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SUPPRESSION OF ALLOGRAFT REJECTION IN THE SPONGE *SUBERITES DOMUNCULA* BY FK506 AND EXPRESSION OF GENES ENCODING FK506-BINDING PROTEINS IN ALLOGRAFTS

WERNER E. G. MÜLLER[1,*], RENATE STEFFEN[1], BERND LORENZ[1], RENATO BATEL[3], MICHAEL KRUSE[2], ANATOLI KRASKO[1], ISABEL M. MÜLLER[1] AND HEINZ C. SCHRÖDER[1]

[1]*Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, D-55099 Mainz, Germany,* [2]*Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany* and [3]*Center for Marine Research, 'Ruder Boskovic' Institute, HR-52210 Rovinj, Croatia*

*e-mail: WMUELLER@mail.UNI-Mainz.DE

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Summary

Porifera (sponges) are, evolutionarily, the oldest metazoan phylum. Recent molecular data suggest that these animals possess molecules similar to and homologous with those of the innate and adaptive immune systems of higher Metazoa. Applying the biological system of parabiosis and the technique of differential display of mRNA, two cDNAs encoding putative FK506-binding proteins were isolated. FK506 is successfully used in clinics as a drug to prevent allograft rejection and is toxic to *Suberites domuncula* cells *in vitro* at doses above 100 ng ml^{-1}. Autograft fusion of transplants from *S. domuncula* was not affected by FK506. Allograft non-fusion was not affected by FK506 at toxic doses; however, at the non-toxic dose of 20 ng ml^{-1}, the allografts fused with each other. It is shown that at the attachment zone in untreated and (particularly drastic) in FK506-treated allografts, expression of the genes encoding the FK506-binding proteins is upregulated. These data indicate that the drug FK506 suppresses allograft rejection in *S. domuncula*, most probably *via* interaction with expression of the gene coding for the FK506-binding proteins.

Key words: allograft, autograft, FK506, FK506-binding protein, sponge, *Suberites domuncula*.

Introduction

It has recently been determined that Porifera (sponges) possess cell–cell and cell–matrix adhesion molecules that group these animals with other metazoan phyla into one taxon (Müller, 1995), which evolved from one common ancestor, the Urmetazoa (Müller and Müller, 2000). These molecules comprise the integrin receptor (Pancer et al., 1997; Wimmer et al., 1999a; Wimmer et al., 1999b), the tyrosine kinase receptor (Schäcke et al., 1994a; Schäcke et al., 1994b), the metabotropic glutamate-like receptor (Perovic et al., 1999) and collagen (Schröder et al., 2000). It is generally accepted that, in Metazoa, in parallel with the evolution of tissue-forming receptors and their corresponding ligands, recognition systems appeared that provide the basis for individuality and immune response (Buss, 1987). Early histocompatibility/incompatibility studies using sponges revealed that grafts between different species (xenografts), e.g. between *Microciona prolifera* and *Haliclona occulata*, reject each other (Moscona, 1968). The immune system in sponges is even more specific; in the classical work of Hildemann et al. (Hildemann et al., 1979), it was demonstrated that allografts from the species *Callyspongia diffusa* reject each other, while autografts fuse compatibly (for reviews, see Pancer et al., 1996; Müller et al., 1999a). In contrast, reports have been published demonstrating that not all sponge allografts reject each other (Curtis et al., 1982; Amano, 1990; Warburton, 1958; Ilan and Loya, 1990).

Sponges are among the longest-studied recognition models in the Metazoa. The earliest studies were performed by Wilson (Wilson, 1907) who described that cells of only the one-species type reaggregate. Until recently, studies on immune-like histoincompatibility reactions were performed solely at the tissue and cellular levels. They conclusively revealed that histoincompatibility reactions between allogeneic donor tissue/cells from *Callyspongia diffusa* resulted in a cytotoxic reaction that occurs over a week for animals, over days for tissue and over hours for cells (Yin and Humphreys, 1996). Elements of the adaptive immunity system comparable with that found in higher vertebrates were identified in grafting experiments performed by Hildemann et al. (Hildemann et al., 1979), who succeeded in showing that after grafting for the second time the sponges react with an elevated rate of rejection in one species over the short term (2 weeks), but failed to do so in another sponge *Hymeniacidon sinapium* (Smith and Hildemann, 1984). The absence of adaptive immunity was

reported by Van de Vyver (Van de Vyver, 1980). It has recently been established, by using molecular biological techniques, that sponges possess, in addition to their innate immunity, elements of an adaptive immunity (for a review, see Müller et al., 1999a).

A closer insight into the phylogenetic relationships between the immune systems of the oldest extant metazoan phylum (the Porifera) and the existing immune mechanisms in Protostomia and Deuterostomia became possible after identification of the potential function of molecules, possibly involved in the immune response, in sponges. Our group has focused on molecules involved in transplantation immunity in sponges, more specifically in the two demosponge species *Suberites domuncula* and *Geodia cydonium*. The data gathered hitherto are reviewed by Müller et al. (Müller et al., 1999a). The results showed that sponges possess key molecules of innate immunity, such as the (2′,5′)oligoadenylate synthetase (Kuusksalu et al., 1995; Kuusksalu et al., 1998; Wiens et al., 1999), which is induced in mammals by the cytokine interferon or by cytokine-like molecules, and also factors similar to those synthesized by cytokine-responsive macrophage molecules, which are presumably involved in the inflammatory response associated with human cardiac transplant rejection (Utans et al., 1995), such as the allograft inflammatory factor 1 (Kruse et al., 1999). In addition, precursors of an adaptive immune system have been identified in sponges, e.g. one molecule that is related to the human pre-B-cell colony-enhancing factor (Müller et al., 1999b) and molecules that contain immunoglobulin-like domains and are highly polymorphic (Pancer et al., 1996; Pancer et al., 1998). These immunoglobulin-like domains have been grouped with the class of variable immunoglobulin-like domains (for a review, see Williams and Barclay, 1988).

