Vol. 178: 205–219, 1999 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published March 17

Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*

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ABSTRACT: In spite of the fact that cells from the phylum Porifera (sponges) contain high levels of telomerase activity, no successful approach to cultivate sponge cells has yet been described. Telomerase is the enzyme which catalyzes the addition of new telomeres onto chromosome ends which have been lost after each round of DNA synthesis. One reason may be seen in the observation that after dissociation the cells lose their telomerase activity. In addition, no nutrients and metabolites have been identified that would stimulate sponge cells to divide. We report here the culture conditions required for the formation of multicellular aggregates from *Suberites domuncula* from dissociated single cells; they are termed primmorphs. These aggregates, formed in seawater supplemented with antibiotics, have a tissue-like appearance; they have been cultured for more than 5 mo. Cross sections through the primmorphs revealed an organized zonation into a distinct unicellular epithelium-like layer of pinacocytes and a central zone composed primarily of spherulous cells. After their association into primmorphs, the cells turn from the telomerase-negative state to the telomerase-positive state. Important is the finding that a major fraction of the cells in the primmorphs undergo DNA synthesis and hence have the capacity to divide. By applying the BrdU (5-bromo-2'-deoxy-uridine)-labeling and detection assay it is demonstrated that up to 33.8% of the cells in the primmorphs are labeled with BrdU after an incubation period of 12 h. It is proposed that the primmorph system described here is a powerful novel model system to study basic mechanisms of cell proliferation and cell death; it can also be used in aquaculture, for the production of bioactive compounds and as a bioindicator system.

KEY WORDS: *Suberites domuncula* · Sponges · Cell culture · Telomerase · Primmorphs · Senescence · Apoptosis · Aquaculture · Bioactive compounds · Bioindicator · Biomarker

INTRODUCTION

The phylum Porifera (sponges) is grouped with the other metazoan phyla into one kingdom (Müller 1995). One key autapomorphic character of Metazoa is, for example, the receptor tyrosine kinase, which is found only in this kingdom (Müller & Schäcke 1996). Within the Metazoa, the Porifera comprise 1 plesiomorphic character which is not found in any higher metazoan phyla; they have in almost all of their cells high levels of telomerase activity (Koziol et al. 1998). In normal somatic cells of higher Metazoa, chromosomes lose about 50 to 200 nucleotides of telomeric sequence per cell division. Hence, synthesis of DNA at chromosome ends by telomerase is necessary for indefinite proliferation of metazoan cells. The fact that sponge cells are provided with high telomerase activity implies that they do not derive from germ- and somatic-cell lineages (see Müller 1998a,b). In higher Metazoa, germ cells are telomerase-positive while somatic cells are telomerase-negative, with the exception of tumor cells which are telomerase-positive (von Lange 1998).

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Nevertheless, until now no report on neoplastic diseases in sponges exists (De-Flora et al. 1995).

Some sponges can reach a life span of more than 1500 yr (Lehnert & Reitner 1997). This fits well with the observation of high levels of telomerase in almost all sponge cells; it may therefore be concluded that sponge cells are immortal. Already in the first report on telomerase activity in sponge cells it was shown that cells which have lost their contact to each other, e.g. after dissociation, turn to the telomerase-negative state (Koziol et al. 1998). Cells kept as single-cell suspension either die, very likely in a process of apoptosis (Wagner et al. 1998), or remain in a non-proliferating dormant state. Furthermore, the fact that all sponge species have a species-specific Bauplan led us to postulate that they are provided with the mechanism of apoptosis to replace a given set of cells at a given time. This assumption was supported by findings indicating that sponge tissue undergoes apoptosis in response to both endogenous (heat-treated bacteria) and exogenous factors (cadmium) (Wagner et al. 1998).

After having proven that sponge cells have high levels of telomerase activity (Koziol et al. 1998) it appeared easily feasible to establish cell cultures. Until now, only sponge-cell maintenance has been achieved from *Hymeniacidon heliophila* (Pomponi & Willoughby 1994), *Latrunculia magnifica* (Ilan et al. 1996) and *Suberites domuncula* (Müller et al. 1996). The cells in suspension do not proliferate (Ilan et al. 1996); therefore the systems can hardly be termed primary cultures (Freshney 1994). In addition, primary single-cell suspensions from sponges also contain Bacteria and Protozoa as verified by earlier experiments (Klautau et al. 1994, Custodio et al. 1995). The reasons for the fact that single cells remain in a resting stage might be seen (1) in the experimental approach to establish a single-cell culture (Pomponi & Willoughby 1994, Ilan et al. 1996) and (2) in the culture conditions. The media used are supplemented with fetal calf/bovine serum (Pomponi & Willoughby 1994, Ilan et al. 1996) under the assumption that the growth factors present in the serum might stimulate cell growth. However, it appears reasonable to accept that sponge cells have receptors on their surface which are activated by ligands different from those acting on mammalian receptors in general and human receptors in particular. Consequently, the growth factors present in calf/bovine serum are not very likely to act on sponge receptors. Furthermore, serum-rich media imply the risk of protozoan contamination, as has been outlined recently (Osinga et al. 1998).

To overcome the blockade of division of sponge cells in culture we have elucidated some metabolic pathways by molecular-biological techniques to obtain first insights into nutritional requirements of the cells. It was found that cells from *Geodia cydonium* are rich in cathepsin (Krasko et al. 1997), a family of enzymes which are localized in lysosomes (Bando et al. 1986). One pathway in macrophages which directs extracellular material to lysosomes starts from cell-surface-bound scavenger (scavenger receptor cysteine-rich repeats, SRCR) receptors (reviewed in Krieger & Herz 1994). It has been reported that macrophage SRCR receptors are involved in clearance of cells which have undergone apoptosis (Platt et al. 1996). Hence, the next step was to analyze if sponge cells contain proteins that feature SRCRs; the cDNAs encoding those polypeptides were recently isolated from *G. cydonium* (Pancer et al. 1997, Pahler et al. 1998a).

Based on previous findings, which indicated that single cells from *Geodia cydonium* and *Suberites domuncula* are telomerase-negative, we concluded that suspension cultures of single sponge cells are more difficult to establish than those with tissue-like aggregates. This implies that cells from sponges will require stimuli resulting from cell-cell and/or cell-matrix contact in order to proliferate. In the present study we detail for the first time data on the successful establishment of sponge cell culture from *S. domuncula*. Detailed experimental data were given recently (Custodio et al. 1998). The culture starts from dissociated single cells, which subsequently form aggregates; in those aggregates the cells start DNA synthesis and proliferate. The aggregates show a tissue-like appearance and can be cultured for more than 5 mo for the *S. domuncula* system, as reported here, or for 8 mo in the experiments with *Polymastia janeirensis* (authors' unpubl. data); the aggregates have been termed primmorphs (Custodio et al. 1998). In addition, data are presented which demonstrate that the proliferating cells in primmorphs can be used for (1) production of bioactive compounds and (2) monitoring of environmental hazards.

ESTABLISHMENT OF PRIMMORPHS

Sponge

Specimens of the marine sponge *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the Northern Adriatic near Rovinj (Croatia), and then kept in aquaria in Mainz (Germany) at a temperature of 16°C.

Media

Natural seawater was obtained from Sigma (Deisenhofen, Germany). The compositions of Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW) and CMFSW containing EDTA (CMFSW-E) have been given earlier

(Rottmann et al. 1987). Where indicated the seawater was supplemented with antibiotics (100 IU ml^{-1} of penicillin and 100 µg ml^{-1} of streptomycin) (seawater/antibiotics).

Dissociation of cells and formation of primmorphs

A schematic outline of the procedure is given in Fig. 1.

