

Matrix-mediated canal formation in primmorphs from the sponge *Suberites domuncula* involves the expression of a CD36 receptor-ligand system

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Summary

Sponges (Porifera), represent the phylogenetically oldest metazoan phylum still extant today. Recently, molecular biological studies provided compelling evidence that these animals share basic receptor/ligand systems, especially those involved in bodyplan formation and in immune recognition, with the higher metazoan phyla. An in vitro cell/organ-like culture system, the primmorphs, has been established that consists of proliferating and differentiating cells, but no canals of the aquiferous system. We show that after the transfer of primmorphs from the demosponge *Suberites domuncula* to a homologous matrix (galectin), canal-like structures are formed in these 3D-cell aggregates. In parallel with the formation of these structures a gene is expressed whose deduced protein falls into the CD36/LIMPII receptor family. The receptor was cloned and found to be strongly expressed after adhesion to the galectin matrix. This process was suppressed if primmorphs were co-incubated with a homologous polypeptide containing the CSVTCG domain, as found in thrombospondin-1 (and related) molecules of vertebrates.

In situ hybridization studies revealed that the *S. domuncula* CD36/LIMPII receptor is localized in the pinacocytes that surround the canals of the sponge. Furthermore, a secondary metabolite from a sponge-associated bacterium was isolated and characterized, the 2-methylthio-1,4-naphthoquinone (MTN). MTN causes inhibition of cell proliferation of vertebrate tumor cells at concentrations of >80 ng/ml. However, doses of only 2 ng are required to potently inhibit angiogenesis in the chick chorio-allantoic membrane assay. At concentrations of 10 ng/ml this compound was also found to suppress the expression of the *S. domuncula* CD36/LIMPII; this result is a first indication that this secondary metabolite has a conserved functional activity: the suppression of the formation of the circulation system, from sponges to vertebrates.

Key words: Sponges, Porifera, *Suberites domuncula*, CD36, Lysosomal integral membrane protein II, Thrombospondin, 2-methylthio-1,4-naphthoquinone, Canal formation, Angiogenesis

Introduction

Sponges (Porifera), as the phylogenetically oldest metazoan phylum still extant today, are the closest relatives of the hypothetical common metazoan ancestor, the Urmetazoa (reviewed by Müller, 2001). Compelling evidence has accumulated during the last few years, especially through cloning of genes and subsequent analysis of their functional roles, that sponges possess the basic structural and functional elements that allow a tuned interaction of their differentiated cells (see Müller, 1997). In particular, the immune molecules and the recently identified apoptotic enzymes and receptors enabled sponges to pass the transition from the colonial stage of organization to an integrated stage (Müller, 2003). Intracellular transcription factors, e.g. the homeodomain molecules such as the LIM homeodomain factors (Wiens et al., 2003) and extracellular matrix molecules with their interacting surface-associated receptors, e.g. integrin and their ligands

(Wimmer et al., 1999; Schütze et al., 2001), provided the molecular platform for the development of a bodyplan (Wiens et al., 2003). The tuned interactions of the differentiated cells, controlled by diverse regulatory molecules, allowed a pattern formation.

Even though nerve cell-like receptors have been identified in sponges (Perović et al., 1999), no nervous system can be detected in Porifera; likewise sponges do not possess a blood circulatory system. Nevertheless sponges have a complex water circulating system connecting inhalant and exhalant canals (Möhn, 1984). Sponges require this aquiferous system for their supply of nutrients (Simpson, 1984) and of oxygen (Gatti et al., 2002). Recent studies demonstrated that the speed of the water current surrounding the animals induces morphogenetic processes. These studies were performed with primmorphs from the demosponge *Suberites domuncula* (Custodio et al., 1998; Müller et al., 1999). Primmorphs are a

special form of 3D-cell aggregates from sponge cells; they consist of proliferating and differentiating sponge cells and are initially formed from dissociated single cells. Under the influence of a strong external water flow, primmorphs start to form canal-like structures, a process that is correlated with the expression of a homeobox gene, *Iroquois* (Perović et al., 2003).

Primmorphs can be used as biofermenters for the production of bioactive secondary metabolites (Müller et al., 2000). So far their size is limited to approximately 20–40 mm, however, one possibility for increasing their size is to induce canal formation by culturing them on a homologous matrix. We demonstrated that primmorphs form canal-like structures with the *S. domuncula* galectin as a matrix (Wiens et al., 2003). Also it is known that growth of mammalian tumors depends on the formation of new capillary blood vessels, as first formulated in 1971 by Folkman (Folkman, 1971). One powerful technique to study angiogenesis in vitro is the use of the 3D-cell cultures, the spheroids (reviewed by Müller-Klieser, 1997). With those spheroids it could be shown that angiogenesis depends on soluble factors, e.g. vascular endothelial growth factors (VEGF) or cytokines, which interact with the corresponding receptors localized on the endothelial cells, e.g. the VEGF receptor (see Brower, 1999).

In an attempt to understand, in molecular terms, the transition of the two growth forms of the sponge primmorphs/aggregates (from spheroid to flat; ball-like to canal-like forms) we investigated mechanisms in sponges that have been previously described in vertebrates. All known receptors and their ligands involved in the genesis of new blood vessels in vertebrate systems are restricted to Metazoa. In the present study we examined whether the CD36-thrombospondin (TSP) system, which is assumed to be phylogenetically old (Calvo et al., 1995) is also involved in canal formation of primmorphs.

The glycoprotein CD36, initially known as glycoprotein IV was first identified biochemically on the platelet membranes (Okumura and Jamieson, 1976) and subsequently was studied at the gene level (Wyler et al., 1991). Together with the membrane glycoprotein CD36/LIMPII analog-1 (CLA-1) (Calvo and Vega, 1993) and the lysosomal integral membrane protein II (LIMPII) (Vega et al., 1991), these molecules form the CD36 family (Calvo et al., 1995). Based on a calibrated molecular clock it was estimated that the ancestor of these molecules emerged around 850 to 650 million years ago (Calvo et al., 1995). The CD36 molecules, as well as the LIMPII transmembrane proteins, function as receptors for the extracellular protein thrombospondin (TSP) (Crombie and Silverstein, 1998). TSP-1 is a 450 kDa trimeric glycoprotein that is a ligand for the CD36 receptor (Asch et al., 1987) and also binds to LIMPII (Crombie and Silverstein, 1998). While it is well established that CD36 is a cell surface receptor (Greenwalt et al., 1992) known to bind to collagen (see Enenstein et al., 1998), LIMPII is integrated into the lysosome membrane (Vega et al., 1991). Since a series of lysosomal receptors, e.g. LAMP-1 or LAMP-2, can be found on activated platelets (Silverstein and Febbraio, 1992) or tumor cells and leukocytes (Fukuda, 1985), it was not surprising that LIMPII also binds to TSP-1 (Crombie and Silverstein, 1998) with the CSVTCG peptide segment that is located within the type 1 repeats of TSP (Li et al., 1993).

The interaction of CD36 members with TSP-1 results

in morphogenetic processes and at the same time modulates/inhibits proliferation and differentiation as well as angiogenesis and cell migration on endothelial cells (Dawson et al., 1997). Prevention of the interaction of CD36 with TSP-1 is one target for an anti-angiogenic tumor therapy (Brower, 1999). Besides natural endogenous inhibitors and the recently discovered metallospondins or synthetic inhibitors, thrombospondin is also considered as a good drug candidate for the 'starvation' of a tumor (Brower, 1999). Recently, also secondary metabolites from sponges, which are characterized by highly diverse chemical structures (reviewed by Sarma et al., 1993) causing powerful and specific bioactivities (Faulkner, 2000), have been found to display anti-angiogenic activity. Among them is aeroplysinin-1, an inhibitor isolated from the sponge *Verongia aerophoba*, which inhibits the epidermal growth factor receptor tyrosine kinase (Kreuter et al., 1990) and also displays anti-angiogenic activity at micromolar concentration (Rodrigues-Nieto et al., 2002).