Expression of these potential immune molecules could be studied using the methods of parabiosis (Pancer et al., 1996; Müller et al., 1999a) or the insertion technique (Pancer et al., 1996). It was found that almost all autografts fused, whereas the allografts were rejected as a result of a coordinated interaction between proapoptotic molecules (e.g. a polypeptide that comprises two DEATH domains) and potential anti-apoptotic molecules (e.g. Bcl-2 homologous proteins; Wiens et al., 2000a; Wiens et al., 2000b). In addition, the data revealed that the level of transcripts for these factors changes during histocompatibility reactions in both autografts and allografts (for a review, see Müller et al., 1999a).

S. domuncula occurs in nature as red, orange, whitish or blue, or as a mixture of these colors (Arndt, 1935). Hence, graft experiments can, as a first approach, be performed without labelling the transplants. Parabionts attach firmly to each other in the initial phase after transplantation, irrespective of whether autogenic or allogenic tissue is used. After approximately 3 days, autografts fuse, while allografts reject each other and usually remain separate under the conditions used here (Müller et al., 1999a). The initial firm contact between allografts has also been reported from the sponge *Callyspongia diffusa* (Yin and Humphreys, 1996).

The aim of the present work was to identify, in allografts of *S. domuncula*, genes that are expressed in the zone of attachment. The technique of differential display of mRNA by means of the polymerase chain reaction (PCR) was employed. This strategy resulted in the isolation of a fragment and subsequently of the complete cDNAs encoding putative FK506-binding proteins, FKB1_SUBDO and FKB2_SUBDO. Finally, it was demonstrated that the immunosuppressant macrolide lactone FK506, also termed tacrolimus (Kino et al., 1987), which has been successfully applied clinically to prevent graft-*versus*-host diseases (for a review, see Jacobson et al., 1998), also effectively prevents allograft rejection in this sponge.

Materials and methods

Chemicals and enzymes

The sources of chemicals and enzymes used have been given previously (Kruse et al., 1997; Wimmer et al., 1999b). Digoxigenin (DIG) DNA labelling kit, DIG-11-dUTP, anti-DIG AP Fab fragments and CDP-Star (disodium 2-chloro-5-{4-methoxyspiro[1,2-dioxetane-3, 2′-(5′-chloro)-tricyclo(3.3.1.1^{3,7})decan]-4-yl}phenyl phosphate) were purchased from Roche Diagnostics (Mannheim, Germany); natural, Ca^{2+}- and Mg^{2+}-containing sea water was obtained from Sigma (Deisenhofen, Germany); Dexon fibres (diameter 0.5 mm; no. REF 051220/6) were obtained from B. Braun (Spangenberg, Germany); and methyl-[^{3}H]thymidine (dThd; specific activity 703 GBq mmol^{-1}) was obtained from Amersham (Amersham, UK).

FK506 (tacrolimus) was a gift of Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). A stock solution of 2 mg ml^{-1} was prepared in dimethyl sulphoxide (DMSO).

Sponge

Live specimens of *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected by SCUBA near Rovinj (Croatia) from depths between 20 and 35 m. The sponges were kept in Mainz (Germany) in aquaria (10^3 l) at 17 °C under continuous aeration for more than 1 month before use in the experiments (Fig. 1A,B). The specimens used for the allograft experiments were collected from different areas (23 km apart), 5 km west of Porec or 8 km west of Rovinj. Control experiments revealed that none of the allografts fused (N=63).

Auto- and allografting procedure

The ‘parabiosis technique’ for grafting was chosen, as described previously (Pancer et al., 1996; Kruse et al., 1999; Fig. 1C). Freshly cut areas of the tissues were brought into contact. Autografts, tissue samples from the same specimen, or allografts, from different individuals, were tied together with Dexon fibre. Both allografts and autografts were suspended on a string in a beaker (1 l) filled with natural sea water supplemented with 0.01 % of foetal calf serum and different concentrations of FK506 (Fig. 1D). The seawater medium was

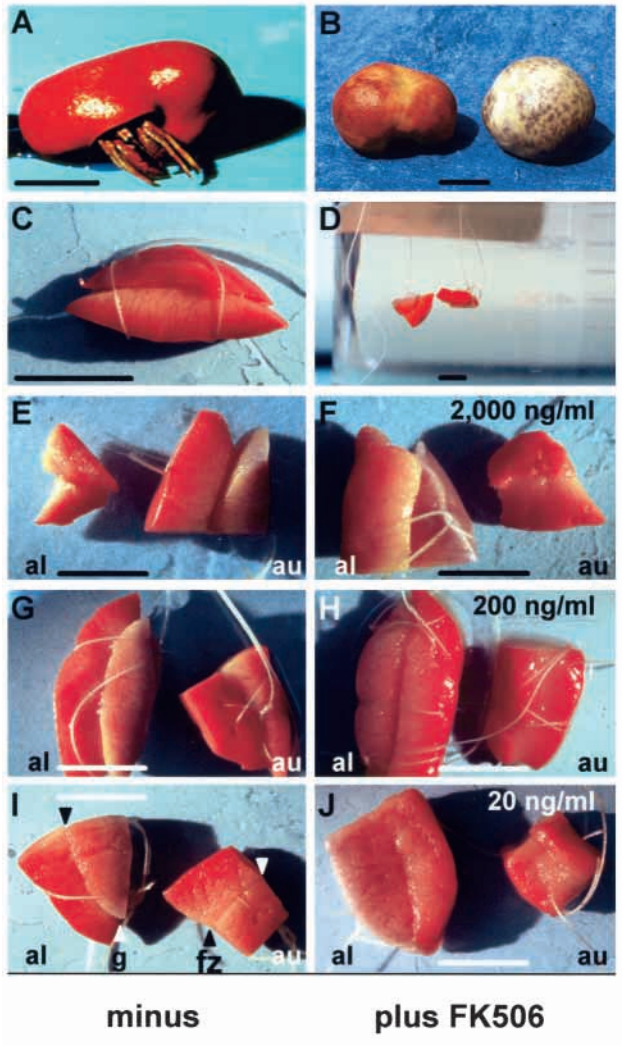

Fig. 1. Effect of FK506 on allograft rejection/non-fusion and autograft fusion of transplants from *Suberites domuncula*. (A) A specimen of *S. domuncula* also showing the hermit crab *Paguristes oculatus*. (B) A red and a blue specimen. (C) Graft tied together with a fibre. (D) Beaker in which two grafts are incubated in sea water, as described in Materials and methods. (E–J) In each panel, two grafts are shown that had been incubated in parallel either without (minus; E,G,I) or in the presence of FK506 (plus FK506; F,H,J); the allograft (al) is shown on the left and the autograft (au) on the right. Three different concentrations of the drug were used: 2000 ng ml^{-1} (F), 200 ng ml^{-1} (H) and 20 ng ml^{-1} (J). In I, the fusion zone (fz) in one autograft and the gap (g) in the allograft are marked (arrowheads). E–J were taken 5 days after grafting. Scale bars, 10 mm.

changed daily. After the indicated times, the grafts were cut at the transplantation area and inspected under an Olympus OSP-MBI binocular microscope.