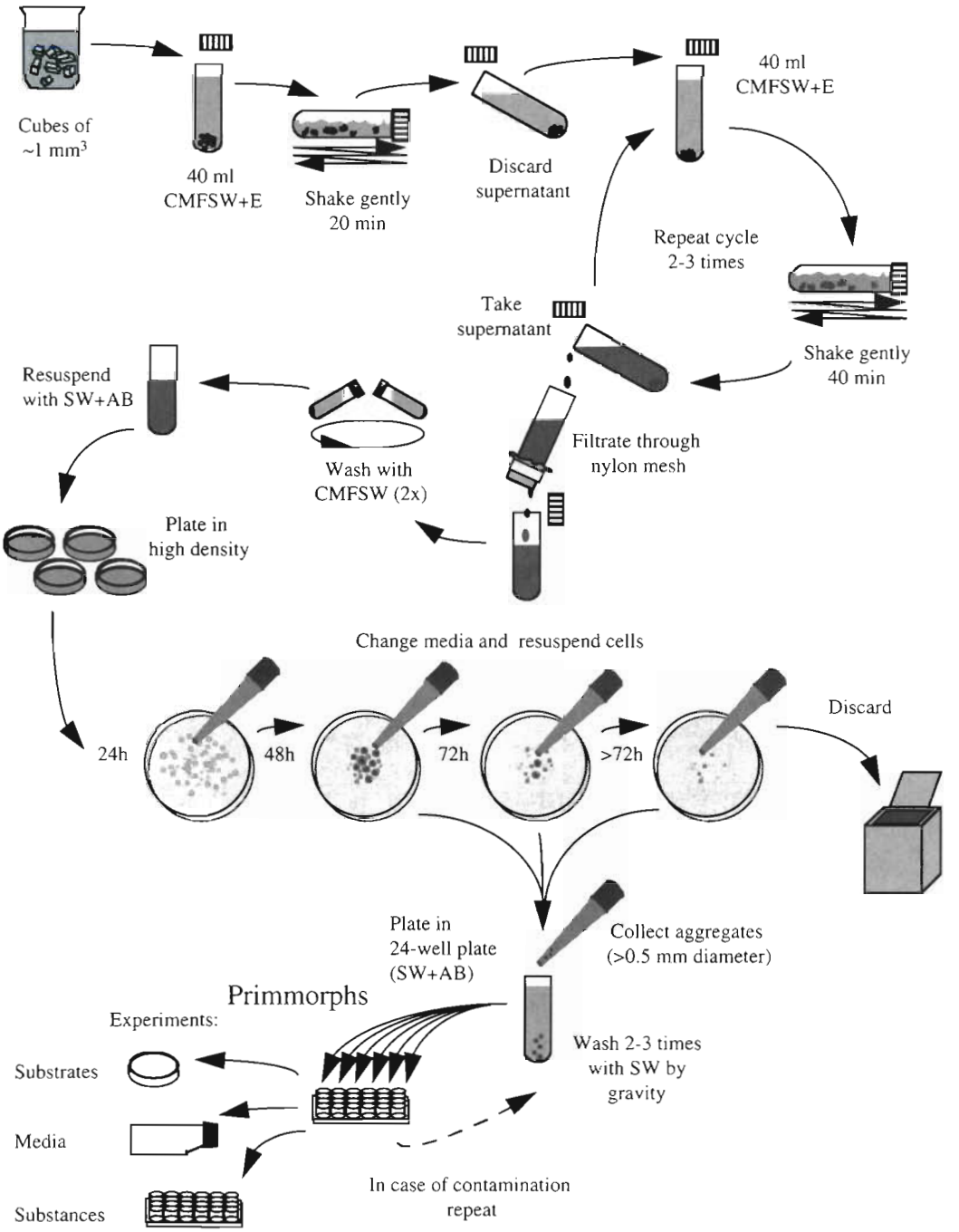


Fig. 1. Scheme for the generation of primmorphs from dissociated cells of the sponge *Suberites domuncula*. Single cells are obtained from tissue by dissociation in CMFSW-E (Ca^{2+}- and Mg^{2+}-free artificial seawater [CMFSW] containing EDTA). After filtration through a nylon net the cells are transferred into CMFSW and plated at high cell density in seawater supplemented with antibiotics (SW+AB). Culture medium is replaced daily by fresh seawater/antibiotics. Primary aggregates of a diameter of at least 0.5 mm are collected and washed twice in seawater by using only gravity for the separation of aggregates and debris. The aggregates obtained are transferred again into new Petri dishes. After their formation, primmorphs are placed into 24-well plates with seawater/antibiotics. After 3 wk the primmorphs can be used for experiments. Details are given in the text

All cell culture dishes and tubes were sterilized and the media were filtrated through 0.2 µm polycarbonate filters. Tissue samples of 4 to 5 cm^3 are submersed in Petri dishes in seawater and cut into 1 mm^3 cubes; these are transferred into 50 ml conical tubes (Falcon no. 2070) filled with CMFSW-E (ratio tissue to medium 1:10). After gentle shaking for 20 to 30 min at 16°C with a rotating agitator, the solution is discarded and new CMFSW-E is added. After continuous shaking for 40 min the supernatant is collected and filtered through a 40 µm mesh nylon net; this process of shaking in CMFSW-E (40 min) and filtration is repeated once. The cells are obtained by centrifugation (500 × *g* for 5 min) and washed twice in CMFSW. The cells in the final pellets are resuspended in seawater/antibiotics to a density of 1.5–2.0 × 10^6 cells ml^{-1} and 6 ml of this suspension is added to 60 mm Petri dishes (Falcon no. 3004). The cultures are kept at 16°C.

Two-thirds of the culture medium is replaced daily by fresh seawater/antibiotics; the cultures in which cell clumps have formed are gently agitated to avoid adhesion of cells to the plate. Primary aggregates of at least 0.5 mm in diameter are collected as soon as they are formed and washed 2 or 3 times in 15 ml tubes (Falcon no. 2096) filled with 10 ml of seawater, using only gravity for the separation of aggregates and debris. The aggregates obtained are transferred again into new Petri dishes (total volume of 6 ml). This collection from the dishes is repeated until no more aggregates are formed or other organisms (mostly protozoans) are observed on the dishes.

After their formation, primmorphs are placed into 24-well plates (Nunclon™, Nunc no. 143982) with 1 ml of seawater/antibiotics and 1 to 2 primmorphs $well^{-1}$. Medium is changed every day during the first 2 wk; later the medium change is necessary only once or twice a week. All pipettings are performed with Pasteur pipettes (diameter of the openings: 2 mm) or plastic tips (with openings cut to diameter of 2 to 3 mm). After at least 3 wk in culture the primmorphs can be used for the experiments, e.g. for testing of suitable substrates or culture media, or for the determination of the effect of substances on cell proliferation and DNA synthesis (e.g. as performed with the potential morphogen the endothelial-monocyte-activating polypeptide; Pahler et al. 1998b).

Histology

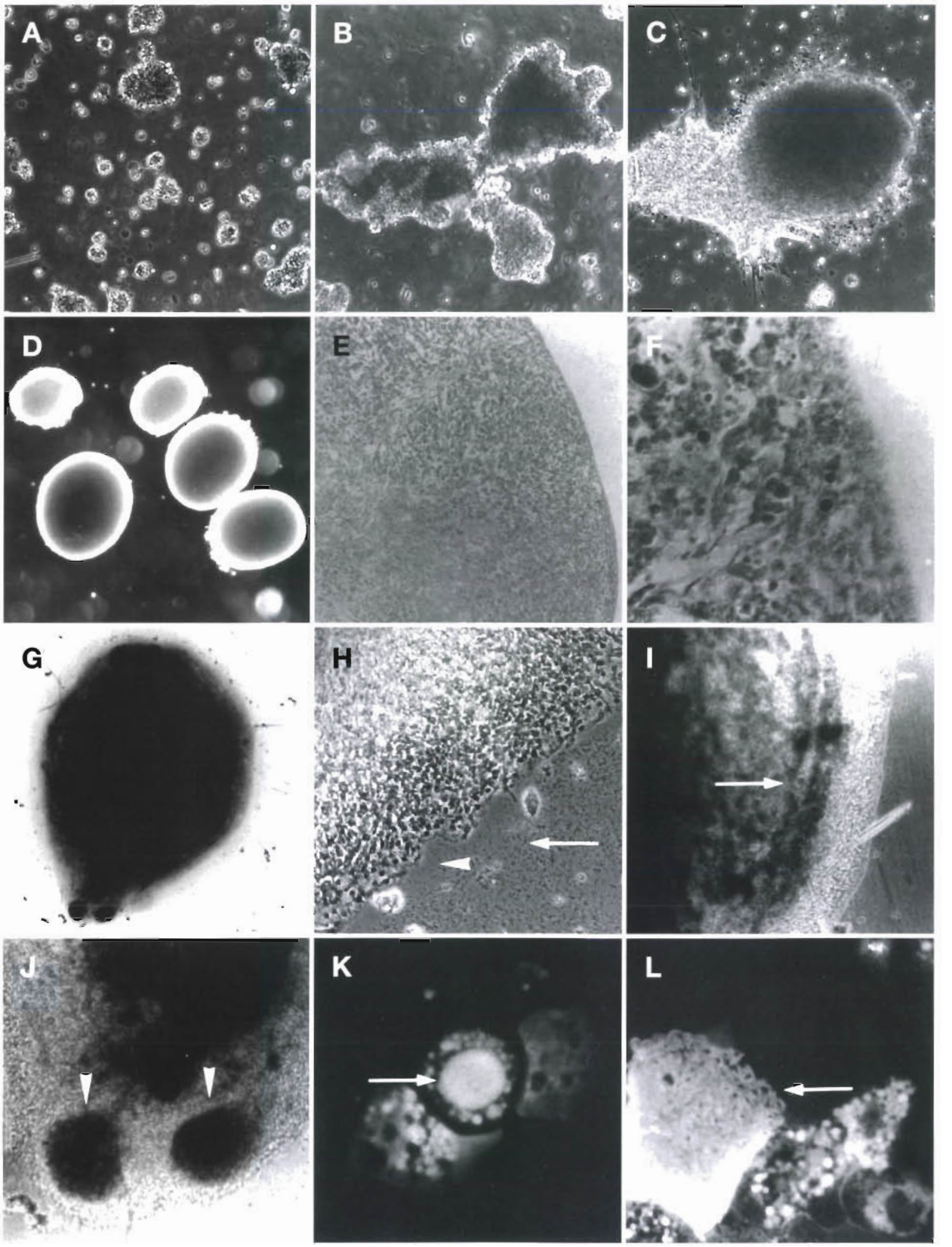
Single cells and small aggregates are obtained by chemical dissociation (Fig. 2A). After 2 washing steps to remove most Protozoa, which preferentially adhere to the plastic surface of the culture dishes, the cells are transferred into seawater/antibiotics. The diameter of the cell aggregates increases steadily during an incubation period of approximately 3 d (Fig. 2B). After a total treatment/incubation period of about 5 d, primmorphs are formed from cell aggregates. During this time the aggregates have an irregular surface (Fig. 2C). Usually after further incubation for 3 to 5 d spherical primmorphs are formed (Fig. 2D). During the phase of primmorph formation they contract to round-shaped bodies, 1 to 2 mm in size, leaving behind detritus and dead cells. In the initial phase the primmorphs remain round-shaped; after an incubation period of longer than 3 to 4 wk approximately half of them attach to the surface of the culture dish.