In the present study we describe how during transformation of round sponge primmorphs to their flat attached growth form with canal-like structures, the CD36/LIMPII receptor is increasingly expressed. This process could be prevented by addition of a polypeptide (TSP-1) consisting of the binding domain to CD36/LIMPII. CD36/LIMPII was cloned and expressed from the *S. domuncula* cDNA. Furthermore, we describe a compound that was isolated from a sponge-associated bacterial strain within our screening program aimed at the identification of novel bioactive natural products in marine organisms by hyphenated HPLC techniques (Bringmann and Lang, 2003). This compound, 2-methylthio-1,4-naphthoquinone (MTN), inhibits canal formation in primmorphs. MTN is a naphthoquinone derivative hitherto known only as a synthetic product (Fieser and Brown, 1949). Sulfur-containing naphthoquinones, but equipped with additional prenyl residues in the 3-position, have been isolated from a thermophilic hydrogen-oxidizing bacterium (Ishii et al., 1987). MTN has been found to exhibit antifungal (Gershon and Shanks, 1975) and antitumor activity (Takano et al., 1960).

Materials and Methods

Chemicals and enzymes

The sources of chemicals and enzymes have been described earlier (Kruse et al., 1997; Wimmer et al., 1999; Krasko et al., 2000).

Sponges

Live specimens of *S. domuncula* (Porifera, Demospongiae, Hadromerida) were collected near Rovinj (Croatia) and subsequently were kept in aquaria in Mainz for more than 4 months prior to use (Le Pennec et al., 2003).

Dissociation of cells and formation of primmorphs

The procedure described for the formation of primmorphs from single cells was applied (Custodio et al., 1998; Müller et al., 1999). Starting from single cells, primmorphs of 3–7 mm are formed after 5 days. For the experiments described here 6-day-old primmorphs were used. They were cultured in natural seawater supplemented with 0.2% of RPMI1640 medium and with the optimal concentration of silicate (60 µM) and Fe³⁺ (30 µM, added as ferric citrate) (Krasko et al., 2002).

After primmorph formation, these three-dimensional aggregates

were transferred to 12-well tissue culture test plates (Nunc-Clon-Surface; Nunc, Wiesbaden; Germany; TPP) either uncoated or coated with recombinant homologous galectin. For coating, 500 µl of a recombinant galectin solution (15 µg/ml) were added per well. After standing for 12 hours at 4°C the plates were washed with seawater and used for the experiments. Subsequently incubation was prolonged for an additional 6 days. During this period the recombinant TSP peptide or MTN was added, as indicated.

In one series of experiments the recombinant TSP peptide was pre-incubated with the recombinant 15 kDa CD36/LIMP-II receptor polypeptide for 3 hours (21°C) prior to the addition to the primmorphs culture.

S. domuncula galectin cDNA and the recombinant protein

The complete cDNA encoding galectin was isolated from *S. domuncula* applying the polymerase chain reaction (PCR) technique (Wiens et al., 2003). The cDNA had a size of 955 nt (excluding the poly(A) tail). The clone was termed *SDGALECI* (accession number AJ493055). For the synthesis of recombinant galectin the *SDGALECI* cDNA was cloned into the pGEX2T plasmid containing the *Schistosoma japonicum* glutathione S-transferase (GST) gene. Expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 hours. The GST fusion protein, termed rGALECI-SUBDO, was purified by affinity chromatography on glutathione Sepharose 4B (Coligan et al., 2000). The fusion protein was cleaved with thrombin (10 units/mg) to separate glutathione S-transferase from the recombinant sponge galectin; the size of the purified galectin has a molecular mass of 22 kDa. This material was used for coating of the cultivation plates.

Isolation of the cDNA encoding the *S. domuncula* CD36/LIMP-II receptor

The complete cDNA encoding the CD36/LIMP-II receptor, *SDCD36/LIMP-II*, was isolated from the cDNA library from *S. domuncula* (Kruse et al., 1997) by PCR technique. The primer was designed against the conserved amino acid residues, found in the 'block A' segment of the lysosomal integral membrane protein II (LIMP-II) (Crombie and Silverstein, 1998). The degenerate forward primer against aa82 to aa91 [human LIMP-II; accession number NP_005497.1 (Calvo et al., 1995)], 5'-GAA/G GAA/G GTA/T/G/C GGA/T/G/C CCA/G/T/C TAC/T-3', was used in the PCR reaction together with the vector primer. The PCR conditions were as follows. Initial denaturation at 95°C for 3 minutes, followed by 30 amplification cycles at 95°C, for 30 seconds, 56°C for 45 seconds, 74°C for 1.5 minutes, and a final extension step at 60°C for 10 minutes. The reaction mixture was as described earlier (Wiens et al., 1998). One fragment of ≈1200 bp was isolated and used to complete the cDNA sequence by screening the library (Ausubel et al., 1995). The clone was termed *SDCD36/LIMP-II*.

Recombinant *S. domuncula* CD36/LIMP-II receptor

To prepare a recombinant part of the CD36/LIMP-II receptor, the sponge cDNA (*SDCD36/LIMP-II*) corresponding to the first 131 aa, including the characteristic domain 'block A' of the predicted protein (open reading frame) was cloned into the pQE vector (Quiagen, Hilden, Germany) and used to transform BL21 strain bacteria, as successfully used previously for the preparation of recombinant sponge proteins (Adell et al., 2003). One positive colony was selected and induced with IPTG for 4 hours at 37°C; cells were collected and the expressed r-CD36/LIMP-II was purified on a Ni-NTA agarose column (Quiagen), according to the manufacturer's instructions. The eluted fraction containing the r-CD36/LIMP-II protein was dialyzed against seawater and used for the incubation/competition studies. The size of the polypeptide, 15 kDa, was verified by gel electrophoresis

in the presence of 12% polyacrylamide containing 0.1% NaDodSO₄ (data not shown).

Northern blotting

RNA was extracted from liquid-nitrogen pulverized sponge tissue with TRIzol Reagent (Gibco-BRL, Grand Island, NY). Then 5 µg total RNA was electrophoresed through a 1% formaldehyde/agarose gel and blotted onto Hybond-N⁺ nylon membrane following the manufacturer's instructions (Amersham, Little Chalfont, Buckinghamshire, UK) (Wiens et al., 1998). Hybridization was performed with 0.5 kb segments of the *SDCD36/LIMP-II* cDNA, the *galectin* (*SDGALECI*) cDNA or the *SDADAMTS* cDNA. The probes were labeled with the PCR-DIG-Probe Synthesis Kit according to the 'Instruction Manual' (Roche). After washing, digoxigenin (DIG)-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase; dilution of 1:10,000) and visualized by chemiluminescence technique using CDP, the chemiluminescence substrate alkaline phosphatase, according to the instructions of the manufacturer (Roche). The signals of the blots were quantified by applying the chemiluminescence procedure of Stanley and Kricka (Stanley and Kricka, 1990); CDP-Star was used as the substrate. The screen was scanned and quantified with the GS-525 Molecular Imager (Bio-Rad, Hercules, CA, USA).