Tissue samples from the contact zones between allografts were taken 0, 10 or 12 h after grafting for the isolation of RNA to identify the level of expression of the genes coding for FK506 binding proteins. In parallel, tissue samples were taken for histological analysis. For each experiment/treatment, at least seven parallel replicates were performed.

Incorporation studies of single cells

The procedure to dissociate cells from *S. domuncula* has been described previously (Müller et al., 1999b; Müller et al., 1999c). In brief, tissue cubes (4–5 cm^3) were dissociated in Ca^{2+}- and Mg^{2+}-free artificial sea water (CMFSW). The cell suspension obtained was centrifuged (3000 ***g***; 5 min) and washed twice with CMFSW. The cells were then resuspended in natural sea water supplemented with antibiotics (penicillin [100 i.u. ml^{-1}] and streptomycin [100 μg ml^{-1}]) and placed into culture Petri chambers (Falcon; diameter 9 cm).

After 2 days in sea water, the cells were incubated with different concentrations of FK506 for 24 h as follows. Samples (5 ml) of 3×10^6 cells were incubated with 925×10^6 Bq of the labelled DNA precursor [^{3}H]deoxythymidine (dThd) for 24 h. Subsequently, the samples were analyzed for radioactivity in the acid-insoluble (DNA) fraction as described previously (Munro and Fleck, 1966; Müller et al., 1977). The amount of radioactivity incorporated was correlated with the amount of protein isolated from the cells used for the measurement.

The protein content was determined using two established methods (Lane, 1957; Lowry et al., 1951) with bovine serum albumin as standard. The values obtained by the two methods differed only by 8 %.

Differential display

Total RNA was extracted from control tissue (non-graft tissue) and from the zone of attachment of allografts 1 day after parabiosis (attachment of two grafts), using TRIzol Reagent (GibcoBRL), as recommended by the manufacturer. Following procedures described previously (Liang and Pardee, 1992; Lohmann et al., 1995; McClelland et al., 1995), total RNA (5 μg) was reverse-transcribed using T_{11}AC as the 3′ primer (Levi et al., 1997). The resulting cDNA was added to the polymerase chain reaction (PCR) using the arbitrary primer GTGATCGCAGG, the T_{11}AC primer in the assay prepared as described previously (Levi et al., 1997) and α-[^{32}P]dATP. After 30 cycles of the PCR reaction at an annealing temperature of 45 °C, the amplified radioactive fragments were separated on a 5 % polyacrylamide sequencing gel (Pancer et al., 1998) and autoradiographed. One fragment of 185 nucleotides, which was expressed only in the contact zone of allografts and not in control tissue, was selected and used to screen the cDNA library. Three separate experiments were performed, and all gave the same results.

Library screening and isolation of two cDNAs encoding putative FK506-binding proteins

The 185 nucleotide fragment expressed only in the contact zone of allografts was used to isolate two related complete cDNAs, *SUBDOFKB1* and *SUBDOFKB2*, encoding the two sponge FK506-binding proteins FKB1_SUBDO and FKB2_SUBDO. The *S. domuncula* cDNA library (Kruse et al., 1997) was used to screen (under lower-stringency hybridization conditions) plaques lifted from 3×10^5 plaque-forming units on nitrocellulose. Filters were hybridized at 37 °C overnight in 35 % formamide, 5× SSC (1× SSC is

0.15 mol l^{-1} sodium chloride, 0.015 mol l^{-1} sodium citrate), 0.02 % SDS, 0.1 % *N*-laurylsarcosine and 1 % blocking reagent (Boehringer Mannheim), containing 10 ng ml^{-1} of DIG-labelled probe. Filters were washed twice in 2× SSC, 0.1 % SDS (5 min, 22 °C), followed by two additional washes in 0.1× SSC, 0.1 % SDS (15 min, 42 °C). Positive clones were detected with an alkaline-phosphatase-conjugated anti-DIG antibody using BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride) as substrate (Blake et al., 1984).

The clones obtained were sequenced using an automatic DNA sequencer (Li-Cor 4200). The longest insert obtained for the shorter species, *SUBDOFKB1*, was 474 nucleotides long [excluding the poly(A) tail] and that for *SUBDOFKB2*, the longer form, was 683 nucleotides long.

Sequence comparisons

The sequences were analyzed using the sequence similarity algorithm Blast (Altschul et al., 1990). Multiple alignment was performed with CLUSTAL W Ver. 1.6 (Thompson et al., 1994), and graphics were prepared with GeneDoc (Nicholas and Nicholas, 1997). The phylogenetic tree was constructed on the basis of amino acid sequence alignments by neighbour-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).

Northern blotting

Sponge tissue samples from the contact zones (from both auto- and allografts) were pulverized in liquid nitrogen, and RNA was extracted using TRIzol reagent. A sample of 5 µg of total RNA was electrophoresed through a 1 % formaldehyde/agarose gel and blotted onto Hybond N^+ membrane following the manufacturer's instructions (Amersham; Little Chalfont, Buckinghamshire, UK; Wiens et al., 1998). Hybridization was performed at lower stringency (two washes with 2× SSC, 0.1 % SDS and two additional washes with 0.2× SSC, 0.1 % SDS at room temperature 20 °C) with two probes: (i) the complete *SUBDOFKB1* cDNA to identify transcripts encoding the FKB1_SUBDO and FKB2_SUBDO molecules; and (ii) as a control, the complete sequence (1.5 kilobase-pairs, kb) of *S. domuncula* β-tubulin (*SDBTUB*; M. Kruse and W. E. G. Müller, manuscript in preparation) was used as a probe for the northern blot experiments.

