Cloning and expression of the putative aggregation factor from the marine sponge *Geodia cydonium*

Joachim Schütze, Anatoli Krasko, Bärbel Diehl-Seifert and Werner E. G. Müller*

Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, D-55099 Mainz, Germany

*Author for correspondence (e-mail: wmueller@mail.uni-mainz.de)

Accepted 23 May 2001

Journal of Cell Science 114, 3189-3198 (2001) © The Company of Biologists Ltd

SUMMARY

Sponges (phylum Porifera) have extensively been used as a model system to study cell-cell interaction on molecular level. Recently, we identified and cloned the putative aggregation receptor (AR) of the sponge *Geodia cydonium*, which interacts in a heterophilic way with the aggregation factor (AF) complex. In the present study, antibodies against this complex have been raised that abolish the adhesion function of the enriched sponge AF, the AF-Fraction 6B. Using this antibody as a tool, a complete 1.7 kb long cDNA, *GEOCYAF*, could be isolated from a cDNA library that encodes the putative AF. Its deduced aa sequence in the N-terminal section comprises high similarity to amphiphysin/BIN1 sequences found in Protostomia and Deuterostomia. However, the C-terminal portion of the sponge sequence lacks the SH3 domain characteristic for amphiphysin/BIN1. The polypeptide with a calculated size of 47 kDa was expressed in *Escherichia coli*. The recombinant, soluble 36 kDa putative AF was prepared and found to compete with the AF complex-associated adhesion protein of the AF-Fraction 6B for the binding sites at the cell surface. Furthermore, the recombinant putative AF was recognized by the antibody used to screen the cDNA library by western blotting. In addition, there is evidence that the recombinant putative AF binds to the *G. cydonium* galectin. It is concluded that the putative *G. cydonium* AF - a further autapomorphic molecule characteristic for Metazoa - binds to the AR present on the cell surface in association with the homologous galectin.

Key words: *Geodia cydonium*, Metazoa, Sponges, Aggregation factor, Aggregation receptor, Adhesion, Evolution

INTRODUCTION

Since 1907, sponges (phylum Porifera) have been traditionally used as a model to study both cell-cell and cell-matrix adhesion (reviewed by Burger and Jumblatt, 1977; Müller, 1982; Wilson, 1907). Two marine demosponges, *Microciona prolifera* and *Geodia cydonium*, have been the most thoroughly studied species. In 1973, two groups isolated and purified both from *M. prolifera* (Henkart et al., 1973) and *G. cydonium* (Müller and Zahn, 1973) the first extracellular particle, termed aggregation factor (AF), which promotes the species-specific aggregation of sponge cells. The AFs were characterized as high molecular weight complexes (M_r 2×10^7 (*M. prolifera*) or sedimentation coefficient s=20, where the rotor velocity ω=3000 (*G. cydonium*)) (Cauldwell et al., 1973; Zahn et al., 1976) that are assembled from a series of proteins that are bound to the core structure both covalently and noncovalently (Müller et al., 1979; Misevic et al., 1982; Wagner-Hülsmann et al., 1996). The AF that mediates cell-cell aggregation depends on the presence of Ca^{2+} (Burger and Jumblatt, 1977; Müller, 1982) and binds to the cell surface-bound aggregation receptor (AR) (Weinbaum and Burger, 1973; Müller et al., 1976; Varner et al., 1988). Two reports have been published suggesting that the AF complex is assembled intracellularly and subsequently released from archaeocytes via excocytosis (Müller et al., 1982) or perhaps by a sulfate-mediated transport (Kuhns et al., 1995).

The major obstacle to the identification of the molecules in the complex and dynamic cell-cell and cell-matrix recognition in sponges was the fact that the underlying molecules involved had not been obtained by molecular cloning. Even until 1994, it remained uncertain if the sponge adhesion molecules display high sequence relationship to functionally related molecules present in higher Metazoa (Gamulin et al., 1994). With the isolation of a galectin (Pfeifer et al., 1993) as the first cell-cell adhesion molecule, and with integrin as the first cell-matrix adhesion receptor in *G. cydonium* (Pancer et al., 1997; Wimmer et al., 1999), it became obvious that sponges contain highly related molecules known to promote adhesion in Protostomia and Deuterostomia. This finding has been taken as one major clue to the now established view that Metazoa, including Porifera, are of monophyletic origin (Müller, 1995).

Earlier, the galectin of *G. cydonium* was cloned; sequence analysis revealed that those aa residues that are involved in galectins from mammalian species in binding to galactose are strikingly conserved in the sponge sequence (Pfeifer et al., 1993). The sponge galectin is one polypeptide that is associated with the adhesion system in sponges. The galectin links the AF complex to the membrane-associated AR (Wagner-Hülsmann et al., 1996). The sponge galectin occurs in at least three different sequence isoforms (Pfeifer et al., 1993; Wagner-Hülsmann et al., 1996; Müller, 2001; accession numbers X93925, AJ400908 and

AJ400909), all of them present in a soluble and a membrane-associated form (Wagner-Hülsmann et al., 1996; Müller et al., 1997).

The putative AR was recently cloned from *G. cydonium* and found to comprise 14 scavenger receptor cysteine-rich (SRCR) domains, six short consensus repeats (SCR), a C-terminal transmembrane domain and a cytoplasmic tail (Blumbach et al., 1998). Competition experiments using recombinant AR or antibodies raised against this receptor suggested that the adhesion molecule present in the enriched AF ('AF-Fraction 6B') binds to the AR. In addition, previous experiments indicated that the strength of binding of the AF-Fraction 6B to the cell surface AR is augmented by galectin (Wagner-Hülsmann et al., 1996). In the AF-complex from *M. prolifera*, Burger's group identified a proteoglycan-like core structure, a protein that had been termed MAFp3; it has subsequently been cloned (Fernandez-Busquets et al., 1996; Fernandez-Busquets and Burger, 1997). MAFp3 is likely to be entrapped into a polysaccharide cover (Fernandez-Busquets et al., 1996). Sequence analysis revealed that the gene encoding MAFp3 is highly polymorphic and might be involved in the cell adhesion system during sponge allogeneic reactions (Fernandez-Busquets and Burger, 1997); nevertheless the structure-function relationship of MAFp3 to sponge cell adhesion remains to be investigated (Fernandez-Busquets et al., 1996). Antibodies raised against the *M. prolifera* MAFp3 protein were found to identify, besides the core protein of the AF, a 68 kDa protein that does not belong to the AF (Fernandez-Busquets et al., 1998). Also, the cDNA libraries from *G. cydonium* were successfully screened for a sequence related to that from *M. prolifera* (Müller et al., 1999). Until now no further proteins have been cloned from the AF-complex even though a series of protein species have been demonstrated on protein level both in *M. prolifera* (Fernandez-Busquets and Burger, 1999) and in *G. cydonium* (Müller, 1982). It has even been suggested that more than one AF complex may exist, or that additional proteins are present on the cell membrane that contribute to the histocompatibility reaction of sponges, or that both scenarios co-exist (Fernandez-Busquets and Burger, 1999). In addition, evidence has been presented indicating that homologous AF complexes interact with each other (see Fernandez-Busquets and Burger, 1999). It should be stressed here that, besides protein-protein interactions, glycan-glycan binding reactions might contribute to AF-mediated cell-cell adhesion reactions (Misevic, 1999).