In situ localization of CD36/LIMP-II receptor

The method of in situ localization applied was based on the procedure described by Polak and McGee (Polak and McGee, 1998) with modifications (Le Pennec et al., 2003; Perović et al., 2003). In brief, siliceous spicules were removed from tissue samples with HF/NH₄F. After washing with Ca²⁺- and Mg²⁺-free artificial seawater (CMFSW) (Rottmann et al., 1987) the sponge pieces were embedded in Tissue-Tek. In contrast to the tissue materials, the primmorphs were not treated with HF/NH₄F but were directly embedded into Tissue-Tek. 8-µm sections through frozen tissue and primmorphs were cut. Cryosections were fixed with paraformaldehyde (4% in CMFSW) for 30 minutes, and then washed with phosphate-buffered saline (PBS) at room temperature. Hybridization was performed in 2× SSC (sodium chloride/sodium citrate), supplemented with 50% formamide. The activation of the DIG-labeled probe (10 pmol/ml) was performed for 1 minute at 95°C in 4× SSC buffer. To each cryosection, 65-70 µl of hybridization buffer, including the probe, was added. Hybridization proceeded overnight in a glass chamber at 45°C. Subsequently the sections were washed at 55°C, 1× 2 minutes in 2× SSC and 3× 20 minutes in 0.2× SSC. Final washes (2× 5 minutes) were with PBT [PBS containing 0.1% (v/v) Triton X-100 and 2 mg/ml of BSA] at room temperature. After blocking [1% blocking reagent for nucleic acids (Roche) in 1× PBS, containing 0.1% (v/v) Tween 20] for 15 minutes at room temperature, the slices were reacted with anti-DIG-Fab fragments conjugated to alkaline phosphatase (dilution 1:100) for 1 hour at 37°C in a humid chamber. After two washes (5 minutes each) with PBT at room temperature and one wash (5 minutes) with the Tris buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl and 50 mM MgCl₂), the sections were incubated with Tris buffer, supplemented with the dye reagents NBT (4-nitro blue tetrazolium chloride) and X-phosphate (Roche) for 45 minutes at 37°C in the dark. After washing the sections for 5 minutes in 1× PBS, they were covered in Glycergel (DAKO, Hamburg, Germany) and analyzed under the microscope.

The DNA probe had a length of 450 bp (nt₁₁₈₀ to nt₁₆₃₀ within the cDNA) and comprised the 3'-terminal region of the ORF of the *SDCD36/LIMP-II* cDNA. PCR was carried out with an initial denaturation at 95°C for 3 minutes, then 35 amplification cycles each at 95°C for 30 seconds, 58°C for 30 seconds, 74°C for 4 minutes, and a final extension step at 72°C for 20 minutes. Labeling was performed by using the DIG Oligonucleotide Labeling Kit (Roche). The antisense-*SDCD36* probe was found to react with cells in the section,

while the sense-*SDCD36* probe, synthesized complementary to the direction of the anti-sense probe, showed no binding. The sections were inspected with a Olympus VANOX AHB3 microscope. The images were recorded with a ColorView 12 camera, applying the Soft Image System analysiS 3.0 (Soft Image System GmbH, Münster, Germany).

Cloning and expression of the CSVTCG cell adhesive motif

Searching the expressed sequence tag (EST) cDNA database of *S. domuncula*, which had been established by our group, several ESTs were detected that had high sequence similarity to the ADAMTS protein, also termed disintegrin/metalloproteinase with thrombospondin motifs. One sequence of 541 nucleotides was extracted and termed *SDADAMTS*; it contained no stop codon. A 348 nt fragment from base pairs 2-349 (see below) was subcloned into the bacterial glutathione S-transferase/oligohistidine/S expression vector pET41a (Novagen, Madison WI, USA) via the *EcoRV* (5' end) and the *XhoI* (3' end) restriction sites. *E. coli*, strain BL21, was transformed with this plasmid and expression of fusion protein was induced for 6 hours at 37°C with 1 mM IPTG (Ausubel et al., 1995). Bacteria from 500 ml cultures were obtained by centrifugation; the fusion protein was extracted and purified first using the His-tag purification kit (Novagen) and subsequently with the glutathione S-transferase-tag purification kit (Pharmacia, Freiburg, Germany) as recommended by the manufacturer. Then the fusion protein was cleaved with the recombinant enterokinase (5 units; Novagen) as recommended. The recombinant ADAMTS molecule, tag-free, was purified in a batch procedure using the glutathione S-transferase-tag purification kit. The purity of the material was checked by 15% polyacrylamide gel containing 0.1% NaDodSO₄ according to the method of Laemmli (Laemmli, 1970). The enriched r-ADAMTS polypeptide was subjected to a refolding process using the Protein Refolding Kit (Novagen). The protein was dialyzed against seawater.

Sequence analysis

The sequence was analyzed using the BLAST (2003) and FASTA (2003) computer programs. Multiple alignments were performed with CLUSTAL W Ver. 1.6 (Thompson et al., 1994). Phylogenetic trees were constructed on the basis of amino acid sequence alignments by neighbor-joining, as implemented in the Neighbor program from the PHYLIP package (Felsenstein, 1993). The distance matrices were calculated using the Dayhoff PAM matrix model as described previously (Dayhoff et al., 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). The graphic presentations were prepared with GeneDoc (Nicholas and Nicholas, 2001).

Isolation and characterization of 2-methylthio-1,4-naphthoquinone (MTN)

A tissue sample from the central core of the sponge *Dysidea avara* was stanced out under sterile conditions and rinsed 3-4 times with sterile seawater. This tissue was squeezed between two sterile glass slides. The resulting extract was further diluted (10^{-1} to 10^{-5}) and a sample of each dilution was plated on agar medium (0.25% peptone, 0.15% yeast extract, 0.15% glycerol, 1.6% agar, 100% seawater). The plates were incubated at 30°C for 24-72 hours. Bacteria were further purified and cultured on agar medium. Eight bacterial strains (D1-D8) were obtained from this sponge specimen.

The bacterial strain D1 was cultured and extracted with n-butanol following the method of Elyakov et al. (Elyakov et al., 1996). 16S rDNA analysis revealed that D1 shares 99% identity to the alpha-proteobacteria MBIC3368 (accession number AF218241). The bacterial isolate was inoculated into conical flasks (1 liter) with 500 ml cultural broth. The culture broth contained peptone (0.25%), yeast

extract (0.15%) and glycerol (0.15%) in seawater. The pH was adjusted to 7.2-7.5. The flasks were incubated at 30°C for 3 days with shaking (100 rpm). After incubation, bacterial cultures [500 ml each (total 2 l)] were mixed with 150 ml of n-butanol. The mixture was kept at 40°C for 24 hours, stirred for 20 minutes, centrifuged and the butanol layer was separated and then evaporated using a rotary evaporator. Dry residue (100-150 mg) was stored below 5°C until further use.

The extract was subjected to gel filtration on Sephadex LH-20 (23×3 cm; eluent methanol; fraction size, 18 ml). Fractions 14 and 15 were evaporated to dryness and further purified using preparative high-performance liquid chromatography (column: Waters Xterra 19×300 mm; solvents: water and acetonitrile, both with 0.05% TFA; gradient: 0 minutes – 10% acetonitrile, 30 minutes – 100% acetonitrile; flow rate: 12 ml/minute). The compound that was eluted after 23 minutes yielded, after drying, 0.5 mg of pale yellowish needles. The substance was identified as 2-methylthio-1,4-naphthoquinone (MTN) by evaluation of NMR and MS spectra: the data show good agreement with those published for MTN (Kametani et al., 1977; Coll et al., 1988). The melting point (mp) was determined to be 176-178°C (Kametani et al.: mp 165-166°C; Coll et al.: mp 185-186°C).

Anti-angiogenic (CAM) assay

The original chick chorio-allantoic membrane (CAM) assay (Crum et al., 1985) was used with modifications (Pathare, 2001). The compound MTN was tested at four different concentrations ranging from 0.25 ng to 1 ng/disc. MTN of the respective concentration (volume 10 µl) was dissolved in 2.5% agar. This solution was air dried on a Teflon-coated tray and dried agar discs of 4 mm diameter were prepared. Fertilized, 5-day incubated eggs were obtained from a central poultry breeding farm (Aarey colony, Mumbai). In the laboratory the surfaces of these eggs were sterilized by wiping them with 70% alcohol and they were kept in an incubator at 38°C. The fertilized eggs were 'candled' so as to locate the position of developing embryo and a window of 1 cm² was marked. The marked window was cut in order to see the embryo and the surrounding blood vessels on CAM. An agar disc impregnated with a known concentration of the compound was placed in the outer third portion of the CAM, in the region of the proliferative capillaries-dendrites. After placing the disc on the CAM, the window was resealed with Parafilm and the eggs were incubated at 38°C for 48 hours. After incubation, eggs were opened and the anti-angiogenic response was assessed by measuring the avascular zone of the CAM beneath the disc. A positive control of a mixture of hydrocortisone (60 µg) and heparin (50 µg) was applied, which showed 100% anti-angiogenic activity. An agar discs with saline was used as a negative control, which did not show activity. At least 20 eggs were used for each dose and the experiments were performed in triplicate to ensure reproducibility.