The chemiluminescence procedure was used to quantify northern blot signals (Stanley and Kricka, 1990); CDP-Star was used as substrate. The screen was scanned with the GS-525 Molecular Imager (Bio-Rad; Hercules, CA, USA).

Histological analysis

Fresh tissue was fixed in 4 % (w/v) paraformaldehyde in Ca^{2+}- and Mg^{2+}-containing artificial sea water (Pancer et al., 1996). After dehydration, the samples were embedded in Technovit 8100 (Beckstead, 1985), according to the manufacturer's instructions. Sections (9 µm thick) were prepared and stained with 5 % Indian ink [Pelikan, Hannover; no. 17 black (221143)] in 10 % methanol (supplemented with 3 % glacial acid) for 1 h and then washed with water. The tissue was inspected using an Olympus AHBT3 microscope.

Results

Cloning of the cDNAs encoding the FK506-binding proteins from S. domuncula

Employing the differential display technique, a fragment of 185 nucleotides could be identified in an RNA preparation isolated from the attachment zone between allografts 1 day after contact; this 185-nucleotide sequence does not appear in the RNA preparation from control untreated *S. domuncula* tissue; a subsequent study using RNA from tissue taken from autografts (1 day after transplantation) likewise did not display this 185-nucleotide sequence (data not shown).

This 185 base pair (bp) fragment was used to screen the cDNA library. Two forms of cDNA were isolated that differed in sequence and length. The cDNA of the shorter form (474 bp) is termed *SUBDOFKB1* (GenBank accession number AJ278329) and that of the longer one (683 bp) *SUBDOFKB2* (GenBank accession number AJ278328). Northern blot analysis identified an RNA species of 0.62 kb for *SUBDOFKB1* and of 0.79 kb for *SUBDOFKB2* (see below), indicating that the full-length clones had been isolated.

The potential open reading frame (ORF) for the protein deduced from *SUBDOFKB1*, FKB1_SUBDO, starts at nucleotides 29–31, ends at stop codon nucleotides 353–355 and encodes a polypeptide of 108 residues (Fig. 2A). On the basis of the presence of the two FKBP-type peptidyl-prolyl *cis–trans* isomerase signatures, the first site is found at amino acid residues 25–40 (VHYTGTLTNGKKFDSS) and the second at amino acid residues 56–84 (VIRGWDEGVAKMS-VGQRAKLTCSSDYAYG), so the polypeptide can be grouped with the FK506-binding proteins, which are *cis–trans* peptidyl-prolyl isomerases (Harding et al., 1989; Fig. 2A). The calculated M_r of FKB1_SUBDO is 11,645. Using the method of Rao and Argos (PC/GENE Data Banks CD Rom, 1995) no transmembrane helix was detected.

The polypeptide deduced from *SUBDOFKB2*, FKB2_SUBDO, is 209 residues long (the ORF extends from amino acid residue 27 to 653 and corresponds to an M_r of 23,163). As for FKB1_SUBDO, the translation initiation site is of average efficiency (Kozak, 1991). In FKB2_SUBDO, the two FKBP-type signatures are found at amino acid residues 54–69 and 84–112 (Fig. 2A). In contrast to FKB1_SUBDO, the polypeptide FKB2_SUBDO shows one transmembrane helix from amino acid residue 8 to 25 (PC/GENE Data Banks CD Rom, 1995).

The sequences were analyzed using the computer program Blast. The sponge sequences share more than 80 % identity (and more than 90 % similarity plus identity) with the related sequences from animals and more than 70 % identity and and more than 80 % similarity with yeast and plant sequences.