In the present study we identified one molecule of the AF complex in the *G. cydonium* system by raising antibodies against the enriched AF-Fraction 6B, which were found to inhibit cell-cell aggregation. The antibodies were used for immunoscreening of the *G. cydonium* cDNA expression library. A cDNA was isolated; the deduced polypeptide, termed putative aggregation factor (putative AF), displayed a distant relationship to amphiphysin II and the bridging integrator protein, two families of molecules that interact with integrin (Wixler et al., 1999). Functional studies revealed that the recombinant, putative AF abolishes the AF-complex-mediated cell adhesion in the homologous system. Consequently, it is concluded that the putative AF described here, is a component that is functionally involved in cell adhesion mediated by the AF-complex.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other enzymes for recombinant DNA techniques and vectors were obtained as described (Wimmer et al., 1999).

Buffers

The descriptions of Ca^{2+}- and Mg^{2+}-free seawater (CMFSW), of CMFSW containing EDTA (CMFSW-E) as well as of Ca^{2+} and Mg^{2+}-containing artificial seawater (ASW) were given earlier (Rottmann et al., 1987).

Sponge and sponge components

Live specimens of *G. cydonium* (Porifera: Demospongiae: Tetractinomorpha: Astrophorida: Geodiidae) were collected near Rovinj, Croatia. The tissue samples were either immediately frozen in liquid nitrogen until use or were immediately processed for immunofluorescence analysis. The preparation of viable cells was described earlier (Müller and Zahn, 1973). The crude extract from *G. cydonium* tissue was obtained by homogenization of 5 g of sponge tissue with 15 ml of a 25 mM of Tris/HCl buffer (pH 7.5, 150 mM NaCl, 20 mM 2-mercaptoethanol, 50 μM phenylmethylsulfonyl fluoride); after centrifugation (90 minutes, 80,000 ***g***), the supernatant obtained was collected. It contained 4.2 mg/ml of protein. Under these conditions the soluble galectins have been obtained (Müller et al., 1997).

Isolation and enrichment of the aggregation factor

The procedure for the isolation of the *G. cydonium* AF was as described (Müller and Zahn, 1973; Conrad et al., 1984; Wagner-Hülsmann et al., 1996). Briefly, crude extract was prepared from 50 g of tissue in CMFSW-E. After centrifugation the cell-free supernatant was supplemented with $CaCl_2$ to precipitate the AF. The suspension was centrifuged and the resulting pellet was treated with CMFSW-E. After centrifugation the supernatant was applied to a Sepharose 6B column and elution was performed with CMFSW. The fractions eluting in the first peak, the void volume, was collected and termed 'AF-Fraction 6B'. The protein concentration in the pooled AF-Fraction 6B was 4.8 mg/ml.

Antibodies

Polyclonal antibodies (PoAb) against the AF-Fraction 6B were raised in female rabbits (White New Zealand). Enriched AF-Fraction 6B (10 μg of protein) was injected at 4-week intervals. After three boosts, serum was collected and the antibodies prepared (Harlow and Lane, 1988). The PoAb selected for these studies were termed PoAb-AF. In control experiments, 100 μl of the PoAb-AF were adsorbed to 50 μg of enriched AF-Fraction 6B (30 minutes; 4°C) prior to its use. Preparation of Fab′ fragments of PoAb-AF was performed as follows. PoAb-AF were purified by affinity chromatography using protein A agarose macrobeads (Harlow and Lane, 1988). IgG molecules were fragmented by enzymic digestion to $(Fab')_2$ fragments using insoluble papain attached to agarose; Fab′ fragments were obtained by subsequent reduction and alkylation (Acheson and Gallin, 1992).

Western blotting

Gel electrophoresis of the protein extracts was performed in polyacrylamide gels (the percentage is given with the respective experiments) containing 0.1% $NaDodSO_4$ (PAGE), as previously descibed (Laemmli, 1970). Protein samples were subjected to gel electrophoresis in the presence of 2-mercaptoethanol and stained with Coomassie brilliant blue. Semi-dry electrotransfer was performed onto PVDF-Immobilon as described (Kyhse-Andersen, 1984). Membranes were processed (Bachmann et al., 1986) and incubated with PoAb-AF (diluted 1:500) for 90 minutes at room temperature.

After blocking the membranes with 5% bovine serum albumin, the immune complexes were visualized by incubation with anti-rabbit IgG (alkaline phosphatase conjugated), followed by staining with 4-nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. In one control experiment, the PoAb-AF (100 μg) was treated with 20 μg of enriched AF-Fraction 6B prior to use in western blots.

Cell aggregation and aggregation inhibition assay

In the standard incubation assay (3 ml volume) a suspension of $25\pm5\times10^6$ cells per ml of ASW was placed into glass tubes and rolled by 35 rpm at 20°C (Müller and Zahn, 1973). Enriched AF-Fraction 6B, galectin and/or Fab′ fragments (PoAb-AF) were added at the indicated concentrations. The suspension was incubated for 60 minutes. The size of the aggregates formed was determined optically and is given in micrometers (Müller et al., 1979).

Following a previously described procedure (Brackenbury et al., 1977), the AF-Fraction 6B (in a 10-fold higher concentration than used in the aggregation assay) was pre-incubated for 30 minutes at 20°C together with 100 μg of the Fab′ fragments and subsequently added to the cells.

In one series of experiments the single cells at a density of $1\pm5\times10^8$ were treated with 0-3 μg/ml of recombinant AF, rAF_GEOCY, in CMFSW for 60 minutes (4°C). The cells were then washed in CMFSW by centrifugation (10 minutes at 800 *g*), adjusted to the cell concentration required for the aggregation assay and incubated in ASW with 100 μg AF-Fraction 6B per assay.

Screening of cDNA library

The cDNA library from *G. cydonium* (Pfeifer et al., 1993) was screened with PoAb-AF, according to a described procedure (Young and Davis, 1983). Positive clones were isolated, rescreened twice and converted into the plasmid vector (pBK-CMV, Stratagene) using the rapid excision kit (Stratagene) according to the manufacturer's instructions. The sequence obtained, termed *GEOCYAF*, was confirmed by screening the library using PCR.

Sequence analysis

The sequences were analyzed using computer programs BLAST (http://.www.ncbi.nlm.nih.gov/BLAST/) and FASTA (http://www.ebi.ac.uk/fasta3). Multiple alignments were performed with CLUSTAL W Ver. 1.6 (Thompson et al., 1994). Phylogenetic trees were constructed on the basis of aa sequence alignments by neighbour-joining, as implemented in the 'Neighbor' program from the PHYLIP package (J. Felsenstein, University of Washington, Seattle). The distance matrices were calculated using the matrix model as described (Dayhoff et al., 1978). The degree of support for internal branches was further assessed by bootstrapping (Felsenstein, 1993). The graphic presentations were prepared with GeneDoc (K. B. Nicholas and H. B. Nicholas Jr (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. Version 1.1.004. http://www.psc.edu/biomed/genedoc). Hydropathicity analysis (window size 15 aa) was performed as described (Klein et al., 1985).

Protein expression

Expression of the *GEOCYAF* gene was performed in *E. coli* using the 'GST (glutathione-*S*-transferase) Fusion system (Amersham) as described (Ausubel et al., 1995; Coligan et al., 2000) and following the instructions of the manufacturer. The *GEOCYAF* clone was introduced into the pGEX2T plasmid containing the *Schistosoma japonicum* glutathione S-transferase gene and expressed with IPTG. The fusion protein was purified by affinity chromatography on glutathione Sepharose 4B (Coligan et al., 2000). If not mentioned otherwise, this recombinant fusion protein, rAF_GEOCY, was used for the experiments. In one series of experiments, the fusion protein was cleaved with thrombin (10 units/mg) to separate glutathione-*S*-transferase from the recombinant sponge putative AF.