Cross sections through the primmorphs were performed. Microscopical analysis of the sections stained with Ziehl's fuchsin (Romeis 1989) revealed that the cells present in the interior are surrounded by an almost complete single-cell layer of epithelium-like cells (Fig. 2E, F). The cells which compose the squamous epithelium of the primmorphs are pinacocytes as judged from their flattened, fusiform extensions and their prominent nucleus (Simpson 1984); the size of the cells ranges from 15 to 20 µm. The cells inside the primmorphs are primarily spherulous cells. They have a diameter of 40 to 45 µm and are characterized by large round vacuoles which occupy most of the space in the cells. The other cells (~55 to 60 µm) may be termed amoebocytes and archaeocytes (Bergquist 1978). The organized arrangement of the cells within the primmorphs distinguishes them from aggregates which are formed from dissociated cells in the presence of the homologous aggregation factor (Müller 1982).

Subcultures of primmorphs

The primmorphs can be kept in culture by constantly renewing the medium, seawater/antibiotics. Under

Fig. 2. *Suberites domuncula*. Formation of primmorphs from sponge cells. (A) Cell aggregates formed after transfer of dissociated cells into seawater/antibiotics (magnification ×100); (B,C) irregular aggregates, after 1 d or 2 d in seawater/antibiotics (×30); (D) primmorphs (×10); (E,F) cross sections through a primmorph, stained with Ziehl's fuchsin (E, ×120 and F, ×700); (G) primmorph after a 1 wk culture period in an aquarium (×20); (H) primmorph surrounded by a relatively particle-free zone (arrowhead) and by a border of detritus (arrow) (×280); (I) canal-like structure (arrow) in a primmorph (×350); (J) 2 cell clumps (arrowheads) within a primmorph presumably involved in egestion (×320); (K,L) capsule (arrow) filled with bacteria and surrounded by sponge cell(s) (×600). With the exception of (K) and (L), all microscopic images were made with visible light; cells in (K) and (L) were inspected by fluorescence microscopy (λ550–600 nm)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such conditions the primmorphs remain in a functional state by synthesizing DNA for more than 5 mo.

Either immediately after formation or after 5 mo the primary primmorphs can be dissociated again using CMFSW-E. The single-cell suspension again forms aggregates and subsequently small primmorphs if they are transferred into seawater/antibiotics; these are termed secondary primmorphs. The kinetics of secondary primmorph formation is identical to that seen for the primary primmorphs. In the absence of Ca^{2+}, using the medium CMFSW, the single cells obtained from primary primmorphs after dissociation readily attach to the surface of glass dishes. For optimal attachment to plastic, the dishes have to be scratched moderately using the tip of a pipette or a plastic rubber (not shown).

Long-term cultivation of primmorphs in the aquarium

Primmorphs are usually kept for at least 4 wk in an air incubator at 16°C. Then they can be transferred into culture chamber slides (Nunc no. 177453) and be cultivated further in aquaria together with other sponges (e.g. *Suberites domuncula*, *Geodia cydonium*), sea urchins (e.g. *Psammechinus microtuberculatus*) or sea cucumbers (e.g. *Cucumaria planci*). The animals in the aquarium (volume ≈ 130 l) are kept in artificial seawater (sea salt 'Tropic Marine' from Dr. Binder GMBH, Wartenberg, Germany). The sponges (usually 20 *S. domuncula* specimens are kept in 1 aquarium) are fed with four 4 ml of phytoplankton ('Marin Niedere Tier Futter' from Amtra Aquaristik, Rodgau, Germany) twice a week; twice a month the seawater is supplemented with vitamins and trace elements ('Vitamine & Mineral Supplement' from Kent Marine, Marietta, GA, USA). In addition, the hermit crab *Pagurites oculatus* (Decapoda: Paguridea) which resides in shells of the mollusc *Trunculariopsis trunculus* (Gastropoda: Muricidae) is present in all specimens of *S. domuncula*; they are fed with 5 g krill and animal plankton ('Krill Pacifica' from Petfood, F. Hundt, Wuppertal, Germany) every 3 d. Under those conditions most of the primmorphs attach to the culture dishes (Fig. 2G) and start to rearrange their cells into a higher organization state. The primmorphs are surrounded by a relatively particle-free zone (arrowhead in Fig. 2H) and a rim of detritus and dead cells (arrow in Fig. 2H) which probably have been extruded by the primmorphs.

In the aquarium the primmorphs form new monaxonial spicules, styles, surrounded by organic matrix material which positively stains with the trichrome stain ASTRIN (Pancer et al. 1996; not shown). In addition, first formation of canals and dermal membrane development is seen after cultivation of the primmorphs in the aquarium for 3 wk (Fig. 2I). Occasionally, cell clumps are found within the primmorphs which probably later are released from them (Fig. 2J). It is intriguing to assume that these clumps contain particles/molecules which have to be eliminated. Sponge cells provided with an egestive function have been described in a series of species (Reiswig & Brown 1977).

CHARACTERIZATION OF PRIMMORPHS

Level of telomerase activity in cells, depending on the culture conditions

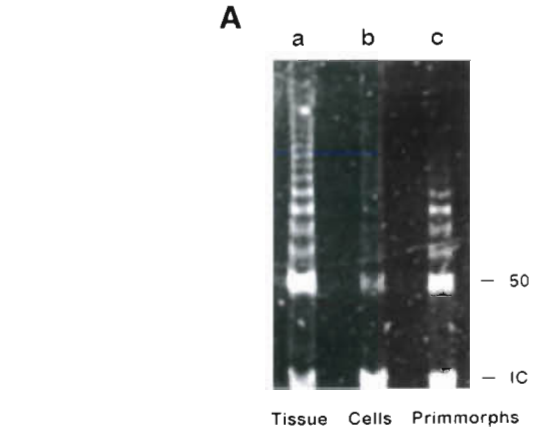
As reported earlier (Koziol et al. 1998) sponge cells undergo a transition from the telomerase-positive to a telomerase-negative state after dissociation into a single-cell suspension. The level of telomerase activity has been determined in cells during formation of primmorphs from a single-cell suspension (Custodio et al. 1998). Cells in natural tissue association contain high levels of telomerase activity; a quantitative analysis revealed an activity of 8.9 TPG (total product generated) units in 5×10^3 cell equivalents (Fig. 3A, lane a). In cells which had been left for 14 h in the dissociated single-cell state, the enzyme level dropped to 0.9 TPG units in 5×10^3 cells (Fig. 3A, lane b). However, in cells from primmorphs (used 10 d after formation from single cells) a telomerase activity of 4.7 TPG units in 5×10^3 cells is seen (Fig. 3A, lane c).

These data confirm that cells lose their telomerase activity if removed from the tissue assembly. As has already been postulated (Koziol et al. 1998, Wagner et al. 1998), single cells will recover after formation of tissue-like bodies, primmorphs, and change from the telomerase-negative to the telomerase-positive state.

Immunocytochemical detection of BrdU incorporation in cells of primmorphs

The BrdU (5-bromo-2'-deoxy-uridine)-labeling and detection assay (Gratzner 1982) was used to demonstrate that the cells organized into primmorphs regain the capacity to proliferate. As recently reported (Custodio et al. 1998) the BrdU-labeling and detection kit from Boehringer Mannheim was used for the studies. As a measure for proliferation the cells are incubated for 12 h in the presence of BrdU. Then the incorporation of BrdU into DNA is detected by an anti-BrdU monoclonal antibody.

The BrdU-positive cells, undergoing DNA synthesis, are stained brownish in their nuclei (Fig. 4B–D). In a