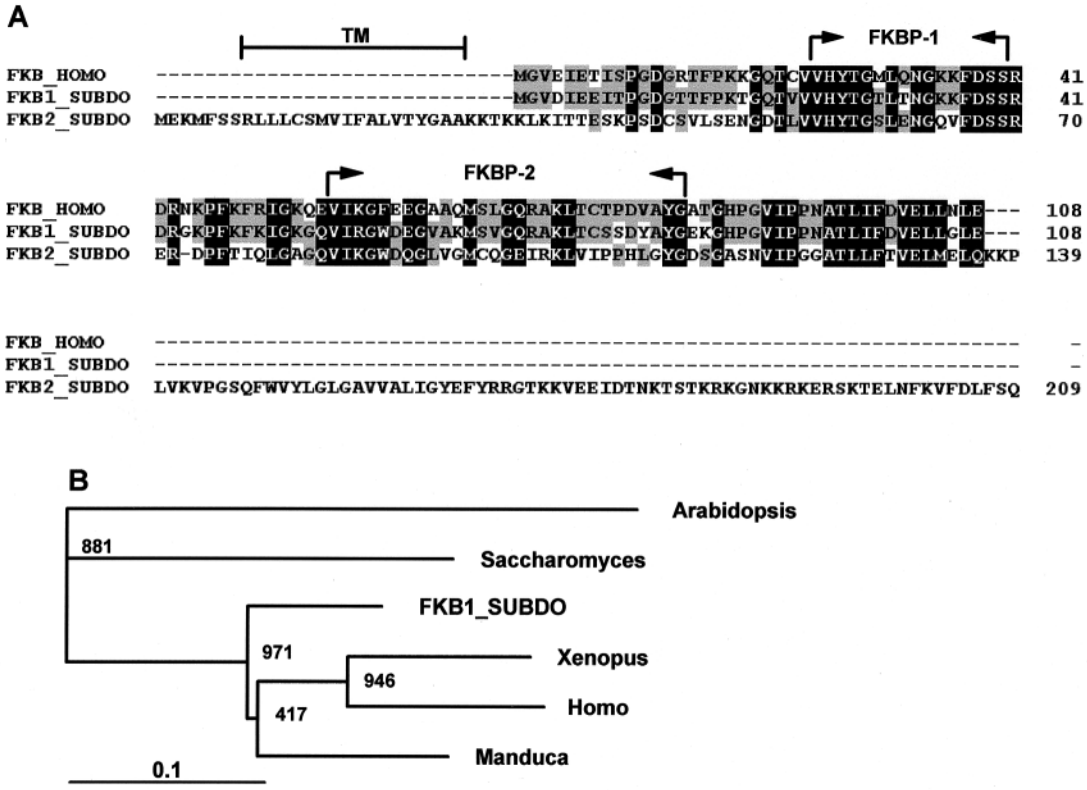

Fig. 2. Putative FK506-binding proteins FKB1_SUBDO and FKB2_SUBDO. (A) Alignment of the deduced sponge sequences, (FKB1_SUBDO and FKB2_SUBDO) with the human FK506-binding protein (FKB_HOMO; GenBank accession number NP_004107). Residues conserved (identical or similar with respect to their physico-chemical properties) in all sequences are shown in white on black and those in at least two sequences in black on gray. The locations of the two FKBP-type peptidyl-prolyl *cis–trans* isomerase signatures 1 and 2 (FKBP-1 and FKBP-2) are marked. In addition, the transmembrane helix (TM) is indicated in FKB2_SUBDO. (B) Rooted phylogenetic tree built by two listed sequences (FKB1_SUBDO and FKB_HOMO; Homo) as well as by the related sequences from *Manduca sexta* (Manduca; AAF16717), *Xenopus laevis* (Xenopus; O42123), *Saccharomyces cerevisiae* (Saccharomyces; NP_014264) and *Arabidopsis thaliana* (Arabidopsis; S72485). The last sequence was used as an outgroup. Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

Similarity to the sequences for monocellular eukaryotes (23 % identity and 30 % similarity; *Trypanosoma cruzi*, accession number Q09734) and to bacteria (23 % identity and 30 % similarity; *Porphyromonas gingivalis* AAD33931) is low. A phylogenetic tree, rooted with the plant FK506-related protein from *Arabidopsis thaliana*, revealed that this sequence, together with the yeast sequence, forms the basis of the tree, while the metazoan FK506-binding proteins form a separate branch with the sponge molecule as the common ancestor (Fig. 2B).

These data show that *S. domuncula* contains two genes encoding FK506 polypeptides. In view of previous data which demonstrated that the drug FK506 blocks a common intermediate step required for the T-cell response to antigen presentation (Schreiber and Crabtree, 1992) and the findings that proliferation of yeast cells can be modulated by FK506 (Cardenas et al., 1994), we attempted to determine whether FK506 exerts (i) a toxic effect on sponge cells and/or (ii) an immunosuppressive effect on *S. domuncula* grafts.

Toxicity studies with FK506

Incorporation studies with [^{3}H]dThd were performed to measure the effects of FK506 on DNA synthesis. The results revealed that cells incubated for 24 h in the absence of the drug incorporated 742±78 cts min^{-1} mg^{-1} protein. When FK506 was added at a concentration of 10 ng ml^{-1}, the amount incorporated did not change significantly (P≈0.1). However, when concentrations of drug above 100 ng ml^{-1} were used, the amount incorporated dropped to 315±45 cts min^{-1} mg^{-1} protein (Fig. 3) (P<0.001). The concentration used for the transplantation studies, 20 ng ml^{-1}, was not inhibitory for incorporation of [^{3}H]dThd into DNA fraction (788±85 c.p.m. mg^{-1} protein). At 200 ng ml^{-1}, the FK506 inhibition of incorporation is pronounced (373±65 c.p.m. mg^{-1} protein) (P<0.001). From these results, we conclude that concentrations below 20 ng ml^{-1} of FK506 are not toxic for *S. domuncula*.

Effect of FK506 on allograft non-fusion and autograft fusion

FK506 has a pronounced effect on allograft non-fusion. First, both auto- and allografts were incubated in sea water in the absence of the drug. In all experiments (N=5), the autografts fused after an incubation period of 5 days

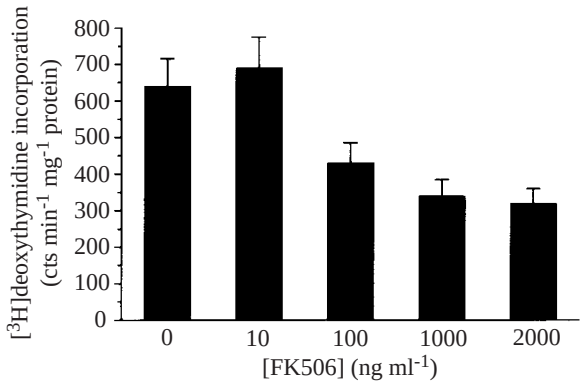

Fig. 3. Effects of FK506 on the incorporation of [^{3}H]deoxythymidine into the acid-insoluble fraction from cells of *Suberites domuncula*. Single cells were incubated in sea water for 2 days and subsequently exposed to different concentrations of FK506 for 24 h, as described in Materials and methods. The acid-insoluble fraction was examined and the incorporation of ^{3}H correlated with the protein content of the sample. Values are means ± S.D. of eight experiments.

(Fig. 1E,G,I). Allografts without FK506 always rejected each other (Fig. 1E,G,I). In the presence of toxic concentrations of FK506 (2000 or 200 ng ml^{-1}), allografts did not fuse and showed a distinct gap (Fig. 1F,H). However, if FK506 was administered at only 20 ng ml^{-1}, a non-toxic concentration, the allografts fused perfectly (Fig. 1J), showing no difference from the fusion of autografts.

To investigate whether this surprising effect, the fusion of allografts in the presence of low concentrations of FK506, is only a transient process and whether the allografts reject each other again later, the transplant incubation periods were extended for up to 10 days and, even after this longer incubation time, no non-fusion could be detected (results not shown).

Microscopic analysis

The attachment sites of autografts and of allografts were inspected microscopically at days 1 and 5 after transplantation. At day 1, the grafts attach to each other and close contact develops in the central part of the grafts. This contact is also caused by the slight pressure on the tissue by the fibres used to fix the parabionts (Fig. 4A). A cleft was seen between the grafts at the rim of the parabionts, irrespective of whether the tissues were from the same or from different individuals (Fig. 4A,C,E,G). In Fig. 4A,C, the transition from the outer 'cleft-zone' to the inner 'attachment zone' is shown.