Histological analysis

Fresh tissue from *G. cydonium* was transferred to isopentane and cooled to –80°C. After transfer for 1 hour into 6% sodium fluoride to dissolve the spicules, the samples were washed in ASW. Then the tissue was fixed in 4% (w/v) paraformaldehyde in CMFSW-E, which was supplemented with 1% (w/v) sodium borohydrate to suppress autofluorescence (Pancer et al., 1996). After a further wash in CMFSW-E the samples were transferred into 20% (w/v) sucrose in CMFSW-E for 10 minutes and then immediately frozen at –80°C in isopentane. The frozen tissue was sectioned in a cryostat at –20°C. Sections, measuring 8 μm, were collected on 3-aminopropyltriethoxy-silane-coated slides (Ohno et al., 1994). After fixation to the slides the cells were made permeable with 0.1% saponin (Hafen et al., 1983), washed in PBS and incubated with PoAb-AF (1:500 dilution) for 30 minutes. The slides were then incubated with FITC-conjugated goat anti-rabbit IgG for 2 hours. The sections were inspected by immunofluorescence with an Olympus AHBT3 microscope.

Binding of recombinant putative AF to *G. cydonium* galectin

To demonstrate that the recombinant putative AF binds to the homologous galectin, rAF_GEOCY was either used directly as fusion protein with the glutathione S-transferase or the fusion protein was used after digestion (see above). The recombinant proteins were size separated on a 12% polyacrylamide gel containing 0.1% $NaDodSO_4$. Subsequently, the proteins were transferred to PVDF-Immobilon membrane and incubated for 2 hours with 400 μg/ml of the crude sponge extract. The blots were washed and finally incubated with the mouse monoclonal antibody (McAb) IIIc8 (1:500 dilution) raised against the *G. cydonium* galectin (Wagner-Hülsmann et al., 1996), anti-galectin McAb. After incubation, the immunocomplex was detected as described above.

Protein content was determined with the Lowry method (Lowry et al., 1951).

RESULTS

Preparation of antibodies against enriched AF from *G. cydonium*

The crude *G. cydonium* AF (Fig. 1A, lane a) was enriched to obtain the AF-Fraction 6B (Fig. 1A, lane b); the latter fraction was used to raise polyclonal antibodies. The antibodies obtained were used in a western blot to identify the protein species in the crude extract that reacted with them. One band corresponding to a size of 36 kDa was obtained (Fig. 1B, lane a). This M_r matches the one that is seen in the enriched AF fraction (Fig. 1A, lane b). In one control experiment, using PoAb-AF that had been adsorbed with enriched AF-Fraction 6B, no band is seen (Fig. 1B, lane b), indicating that the immune reaction with the 36 kDa protein is specific.

Effect of the antibodies against AF-Fraction 6B on cell aggregation

The AF-Fraction 6B causes cell-cell aggregation in a concentration-dependent manner. As shown in Fig. 2A, a significant increase in the extent of aggregation is seen at a concentration of >3 μg/assay ($P<0.001$). After addition of 100 μg/assay the average size of the aggregates increased to 2,300±250 μm during the 60-minute incubation period (Fig. 2A). After addition of the recombinant putative AF, rAF_GEOCY, which had been preincubated with Fab′ fragments obtained from PoAb-AF, the size of the aggregates strongly decreased to 320±70 μm (Fig. 2A).

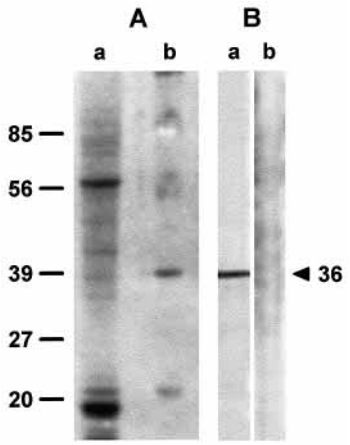

Fig. 1. Identification of the protein species in the *G. cydonium* AF that reacted with the polyclonal antibodies PoAb-AF. The antibodies were raised against the enriched AF, AF-Fraction 6B. (A) The proteins (10 μg of protein per lane) present in the crude AF (lane a) as well as in AF-Fraction 6B (lane b) were size-separated by PAGE using an 8% polyacrylamide gel, and stained with Coomasie brilliant blue. (B) Western blot analysis of the AF-Fraction 6B using PoAb-AF. The proteins in the AF fraction were transferred to Immobilon sheets, and incubated with either the untreated PoAb-AF (lane a) or with the PoAb-AF, adsorbed with AF-Fraction 6B. The immunocomplex has been visualized by a labeled secondary antibody. For further data see Materials and Methods.

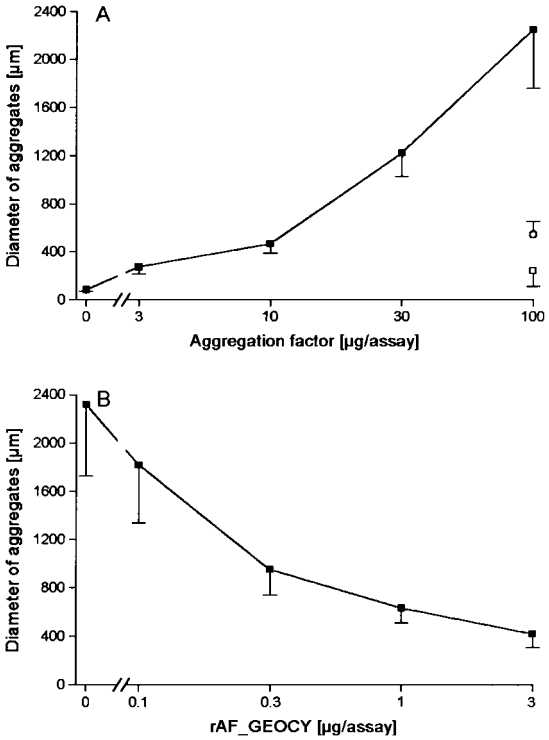

Fig. 2. Aggregation-promoting activity of AF-Fraction 6B. (A) Effect of AF-Fraction 6B on cell reaggregation. *G. cydonium* cells have been incubated with increasing concentrations of AF-Fraction 6B as indicated. After the chosen incubation period, 60 min, the size of the aggregates was determined. The AF was added either directly (■) or after preincubation with 100 μg of Fab′ fragments from the PoAb-AF (□). In a further series of experiments, 3 μg/assay of the recombinant putative AF related molecule, rAF_GEOCY have been added to the single cell suspension (30 minutes); subsequently, 100 μg/assay of AF-Fraction 6B was added and the size of the aggregates determined after 60 minutes (○). (B) Influence of rAF_GEOCY on AF-mediated cell aggregation. Single cell suspensions were treated with 0-3 μg/assay of rAF_GEOCY as described in Materials and Methods. Then the cells were washed and incubated in ASW for 60 minutes with 100 μg AF-Fraction 6B per assay.

The aggregation-promoting activity of the AF-Fraction 6B is also documented by micrographs. If the AF-Fraction 6B at a concentration of 100 μg/assay is added to single cells (Fig. 3A), the size of the aggregates increases to >2000 μm (Fig. 3B). However, if the AF-Fraction 6B was preincubated with Fab′ fragments of PoAb-AF before addition to the cells, only small aggregates are seen (Fig. 3C). If the cells are incubated first with the recombinant AF, rAF_GEOCY (see below), and subsequently with the native AF-Fraction 6B, then the size of the aggregates is also drastically reduced (Fig. 3D).