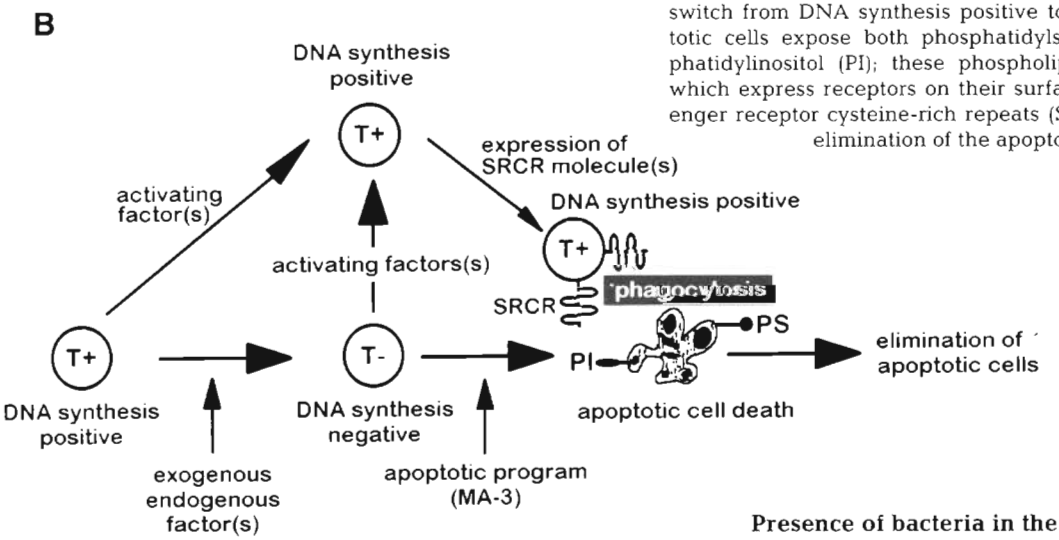


Fig. 3. *Suberites domuncula*. Telomerase and its potential role in sponge cells. (A) Telomerase activity was determined in cells present (1) in tissue (lane a), (2) in the dissociated single-cell state (the cells were analyzed 14 h after dissociation) (lane b) and (3) in primmorphs (lane c). Defined amounts of tissue, corresponding to 5×10^3 cell equivalents, were assayed in the TRAP assay (Koziol et al. 1997). After PCR amplification the products were resolved in a non-denaturing polyacrylamide gel and the gels were stained with SYBR Green I to detect the DNA fragments. IC: internal control in the assay. The products of the telomerase reaction are visualized as a ladder of oligonucleotides with 6 base increments starting at 50 nucleotides: 50 (marked), 56, 62, 68, etc. (B) Proposed scheme for the explanation of the transition of telomerase-positive (T^+) (and DNA synthesis-positive) to telomerase-negative (T^-) (DNA synthesis-negative) cells, with the consequence of apoptotic cell death. It is outlined that exogenous and/or endogenous factor(s) cause the transition from T^+ to T^- cells. During this process the apoptotic program is initiated and the cells lose the ability to undergo DNA synthesis; the cells switch from DNA synthesis positive to negative. The apoptotic cells expose both phosphatidylserine (PS) and phosphatidylinositol (PI); these phospholipids bind to T^+ cells which express receptors on their surface composed of scavenger receptor cysteine-rich repeats (SRCR), resulting in an elimination of the apoptotic cells

control assay the antibody recognizing BrdU was omitted; under this condition no staining is observed (Fig. 4A).

Suspensions of dissociated cells which had been kept for 1 d in CMFSW-E did not contain any cell which underwent DNA synthesis (Table 1). The percentage of BrdU-positive cells present in cell aggregates formed from single cells after 1 d in culture is low; only 6.5% were counted to be positive. In contrast, the number of DNA-synthesizing/proliferating cells present in primmorphs is high. As summarized in Table 1 the number of BrdU-positive cells in primary primmorphs is 33.8% and in 'older' primmorphs, after 1 mo in culture, 22.3%. These data document that cells which are reorganized in tissue-like primmorphs undergo DNA synthesis and very likely subsequently also cell division.

Presence of bacteria in the primmorphs

Most sponge species, perhaps with the exception of the calcareous sponges, live in symbiosis/commensalism with bacteria, cyanobacteria and zooxanthellae

Table 1. *Suberites domuncula*. Analysis of cells for DNA synthesis. Single-cell suspensions or primmorphs were incubated with BrdU and the incorporated nucleotides were visualized immunologically using anti-BrdU monoclonal antibody. The percentage of BrdU-positive cells is given. The analysis was performed with (1) dissociated cells which had been kept for 1 d in CMFSW-E, (2) cell aggregates formed from single cells after 1 d in culture with seawater, (3) primmorphs (after formation for 3 d) and (4) primmorphs (after 1 mo). Per assay, 300 cells were counted

Cells analyzed	Percentage of BrdU-positive cells
Dissociated cells (after 24 h)	0
Aggregates (after 24 h)	6.5
Primmorphs (after 3 d)	33.8
Primmorphs (after 1 mo)	22.3

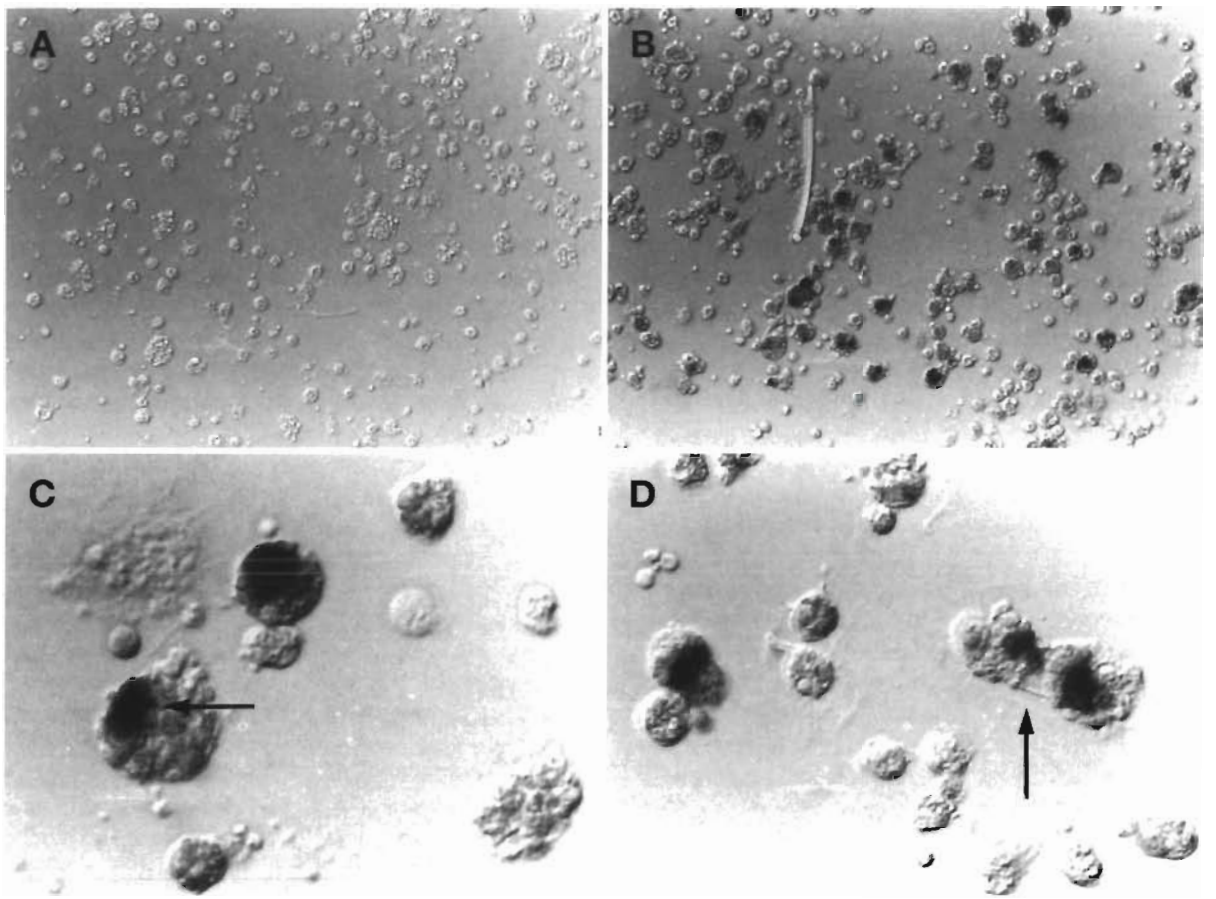


Fig. 4. *Suberites domuncula*. Immunocytochemical detection of proliferating (BrdU-labelled) cells from primmorphs. After incubation of the primmorphs with BrdU the cells were dissociated and subjected to staining with anti-BrdU monoclonal antibody. (A) Control in which the antibody against BrdU was omitted. (B–D) Dark brownish stained nuclei are those which have incorporated BrdU. In (C) the arrow marks a BrdU-positive cell; in (D) one cell is seen which has undergone division during the labelling period (arrow). Magnifications: (A)×50, (B)×100, (C,D)×700

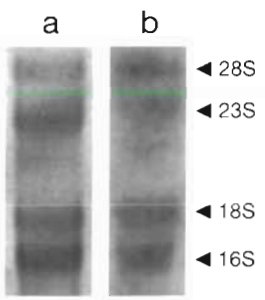


Fig. 5. *Suberites domuncula*. Pattern of total RNA from (1) tissue of *Suberites domuncula* (lane a) as well as (2) primmorphs (1 mo after formation) (lane b). After isolation, 5 µg of total RNA from tissue and 2 µg of RNA from primmorphs were size-separated by electrophoresis through 1% formaldehyde/agarose gel and then stained with ethidium bromide (1 µg ml^{-1})

(reviewed in Simpson 1984). In all species where bacteria have been identified they are scattered within the mesohyl (mesenchyme) or are present intracellularly (reviewed in Simpson 1984). In *Suberites domuncula* the bacteria are found in clusters which are compartmented in capsules formed from unknown material (Fig. 2K,L). Striking is the fact that the capsules remain in intimate contact with sponge cells even after dissociation (Fig 2K, L). The capsules containing the bacteria are found both with dissociated cells from tissue (Fig. 2K) and from primmorphs (Fig. 2L).

The total RNA from sponge tissue as well as from primmorphs was isolated, size-separated (Wiens et al. 1998) and stained with ethidium bromide (Ausubel et al. 1995). The RNA pattern revealed 4 major bands of sizes of 28S, 23S, 18S and 16S, found both in the tissue (Fig. 5, lane a) and in the primmorphs (Fig. 5, lane b). The 28S RNA represents the major rRNA species from the large subunit and 18S RNA the major species from the small subunit known from higher eukaryotic cells, while the 23S RNA and the 16S RNA correspond to the major rRNAs from bacteria.

These 2 sets of data indicate that bacteria are present in tissue from the sponge *Suberites domuncula* as well as in primmorphs obtained from cells of the same species. It should be stressed that no sign of bacterial

infection is seen in the primmorphs; it appears most likely that the growth of the microbes is controlled by the sponge cells and hence supports the view that some sponges live in symbiosis with a distinct bacterial flora (Müller et al. 1981, Althoff et al. 1998).