At day 5, a clear distinction between the reaction of the auto- and allografts becomes obvious. While the autografts fused, both in the presence and in the absence of FK506 (Fig. 4B,F), a narrow gap remained between the allografts not treated with FK506 (Fig. 4D), and the tissue samples detached after removal of the Dexon fibres. However, the allografts that had been treated with 20 ng ml^{-1} of FK506 fused and showed a continuous distribution of cells in the tissue (Fig. 4H).

Expression of the FK506-binding proteins

From an earlier study with monkey cells, it is known that expression of the FK506-binding protein can be modulated by glucocorticoids, e.g. dexamethasone (Reynolds et al., 1998), which are known to act also as potent suppressors of the cellular immune response in allografts/xenografts (Hudde et al., 1999; Nielsen, 2000). Therefore, the level of expression was determined in tissue from the attachment zone 12 h after grafting. The sizes of the two transcripts, *SUBDOFKB1* and *SUBDOFKB2*, were determined by northern blotting (at low stringency); the blot of *SUBDOFKB1* showed a band at 0.62 kb, and the blot of *SUBDOFKB2* showed a band at 0.79 kb (Fig. 5A). The level of expression of the shorter form of the FK506-binding protein (*SUBDOFKB1*) was approximately

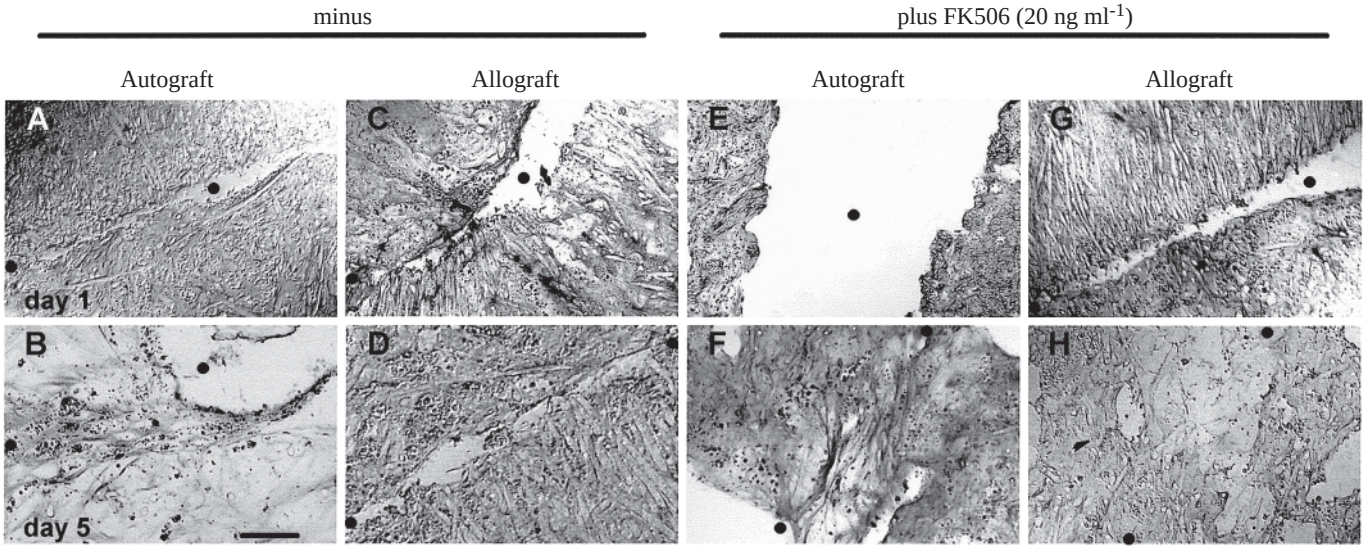

Fig. 4. Microscopic analysis of auto- and allografts in the absence (minus; A–D) or presence of FK506 (20 ng ml^{-1}; E–H). Grafts 1 day (A,C,E,G) or 5 days (B,D,F,H) after the transplantation are shown. The respective auto- (A,B,E,F) and allografts (C,D,G,H) are also indicated on the top of the figures. The zones between the grafts are marked with filled circles. Scale bar, 1 mm.

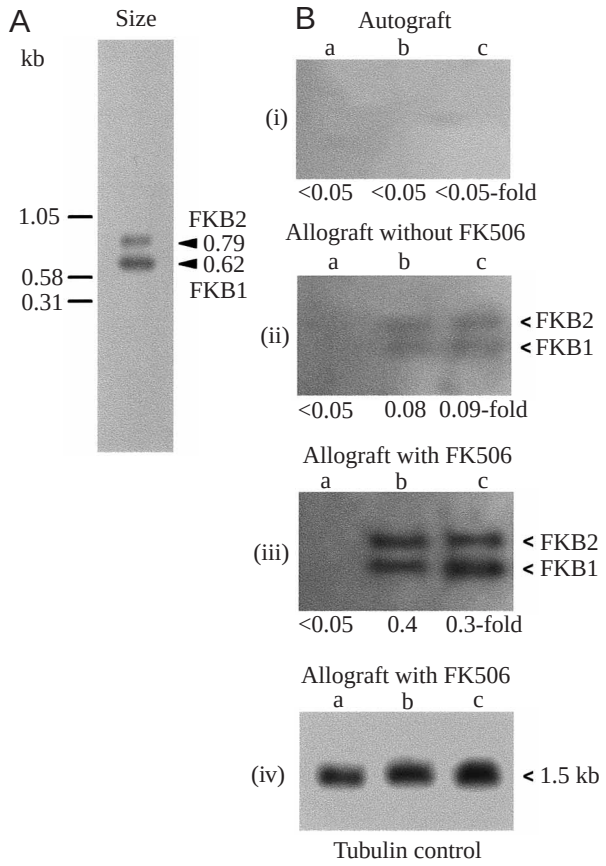

Fig. 5. Transcripts for the two FK506-binding proteins FKB1_SUBDO and FKB2_SUBDO from *Suberites domuncula*, identified using northern blotting. (A) Determination of the size of the two transcripts, *SUBDOFKB1* (FKB1; 0.62 kb) and *SUBDOFKB2* (FKB2; 0.79 kb); the positions of size markers (kb) are given on the left. (B) RNA from transplants was obtained as described in Materials and methods. (i) Autografts (not treated with FK506), (ii) allografts (untreated) and (iii) allografts (treated with 20 ng ml^{-1} of FK506) were taken, RNA was extracted, and northern blots were performed. RNA was extracted from the graft zones, 0 (lane a), 10 (lane b) or 12 h (lane c) after the start of the transplantation experiments. The intensities of the bands corresponding to *SUBDOFKB1* (FKB1) and *SUBDOFKB2* (FKB2) were standardized against those seen with β-tubulin, as described in Materials and methods. (iv) In parallel, RNA from allografts (treated with 20 ng ml^{-1} FK506) were size-separated and analyzed with the *S. domuncula* β-tubulin probe *SDBTUB*.

five times higher (Fig. 5Biii; comparison between the signal intensities of FKB1 and FKB2) than that observed for the longer form (*SUBDOFKB2*), which we attribute to the fact that *SUBDOFKB1* cDNA (shorter form) was used as the probe for the hybridization experiments.