From these data we conclude that the antibodies raised against the AF-Fraction 6B are directed against the binding protein present in the AF complex, the AF-Fraction 6B. Furthermore, the recombinant AF protein has the ability to bind to the membrane of the cells and competes with the AF protein present in the native AF-Fraction 6B for the binding site at the cell surface (see also below).

Immunohistological analysis

Sections through tissue from of *G. cydonium* were prepared and reacted with antibodies against the AF-Fraction 6B, PoAb-AF. The immunocomplexes were visualized with a secondary FITC-labeled antibody. As shown in Fig. 4A the cells in the mesohyl of the sponge were brightly stained, whereas, in the control sections that were treated with only the secondary antibody (Fig. 4B), a scattered, faint staining was visible, due to residual autofluorescence. The autofluorescence could be largely abolished by sodium borohydrate, as described under Materials and Methods.

Immunoscreening and primary structure of the putative AF

PoAb-AF were used to immunoscreen the *G. cydonium* cDNA library as described in Materials and Methods. After screening the recombinant plaques, five immuno-reactive identical clones were isolated.

The 1710 bp long nucleotide sequence, termed *GEOCYAF*, has an open reading frame (ORF) of 1242 nt (accession number AJ311598). Northern blot analysis revealed a single band of ≈1.7 kb, indicating that the clone is of full length (not shown). The translation product of *GEOCYAF* has 414 aa and was named AF_GEOCY, (Fig. 5A) has a calculated size of M_r 46,558 and a pI of 4.75; according to its computed instability index of 65.7 it is a predicted to be an unstable protein (PC/GENE (1995). *Data Banks CD-ROM; Release 6.85*. (A. Bairoch, University of Geneva, Switzerland, IntelliGenetics, Inc. Mountain View, CA). One potential N-glycosylation site is found at residue Asn61 (Isrec-Server (2001) http://www.isrec.isb-sib.ch/cgi-bin/PFSCAN_form_parser).

The sponge putative AF molecule shares 28 (29)% identical and 44 (45)% similar (identical plus physico-chemcial related aa) aa with human amphiphysin II (Ramjaun et al., 1997;

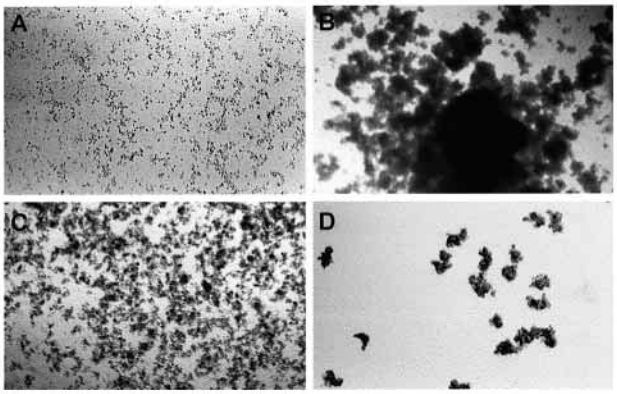

Fig. 3. Inhibition of AF-mediated cell aggregation. A single cell suspension of *G. cydonium* (A) has been treated for 60 minutes in the presence of 100 μg/assay of AF-Fraction 6B (B). (C) Addition of 100 μg Fab′ fragments obtained from PoAb-AF to the AF-Fraction 6B 30 minutes before the incubation period for 60 minutes with the cell suspension. (D) The cell suspension was incubated first with 3 μg/assay of recombinant rAF_GEOCY; after a washing step, 100 μg/assay of AF-Fraction 6B was added and incubation proceeded for 60 minutes. Magnification ×50 (A); ×5 (B-D).

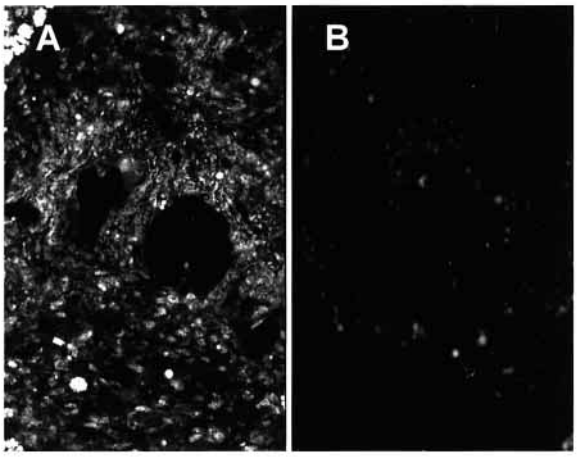

Fig. 4. Immunohistological analysis of *G. cydonium* AF by using antibodies raised against the AF-Fraction 6B. Cryosections through the sponge were obtained and stained in A with the polyclonal PoAb-AF and subsequently with a FITC-labeled anti-rabbit antibody. In B the specimens were reacted only with the labeled secondary antibody. Further data are given in Materials and Methods. Magnification ×50.

AF004015) and human bridging integrator protein-1 BIN1 (Sakamuro et al., 1996; U84004). However, only the first 260 aa contribute to this similarity with 37% identical and 60% similar aa with respect to both human sequences, whereas the C-terminal stretch of the sponge protein has only a low sequence relationship with 13% identical and 24% similar aa (Fig. 5A). In the C-terminal segments of amphiphysin II and BIN1, the SH3 domain is present in both human sequences (Ramjaun et al., 1997), whereas it is absent in the sponge putative AF (Fig. 5A). Additionally, the Pro-rich sequence, which spans in amphiphysin from aa_{290} to aa_{349}, is not present and the nuclear localization signal, which is present in BIN1, is lacking in the putative AF (Fig. 5A). The SH3 region is presumably involved in protein:protein interaction via binding to Pro-rich peptides (Morton and Campbell, 1994); amphiphysin and BIN1 evidence indicates that these proteins associate with other intracellular proteins, such as clathrin (Ramjaun et al., 1997). The sponge sequence, however, displays a Gly-rich segment at the N-terminus that is not found in amphiphysin/BIN1. No eukaryotic secretory signal peptide could be predicted for the sponge sequence (SignalP V2.0.b2 WWW Prediction Server, http://www.cbs.dtu.dk/services/SignalP V2.0/). However, hydropathicity analysis (Kyte and Doolittle, 1982) revealed that the sponge putative AF could be classified as a peripheral, membrane-associated molecule.

Phylogenetic analyses of the putative AF

Searching the databank (BLAST databank) with the deduced *G. cydonium* putative AF revealed highest similarity with the deuterostomian (*Homo sapiens*) amphiphysin and BIN1 sequences with significance scores of 10^{-55} and alignment scores (in bits) of >200, as found in the respective BLASTP report (Coligan et al., 2000). A moderate similarity is also present between the sponge putative AF and the protostomian sequences of *Drosophila melanogaster* amphiphysin (10^{-45}, 178; accession number AJ242855.1) and of the *Caenorhabditis elegans* amphiphysin-like protein (10^{-37}, 152; Z68217). In this context it must be mentioned that, in contrast to the sponge sequence, the *D. melanogaster* amphiphysin contains an SH3 domain. Only distantly related are the viability/starvation protein RVS161 from *Saccharomyces cerevisiae* (10^{-10}, 64; NP_009935), and the hypothetical protein MDF20 from *Arabidopsis thaliana* (10^{-4}, 44; AB009050-BA000015).

After alignment, a phylogenetic tree was constructed, using the plant sequence as an outgroup (Fig. 5B). It shows that the amphiphysin/BIN molecules from Metazoa form one branch and exclude the yeast sequence. The tree suggests that the sponge sequence displays a higher similarity to the human sequences; however, this overall higher similarity is due to the closer similarity of the sponge putative AF to the two human sequences with respect only to the N-terminal part of the sequence. With respect to the C-terminus, the sponge sequence is ancestral since the protostomian sequences share the functional important SH3 domain with the human amphiphysin/BIN molecules.