Cytotoxic activity

Four permanent mammalian tumor cell lines were applied to determine the cytotoxic effect caused by MTN: murine leukemic lymphoblasts L5178y (ATCC CRL 1722), rat adrenal pheochromocytoma cells PC-12 (ATCC CRL 1721), human cervix HeLa S3 cells (ATCC CCL 2.2) and the rat embryonal fibroblast cells Rat1 (Klock et al., 1998). The tumor cells were grown as described previously (Müller et al., 1977; Bartl et al., 2001) with the modification that RPMI 1640 medium, enriched with 10% fetal calf serum was used. The cells were grown in a humidified atmosphere of 5% CO₂ and 95% air.

The cell viability assay was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) method as described previously (Tagliatela et al., 1977). The cells were seeded

in 96-well plates at a density of 10×10^4 cells per well with or without the compound. After 72 hours, plates were read on the microplate reader (model 450; Bio-Rad) at a test wavelength of 595 nm. Ten parallel assays were performed for every concentration of MTN. The ED_{50} was determined by logit regression (Sachs, 1974).

Results

Canal formation in primmorphs

As outlined previously (Wiens et al., 2003), canal formation was induced in primmorphs from *S. domuncula* by cultivating them on a galectin-coated matrix. The primmorphs were obtained from dissociated single cells (Fig. 1A) in seawater, supplemented with RPMI, silicate and Fe^{3+} , as described in Materials and Methods. After 3 days aggregates formed that still had a rough surface, but are not composed of the characteristic pinacocyte layer (Fig. 1B) (Le Pennec et al., 2003). However, after 5 days a smooth surface was formed around the non-attached primmorphs, which is composed of pinacocytes (Fig. 1C). After 6 days, the primmorphs remained either in the uncoated plates for an additional 6 days (Fig. 1F), in which case they did not change their spherical appearance and remained non-attached, or, they were cultured on galectin-coated plates for a subsequent 6 days, when the primmorphs attached to the surface, flattened and formed canal-like structures (Fig. 1D,E).

S. domuncula CD36/LIMP-II receptor

To determine if an expression of the CD36/LIMP-II receptor occurs during the canal formation the respective cDNA had to be cloned by PCR. As described in Materials and Methods one degenerate primer was designed against the conserved domain block A (Crombie and Silverstein, 1998). One cDNA was obtained and termed *SDCD36/LIMP-II*; its deduced amino acid (aa) sequence characterizes it as a member of the CD36 family. The cDNA *SDCD36/LIMP-II* is 1632 nt long (without the poly(A) tail), and the size determined by northern blotting was found to be 1.7 kb. The open reading frame, from nt₅₈₋₆₀ to nt₁₅₂₈₋₁₅₃₀ (stop) encodes a deduced polypeptide with 490 aa and a calculated size of 55,697 Da; the polypeptide was called CD36/LIMP-II receptor (CD36/LIMP-II_{SD}; Fig. 2A).

Comparing the sponge CD36/LIMP-II receptor with the related proteins, the transmembrane region at the C terminus, ranging in CD36/LIMP-II_{SD} from aa₄₃₇ to aa₄₆₂ (Fig. 2A) can

be delimited (Kyte and Doolittle, 1982). There are also four characteristic internal potential *N*-linked glycosylation sites, which are highlighted in Fig. 2A.

Phylogenetic analysis of *S. domuncula* CD36/LIMP-II receptor

Molecules of the CD36 family are restricted solely to Metazoa (Calvo et al., 1995). Therefore it was interesting to define the potential ancestral member from a sponge. Only sequences that had the highest similarity scores with the sponge molecule in the Blast-search were included in this analysis. Sequences from human, as a representative for Deuterostomia (LIMP-II), and those from *Drosophila melanogaster* (the epithelial membrane protein CG2727-PA) and *Caenorhabditis elegans* (the CD36 family member), as members of the protostomians, were used. After alignment the tree was constructed and rooted with the closest related plant sequence. All metazoan sequences selected cluster together, while the yeast and plant sequences were shown to be only distantly related (Fig. 2B). Approximately 20% identical and 38% similar amino acids were found among the metazoan proteins, while the relationship with the yeast and the plant molecules is low (<10%/<20%, respectively).

Calvo et al. (Calvo et al., 1995) proposed that the CD36 family might have an ancestor that existed prior to the protostomian-deuterostomian split, around 650 to 850 million years ago. Therefore, the sponge CD36/LIMP-II receptor was compared with the other known members from human and demonstrated in a phylogenetic, slanted cladogram (Fig. 2C); the identity/similarity scores are approximately 20%/40%. With the sponge CD36/LIMP-II receptor as an outgroup the next closest related molecule is LIMP-II followed by the other members of the family including glycoprotein CLA-1 and CD36. This branching order supports the prediction (Calvo et al., 1995).

CSVTCG-containing peptide

An EST/cDNA that encodes a putative protein with similarity to the ADAMTS protein (*SDADAMTS*) was selected from the database of *S. domuncula*. This molecule contains in the deduced polypeptide the CSVTCG sequence, known to interact both with the CD36 receptor and the LIMP-II receptor (Li et al., 1993; Crombie and Silverstein, 1998). The fragment of the deduced sponge protein has high sequence similarity to the

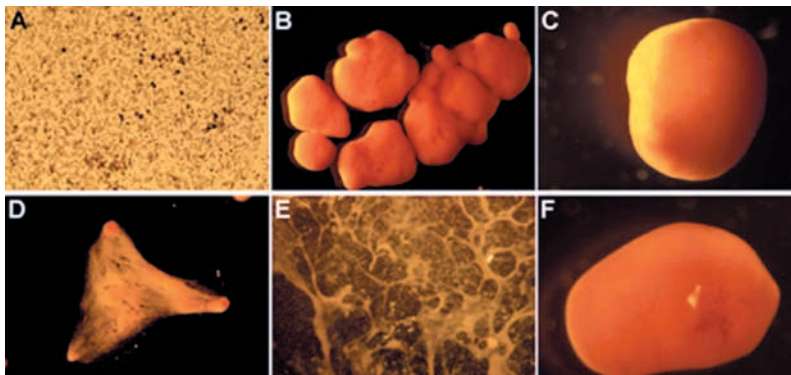


Fig. 1. Formation of canal-like structures in primmorphs from *S. domuncula*. (A) Dissociated cells were obtained and (B) transferred to seawater-based medium; after 3 days aggregates with a rough surface were formed. (C) After a prolongation of the incubation for 6 days round primmorphs with a smooth surface (surrounded by choanocytes) were formed. (D) A subsequent transfer of the primmorphs onto galectin-coated culture dishes resulted, after six days (total incubation period of 12 days), in the formation of canal-like structures. (E) A higher magnification of the structures. (F) A primmorph that was cultured for the complete incubation period of 12 days in uncoated culture dishes; these structures remained in the non-attached state and were roughly spherical. Original magnifications: (A) $\times 50$; (B,C,F) $\times 10$; (D) $\times 5$; (E) $\times 50$.