Expression of a sponge gene as a marker for the origin of the cells

Considering (1) the fact that in the past the cell cultures that were attempted from sponges have not been tested for their true sponge origin and (2) the risk of a sudden appearance of eukaryotic microorganisms of the phylum Labirinthulomycota in those cultures (Ilan et al. 1996), a molecular marker for an unequivocal idenfication of the cells in the *Suberites domuncula* primmorph culture was used. For these experiments we selected a gene which has been shown to be relevant for environmental monitoring, a member of the cytochrome P450 superfamily (Goksøyr & Förlin 1992). The cytochrome P-450 proteins (CYPs) belong to a superfamily of structurally and functionally related hemoproteins, NAD(P)H-dependent monooxygenases, that metabolize numerous endogenous but also exogenous substrates, e.g. steroids, fatty acids, drugs, carcinogens, and peptides (Gonzalez 1989). The prominent subfamilies are CYP1A (inducers: aromatic hydrocarbons), CYP2B (phenobarbital), CYP3A (glucocorticoids), and CYP4A (clofibrate) (Nebert et al. 1991). The specific gene from *S. domuncula* belongs to the latter subfamily. It is termed *SDCYP4* (accession number Y17816).

The gene *SDCYP4* was isolated from a *Suberites domuncula* cDNA library in λZAP Express™ (Kruse et al. 1997) by application of the polymerase chain reaction (PCR). The forward primer used was 5'-GAA/G AGT/C CAA/G GAU/C CGI CTI AGG/A ACI GAA/G-3' (where I = inosine) in conjunction with the 3'-end vector-specific primer T7. The degenerate primer was designed against the conserved amino acid (aa) stretch present in the vertebrate *CYP4* sequences (with respect to the human gene this segment is located between aa_{283} and aa_{291}; EYQERCRQE; CYP4_HUMAN; accession number 3012097). Details will be given elsewhere (Wiens et al. unpubl.). The PCR fragment obtained was used to isolate the cDNA from the library (Ausubel et al. 1995). The longest insert obtained had a size of 1810 nucleotides [excluding the poly(A) tail].

The deduced aa sequence, named CYP_SD, 481 aa in size (Fig. 6A), has a calculated polypeptide mass of 55 334 kDa and an isoelectric point of 5.83; the instability index is computed to be 46.2, classifying this protein as unstable (PC/GENE 1995; Physchem program). The sequence was analyzed using the computer program BLAST (1997; www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast). Highest similarity was found to sequences encoding cytochrome *P*-450 proteins of subfamily 4 from higher metazoan phyla. The degrees of identity (similarity) were ~17% (~33%) for the CYP4A or CYP4A-related sequences. A phylogenetic tree (Fig. 6B) was constructed using human, rat, *Drosophila melanogaster*, cockroach, and *Caenorhabditis elegans* sequences. The related sequence from *Mycobacterium tuberculosis* was added and was used as outgroup to obtain a clearer phylogenetic position of the sponge polypeptide. The tree indicates that the sponge sequence forms the basis of the metazoan sequences (Fig. 6B). The order of branching is statistically robust as assessed by bootstrap analysis. This finding again demonstrates that cDNAs from sponges which encode putative proteins are phylogenetically the oldest within the metazoan kingdom (Müller 1998b).

As known from other CYP polypeptides, the sponge sequence also comprises a highly hydrophobic N-terminal segment (Fig. 6A), which has been attributed to the presence of an uncleavable membrane-directing signal sequence (Bar-Nun et al. 1980). The further conserved region within the CYP family is present in the *Suberites domuncula* sequence around Cys-426 (Hardwick et al. 1987) within the stretch **FGV**HSR-**RKC**P**G**YLF**S**YF**E**VG**V** (the conserved aa residues are in bold and underlined); this part of the sequence is very likely involved in binding of the heme ion (Gonzalez 1989).

The size of the sponge CYP mRNA is approximately 1.9 kb, as identified by Northern Blot analysis (Fig. 6C). RNA was extracted from tissue of *Suberites domuncula* as well as from primmorphs of the same sponge species. The Northern Blot experiment using *SDCYP4* as a probe revealed that in both samples only 1 band is detected with a size of 1.9 kb (Fig. 6C, lane a and b), demonstrating that the cells present in the primmorphs originate from *S. domuncula*.

Preservation and storage of sponge cells

For future application of the primmorphs in aquaculture and for the production of bioactive compounds it is essential to establish techniques to preserve and store living sponge cells. For mammalian cells the technique of cryopreservation is well established (Celis 1998). The procedure used to freeze the sponge cells is in the process of being optimized.

The storage of primmorphs or of single cells is feasible, as determined for *Suberites domuncula*. If the samples had been transferred into seawater and kept at 4°C, storage and hence shipping is possible for

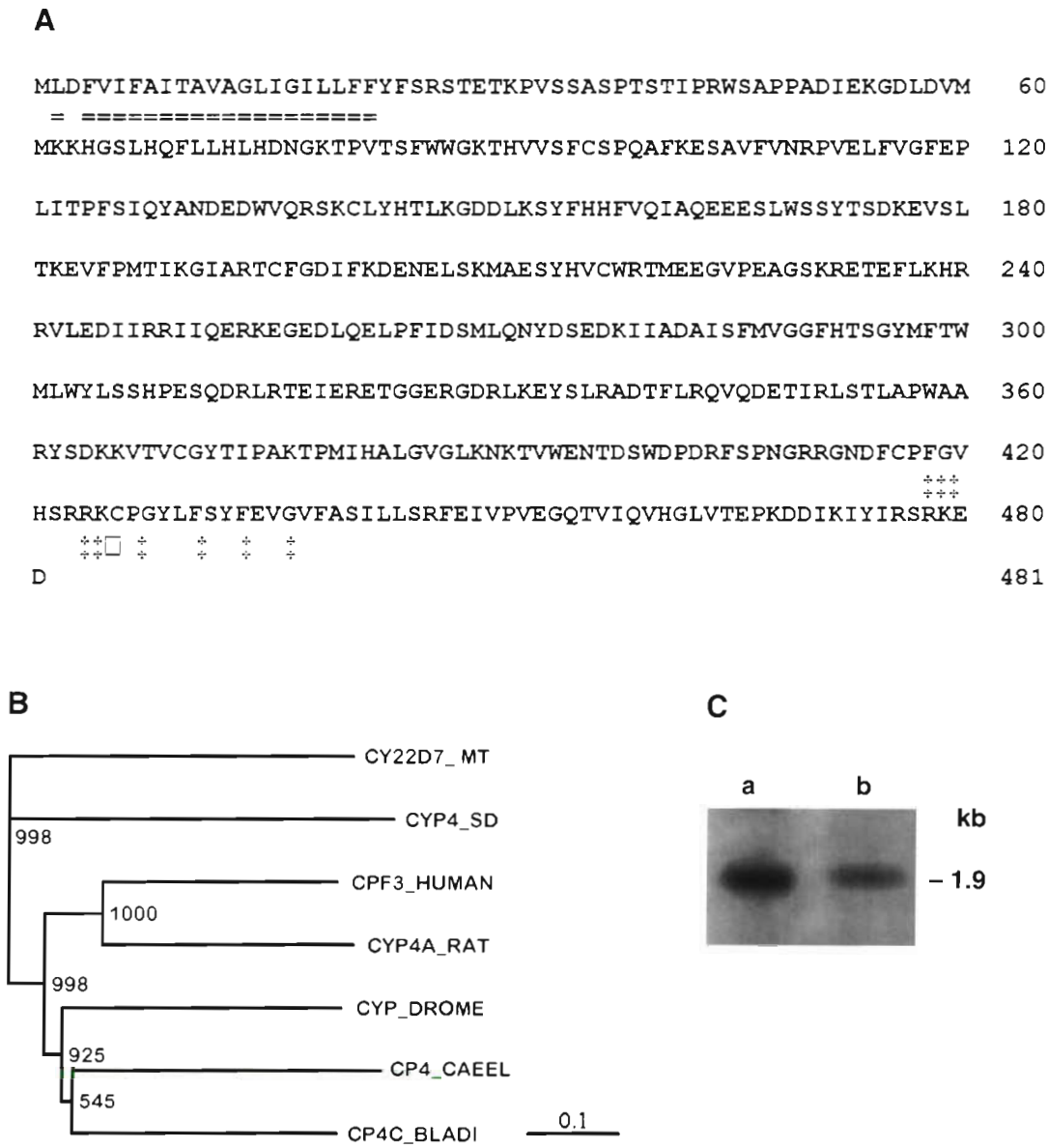


Fig. 6. *Suberites domuncula*. Sponge cytochrome P-450 4. (A) Deduced aa sequence CYP_SD, from the cDNA *SDCYP4*. Doubly underlined residues near the N-terminus indicate a hydrophobic region characteristic for microsomal CYPs. The hem-binding cysteine (position 426, $\underline{\underline{\text{C}}}$) is surrounded by highly conserved aa residues (÷). (B) The phylogenetic tree (rooted) of sponge CYP_SD protein was computed with related sequences from human [HCPF3_HUMAN; accession number AC004523], rat [CYP4A_RAT; M57718], *Drosophila melanogaster* [CYP_DROME; AF017017], the tropical cockroach *Blaberus discoidalis* [CP4C_BLADI; P29981], *Caenorhabditis elegans* [CP4_CAEEL; Z99102] and *Mycobacterium tuberculosis* [CY22D7_MT; Z83866]; the latter sequence was used as outgroup. The phylogenetic tree was constructed on the basis of aa sequence alignment by neighbour-joining, applying the 'Neighbor' program from the PHYLIP package (Saitou & Nei 1987, Felsenstein 1993). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein 1993). The distance matrix was calculated as described in Dayhoff et al. (1978). The numbers at the nodes refer to the level of confidence as determined by bootstrap analysis (1000 bootstrap replicates). Scale bar indicates an evolutionary distance of 0.1 aa substitutions per position in the sequence. (C) Northern blot analysis to determine the size of the transcripts of the mRNA encoding the sponge CYP_SD. RNA from sponge tissue (lane a) and primmorphs (4 wk in culture; lane b) was isolated, size-separated, blot-transferred and hybridized with the antisense DIG (digoxigenin)-labeled *SDCYP4* probe. 5 µg (1 µg) of total RNA from sponge tissue (primmorphs) was analyzed. The experimental conditions have been given previously (Wiens et al. 1998)

longer than 10 d without significant loss of the ability to synthesize DNA (in primmorphs) or to form primmorphs (from single cells).