Recombinant putative AF

A 1133 nt-long segment, corresponding to nt_{148} to nt_{1281} (the start ATG of ORF is located at $nt_{40\text{-}42}$), of the cDNA *GEOCYAF* was expressed as a GST fusion protein in *E. coli*. After induction of the β-galactoside promotor with IPTG, one band of 62 kDa became strongly visible in the bacterial lysate after $NaDodSO_4$-PAGE (Fig. 6A, lanes b,c versus lane a). After purification, the 62 kDa fusion protein was obtained (Fig. 6A, lane c), comprising the 26 kDa GST moiety (Coligan et al., 2000) and the 36 kDa *GEOCYAF* fragment.

The recombinant putative AF, rAF_GEOCY, was identified by western blotting. The fusion protein was purified, size separated and subjected to western blotting. This study revealed that the PoAb-AF recognized the 62 kDa rAF_GEOCY (Fig. 6B, lane b); control experiments established that the PoAb-AF that had been adsorbed with AF-Fraction 6B did not detect the 62 kDa protein on the membrane (Fig. 6B, lane a).

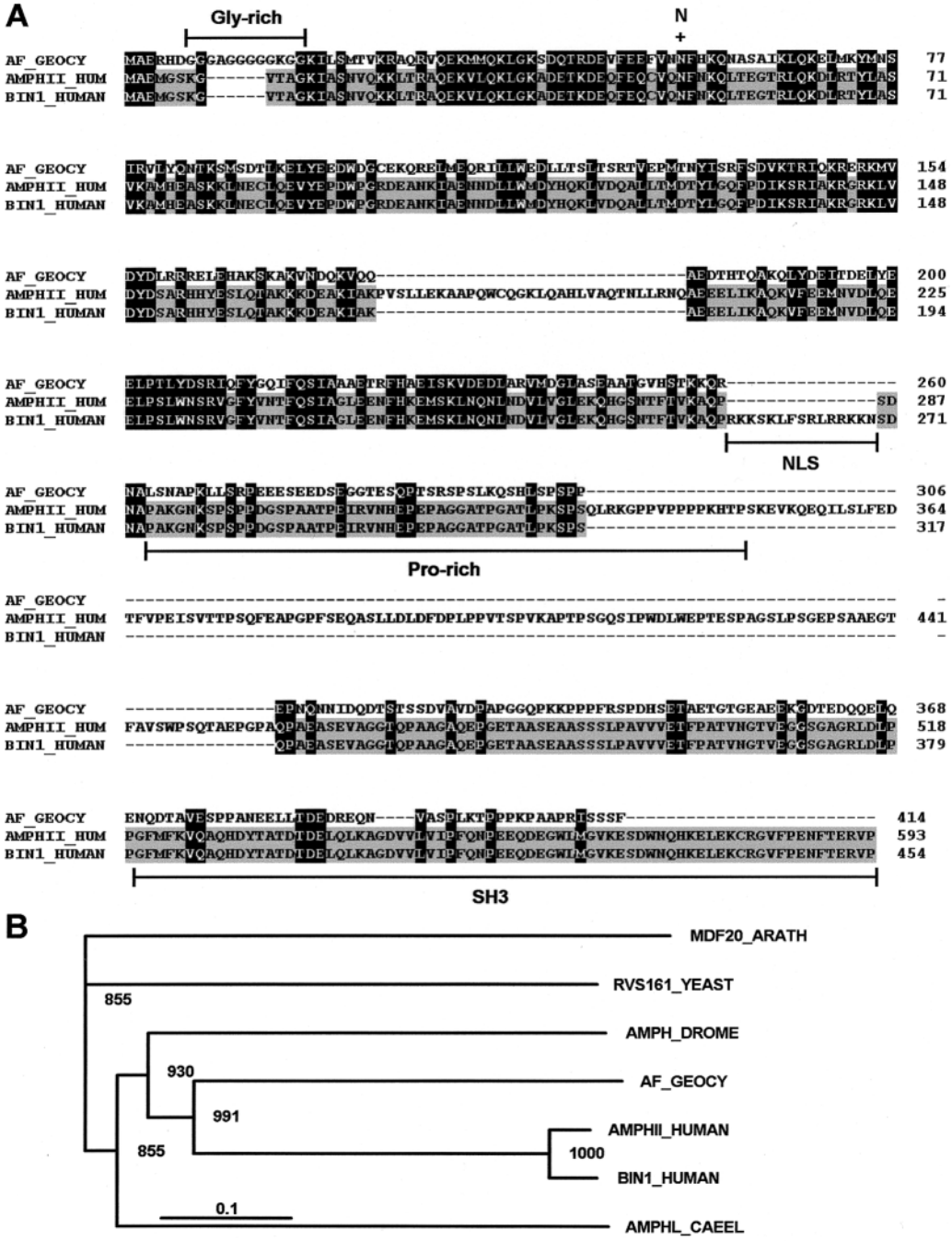

Fig. 5. Sponge AF and its phylogenetic relationships. (A) Alignments of the *G. cydonium* AF protein, AF_GEOCY, deduced from the cDNA *GEOCYAF*, with the following sequences: the human bridging integrator protein-1 (BIN1) gene (BIN1_HUMAN; accession number U68485) and human amphiphysin II (AMPHII_HUMAN; AF004015). Residues conserved (similar or related with respect to their similar physico-chemical properties) in all sequences are shown in white on black and those in at least two sequences in black on gray. The characteristic nuclear localization signal (NLS) present in amphiphysin and the Pro-rich region together with the SH3 domain in both human sequences are marked. The sponge sequence comprises a Gly-rich segment. The single N-glycosylation site within the sequences is marked (N/+). (B) The phylogenetic relationship of the sponge AF protein sequence with the sequence listed under A together with: the reduced viability/starvation protein from *S. cerevisiae* (RVS161_YEAST; NP_009935), amphiphysin from *D. melanogaster* (AMPH_DROME; AJ242855.1 - GI:5102563) and the amphiphysin like protein from *C. elegans* (AMPHL_CAEEL; Z68217). The hypothetical protein MDF20 from *A. thaliana* (MDF20_ARATH; AB009050-BA000015) was used as outgroup. 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.

Functional study with the recombinant putative AF

Incubation of the rAF_GEOCY polypeptide with single cells from *G. cydonium* prior to the addition of the AF-Fraction 6B resulted in a strong reduction of the size of the aggregates formed in the presence of the high concentration of AF-Fraction 6B (100 μg/assay). In the absence of rAF_GEOCY, the aggregates measure 2,300±250 μm is measured, whereas, after a preincubation with 3 μg of rAF_GEOCY per assay and a subsequent incubation with 100 μg AF-Fraction 6B/assay, the size of the aggregates is reduced to 430±65 μm (Fig. 2A; Fig. 3D). In a further series of experiments the cells were pretreated with increasing concentrations of rAF_GEOCY. After one washing step the cells were incubated for 60 minutes with AF-Fraction 6B. The results show that the recombinant rAF_GEOCY at a concentration of 0.3 μg/assay and higher, significantly reduced the size of the aggregates formed after 60 minutes (Fig. 2B).

From these data we conclude that the recombinant putative AF from *G. cydonium* competes with the binding protein present in the AF complex (AF-Fraction 6B) for the cell membrane-associated AR/galectin molecule(s).