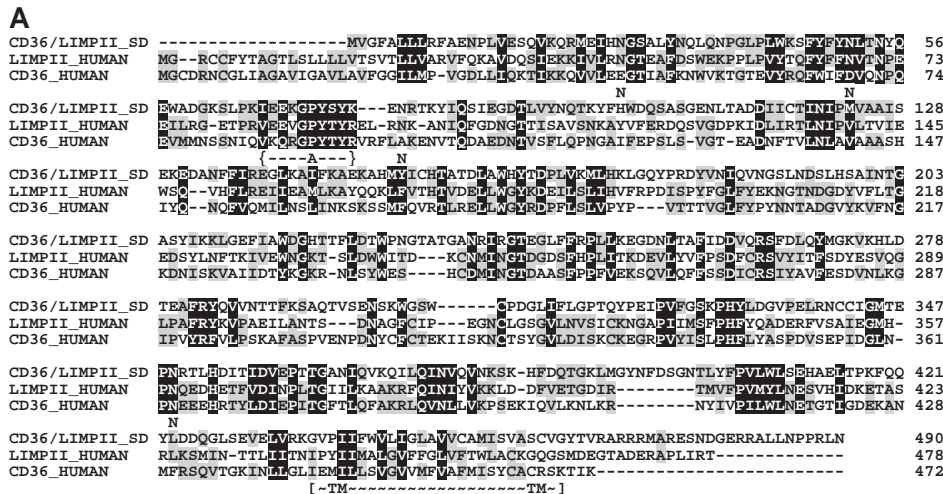
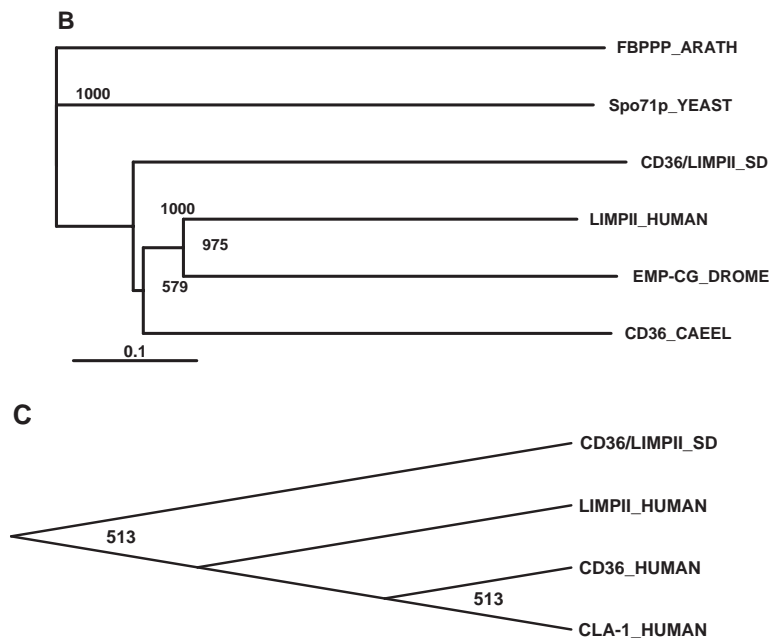


Fig. 2. *S. domuncula* CD36/LIMPII receptor. (A) The deduced *S. domuncula* protein (CD36/LIMPII_SD) is aligned with the human lysosomal integral membrane protein II (LIMPII) (LIMPII_HUMAN; NP_005497.1) (Calvo et al., 1995), and the human CD36 platelet glycoprotein IV (CD36_HUMAN; P16671) (Oquendo et al., 1989). Amino acids, similar among all sequences, are in black boxes and those conserved in at least two sequences are in shaded boxes. The amino acid residues, characteristic for conserved domain 'block A' ([-A-]) and the transmembrane region ([-TM-]) as well as those which represent the internal potential *N*-linked glycosylation sites, are marked. (B) Phylogenetic tree constructed from the above mentioned sponge

sequence and the two human sequences together with the epithelial membrane protein CG2727-PA from *Drosophila melanogaster* (EMP-CG_DROME; NP_523859.2), the CD36 family member from *Caenorhabditis elegans* (CD36_CAEEL; NP_499625.1), Spo71p sequence from *Saccharomyces cerevisiae* (Spo71p_YEAST; NP_010389.1) (Jacq et al., 1997) and the fructose-bisphosphatase precursor protein At3g54050.1 from *Arabidopsis thaliana* (FBPPP_ARATH; NP_190973.1). After alignment the tree was built and rooted using the plant sequence as an outgroup. Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence. (C) Comparison of the *S. domuncula* CD36/LIMPII receptor molecule with the related human sequences: the lysosomal integral membrane protein II (LIMPII_HUMAN), membrane glycoprotein CLA-1 protein (CLA-1_HUMAN; A48528) (Calvo et al., 1993) and CD36 (CD36_HUMAN). A phylogenetic cladogram (slanted), was constructed and, after alignment, rooted with the sponge CD36/LIMPII receptor. The analysis was performed by neighbor-joining as described under Materials and Methods. The numbers at the nodes are an indication of the level of confidence – given as a percentage – for the branches as determined by bootstrap analysis [1000 bootstrap replicates]. Note: The cDNA sequences from *Suberites domuncula* have been deposited in EMBL/GenBank databases; cDNA for the CD36/LIMPII receptor is under the accession number AJ558195.



metazoan proteins termed 'disintegrin/metalloproteinase with thrombospondin motifs' (ADAMTS). The polypeptide used here was most similar to ADAMTS-9 (Q9P2N4); it shared 20% identical and 30% similar aa with the human ADAMTS-9 precursor (Clark et al., 2000) (Fig. 3). A stretch of 348 nt within *SDADAMTS* was selected from the cDNA for the preparation of the recombinant polypeptide. The 116 aa long peptide has a calculated size of 12,749 Da (Fig. 4).

Expression of the CD36/LIMPII receptor gene in primmorphs

Northern blotting was applied to semi-quantitatively measure the expression of the gene encoding the CD36/LIMPII receptor. After extraction of RNA and application of the same amount of RNA per sample onto the gel it became obvious that the single cells (Fig. 5A; time 0), as well as the primmorphs

that were cultured on uncoated dishes for 6 days, showed only a low expression of the CD36/LIMPII receptor gene; the transcript size was 1.7 kb. However, if the primmorphs were transferred after 6 days into culture dishes that had been coated with galectin, a strong increase of expression was seen. The highest steady-state expression of 5.1-fold increase (compared to the expression measured at day 0) was seen 3 days after the transfer (total incubation period of 9 days); almost the same level of expression (4.5 fold) was seen after an incubation period of 6 days on the galectin matrix (Fig. 5A).

In contrast, if the primmorphs were left on uncoated dishes for an additional 6 days after the initial incubation period, they remained unattached and did not show marked changes in the expression of the respective genes; the expression was estimated to be 1.9-fold higher than the control level. Interestingly the primmorphs that were transferred after 6 days onto galectin-coated dishes and were treated additionally with

ADAMTS_SD	ARGGGQCDR---CRMAEPEDVTRCSVTCEGSRSRSEVNCFSRDNPQVTEEGFCRSDTLLDTRO	62
ADAMTS-9_HUMAN	PVAKERCSVTPCQMKAL--DWSSCSVTCEGSRATROVMGVNYS--HVIDRSECDQDYIPENDQ	1288
{-expression-}-----[CD36BD]-----		
ADAMTS_SD	NCVIFNCFGSCVFEPSCRVLAALNLCK---EERFMSTTVQCCMSCFLQAPITLPP-----FT	118
ADAMTS-9_HUMAN	DCSMSPCFORTPDGSLAQHPFQNEFYRPSASPSRTHVLGGNQWRGCEWGCSSICAGGSQRRVV	1353
-----expression-}		
ADAMTS_SD	PLSEVNVTLDMDTYTVPMGMDLEVCCVNAETORFLNAVFTCT--FTADTDGEMFVGNDEVGEMKG	181
ADAMTS-9_HUMAN	VCQDENGYTANDCVERIKPDEQRACESSGPCPWAYGNWGECKLGGGGHRTRLVVCQRSGERFP	1418

Fig. 3. Alignment of the *S. domuncula* peptide fragment (ADAMTS_SD), deduced from the EST *SDADAMTS*, with the human ADAMTS-9 precursor, a disintegrin and metalloproteinase with thrombospondin motifs 9 (ADAMTS-9_HUMAN, Q9P2N4) (Clark et al., 2000). The numbers at the human sequence refer to the complete sequence. The sponge polypeptide consists of the CSVTCEG peptide domain that bind to the CD36 receptors (CD36BD). The borders of the sequence that were used for the preparation of the recombinant protein are given {-expression-}.