APPLICATION OF PRIMMORPHS

Bioindicator

The approximately 5000 currently known species belonging to the Porifera (sponges) constitute the most dominant animal phylum found in the marine hard-substrate benthos (Sarà & Vacelet 1973). They are sessile filter-feeders and are exposed to the aqueous environment in a manner not observed in any other metazoan phylum. As an example a sponge specimen of 1 kg may filter ~24 000 l d^{-1} (Vogel 1977). Therefore, it can be predicted, and it has been proven, that sponges have developed efficient strategies to resist unfavourable environmental loads (Müller & Müller 1998).

Recently, the 2 sponges *Suberites domuncula* and *Geodia cydonium* were shown to be suitable bioindicator organisms to monitor dissolved heavy metals, e.g. cadmium (Müller et al. 1998, Wagner et al. 1998) or polychlorinated biphenyls (Wiens et al. 1998). As biomarkers for the detection of environmental stress the heat shock proteins, HSP70 (Koziol et al. 1996, Schröder et al. 1999) and DnaJ (Koziol et al. 1997), or putative SOS proteins, e.g. the AidB-like protein (Krasko et al. 1998), were used.

From mammalian systems it is known that Cd causes an impairment of DNA synthesis (Beyersmann & Hechtenberg 1997). Therefore, we investigated if in the primmorph system Cd causes a reduction of DNA synthesis as well. Four week old primmorphs were exposed to between 100 ng ml^{-1} (corresponding to 163.2 ng ml^{-1} of $CdCl_2$) and 3 µg ml^{-1} of Cd in seawater/antibiotics for 0 to 3 d in 24-well plates. At a concentration of 1 µg ml^{-1} a reduction by 63% of the extent of DNA synthesis is seen after 2 d if compared with the controls (Fig. 7A). In a concentration-dependent study it was found that 0.3 µg ml^{-1} of Cd causes a reduction of DNA synthesis by 74%, a value of inhibition which increases further at higher Cd concentrations during the 2 d incubation period (Fig. 7B).

In the natural marine environment the Cd concentration reaches values between 0.1 and 0.5 µg l^{-1} (Pula area; south of Rovinj; Croatia) (Mikulic 1994). Chemical analyses revealed that in this area specimens of *Suberites domuncula* have a Cd concentration between 0.3 and 7.00 mg kg^{-1} (Müller et al. 1998). This means that this species can accumulate Cd to a value of up to 70 000-fold that in the field. In addition, these data show that sponges in general and the species *S. domuncula* in particular are suitable monitoring organisms for the detection of a chronic exposure to relatively small cadmium concentrations. Experiments are planned to use primmorphs from *S. domuncula* as a bioindicator system for further monitoring studies; as parameter for the degree of stress the extent of DNA synthesis will be chosen.

Production of bioactive compounds

Sponges are known to be rich sources for bioactive compounds (Sarma et al. 1993). It is still under discus-

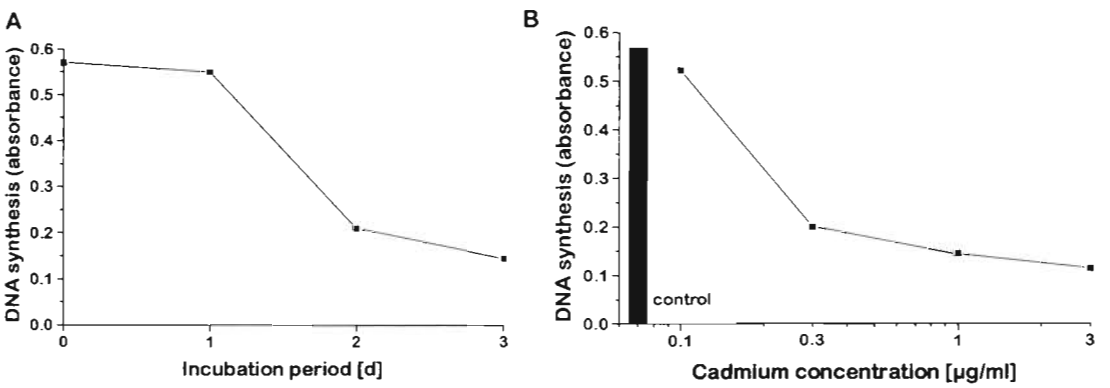


Fig. 7. *Suberites domuncula*. Effect of Cd on the extent of DNA synthesis in primmorphs. Four week old primmorphs were exposed to different concentrations of Cd. (A) Time-dependent reduction of DNA synthesis in primmorphs during an incubation period of 0 to 3 d at 1 µg ml^{-1} of Cd (B); effect of different Cd concentrations on primmorphs. Then the primmorphs were incubated with BrdU for 12 h. The cells were dissociated in CMFSW-E and placed into 96-well plates and dried; 5×10^3 cells were added per well. After fixation the samples were incubated with anti-BrdU mouse monoclonal antibody. The amount of BrdU incorporated into DNA was quantitatively assessed using the 'Cell Proliferation ELISA (BrdU, colorimetric) Kit' from Boehringer Mannheim

sion if the sponge cells, the bacteria or both partners produce these compounds (Proksch 1994). Some of those, e.g. the nucleotides arabinosyluridine (Bergmann & Feeney 1951), arabinosylcytosine (Müller et al. 1972, 1977) and arabinosyladenine (Müller et al. 1975), are now used in medical practice. However, a wider application of the bioactive secondary metabolites from sponge which cannot be chemically synthesized for potential human use is hampered by the fact that the amount of defined starting material is limited. Two solutions are available: First, cultivation of intact sponges taken from the sea in tanks — this is no problem for freshwater sponges (Weissenfels & Langenbruch 1985) but difficult for marine sponges (Osinga et al. 1998) — or in the field (Sendler 1912). Second, *in vitro* cultivation of sponge cells, if possible with their unicellular symbionts/commensals, as described here. Then sponges can be selected as sustainable sources for a wider use in the isolation of bioactive compounds for therapeutical purposes. In the case of toxic proteins these difficulties do not exist. The proteins can be prepared recombinantly from all sponge species, e.g. as already demonstrated for cathepsin from *Geodia cydonium* (Krasko et al. 1997).

Until now 1 bioactive compound has been isolated from *Suberites domuncula* which displays hemolytic activity, the toxic protein suberitine (Cariello & Zanetti 1979). By the test system described (Cariello & Zanetti 1979), the titer of this compound was determined in the crude extract as well as in primmorphs (cultured for 5 wk) from this sponge species. The determination revealed a hemolytic activity in the crude extract from the tissue of 4.5 TU mg^{-1} (TU: titer units) and from primmorphs of 3.5 TU mg^{-1}.

It is not known if suberitine is produced from sponge cells or from bacteria associated with them. However, these data indicate that also primmorphs retain the ability to synthesize at least bioactive proteins. It will be studied in the future whether tissue from *Suberites domuncula* produces low molecular weight bioactive compounds; if so, study can be made to determine if these compounds are produced by the primmorphs as well.

Aquaculture

It has been mentioned above that the difficulties in the isolation and production of secondary metabolites might be overcome by cultivation of sponges collected from the field or raised from single cells via the primmorph stage. These procedures, if they are successful, will allow a controlled addition of nutrients and symbionts/commensals, as well as of growth factors, especially if the cultures are performed in the laboratory. The cloning of a first potential morphogen, an endothelial-monocyte-activating polypeptide, from a sponge has been achieved (Pahler et al. 1998b), and the recombinant protein will be available soon. Hence, especially with the availability of the primmorph technology, it will be possible within the next few years to use sponges which are raised in aquaculture at an industrial scale for pharmaceutical purposes.

DISCUSSION

From invertebrate phyla only cell cultures from insects are well established (Celis 1998). Until now no procedure was published which unequivocally documents sponge cells in culture; a definite growth of the cells or the process of DNA synthesis has not been reported. As outlined in the 'Introduction' the sponge cells were only maintained for a limited period of time. In experiments in our laboratory we also failed to compose a culture medium for single cells in suspension culture.