Binding of the *G. cydonium* galectin to the recombinant putative AF

A modified western blotting approach was chosen to clarify if the recombinant putative AF, AF_GEOCY binds to the

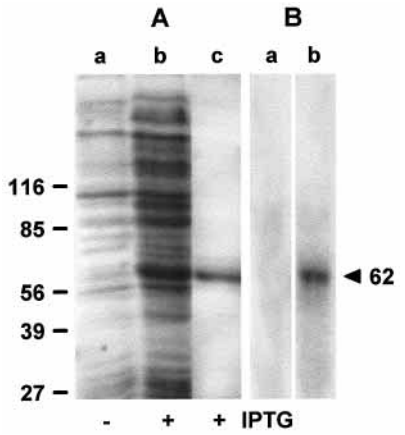

Fig. 6. Expression of the putative AF. (A) NaDodSO$_4$-PAGE gel stained with Coomassie brilliant blue, showing the expression of the GST-AF_GEOCY; lane a, total lysate before induction with IPTG; lane b, lysate obtained from cells after IPTG induction for 6 hours; lane c, fusion protein after purification on glutathione Sepharose. The position of the fusion protein is indicated by the arrow head. (B) Western blot analysis of the purified rAF_GEOCY fusion protein. The fraction was size-fractionated by NaDodSO$_4$-PAGE transferred to Immobilon sheets, and incubated with PoAb-AF; the immunocomplexes were visualized by labeling with the secondary antibody (lane b). In one control experiment (lane a), the PoAb-AF was pretreated with AF-Fraction 6B as described in Materials and Methods.

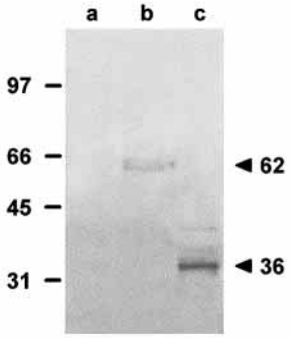

Fig. 7. Binding of the recombinant putative AF, rAF_GEOCY, to the *G. cydonium* galectin. The recombinant putative AF, either as the fusion protein with glutathione S-transferase (lane b), or after cleavage (lane c) were subjected to PAGE. In lane a, no protein was loaded onto the gel. After separation, the proteins were transferred and the membranes incubated with crude sponge extract. Subsequently, the blots were incubated with the anti-galectin McAb IIIc8; the immunocomplexes were identified by labeled secondary antibodies as described in Materials and Methods.

sponge galectin. Therefore, the undigested (Fig. 7, lane b) as well as the digested rAF_GEOCY (Fig. 7, lane c) was applied onto the gel. After PAGE and subsequent incubation of the blot with crude extract that contains the galectin, the binding of the rAF_GEOCY to the galectin was demonstrated by the anti-galectin McAb IIIc8. In this approach the antibody recognized the galectin:full-size fusion protein (M_r 62 kDa; Fig. 7, lane b) or the processed form (M_r 36 kDa; Fig. 7, lane c).

DISCUSSION

In contrast to phylogenetically younger metazoan phyla sponges, *G. cydonium* (Müller and Zahn, 1973) and *M. prolifera* (Henkart et al., 1973) possess a specialized adhesion system that is constructed of the two component system, the AF and the AR (Weinbaum and Burger, 1973). Cloning studies of the sponge AR, from *G. cydonium*, identified it as a complex SRCR/SCR membrane receptor (Blumbach et al., 1998). As outlined earlier, the sponge AF is a multiprotein complex, which is built from a core structure that appears as 'sunbursts' and a series of proteins associated with it (Müller and Zahn, 1973; Cauldwell et al., 1973). The first protein of the AF-complex, the proteoglycan-like core structure, was cloned from *M. prolifera* (Fernandez-Busquets et al., 1996; Fernandez-Busquets and Burger, 1997) and later from *G. cydonium* (Müller et al., 1999). In *G. cydonium*, this AF-associated protein with a M_r of 86 kDa comprises high sequence similarity to selectin molecules found in Protostomia and Deuterostomia (Müller et al., 1999; W.E.G.M., unpublished). No sequence similarity has been found to proteins from *A. thaliana* or *C. elegans*.

In *G. cydonium* the AF is known to associate with the AR; the strength of this binding is enhanced by galectin (Pfeifer et al., 1993; Wagner-Hülsmann et al., 1996). In the present study, antibodies were raised against the AF complex; the high molecular weight fraction AF-Fraction 6B was used to identify the molecule that binds to galectin. This approach resulted in the isolation of a cDNA encoding a protein termed putative AF, AF_GEOCY. The molecule was expressed and the recombinant protein shown to inhibit the AF-Fraction 6B-mediated cell-cell interaction. This finding indicates, first, that the cloned putative AF is involved in cell adhesion and, second, that the recombinant, putative AF competes with the particle (core structure)-associated adhesion molecule for the binding site at the cell surface. The latter result can be explained by the assumption that the recombinant putative AF has bifunctional activity: to bind to the AR/galectin at the cell surface and to associate with the AF core structure.

In an approach to demonstrate that the putative AF binds to the cell surface-AR via galectin, western blot studies have been performed. These studies revealed that the putative AF binds to galectin present in a crude extract obtained from the same sponge species. Hence, the following schematic model for the AF-mediated cell-cell interaction can be outlined. The core structure of the sponge AF is associated with at least two adhesion-promoting proteins: the 86 kDa selectin-related molecule and the 36 kDa putative AF. For the latter molecule, it is demonstrated in the present study that it associates with galectin, which in turn, as reported earlier, binds to the AR (Wagner-Hülsmann et al., 1996) (Fig. 8). The *G. cydonium* galectin molecules have only one carbohydrate binding site that is specific for galactans (Müller et al., 1997); galectin-galactan interaction requires Ca^{2+} for full activity (Diehl-Seifert et al., 1985). Consequently, it might be adopted that two galectins are involved in the bridging of the AR to the putative AF. Both the putative AF (one N-glycosylation site) and the aggregation receptor (24 N-glycosylation sites of the large membrane-spanning isoform; accession number Y14953) from *G. cydonium* have potential glycosylation sites that could harbor oligosaccharide side-chains. However, it is more likely

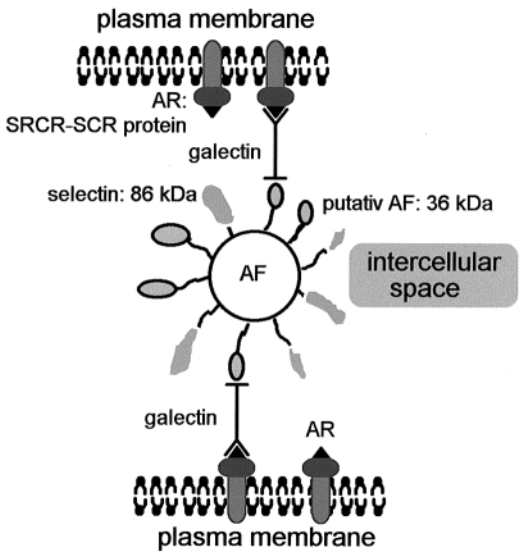

Fig. 8. Schematic model of the AF-mediated cell-cell recognition in *G. cydonium*. The SRCR/SCR membrane receptor (AR) is inserted into the plasma membrane. Operationally, it is suggested that one galectin molecule binds to the AR. This galectin might bind either directly, as shown here, or after 'dimer' formation in the presence of Ca^{2+} to the putative 36 kDa AF. Based on the experiments described, it is proposed that galectin links the AR via the putative AF protein to the AF core structure. A second putative protein is known, which is likely to bind to the AF core structure, the 86 kDa selectin.

that only one molecule of galectin is involved in this binding since the putative AF, obtained in a recombinant form from *E. coli*, which has been applied here, can bind to the galectin. It must be stressed that there is very limited knowledge of the type of glycosylation of sponge proteins as well as of the composition of the carbohydrate chains in Porifera.