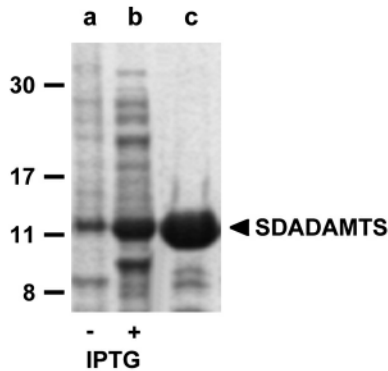


Fig. 4. Expression of a part of the *S. domuncula* *SDADAMTS* sequence including the CD36 binding motif. As outlined under Materials and Methods the fragment was subcloned into the bacterial expression vector pET41a and expression was performed in *E. coli* BL21 using IPTG. Lysates from non-induced (-IPTG; lane a) as well as from induced bacterial cultures (+IPTG; lane b) were prepared and analyzed using 15% polyacrylamide gel containing NaDodSO₄; the gel was stained with Coomassie Brilliant Blue. The protein extract containing the recombinant *SDADAMTS* was purified/enriched by affinity chromatography (lane c).

10 µg/ml of recombinant *S. domuncula* ADAMTS during this period had a much lower expression (2.6 fold; Fig. 5A). In a control experiment, the primmorphs were co-incubated with a further *S. domuncula*-derived recombinant peptide, a segment from the heat shock protein 70 (Kozioł et al., 1997) of a similar size of 13 kDa. Under these conditions no change in the high expression of *CD36/LIMP2* receptor was seen (not shown).

In parallel, the expression of the *S. domuncula* *galectin* gene was monitored. As shown in Fig. 5B, the 1.1 kb *galectin* transcripts remained almost at the same steady-state level, irrespective of the cultivation/treatment in (un)coated dishes or co-incubation with the recombinant ADAMTS peptide. Five experiments for each treatment were performed, with the same outcome.

In one series of experiments the primmorphs (developed on galectin-coated dishes) were incubated with the recombinant *SDADAMTS* only or with the recombinant *SDADAMTS* that had been pre-incubated with equal amounts of recombinant *CD36/LIMP2* receptor. Subsequently, a northern blot experiment was performed. The results showed again that the primmorphs, incubated with *SDADAMTS* alone show a low expression of the *CD36/LIMP2* receptor gene (0.2 fold with respect to the expression level seen in the absence of the

peptide), while *SDADAMTS* which had been co-incubated with the recombinant *CD36/LIMP2* receptor showed an almost normal expression level (0.8 fold; Fig. 5E).

In situ localization of cells expressing *CD36/LIMP2* receptor in tissue and primmorphs

The results described above suggest a morphogenetic role of galectin in primmorphs; after attachment to the substrate the primmorphs formed canal-like structures. Therefore, it was advisable to determine those cells that express high levels of transcripts encoding the *CD36/LIMP2* receptor. Recently we established the technique of in situ hybridization for sponge tissue/primmorphs (Perović et al., 2003), which we applied for this purpose. By this approach with the antisense *SDCD36/LIMP2* cDNA as a probe it was found that only cells that surround the aquiferous canals within the sponge tissue could be stained (Fig. 6A,C). In contrast, the sense *SDCD36/LIMP2* probe did not react with any sponge cells (Fig. 6B,D).

Isolation and characterization of MTN

The extract of a sponge-associated bacterium (an alpha-proteobacterium) isolated from the sponge *D. avara* exhibited considerable anti-angiogenic activity in the CAM assay. Therefore, the active compound was isolated using gel filtration on Sephadex LH-20 and reversed-phase preparative HPLC in a bioactivity-guided manner. The compound thus obtained was readily identified using MS and NMR spectroscopy as 2-methylthio-1,4-naphthoquinone (MTN; Fig. 7).

Anti-angiogenic activity using the CAM assay

To test the compound for anti-angiogenic activity in vivo, the chick chorio-allantoic membrane (CAM) assay was applied (Auerbach et al., 2003).

Our studies reveal that MTN is a potent angiogenesis inhibitor. It showed 100% activity at a concentration 1 ng/disc. This compound causes discontinuous and disrupted blood vessels in a wide area around the disc. The normal embryogenesis pattern of the chick embryo is shown in Fig. 8A. However, if MTN was added at a dose of 0.25 ng (per disc) (Fig. 8B), or of 1 ng (Fig. 8C), a distinct avascular zone at the place of application is seen. A quantitative analysis showed that at a dose of 0.25 ng (per disc) 20% of the embryos showed a significant anti-angiogenic effect ($n=20$); at the higher doses of 0.50 ng to 1 ng (per disc) of MTN, the percentage of embryos with a significant anti-angiogenic effect reached 100%. The effect of MTN on the viability of L5178y leukemic

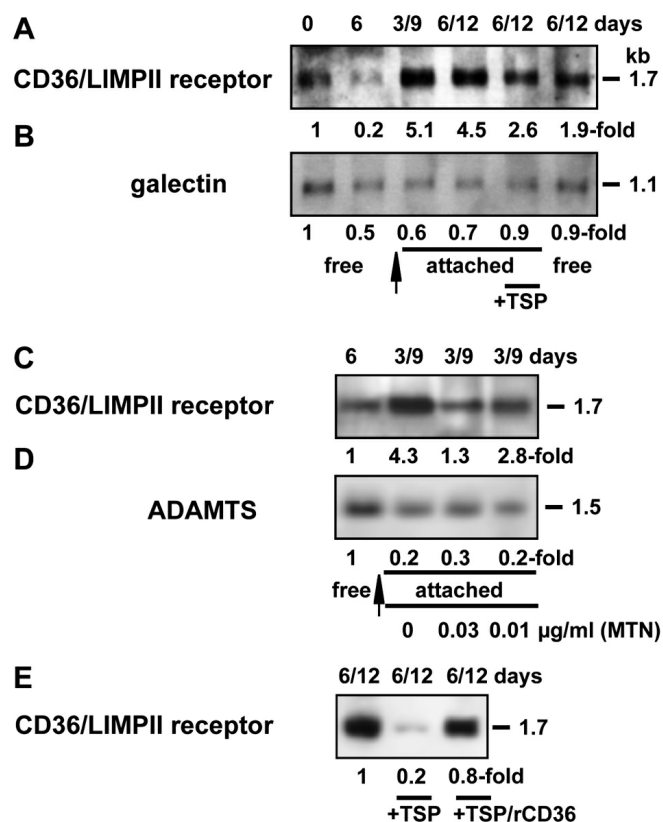


Fig. 5. Expression of the genes encoding the CD36/LIMP-II receptor, galectin as well as ADAMTS in primmorphs in response to different cultivation matrices. (A) The dissociated cells (time 0) were transferred into a seawater-based medium and cultured in uncoated dishes for 6 days (free). Then the primmorphs were transferred onto galectin-coated dishes for either 3 days (total period of incubation: 9 days) or 6 (12) days during which time they attached (attached: arrow). In a parallel series of experiments the primmorphs remained in uncoated dishes where they remained in the free state for 6 (12) days (free). Furthermore, the primmorphs that had been cultured on galectin-coated dishes were co-incubated for 6 (12) days with 10 µg/ml of the recombinant SDADAMTS peptide, containing the CSVTCT motif [+TSP]. RNA was isolated from the respective primmorphs and the same amount of RNA was loaded onto the gels and size separated. After blot transfer the filters were probed with the *CD36/LIMP-II receptor* cDNA. (B) In parallel, the blot was probed with the *galectin* cDNA. (C) Effect of MTN on primmorphs that had been transferred to galectin-coated dishes for 3 (9) days. Either no compound was added to the cultures, or MTN was added at a concentration of 0.03 or 0.01 µg/ml to the assays for this incubation period. The blot was developed with the *CD36/LIMP-II receptor* cDNA. (D) In parallel, the blot was incubated with the *SDADAMTS* probe. (E) As a control to show the interaction between the recombinant SDADAMTS peptide and the CD36/LIMP-II receptor, this peptide was pre-incubated with equal concentrations of the recombinant CD36/LIMP-II receptor polypeptide as described under Materials and Methods (+TSP/rCD36). The primmorphs were incubated on galectin-coated dishes for 6 (total incubation period of 12) days either in the absence of any recombinant polypeptide, or in the presence of 10 µg/ml recombinant SDADAMTS (+TSP), or in the presence of 10 µg/ml recombinant SDADAMTS together with 10 µg/ml recombinant CD36/LIMP-II receptor (+TSP/rCD36). The relative degree of expression was correlated with that seen at the related control time point (day 0 or day 6, as indicated); this value is set to 1.

