteins were compared with the related nocturnin and nocturnin-related sequences from mammals: Pan troglodytes (NOC_PAN; NCBI reference sequence XP_003310517.1), Cricetulus griseus (NOC_CRIGRI; NCBI reference sequence EGW07004.1), Omithorhynchus anatinus (NOC_ORNITHO; NCBI reference sequence XP_001509853.2); other deuterostomes: Danio rerio (NOC2_DANIO; NCBI reference sequence NP_697426.2), Xenopus laevis (NOC4_XENLA; NCBI reference sequence NP_001089262.1), Branchiostoma floridae (NOC_BRAFLO; NCBI reference sequence XP_002609167.1); protostomes: Tribolium castaneum (NOC_TRIBO; NCBI reference sequence XP_974729.2) and Drosophila melanogaster (CURLe_DROME; NCBI reference sequence NP_001097747.1); as well as the yeast CCR4/NOT homologue family member from Saccharomyces cerevisiae (CCR4_YEAST; NCBI reference sequence NP_001023608.1). The plant putative carbon catabolite repressor protein, 4-like 1 protein, from Arabidopsis thaliana (F4J8R6_ARATH; NCBI reference sequence NP_191415.2) was used as an outgroup to root the tree. Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence.

was used for transfection. The part of the nt sequence (nt 74–1015) coding for the complete enzyme part (aa 1–314), was amplified with a combination of the forward primer, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTA-3′ and the reverse primer, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTA-3′ (underlined is the attB recombination site). The attB PCR product [1006 nt (942 nt for the nocturnin gene and 64 nt for the attb recombination site)] obtained was purified from the gel and used for transformation of TOP10 cells. After recombination and induction with L-arabinose the recombinant protein was isolated from the bacteria and purified as described above. After unfolding and refolding of the nocturnin the recombinant protein was checked by SDS/PAGE for purity, which was >97%, and for size, which was 37.5 kDa. The enzyme protein was termed rNOCPAE_SUBDO.

Preparation of antibodies

The purified recombinant nocturnin protein (rNOC_SUBDO) was used for the production of polyclonal antibodies (PoAb), as described previously [4]. Female rabbits (White New Zealand) were immunized three times with 30 μg/boost. Following collection of the serum the titre of the PoAb (termed PoAb-rNOC_SUBDO) was determined and found to be >1:5000. For the control experiments, 100 μl of the PoAb preparation were incubated with 20 μg of the antigen (rNOC_SUBDO) for an incubation period of 1 h (4 °C). The adsorbed preparation failed to recognize the antigen as analysed both by Western blotting (results are not shown) and by immunofluorescence (see Figure 4K of the main text). As a further control pre-immune serum was used (see Figure 4L of the main text).

SDS/PAGE and Western blot analysis

SDS-PAGE was performed as follows. Samples of 5–8 μg of protein were mixed with loading buffer (Roti-Load, Carl Roth), boiled for 8 min and subjected to SDS/PAGE (12% gel) as described previously [5]. The gels were stained with Coomassie Brilliant Blue. The protein size standard “Dual Color” (Carl Roth) was used to estimate protein sizes.

For the Western blot analyses, size-separated proteins were transferred to PVDF-Immobilon membranes. The membranes were then blocked at room temperature (20°C) with Blocking solution [1% (v/v) in TBS-T buffer; 20 mM Tris/HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20; Roche].
Following consecutive incubations with PoAb-rNOCSUBDO [1:3000 dilution in TBS-T, supplemented with 0.1% (v/v) Blocking solution], alkaline phosphatase-conjugated species-specific secondary antibodies (1:4000 dilution), and Nitro Blue Tetrizolum/BCIP (5-bromo-4-chloroindol-3-yl phosphate) (Invitrogen), proteins were detected colorimetrically. The Ultra-Low Range Marker (M3S46, Sigma) was used to determine the apparent size of the peptide.

RESULTS

Sequence analysis and phylogenetic relationship of sponge nocturnin

The complete SDNOC sequence was obtained using an EST tag from the SpongeBase as the start by application of the 3′- and 5′-racing technique. The nucleotide sequence, encoding the complete ORF, was obtained and spanned 1066 bp; the cDNA was termed SDNOC. The ORF nt 74–76 to nt 1016–1018 encoded a 314 aa-long polypeptide, termed NOC_SUBDO, with a size of 34836 Da and a pI of 5.53 (Figure S1). The cDNA/ORF was complete as proven by Northern blot analysis (1.2 kb; results not shown). The deduced sponge protein was termed NOC_SUBDO. The similarity/identity of the sponge nocturnin polypeptide to the corresponding human sequence (NCBI reference sequence NP_036250) was 39% and to the closer related sequence from the cnidarian Nematostella vectensis was 50%/35% (NCBI reference sequence XP_001626780). The sequence NOC_SUBDO comprises the domain (aa 22–300), characteristic for the exonuclease-endonuclease-phosphatase domain superfamily. Enzymes included in this group share a common catalytic mechanism of cleaving phosphodiester bonds [6]; the high score is reflected by the E-value [7] of 1.94e−96.