For the sponge *M. prolifera*, a slightly different model has been predicted (Fernandez-Busquets and Burger, 1999). The central core structure of the AF was shown to associate with the Na^+-Ca^{2+} exchange protein/selectin molecule, which is bound in a Ca^{2+}-dependent way with another AF particle. This complex interacts with the AR of the plasma membrane. Interestingly, the *M. prolifera* Na^+-Ca^{2+} exchange protein/selectin molecule is polymorphic, suggesting an involvement of the sponge AF in histocompatibility (Fernandez-Busquets and Burger, 1999).

Sequence analysis of the putative AF indicates that the N-terminal part is highly similar to the human amphiphysin II and BIN1 protein. However, in the C-terminal portion of the sponge molecule, the SH3 domain, likely to be involved in amphiphysin/BIN1 protein-protein interaction, is lacking. In this region the putative AF shares no obvious relationship to non-sponge molecules. It should be noted that the sponge putative AF with its distant relationship to amphiphysin/BIN1 shares only a low similarity to a *S. cerevisiae* molecule and only a very distant similarity to an *A. thaliana* molecule. This result corroborates earlier data that indicate a common ancestry of Metazoa and Yeast and only a distant relationship with Viridiplantae (Müller, 2001). As outlined in the 'Introduction', the sponge AF is assumed to be released from the cells via exocytosis. Hence it might be assumed that the putative AF and the amphiphysin/BIN1 molecules share a common ancestor molecule that might be involved in plasma membrane fusion processes. The size of the putative AF, after identification by PAGE/western blots of the protein both in the AF-Fraction 6B and in its recombinant form is 36 kDa. By contrast, the M_r for the predicted polypeptide corresponding to the ORF from the cDNA *GEOCYAF* is 47 kDa. Since the antibody used for the detection of the native as well as the recombinant proteins was the same as the one used for the identification of the cDNA, it can be assumed that either the protein is processed after translation, or the PAGE migration behavior of the putative AF is unusual. The latter explanation appears more likely in view of the fact that over 55% of the putative polypeptide exists in the stable helical form, as predicted by secondary structure analysis (Garnier et al., 1978).

In conclusion, it is demonstrated that the sponge putative AF is a relevant molecule involved in the heterophilic cell-cell interaction in sponges. This molecule comprises a sequence similarity to molecules of other metazoan phyla (e.g. to amphiphysin/BIN1), but no significant relationship to yeast or plant molecules. This finding supports the view that the adhesion molecules in Metazoa, with Porifera as the phylogenetically oldest phylum, represent evolutionary novelties that contributed to the successful transition to Metazoa. It remains to be studied if the adhesion molecules or the subsequent molecules, involved in the signal transduction pathway (Rottmann et al., 1987) evolved separately or in parallel. Recent data with immune molecules, the ITAM/ITiM-motif-containing receptors and their regulatory kinase Syk, again autapomorphic characters of Metazoa, suggest a co-evolution (W.E.G.M., unpublished). Taken together, the data reported here demonstrate that the putative AF of *G. cydonium* is a component which is functionally involved in cell adhesion, and is associated with the AF-complex. These data complement earlier research activities with the sponge *M. prolifera*, which revealed that the core structure of the AF-complex is composed of a polymorphic, apparently sponge-specific, MAFp3 protein (Fernandez-Busquets and Burger, 1999).

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Mü 348/12-5) and the International Human Frontier Science Program (RG-333/96-M). The sequence reported here is in the EMBL data base; *Geodia cydonium* putative aggregation factor, accession number AJ311598.

REFERENCES

Acheson, A. and Gallin, W. J. (1992). Generation and characterization of function-blocking anti-cell adhesion molecule antibodies. In *Cell-Cell Interactions*. (ed. B. R. Stevenson and W. J. Gallin), pp. 31-54. IRL-Press, Oxford.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidmann, J. G. and Struhl, K. (1995). *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

Bachmann, M., Mayet, W. J., Schröder, H. C., Pfeifer, K., Meyer zum Büschenfelde, K.-H. and Müller, W. E. G. (1986). Association of La and Ro antigen with intracellular structures in HEp-2 carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **83**, 7770-7774.

Blumbach, B., Pancer, Z., Diehl-Seifert, B., Steffen, R., Münkner, J., Müller, I. and Müller, W. E. G. (1998). The putative sponge aggregation receptor: isolation and characterization of a molecule composed of scavenger receptor cysteine-rich domains and short consensus repeats. *J. Cell Sci*. **111**, 2635-2644.

Brackenbury, R., Thiery, J. P., Rutishauser, U. and Edelman, G. M. (1977). Adhesion among neuronal cells of the chick embryo. *J. Biol. Chem. 252*, 6835-6840.

Burger, M. M. and Jumblatt, J. (1977). Membrane involvement in cell-cell interactions: a two component model system for cellular recognition that does not require live cells. In *Cell and Tissue Interactions* (ed. J. W. Lash and M. M. Burger), pp. 155-172. Raven Press, New York.

Cauldwell, C., Henkart, P. and Humphreys, T. (1973). Physical properties of sponge aggregation factor: a unique proteoglycan complex. *Biochem.* **12**, 3051-3055.

Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (2000). *Current Protocols in Immunology* (John Wiley, New York), pp. 8.4.1-8.4.21.

Conrad, J., Uhlenbruck, G., Zahn, R. K., Kurelec, B., Jericevic, B. and Müller, W. E. G. (1984). The role of lectin I and of glycoconjugates in recognition of cells from the siliceous sponge *Geodia cydonium*. *Biol. Cell*. **51**, 287-294.

Diehl-Seifert, B., Uhlenbruck, G., Geisert, M., Zahn, R. K. and Müller, W. E. G. (1985). Physicochemical and functional characterization of the polymerization process of the *Geodia cydonium* lectin. *Eur. J. Biochem.* **147**, 517-523.

Dayhoff, M. O., Schwartz, R. M. and Orcutt, B. C. (1978). A model of evolutionary change in protein. In *Atlas of Protein Sequence and Structure* (ed. M. O. Dayhoff), pp. 345-352. National Biomedical Research Foundation, Washington DC.

Fernandez-Busquets, X. and Burger, M. M. (1997). The main protein of the aggregation factor responsible for species-specific cell adhesion in the marine sponge *Microciona prolifera* is highly polymorphic. *J. Biol. Chem.* **272**, 27839-27847.

Fernandez-Busquets, X. and Burger, M. M. (1999). Cell adhesion and histocompatibility in sponges. *Microsc. Res. Tech.* **44**, 204-218.

Fernandez-Busquets, X., Kammerer, R. A. and Burger, M. M. (1996). A 25 kDa protein is the basic unit of the core from the 2×10^4-kDa aggregation factor responsible for species-specific cell adhesion in the marine sponge *Microciona prolifera*. *J. Biol. Chem.* **271**, 23558-23565.

Fernandez-Busquets, X., Gerosa, D., Hess, D. and Burger, M. M. (1998). Accumulation in marine sponge grafts of the mRNA encoding the main proteins of the cell adhesion system. *J. Biol. Chem.* **273**, 29545-29553.