In a rational approach we could demonstrate that after dissociation the sponge cells lose telomerase activity and as a consequence their intriguing property to be (to a large extent) immortal (Koziol et al. 1998, Wagner et al. 1998). In addition, in a series of approaches we failed to identify supplements for the culture medium with respect to growth factors known to be required for invertebrate and lower vertebrate cell growth (data not given). Based on these facts we postulate that for an efficient culture of dividing sponge cells, the following prerequisites are necessary: (1) viable cells, (2) provision of cell-cell and/or cell-matrix contact and (3) suitable nutrients (perhaps delivered as debris from dead homologous cells). These criteria have been met with the establishment of primmorphs. Knowing from earlier studies that sponges live in a symbiotic and/or commensalic relationship with bacteria (Müller et al. 1981, Althoff et al. 1998) and/or algae (Gilbert & Allen 1973) it was reasonable to develop a procedure by which sponge cells retain the ability to maintain the relationship to bacteria and — if present — also to algae. Therefore, the cells were dissociated and allowed to reaggregate under conditions which facilitate reaggregation of cells with their potential symbionts and allow the extrusion of cell debris and — very likely — also foreign pro- and/or eukaryotes. The cell debris was observed to be deposited at the rim of the free-floating primmorphs, while the foreign pro- and/or eukaryotes were found to attach to the surface of the culture dishes.

The sponge cells, assembled in primmorphs, become telomerase-positive and show DNA synthesis. Hence they regain the prerequisites for cell growth, perhaps

for unlimited proliferation. One cause for this transition from the telomerase-negative to telomerase-positive state must be the fact that the cells have recovered the physiological contact to the neighbouring cells. In addition, they can attach to the homologous extracellular matrix and as a consequence can arrange a functional organization.

The observation that the primmorphs comprise cells which undergo DNA synthesis even if they have been cultivated in seawater/antibiotics without any further supplements was unexpected. This observation can be explained if the assumption that some cells within the primmorphs suffer death and their resulting fragments are taken up by phagocytosis is adopted. It is well established that archaeocytes, choanocytes or spherulous cells are active in the phagocytosis of cell debris (see Simpson 1984), a histological finding which is also supported by enzymatic data (Krasko et al. 1997). Furthermore, it has been shown that in sponges spherulous cells have the ability to secrete the organic content from their vacuoles (Garrone 1978), which supposedly can also be used as nutrients for the surviving cells. The fact that under the conditions described here no nutrients have to be added to the primmorphs in the seawater medium does not exclude future potentially successful attempts to identify suitable growth factors for a sponge-cell culture. Studies in this direction are in progress.

Based on the data presented it is postulated that exogenous and/or endogenous factor(s) cause a transition of telomerase-positive (DNA synthesis-positive) to telomerase-negative (DNA synthesis-negative) cells with the consequence of an induction of apoptosis. Recent data support this notion (Wagner et al. 1998). The first apoptotic gene, MA-3, has been identified in the sponge *Geodia cydonium* (Wagner et al. 1998). Based on the data available it appears likely that the process of apoptosis can be induced by the loss of binding of the integrin receptor to the extracellular matrix, as demonstrated in vertebrate systems (Meredith et al. 1993). From studies with mammalian cells it is known that the phospholipids phosphatidylserine and phosphatidylinositol are exposed on apoptotic cells (Rigotti et al. 1995). These ligands bind to receptors displaying SRCR (Krieger & Herz 1994); therefore, it can be assumed that cells which expose the SRCR receptors and are telomerase-positive (DNA synthesis-positive) phagocytose telomerase-negative (DNA synthesis-negative) cells (Fig. 3B). SRCR receptors have already been identified in sponges (Pancer et al. 1997, Pahler et al. 1998a). Until now, no experimental data are available on factors which might be involved in the expression of the SRCR receptors (Fig. 3B). Hence, it can be suggested that both apoptotic cells and bacteria or other organisms which act as pathogens for sponges are eliminated via binding to scavenger (SRCR) receptors and serve as suitable nutrients for the support of the cell metabolism and as a signal for the initiation of division in cells organized in sponge primmorphs. In addition, it is evident that the sponge cells require cell-cell contact for DNA synthesis and growth. The primmorphs system described here can be considered to be a powerful novel model system to study basic mechanisms of cell proliferation and cell death.

To conclude, the primmorph system described by Custodio et al. (1998) can be used in the future for a variety of applications in the following main directions: (1) as a bioreactor to produce bioactive compounds from sponges and (2) in environmental monitoring, as well as (3) for the detection of potential cytostatic compounds causing a transition from telomerase-positive to telomerase-negative cells. Major tasks for the future are the establishment of continuous cell lines and cell clones from sponges and the further elucidation of the interaction between sponge cells and their symbionts/commensals on a biochemical level. In addition, proliferation factors have to be discovered and feeding of the cultures, the development of defined culture media, needs further investigation.

Acknowledgements. This work was supported by grants from the Bundesministerium für Bildung und Forschung (Project 'Cell Culture of Sponges'; project no. 03F0197A; W.E.G.M.), the International Human Frontier Science Program (RG-333/96-M; W.E.G.M.) and from CNPq and PRONEX of the Brazilian Government (R.B.).

Disclosure statement. A patent has been applied for entitled 'Herstellung von Primmorphe® aus dissoziierten Zellen von Schwämmen, Korallen und weiteren Invertebraten zur Produktion und Detektion von bioaktiven Substanzen, zur Detektion von Umweltgiften und zur Kultivierung dieser Tiere in Aquarien und im Freiland'; (AZ 198 24 384).

LITERATURE CITED

Althoff K, Schütt C, Krasko A, Steffen R, Batel R, Müller WEG (1998) Evidence for a symbiosis between bacteria of the genus Rhodobacter and the marine sponge *Halichondria panicea*: harbor also for putatively-toxic bacteria? Mar Biol 130:529–536

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidmann JG, Struhl K (1995) Current protocols in molecular biology. John Wiley and Sons, New York

Bando Y, Kominami E, Katanuma N (1986) Purification and tissue distribution of rat cathepsin L. J Biochem 100: 35–42

Bar-Nun S, Kreibich G, Adesnik M, Alterman M, Negishi M, Sabatini DD (1980) Synthesis and insertion of cytochrome *P*-450 into endoplasmic reticulum membrane. Proc Natl Acad Sci USA 77:965–969

Bergmann W, Feeney RJ (1951) Contribution to the study of marine sponges. 32. The nucleosides of sponges. J Org Chem 16:981–987