The levels of expression of the two genes, which encode proteins thought to bind FK506, were then determined in the attachment zones of the grafts. Tissue samples were taken 12 h after grafting, the time at which the transient attachment of the transplants was observed (see above). As shown in Fig. 5B, no expression of *SUBDOFKB1* and *SUBDOFKB2* could be seen in autografts (Fig. 5Bi). The intensity of the bands obtained after hybridization with *SUBDOFKB1* and with the β-tubulin probe were quantified. The values for *SUBDOFKB1* and *SUBDOFKB2* were correlated with the intensities seen with the β-tubulin probe to normalize for the amount of RNA applied to the gels. Using this semi-quantitative determination, it became evident that in untreated allografts the levels of the two transcripts increase and reach a value of 0.08 with respect to the steady-state level of β-tubulin (10 h after the start of the grafting experiment) and 0.09 (after 12 h; Fig. 5Bii). In contrast, the RNA samples isolated from allografts treated with 20 ng ml^{-1} FK506 showed dramatic upregulation of both *SUBDOFKB1* and *SUBDOFKB2* gene expression; levels of 0.4 (10 h after the start of the grafting experiment) and 0.3 (after 12 h) were measured (Fig. 5Biii). In parallel, RNA was probed in northern blot experiments with the tubulin probe from *S. domuncula*, *SDBTUB*. As documented in Fig. 5Biv, using RNA from experiments with FK506-treated tissue, the signal observed was almost identical in the samples, irrespective of the duration of treatment with FK506.

These data indicate that in allografts FK506 induces expression of the two sponge FK506-binding proteins.

Discussion

In the last 3 years, it has become apparent that sponges express molecules that display major sequence similarity to deuterostomian/vertebrate functional molecules that are likely to be involved in immune reaction (see Introduction). In functional studies with sponge auto- and allografts, it could be documented that some of those molecules, isolated from the demosponges *S. domuncula* and *G. cydonium*, function in a manner comparable with mammalian immune system molecules and, hence, are (very likely) homologous, e.g. the cytokine-like molecules allograft inflammatory factor 1 or glutathione peroxidase produced during the graft response (Kruse et al., 1999).

In the present study, the technique of differential display was chosen to identify molecules that are newly expressed in the attachment zone of allografts. These molecules were identified as FK506-binding proteins on the basis of their high sequence similarity to related molecules from the Metazoa, yeast and plants. These molecules function in those organisms as peptidyl-prolyl *cis–trans* isomerase, an enzyme that is assumed to be involved in the folding of proteins (Liu et al., 1990). Furthermore, the peptidyl-prolyl *cis–trans* isomerase(s) apparently carry out a series of other biological functions that can be predicted also to be crucial for sponge metabolism, channel gating, association with cellular receptors, association with receptors involved in immune response, gene transcription or cell cycle control (as summarized by Galat and Rivière, 1998). In addition, in vertebrates, it has been shown that FK506 binds to this isomerase and it was therefore termed FK506-binding protein or immunophilin (Mouzaki and Rungger, 1994; Shaw et al., 1995). The complex formed (FK506/FK506-binding protein) inhibits the phosphorylase

activity of calcineurin (Liu et al., 1991), thus preventing translocation of the nuclear factor of activated T-cells (NFAT) into the nucleus. In this way, FK506 inhibits interleukin-2 gene expression and prevents the transformation of precursor helper T lymphocytes into antigen-conditioned helper T lymphocytes (Mouzaki and Rungger, 1994; Shaw et al., 1995; for a review, see Jacobson et al., 1998).

Two genes encoding the FK506-binding proteins have been isolated from *S. domuncula* and extend our understanding of the role of FK506. The polypeptides deduced from the two genes comprise two characteristic peptidyl-prolyl *cis–trans* isomerase signatures. The sequences display an overall high sequence similarity to mammalian, yeast and plant polypeptides (>70 % identity and >80 % similarity). In *S. domuncula*, two forms of FK506-binding protein are present: one that has no transmembrane region and is apparently a cytosolic protein, and a second that contains a transmembrane segment. The existence of more than one form of FK506-binding proteins has also been reported for other metazoan organisms, such as human (Jin et al., 1991). Future studies must clarify whether the two sponge genes have different roles; at present, however, on the basis of the same expression pattern in the graft attachment zone, we have no evidence of different functions, at least with respect to the immune response.

It is now accepted that all metazoan phyla, including the Porifera, evolved from one common ancestor (Müller, 1995); this view is based on the fact that sponges comprise homologous cell–cell/matrix adhesion systems, e.g. the integrin-mediated adhesion receptor (Wimmer et al., 1999a; Wimmer et al., 1999b), and possess molecules likely to be involved in immune reaction, e.g. the cytokine-related molecules and the polymorphic immunoglobulin domains (for a review, see Müller et al., 1999a), which are very similar to those of higher metazoan animals. We wished to determine whether immune reactions in sponges can be modulated by drugs that have successfully been applied in higher metazoan phyla, and especially in humans. We therefore investigated whether allograft rejection in sponges is suppressed, as in mammalian systems, by the drug FK506. This macrolide lactone compound was selected since it was the most promising candidate for potential binding to the cloned FK506-binding proteins and because it might initiate immunosuppression.