Gamulin, V., Rinkevich, B., Schäcke, H., Kruse, M., Müller, I. M. and Müller, W. E. G. (1994). Cell adhesion receptors and nuclear receptors are higly conserved from the lowest Metazoa (marine sponges) to vertebrates. *Biol. Chem. Hoppe-Seyler* **375**, 583-588.

Garnier, J., Osguthorpe, D. J. and Robson, B (1978). Analysis of the accuracy and implications of simple methods for predicting secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97-120.

Hafen, E., Levine, M., Garber, R. L. and Gehring, W. L. (1983). An improved *in situ* hybridization method for the detection of cellular RNAs in *Drosophila* tissue sections and its application for localizing transcripts of the homeotic antennapedia complex. *EMBO J.* **2**, 617-623.

Harlow, E. and Lane, D. (1988). A*ntibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Henkart, P., Humphreys, S. and Humphreys, T. (1973). Characterization of sponge aggregation factor. A unique proteoglycan complex. *Biochemistry* **12**, 3045-3050.

Klein, P., Kanehisa, M. and DeLisi, C. (1985). The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta* **815**, 468-476.

Kuhns, W. J., Popescu, O., Burger, M. M. and Misevic, G. (1995). Sulfate restriction induces hypersecretion of the adhesion proteoglycan and cell hypomotility associated with increased $^{35}SO_4^{2-}$ uptake and expression of a band 3 like protein in the marine sponge *Microciona prolifera*. *J. Cell. Biochem*. **57**, 71-89.

Kyhse-Andersen, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203-209.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **157**, 105-132.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

McPherson, P. S., Garcia, E., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauernfeind, R., Nemoto, Y. and De Camilli, P. (1996). A presynaptic inositol-5-phosphatase. *Nature* **379**, 353-357.

Misevic, G. N. (1999). Molecular self-recognition and adhesion via proteoglycan to proteoglycan interactions as a pathway to multicellularity: atomic force microscopy and color coded bead measurements in sponges. *Microsc. Res. Tech.* **44**, 304-309.

Misevic, G. N., Jumblatt, J. E. and Burger, M. M. (1982). Cell binding fragments from a sponge proteoglycan-like aggregation factor. *J. Biol. Chem.* **257**, 46931-46936.

Morton, C. J. and Campbell, I. D. (1994). SH3 domains. Molecular 'Velcro'. *Curr. Biol.* **4**, 615-617.

Müller, W. E. G. (1982). Cell membranes in sponges. *Int. Rev. Cytol.* **77**, 129-181.

Müller, W. E. G. (1995). Molecular phylogeny of Metazoa (animals): Monophyletic origin. *Naturwiss.* **82**, 36-38.

Müller, W. E. G. (2001). How was metazoan threshold crossed: the hypothetical Urmetazoa. *Comp. Biochem. Physiol*. **129(A)**, 433-460.

Müller, W. E. G. and Zahn, R. K. (1973). Purification and characterization of a species-specific aggregation factor in sponges. *Exp. Cell Res*. **80**, 95-104.

Müller, W. E. G., Müller, I., Zahn, R. K. and Kurelec, B. (1976). Species-specific aggregation factor in sponges. VI. Aggregation receptor from the cell surface. *J. Cell Sci.* **21**, 227-241.

Müller, W. E. G., Zahn, R. K., Arendes, J., Kurelec, B., Steffen, R. and Müller, I. (1979). Aggregation of sponge cells. Self-aggregation of the circular proteid particle. *Biochim. Biophys. Acta* **551**, 363-367.

Müller, W. E. G., Zahn, R. K., Bernd, A., Dawes, K., Müller, I., Uhlenbruck, G. and Dorn, A. (1982). Molecular basis for cell-cell recognition: isolation, synthesis and function of an aggregation complex from the sponge *Geodia cydonium*. In *Cellular Recognition* (New Series). Vol. 3 (ed. W. A. Frazier, L. Glaser and D. J. Gottlieb), pp. 809-816. UCLA Symposia on Molecular and Cellular Biology, A. R. Liss, New York.

Müller, W. E. G., Blumbach, B., Wagner-Hülsmann, C. and Lessel, U. (1997). Galectins in the phylogenetically oldest Metazoa, the sponges (Porifera). *Trends Glycosci. Glycotechnol*. **9**, 123-130.

Müller, W. E. G., Koziol, C., Müller, I. M. and Wiens, M. (1999). Towards an understanding of the molecular basis of immune responses in sponges: the marine demosponge *Geodia cydonium* as a model. *Microsc. Res. Tech.* **44**, 219-236.

Ohno, S., Terada, N., Fujii, Y. and Ueda, H. (1994). Membrane skeleton in fresh unfixed erythrocytes as revealed by a rapid-freezing and deep-etching method. *J. Anat.* **185**, 415-420.

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

Pancer, Z., Kruse, M., Müller, I. and Müller, W. E. G. (1997). On the origin of adhesion receptors of metazoa: cloning of the integrin α subunit cDNA from the sponge *Geodia cydonium*. *Mol. Biol. Evol*. **14**, 391-398.

Pfeifer, K., Haasemann, M., Gamulin, V., Bretting, H., Fahrenholz, F. and Müller, W. E. G. (1993). S-type lectins occur also in invertebrates: high conservation of the carbohydrate recognition domain in the lectin genes from the marine sponge *Geodia cydonium*. *Glycobiol*. **3**, 179-184.

Ramjaun, A. R., Micheva, K. D., Bouchelet, I. and McPherson, P. S. (1997). Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem.* **272**, 16700-16706.

Rottmann, M., Schröder, H. C., Gramzow, M., Renneisen, K., Kurelec, B., Dorn, A., Friese, U. and Müller, W. E. G. (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.

Sakamuro, D., Elliott, K. J., Wechsler-Reya, R. and Prendergast, G. C. (1996). BIN1 a novel MYC-interacting protein with features of a toumor suppressor. *Nat. Genet.* **14**, 69-77.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. **22**, 4673-4680.

Varner, J., Burger, M. M. and Kaufman, J. (1988). Two cell surface proteins bind the sponge *Microciona prolifera* aggregation factor. *J. Biol. Chem*. **263**, 8498-8508.

Wagner-Hülsmann, C., Bachinski, N., Diehl-Seifert, B., Blumbach, B., Steffen, R., Pancer, Z. and Müller, W. E. G. (1996). A galectin links the

aggregation factor to cells in the sponge (*Geodia cydonium*) system. *Glycobiol*. **6**, 785-793.

Weinbaum, G. and Burger, M. M. (1973). A two-component system for surface guided reassociation of animal cells. *Nature* **244**, 510-512.

Wilson, H. V. (1907). On some phenomena of coalescence and regeneration in sponges. *J. Exp. Zool.* **5**, 245-258.

Wimmer, W., Perovic, S., Kruse, M., Krasko, A., Batel, R. and Müller, W. E. G. (1999). Origin of the integrin-mediated signal transduction: functional studies with cell cultures from the sponge *Suberites domuncula*. *Eur. J. Biochem.* **260**, 156-165.

Wixler, V., Laplantine, E., Geerts, D., Sonnenberg, A., Petersohn, D., Eckes, B., Paulsson, M. and Aumailley, M. (1999). Identification of novel interaction parameters for the conserved membrane proximal region of alpha-integrin cytoplasmic domain. *FEBS Lett*. **445**, 351-355.

Young, R. A. and Davis, R. W. (1983). Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80**, 1194-1198.

Zahn, R. K., Müller, W. E. G., Reinmüller, J., Geisert, M., Michaelis, M., Pondeljak, V. and Beyer, R. (1976). Species-specific aggregation factor in sponges. Characterization of the large circular proteid particle. *Cell. Differ.* **5**, 129-137.