Recombinant SDNOC and antibodies raised against it

A part of the cDNA comprising the ORF for nocturnin (NOC_SUBDO) was expressed in E. coli cells. The 30-kDa protein (representing the recombinant protein) was already dominant in non-purified bacterial extract (Figure S2, lane a), collected 12 h after induction with L-arabinose, as analysed by SDS/PAGE. The migration of the recombinant protein was found to be slightly faster (calculated size of 30 kDa) than that of the expected one from the deduced 34.1 kDa large protein. We attribute this difference to a more globular shape of the recombinant protein. After two purification steps by affinity chromatography of the His-tagged rNOC_SUBDO protein on a Ni-IDA (Ni2+-iminodiacetic acid) column (Figure S2, lanes b and c), the protein sample showed a purity of >95%.

This protein sample (rNOC_SUBDO) was used to raise polyclonal antibodies (PoAb-rNOCSUBDO). The serum recognized the 30-kDa recombinant protein after size separation by SDS/PAGE, followed by Western blotting (Figure S2, lane d). This protein did not cross-react with a pre-immune serum (Figure S2, lane e).

Sequence analysis and phylogenetic relationship of sponge glycogenin

The sponge cDNA was completed and the ORF was deduced. From the 1242-nt long sequence the ORF (nt 25–27 to nt 1037–1039) encoding the polypeptide GLYG_SUBDO was deduced. The calculated size of the protein is 37023 Da with a pI of 5.45. The glycogenin polypeptide deduced by sponge shares the characteristic domains with the glycogenin polypeptides from other metazoans [8,9] (Figure S3): (i) the sequence comprises the glycosyl transferase family 8 segment (aa 1–253), which is characterized by the presence of a tyrosine residue (aa 193), allowing the self-glycosylation reaction, the conserved DxD motif (aa 100/102), required for the co-ordination of the catalytic divalent cation (most often Mn2+), and together with the N-terminal β-α-β Rossmann-like fold, harbouring the nucleotide-binding domain of glycosyltransferases; and (ii) the glycosyl transferase family 8 segment, spanning aa 7–218, mediates the transfer of sugar moieties from an activated donor molecule to a specific acceptor molecule under the formation of a glycosidic bond.
Figure S3  The sponge glycogenin from *S. domuncula* (GYG_SUBDO)

(A) The deduced polypeptide was compared with the human (GLG1-2_HOM; NCBI reference sequence NP_001171649) and the sponge glycogenin from *Amphimedon queenslandica* (GYG_AMPHI; NCBI reference sequence XP_003383748.1). The borders for the two typical glycogenin domains, the segment characteristic for the glycosyl transferase family 8 (-GT8_Glycogenin-) and the region for the glycosyl transferase family 8 segment (∼GT8∼) are given. Within these regions the locations of the tyrosine residue and the conserved DxD motif (aa 100/102) are shown. (B) The glycogenin sequences are compared with the cnidarian glycogenin from *Hydra magnipapillata* (GYG_HYDRA; NCBI reference sequence XP_002161422.1) and the protostomian glycogenin from *Drosophila melanogaster* (GYG_DROME; NCBI reference sequence NP_726040.1), as well as the deuterostomian sequences from *Branchiostoma floridae* (GYG_BRAFLO; NCBI reference sequence XP_002588659.1), from the fish *Danio rerio* (GYG_DANIO; NCBI reference sequence NP_001002062.1) and the bird *Gallus gallus* (GYG_GALLUS; NCBI reference sequence NP_001006558.1). In addition, the yeast sequence Glg1p from *Saccharomyces cerevisiae* (GLG1P_YEAST; NCBI reference sequence EGA81916.1) is included together with the plant glycogenin-like starch initiation protein 6 from *Arabidopsis thaliana* (GLCl_ARATH; NCBI reference sequence NP_197349.2). The latter sequence was used as an outgroup.

REFERENCES