The results presented show that, at concentrations above 100 ng ml^{-1}, FK506 causes a significant inhibition (P<0.001; Fig. 3) of DNA synthesis *in vitro* in *S. domuncula* cells. At higher doses, toxic effects have been reported in clinical transplantation studies (Pizzolato et al., 1998). This toxic effect is attributable not to inhibition of the isomerase but to adverse effects on the calcineurin-mediated signal transduction system, resulting in reduced cell survival and T-cell activation (for a review, see Cardenas et al., 1999).

The effect of FK506 on the process of fusion of allografts in *S. domuncula* has been studied within the concentration range used for the *in vitro* experiments. The results show that, at concentrations of FK506 in the surrounding medium above 200 ng ml^{-1}, all allografts were rejected. At the high concentration of 2000 ng ml^{-1}, toxic effects on the grafts were also detected; a decrease in DNA content of the tissue was measured, which can be taken to indicate a loss of cells (data not shown). However, if the non-toxic concentration of 20 ng ml^{-1} was applied, fusion of the allografts occurred, and persisted for at least 10 days. Within such a long transplantation period, untreated allografts are either split into two separate graft units (for a review, see Müller et al., 1999a), or one graft undergoes apoptotic death as a result of downregulation of the expression of anti-apoptotic genes such as *Bcl-2* (Wiens et al., 2000a; Wiens et al., 2000b). It has recently been shown in the *Geodia cydonium* allograft system that the grafts undergo apoptosis as a result of upregulation of the genes coding for DEATH domain-containing protein and for an enzyme involved in inflammation, the LTB_4 12-hydroxy-dehydrogenase (Wiens et al., 2000b).

The concentration of FK506 used clinically to suppress allograft rejection ranges from 300 to 20 ng kg^{-1} (for a review, see Laskow et al., 1998). In the studies presented here, it was found that the rejection/non-fusion process could be abolished by addition of 20 ng ml^{-1} FK506 to the medium surrounding the grafts. In humans, the drug is unevenly distributed among organs (for a review, see Jacobson et al., 1998), so that a higher overall drug concentration is required. In contrast in sponges, which are not compartmented into organs (Simpson, 1984), FK506 is probably distributed homogeneously.

In addition, we found that, in the attachment zone of FK506-untreated and FK506-treated allografts, the genes encoding the two binding proteins for FK506 are upregulated. In the absence of the drug, this expression is low, but the steady-state levels of the transcripts for the FK506-binding proteins 1 and 2 in the allograft zones are elevated and reach levels of 30 % of the levels of β-tubulin transcripts. At present, we explain this observation by assuming that, in the presence of the drug, the FK506-binding proteins become upregulated, perhaps to support cell survival and/or to promote immune activation of the cells. It is conceivable that overexpression of the FK506-binding proteins causes a reduction/abolition of the intracellular inhibitory response to FK506 and perhaps even an augmentation of calcineurin-mediated cell activation. However, calcineurin in mammalian cells, or in yeast, fulfils several roles that are under intense investigation (for a review, see Cardenas et al., 1999). In the yeast system, it has been elegantly demonstrated that inhibition of the FK506-mediated calcineurin signal-transduction system results in reduced cell survival during cation stress (for a review, see Cardenas et al., 1998).

In earlier studies using the sponge *Callyspongia diffusa*, it was reported that, when tissue pieces from different specimens are attached to each other, they form a contact zone in which cytotoxic reactions proceed (Yin and Humphreys, 1996). The dominant cell types involved in this cytotoxic recognition process are the gray cells. These authors propose the following series of events that result finally in the histoincompatibility reactions: recognition of transplants; generation of signals that suppress cell aggregation; accumulation of gray cells at the

attachment zone; initiation of cytotoxic processes. Our results support this observation, indicating that, shortly after transplantation, an accumulation of cells occurs at the attachment zone between allografts. We have not yet tried to identify this cell type unequivocally since the characterization of sponge cells is difficult. However, on the basis of the intense fluorescence of the cells within the attachment zone, a characteristic feature of gray cells (Kuhns et al., 1980), it appears likely that they are indeed gray cells (data not shown).

There are intriguing recent findings indicating that, in contrast to the reaction in the attachment zone of autografts of the sponge *S. domuncula*, the expression of genes for two cytokines is upregulated in allografts. Expression of the allograft inflammatory factor 1, which displays high sequence similarity to the corresponding factor from vertebrates, increases strongly during the first days after transplantation in allografts; in autografts, no change in expression was observed (Kruse et al., 1999). The expression of a second cytokine, the endothelial-monocyte-activating polypeptide (Pahler et al., 1998), was also found to be upregulated in the attachment zone only of allografts of *S. domuncula* (M. Kruse and W. E. G. Müller, in preparation). In mammalian systems, these two factors are highly expressed in activated rat macrophages and monocytes (Schlüsener et al., 1999), an effect that was blocked by the glucocorticoid dexamethasone. Dexamethasone also suppresses allograft rejection in humans (Hudde et al., 1999). Studies are in progress to determine whether the expression pattern of these two cytokines can be modulated (a downregulation is expected) by FK506.

Taken together, these data show that sponges possess an immune system that shares high structural and functional similarities with those of higher metazoan phyla. Sponges contain molecules likely to be involved in immune reactions that share sequence similarity with those found in deuterostomian Metazoa, e.g. apoptotic molecules (DEATH domain-containing molecules; Wiens et al., 2000a; Wiens et al., 2000b), proteins involved in innate immunity, (2′,5′)oligoadenylate synthetase (Wiens et al., 1999), or cytokines (pre-B-cell related colony-enhancing factor; Müller et al., 1999b). Here, we have shown that the drug FK506 used in human therapy for the prevention of allograft rejection functions in sponges in a similar or the same manner. This surprising finding supports a recent study showing that histoincompatibility in the sponge *Microciona prolifera* is suppressed by the immunosuppressant cyclosporin A (Humphreys, 1999). These new findings support earlier proposals that factors involved in human diseases, e.g. myotrophin in cardiovascular disease (Schröder et al., 2000), are highly conserved and occur in the phylogenetically oldest animal phylum. Our data now add the fact that a drug, FK506, successfully prevents a disease-related process in sponges. On the basis of these data, we propose that sponges, which branched off first from the common ancestor of all Metazoa (Müller, 1995), possess key pathways for disease control seen in mammals and current species, and respond to drugs successfully used to the treat disorders in higher mammals.

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