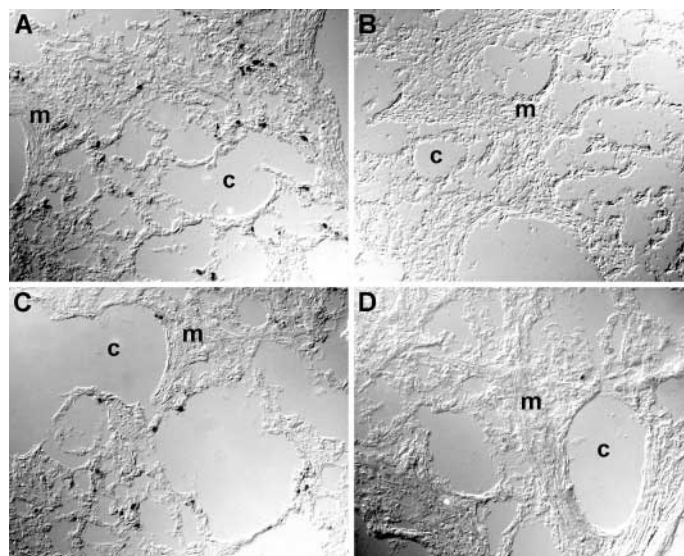


Fig. 6. In situ localization of cells expressing the CD36/LIMP-II receptor in tissue of *S. domuncula*. Cryosections of tissue were hybridized with DIG-labeled *SDCD36/LIMP-II*. Subsequently, the specimens were incubated with anti-digoxigenin/alkaline phosphatase and the signals were detected with NBT/X-phosphate as described under Materials and Methods. (A,C) hybridization with antisense *SDCD36/LIMP-II*; (B,D) hybridization with sense *SDCD36/LIMP-II*. Canals (c) of the aquiferous system within the mesohyl (m) are shown. The canals are lined up by an epithelial layer formed from pinacocytes, which are *SDCD36/LIMP-II*-positive cells. Magnifications: (A,B) ×50; (C,D) ×100.

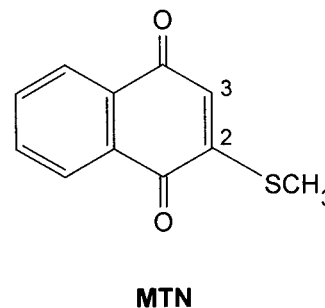


Fig. 7. Structure of 2-methylthio-1,4-naphthoquinone (MTN).

cells, PC-12 pheochromocytoma cells, HeLa S3 human cervix tumor cells and Rat1 fibroblasts was examined. L5178y cells were inhibited by MTN with an ED₅₀ value of 0.08±0.02 µg/ml. The effect on the other tumor cells was less strong; the ED₅₀ value was determined to be > 0.3 µg/ml.

Effect of MTN on the expression of the CD36-like receptor in *S. domuncula*

Northern blot analysis was applied to determine if MTN also causes a modulating effect on the expression of the CD36/LIMP-II receptor gene in the sponge primmorph system. Primmorphs cultured for 3 days on the galectin matrix in the presence of a low concentration of <0.01 µg/ml MTN showed a strong reduction of the steady-state level of this gene (Fig.

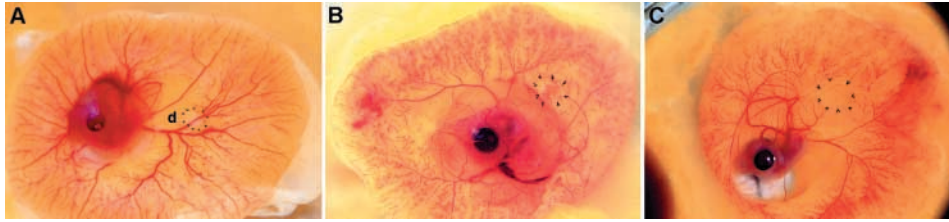


Fig. 8. Effect of MTN on vascularization in chick embryos. (A) Control chick CAM after incubation with an untreated agar disc for 48 hours. (B) CAM incubated for the same period with 0.25 ng MTN/disc and (C) with 1 ng MTN/disc. A distinct disorganization of the vessel formation, avascular zone, is seen at the locations where the discs were placed (d; arrowheads). Magnifications: $\times 10$.

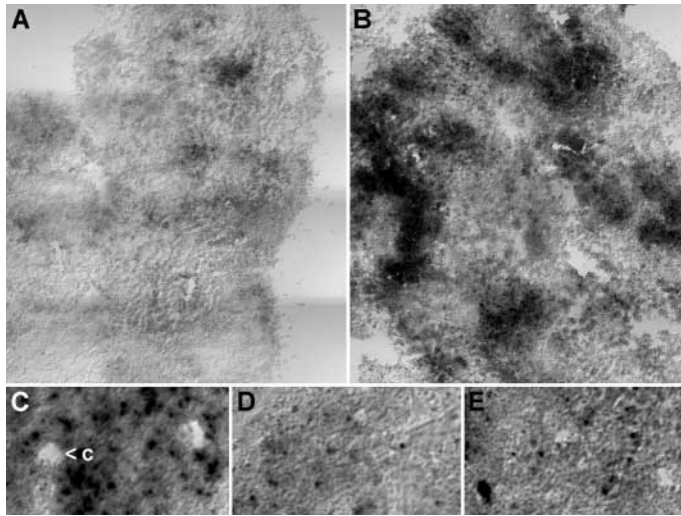


Fig. 9. Modulation of *CD36/LIMP2 receptor* gene expression in primmorphs; analysis was performed by in situ hybridization. (A) Primmorphs were cultured for 6 plus 3 days in uncoated culture dishes; then the in situ analysis was performed. (B) Primmorphs, cultured for 6 days in uncoated dishes and subsequently for 3 days on the galectin matrix. (C) Higher magnification of an area in primmorphs, grown on galectin. An intense staining of the cells, especially around the canal-like structures (c) is seen. (D,E) Primmorphs that had been cultured on galectin but in addition either in the presence of 10 $\mu\text{g/ml}$ of the recombinant SDADAMTS (D), or 0.03 $\mu\text{g/ml}$ of MTN (E). After the 3 days incubation the primmorphs were analyzed by in situ hybridization with the antisense probe *SDCD36/LIMP2*. Magnifications: (A,B) $\times 5$; (C-E) $\times 15$.

5C); the expression level dropped from 4.3 fold (in the absence of MTN) to 1.3 fold (with 0.03 $\mu\text{g/ml}$ MTN).

Reduction of expression of the CD36-like receptor in primmorphs by ADAMTS and MTN

The expression of the *CD36/LIMP2 receptor* gene was analyzed after treatment with ADAMTS and MTN by in situ hybridization. The expression was monitored in primmorphs grown in culture plates, without galectin or on plates coated with recombinant galectin. After an incubation period of 3 days on galectin, the expression of *CD36/LIMP2 receptor* in the primmorphs strongly increased (Fig. 9B,C) in contrast to the expression seen in primmorphs that remained in uncoated dishes (Fig. 9A).

The increase in expression was observed especially in the

areas that surround the canal-like structures (Fig. 9C). The increase of *CD36/LIMP2 receptor* gene expression was strongly reduced in the primmorphs after incubation with the recombinant *S. domuncula* ADAMTS (Fig. 9D). A likewise strong reduction of the expression was seen if primmorphs were incubated on galectin together with 0.03 $\mu\text{g/ml}$ of MTN (Fig. 9E). Additionally the number and diameter of canal-like structures in primmorphs cultured in the presence of recombinant ADAMTS or MTN (Fig. 9D,E) was reduced.