Bergquist PL (1978) Sponges. Hutchinson of London, London

Beyersmann D, Hechtenberg S (1997) Cadmium, gene regu-

lation, and cellular signalling in mammalian cells. Toxicol Appl Pharmacol 144:247–261
Cariello L, Zanetti L (1979) Suberitine, the toxic protein from the marine sponge, *Suberites domuncula*. Comp Biochem Physiol 64C:15–19
Celis JE (1998) Cell biology — a laboratory manual. Academic Press, San Diego
Custodio MR, Imsiecke G, Borojevic R, Rinkevich B, Rogerson A, Müller WEG (1995) Evolution of cell adhesion systems: evidence for Arg-Gly-Asp mediated adhesion in the protozoan *Neoparamoeba aestuarina*. J Protozool Eukaryot Res 42:721–724
Custodio MR, Prokic I, Steffen R, Koziol C, Borojevic R, Brümmer F, Nickel M, Müller WEG (1998) Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death. Mech Ageing Dev 105:45–59
Dayhof MO, Schwartz RM, Orcutt BC (1978) A model of evolutionary change in protein. In: Dayhoff MO (ed) Atlas of protein sequence and structure, Vol 5, Suppl 3. National Biomedical Research Foundation, Washington, DC, p 345–352
De-Flora S, Bagnasco M, Bennicelli C, Camoirano A, Bojnemirski A, Kurelec B (1995) Biotransformation of genotoxic agents in marine sponges. Mechanisms and modulation. Mutagenesis 10:357–364
Felsenstein J (1993) PHYLIP, ver. 3.5. University of Washington, Seattle
Freshney RI (1994) Culture of animal cells, 3rd edn. Wiley-Liss, p 7
Garrone R (1978) Phylogenesis of connective tissue. Karger Verlag, Basel
Gilbert JJ, Allen HL (1973) Chlorophyll and primary productivity of some green, freshwater sponges. Int Rev Ges Hydrobiol 58:633–658
Goksøyr A, Förlin L (1992) The cytochrome *P*-450 system in fish, aquatic toxicology and environmental monitoring. Aquat Toxicol 22:287–312
Gonzalez FJ (1989) The molecular biology of cytochrome *P*-450s. Pharmacol Rev 40:243–288
Gratzner HG (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218:474–475
Hardwick JP, Song BJ, Huberman E, Gonzalez FJ (1987) Isolation, complementary DNA sequence, and regulation of rat hepatic lauric acid ω-hydroxylase (cytochrome *P*-$450_{LA\omega}$). J Biol Chem 262:801–810
Ilan M, Contini H, Carmeli S, Rinkevich B (1996) Progress towards cell cultures from marine sponge that produce bioactive compounds. J Mar Biotechnol 4:145–149
Klautau M, Custodio MR, Borojevic R (1994) *In vitro* culture of primary cell lines from marine sponges. In: van Soest R, Balkema AA (eds) Sponges in time and space. Brookfield, Rotterdam, p 401–406
Koziol C, Wagner-Hülsmann C, Cetkovic H, Gamulin V, Kruse M, Pancer Z, Schäcke H, Müller WEG (1996) Cloning of the heat-inducible biomarker, the cDNA encoding the 70-kDa heat shock protein, from the marine sponge *Geodia cydonium*: response to natural stressors. Mar Ecol Prog Ser 136:153–161
Koziol C, Batel R, Arinc E, Schröder HC, Müller WEG (1997) Expression of the potential biomarker heat shock protein 70 and its regulator, the metazoan DnaJ homolog, by temperature stress in the sponge *Geodia cydonium*. Mar Ecol Prog Ser 154:261–268
Koziol C, Borojevic R, Steffen R, Müller WEG (1998) Sponges (Porifera) model systems to study the shift from immortal- to senescent somatic cells: the telomerase activity in somatic cells. Mech Ageing Dev 100:107–120
Krasko A, Gamulin V, Seack J, Steffen R, Schröder HC, Müller WEG (1997) Cathepsin, a major protease of the marine sponge *Geodia cydonium*: purification of the enzyme and molecular cloning of cDNA. Mol Mar Biol Biotechnol 6:296–307
Krasko A, Schröder HC, Hassanein HMA, Batel R, Müller IM, Müller WEG (1998) Identification and expression of the SOS-response, *aidB*-like, gene in the marine sponge *Geodia cydonium*: implication for the phylogenetic relationships of metazoan Acyl-CoA dehydrogenases and Acyl-CoA oxidases. J Mol Evol 47:343–352
Krieger M, Herz J (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annu Rev Biochem 63:601–637
Kruse M, Müller IM, Müller WEG (1997) Early evolution of metazoan serine/threonine- and tyrosine kinases: identification of selected kinases in marine sponges. Mol Biol Evol 14:1326–1334
Lehnert H, Reitner J (1997) Lebensdauer und Regeneration bei *Ceratoporella nicholsoni* (Hickson, 1911) and *Spirastrella* (Acanthochaetetes) *wellsi* (Hartman & Goreau, 1975). Geol Bl NO-Bayern 47:265–272
Meredith JE, Fazeli B, Schwartz MA (1993) The extracellular matrix as a cell survival factor. Mol Biol Cell 4:953–961
Mikulic N (ed) (1994) Monitoring programme of the Eastern Adriatic Coastal Area (1983–1991). United Nations Environmental Programme, MAP Tech Rep Ser 86
Müller WEG (1982) Cell membranes in sponges. Int Rev Cytol 77:129–181
Müller WEG (1995) Molecular phylogeny of metazoa (animals): monophyletic origin. Naturwissenschaften 82: 321–329
Müller WEG (1998a) Molecular phylogeny of Eumetazoa: experimental evidence for monophyly of animals based on genes in sponges (Porifera). Prog Mol Subcell Biol 19:98–132
Müller WEG (1998b) Origin of Metazoa: sponges as living fossils. Naturwissenschaften 85:11–25
Müller WEG, Müller I (1998) Sponge cells and tissue as biological monitors of aquatic pollution. In: Wells PG, Lee K, Blaise C (eds) Microscale testing in aquatic toxicology — advances, techniques and practice. CRC Lewis Publishers, Boca Raton, FL, p 97–112
Müller WEG, Schäcke H (1996) Characterization of the receptor protein-tyrosine kinase gene from the marine sponge *Geodia cydonium*. Prog Mol Subcell Biol 17:183–208
Müller WEG, Yamazaki Z, Sögtrop HH, Zahn RK (1972) Action of 1-β-D-arabinofuranosylcytosine on mammalian tumor cells. 2. Inhibition of mammalian and oncogenic viral polymerases. Eur J Cancer 8:421–428
Müller WEG, Rohde HJ, Beyer R, Maidhof A, Lachmann M, Taschner H, Zahn RK (1975) Mode of action of 9-β-D-arabinofuranosyladenine on the synthesis of DNA, RNA and protein *in vivo* and *in vitro*. Cancer Res 35:2160–2168
Müller WEG, Zahn RK, Bittlingmeier K, Falke D (1977) Inhibition of herpesvirus DNA-synthesis by 9-β-D-arabinofuranosyladenosine *in vitro* and *in vivo*. Ann N Y Acad Sci 284:34–48
Müller WEG, Zahn RK, Kurelec B, Lucu C, Müller I, Uhlenbruck G (1981) Lectin, a possible basis for symbiosis between bacteria and sponges. J Bacteriol 145:548–558
Müller WEG, Steffen R, Rinkevich B, Matranga V, Kurelec B (1996) The multixenobiotic resistance mechanism in the marine sponge *Suberites domuncula*: its potential applica-

bility for the evaluation of environmental pollution by toxic compounds. Mar Biol 125:165–170

Müller WEG, Batel R, Lacorn M, Steinhart H, Simat T, Lauenroth S, Hassanein H, Schröder HC (1998) Accumulation of cadmium and zinc in the marine sponge *Suberites domuncula* and its potential consequences on single-strand breaks and on expression of heat-shock protein: a natural field study. Mar Ecol Prog Ser 167:127–135

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez F, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ (1991) The *P*-450 superfamily: update on new sequence, gene mapping, and recommended nomenclature. DNA Cell Biol 10:1–4

Osinga R, Tramper J, Wijffels RH (1998) Cultivation of marine sponges for metabolite production: application for biotechnology? Trends Biotechnol 16:130–134

Pahler S, Blumbach B, Müller IM, Müller WEG (1998a) Putative multiadhesive protein from the marine sponge *Geodia cydonium*: cloning of the cDNA encoding a fibronectin-, an SRCR- as well as a complement control protein module. J Exp Zool 282:332–343

Pahler S, Krasko A, Schütze J, Müller IM, Müller WEG (1998b) Isolation and characterization of a cDNA encoding a potential morphogen from the marine sponge, *Geodia cydonium*, that is conserved in higher metazoans. Proc R Soc Lond B 265:421–425

Pancer Z, Kruse M, Schäcke H, Scheffer U, Steffen R, Kovács P, Müller WEG (1996) Polymorphism in the immunoglobulin-like domains of the receptor tyrosine kinase from the sponge *Geodia cydonium*. Cell Adhesion Commun 4:327–339

Pancer Z, Münkner J, Müller I, Müller WEG (1997) A novel member of an ancient superfamily: sponge (*Geodia cydonium*, Porifera) putative protein that features scavenger receptor cysteine-rich repeats. Gene 193:211–218

PC/GENE (1995) Data Banks CD-ROM; Release 14.0. IntelliGenetics, Inc, Mountain View, CA

Platt N, Suzuki H, Kurihara Y, Kodama T, Gordon S (1996) Role of the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. Proc Natl Acad Sci USA 93:12456–12460

Pomponi SA, Willoughby R (1994) Sponge cell culture for production of bioactive metabolites. In: van Soest R, Balkema AA (eds) Sponges in time and space. Brookfield, Rotterdam, p 395–400

Proksch P (1994) Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. Toxicon 32:639–655

Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Reiswig H, Brown M (1977) The central cells of sponges. Zoomorphology 88:81–94

Rigotti A, Acton SL, Krieger M (1995) The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. J Biol Chem 270:16221–16224

Romeis B (1989) Mikroskopische Technik. Urban und Schwarzenberg, Munich

Rottmann M, Schröder HC, Gramzow M, Renneisen K, Kurelec B, Dorn A, Friese U, Müller WEG (1987) Specific phosphorylation of proteins in pore complex-laminae from the sponge *Geodia cydonium* by the homologous aggregation factor and phorbol ester. Role of protein kinase C in the phosphorylation of DNA topoisomerase II. EMBO J 6:3939–3944

Saitou N, Nei M (1987) The neighbor-joining methods: a new method for constructing phylogenetic trees. Mol Biol Evol 4:406–425

Sarà M, Vacelet J (1973) Ecologie des Démosponges. In: Grassé PP (ed) Traité de zoologie: spongiaires, Tome III (1). Masson, Paris, p 462–576

Sarma AS, Daum T, Müller WEG (1993) Secondary metabolites from marine sponges. Akademie gemeinnütziger Wissenschaften zu Erfurt, Ullstein-Mosby Verlag, Berlin

Schröder HC, Hassanein HMA, Lauenroth S, Koziol C, Mohamed TAAA, Lacorn M, Steinhart H, Batel R, Müller WEG (1999) Induction of DNA strand breaks and expression of HSP70 and GRP78 homolog by cadmium in the marine sponge *Suberites domuncula*. Arch Environ Contam Toxicol 36:47–55

Sendler A (1912) Ein Studienaufenthalt auf der Zoologischen Station in Rovigno. F Kaufmann & Co, Frankfurt

Simpson TL (1984) The cell biology of sponges. Springer-Verlag, New York

Vogel S (1977) Current-induced flow through living sponges in nature. Proc Natl Acad Sci USA 74:2069–2071

von Lange T (1998) Telomeres and senescence: ending the debate. Science 279:334–335

Wagner C, Steffen R, Koziol C, Batel R, Lacorn M, Steinhart H, Simat T, Müller WEG (1998) Apoptosis in marine sponges: a biomarker for environmental stress (cadmium and bacteria). Mar Biol 131:411–421

Weissenfels N, Langenbruch PF (1985) Langzeitkulturen von Süßwasserschwämmen (Porifera, Spongillidae) unter Laborbedingungen. Zoomorphology 105:12–15

Wiens M, Koziol C, Hassanein HMA, Batel R, Müller WEG (1998) Expression of the chaperones 14-3-3 and HSP70 induced by PCB 118 (2,3',4,4',5-pentachlorobiphenyl) in the marine sponge *Geodia cydonium*. Mar Ecol Prog Ser 165:247–257

Submitted: July 3, 1998; Accepted: November 3, 1998
Proofs received from author(s): February 2, 1999