Expression of *ADAMTS* in primmorphs in dependence on MTN

Finally, the expression of *SDADAMTS* in primmorphs was determined. Northern blot experiments show that the expression of *SDADAMTS* is strongly downregulated in primmorphs cultured on the galectin matrix. The expression value drops to 0.2 fold compared to the expression measured at day 6 of incubation in uncoated dishes (Fig. 5D). This low level of expression remained unchanged if the primmorphs were co-incubated with 0.01 $\mu\text{g/ml}$ or 0.01 $\mu\text{g/ml}$ of MTN (Fig. 5D).

Discussion

Until recently the factors controlling the induction and formation of the complex aquiferous canal in sponges were unknown. With the elucidation that sponges, such as *S. domuncula*, can express homeobox genes, e.g. *Iroquois*, in response to extracellular conditions of the aqueous milieu (Perović et al., 2003), it became clear that oxygen has also a morphogenetic function. Having established that the *Iroquois* transcription factor is, after its induction through oxygen, crucial for the formation of the sponge circulatory system, we focused in the present study on comparable roles of extracellular matrix molecules.

In response to the extracellular adhesion molecule, galectin, the 3D-cell aggregates (primmorphs) from *S. domuncula* attach and start to arrange their cells in canal-like structures. The ability of *S. domuncula* to react to a homologous extracellular matrix by a morphogenetic effect as reported here and also recently by Adell et al. (Adell et al., 2003) and Wiens et al. (Wiens et al., 2003) is shared with the metazoan systems (Reed, 1990). The dramatic change in the organization pattern of the primmorphs, from spherical non-attached aggregates to flat more complex cellular entities is very reminiscent of mammalian spheroids induced to angiogenesis (Wartenberg et al., 2001). One of the evolutionary oldest angiogenesis-

controlling systems in Metazoa is CD36 and its ligand TSP (Calvo et al., 1995; Brower, 1999). No molecules related to the CD36 receptor or for the soluble TSP have been detected until now in non-metazoans. A comparison of CD36(-related) sequences from Metazoa, deposited in the database, with the sponge CD36/LIMP2 receptor gives alignment scores ' E ' (Expect value) (Coligan et al., 2000) of $<e^{-21}$; in contrast, the scores between the sponge molecule and the closest related molecules from yeast ($E=0.21$; Spo71p sequence) and plant ($E=3.1$; fructose biphosphatase) show a more distant relationship. In the same way, the similarity between the *S. domuncula*-deduced ADAMTS peptide fragment to related metazoan molecules is high ($E<e^{-4}$) compared to *Saccharomyces cerevisiae* ($E=3.4$; mitochondrial ribosomal protein) or *Arabidopsis thaliana* ($E=0.57$; glycine hydroxymethyltransferase) molecules (Fig. 2).

It is most surprising that the expression of the CD36/LIMP2 receptor from *S. domuncula* is upregulated after the structural rearrangement of the primmorphs. In two sets of experiments the role of the sponge CD36/LIMP2 receptor as a molecule involved in the induction of the canal-like structures has been supported. In situ hybridization studies showed that the cells positive for the CD36/LIMP2 receptor are located within the endopinacoderm, a cell layer that surrounds the aquiferous canals of intact sponges. The omnipotent stem cells, which are characterized by the expression of *noggin* transcripts (Schröder et al., 2004) are located in this epithelial layer. It is reasonable to conclude that the pinacoderm harbors not only (stem) cells from which the differentiated cells within the mesohyl compartment of the sponge originate but also those that give rise to new canals. In comparison, CD36 was demonstrated to be localized in the intima region of arteries (Nakagawa-Toyama et al., 2001).

In mammalian systems vascularization, the genesis of vessels, is inhibited by the angiogenesis-inhibitor TSP-1. The binding domain of TSP-1 for CD36 has been narrowed down to the sequence CSVTCG (Li et al., 1993). A peptide from a *S. domuncula* sequence related to the ADAMTS molecules from vertebrates, was prepared; it significantly prevented the galectin-mediated upregulation of the expression of sponge *CD36/LIMP2 receptor*. It remains to be determined whether the CSVTCG motif interacts directly with the existing CD36/LIMP2 receptor molecules on the cell surface and thereby prevents the increased gene expression. This appears to be most likely in view of the existing data in other models and the competition experiment reported in the present study. Alternatively, the peptide may bind to another receptor that controls the expression of *CD36/LIMP2*, e.g. integrin, which had been identified in *S. domuncula* (Wimmer et al., 1999). Therefore, binding studies of the recombinant sponge CD36/LIMP2 receptor and the CSVTCG are in progress. It should be stressed that no other genes encoding a CD36- or CD36-related protein than that described here exist in the *S. domuncula* EST library elaborated by us. Moreover, a screening with primers used in the present study and with other degenerate primers designed against CD36 did not result in the identification of a further, different cDNA for a putative CD36 receptor molecule in *S. domuncula*.

Our results indicate that the canal formation in sponges is regulated by the tuned interaction between the CD36 receptor and the TSP-1 peptide. For a more direct proof of this

assumption, inhibition studies with a secondary metabolite from a sponge were performed. MTN (2-methylthio-1,4-naphthoquinone) was isolated from a bacterial strain isolated from the sponge *D. avara*. MTN was obtained by a bioassay-guided isolation. Our results now establish that MTN, previously known only as a synthetic product (Fieser and Brown, 1949) occurs also as a natural product. Along with other small naphthoquinone derivatives, MTN displays remarkable bioactivities: antifungal (Gershon and Shanks, 1975) and antitumor actions (Takano et al., 1960) of MTN had been described. The finding that MTN causes a strong anti-angiogenic activity at very low doses is new and may qualify the compound for further therapy-oriented studies.

If MTN is added to primmorphs cultured on galectin the formation of the canal-like structures is prevented. The cells in such primmorphs remain in the spherical arrangement and no canals are seen. This effect occurs at a low concentration of $<0.03 \mu\text{g/ml}$. In parallel, the effect of MTN on the vessel formation in the chick chorio-allantoic membrane assay was determined. The alteration/prevention of the angiogenesis was achieved in the CAM assay at a dose of 1 ng/disc (5 pmol/disc). This potency is strong in comparison to other natural secondary metabolites, e.g. aeropysinin (Rodrigues-Nieto et al., 2001), for which an anti-angiogenic activity was found at a dose of 14 nmol/disc. Subsequent cytotoxicity studies with mammalian tumor cells and MTN allowed an estimation of the cell growth inhibitory effect of this compound. In general, the cytotoxic activity of MTN was strong, but did not reach the level seen for the inhibition in the CAM assay. Using the murine leukemic lymphoblast cells L5178y, the threshold below which no cytotoxicity is seen is 30 ng/ml (150 nM). In comparison the cytotoxicity to rat adrenal pheochromocytoma PC-12 cells, or human cervix HeLa S3 cells, caused by MTN is even weaker; no effect on cell proliferation is seen below 300 ng/ml. Further investigations will include inhibition studies with endothelial mammalian cells.

We also investigated if MTN causes a modulating effect on the expression of the CD36 receptor (CD36/LIMP2 receptor)-ligand (TSP) system in sponges. The expression of the *CD36/LIMP2 receptor* gene is downregulated after incubation at a MTN concentration of $0.01 \mu\text{g/ml}$. Hence MTN shows the same vessel-inhibitory effect in both the vertebrate model and a the sponge system.

In summary, the major outcome of the present study is the finding that the CD36-thrombospondin system, hitherto known to be involved in tumor angiogenesis of vertebrates, might have an important role during formation of canals of the aquiferous system in sponges. This result has considerable impact on our understanding of the evolution of diseases in metazoans and the conservation of the pathways involved in development and host defense systems in multicellular animals. Recently, we already presented evidence that secondary metabolites produced by sponges or their associated microorganisms influence immune reactions, e.g. rejection of allogeneic tissue, in the same way as in vertebrates, including human. A striking example is myotrophin. In myocytes from mammals this protein stimulates protein synthesis (Sen et al., 1990), suggesting a crucial role in (reviewed by Sil et al., 1998). In the sponge system myotrophin has been demonstrated to likewise stimulate overall protein synthesis (Schröder et al., 2000).

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