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Review

Circular proteoglycans from sponges: first members of the spongican family

X. Fernàndez-Busquets[a,*] and M. M. Burger[b]

[a] Research Center for Bioelectronics and Nanobioscience, Barcelona Science Park, University of Barcelona, Josep Samitier 1–5, E-08028 Barcelona (Spain), Fax: +34934037225, e-mail: busquets@qf.ub.es
[b] Novartis International AG, CH-4002 Basel (Switzerland)

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Abstract. Species-specific cell adhesion in marine sponges is mediated by a new family of modular proteoglycans whose general supramolecular structure resembles that of hyalectans. However, neither their protein nor their glycan moieties have significant sequence homology to other proteoglycans, despite having protein subunits equivalent to link proteins and to proteoglycan monomer core proteins, and glycan subunits equivalent to hyaluronan and to the glycosaminoglycans of hyalectans. In some species, these molecular components are assembled into a structure with a circular core formed by the link protein- and hyaluronan-like subunits. Besides their involvement in cell adhesion, these sponge proteoglycans, for which we propose the term spongicans, participate in signal transduction processes and are suspected to play a role in sponge self-nonself recognition. Their in vivo roles and the mild methods used to purify large amounts of functionally active spongicans make them ideal models to study the functions and possible new applications of proteoglycans in biomedical research.

Key words. Proteoglycan; hyaluronan; cell adhesion; Porifera; invertebrate histocompatibility; glycosaminoglycan; self-nonself recognition; atomic force microscope.

'*In the water are many creatures that live in close adhesion to an external object (…). And, by the way, the sponge appears to be endowed with a certain sensibility: as a proof of which it is alleged that the difficulty in detaching it from its moorings is increased if the movement to detach it be not covertly applied.*' From Aristotle's *History of Animals,* 350 BC [1].

Introduction

Proteoglycans (PGs) are a diverse group of macromolecules composed of one or more glycosaminoglycan (GAG) chains covalently bound to a protein core [2]. There are four main GAG types: heparin/heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA). Each GAG is a linear polymer of a disaccharide that (i) in heparin, HS, and HA consists of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid, (ii) in CS and DS of *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid, and (iii) in KS of GlcNAc and D-galactose. Except for HA, GAG chains are covalently bound to proteins and are variably sulfated. The number of GAG chain substituents (generally *O*-linked on serine residues) on a protein core may vary from 1 to >100. In addition to the GAG chains, most core proteins carry *N*- and/or *O*-linked oligosaccharides. Serine residues amenable to glycosylation (initiated by xylosylation) [3] would generally occur in SGXG amino acid sequences (where X is variable), usually preceded by a few acidic residues. However, there are numerous exceptions to this rule [4, 5], thus undermining the notion of a universal consensus sequence for GAG attachment.

* Corresponding author.

The enormous diversity in both the protein and the GAG moieties of PGs has often hampered classification efforts, although enough PGs have now been characterized to attempt groupings that generally divide them into five main categories [4–10]. Within the extracellular PGs are the modular PGs that include (i) basement membrane heparan sulfate proteoglycans (HSPGs) like perlecan, and (ii) hyalectans: large, extracellular aggregating PGs with multiple GAG chains that associate in multiple copies with HA (e.g., aggrecan, versican, neurocan, and brevican). The third category of extracellular PGs is formed by the (iii) small connective tissue PGs with one or two GAG chains, best represented by biglycan, decorin, and fibromodulin. The last two categories are (iv) cell surface PGs (syndecans and glypicans), and (v) intracellular PGs (serglycin).

Numerous PG species, however, will not fit into this scheme, since their core proteins bear no resemblance to those of any of the model PGs [5]. In this review, we will argue in favor of the addition of a new category of modular PGs, the first members of which are a family of marine sponge PGs termed aggregation factors (AFs), whose supramolecular structure has recently been cracked. To illustrate the traits that make AFs different from all other described PGs, we start with a brief overview of those properties of the modular PGs that will be relevant for the discussion of the characteristics of this group of sponge PGs, for which we propose the name spongicans.

Structure of modular proteoglycans

Hyalectans

A trait shared by these PGs is a multidomain core protein structure, with two external regions, one that binds HA and one that contains a C-type lectin-like domain, separated by a central region that carries most of the GAG chains [11]. One member, aggrecan, has a third globular domain linked to the HA-binding domain by an intervening peptide region (fig. 1). Endoproteolytic cleavage of the protein cores has been described for neurocan and brevican [7, 12], thus suggesting that proteolytic processing of hyalectans is a general feature required for their function. Analysis of the genomic organization of the hyalectan genes indicates that they are modular and have utilized exon shuffling and duplication during evolution [8, 13]. A salient characteristic of the hyalectan genes is that all of the introns between those protein modules that are considered to fold autonomously are in phase I, i.e., placed between the first and second nucleotides of a codon [14], thereby allowing alternative splicing events to occur without altering the reading frame. Alternate exon usage is widely used for increasing coding diversity within genes of extracellular matrix (ECM) proteins [15, 16]. The 3′ end of the hyalectan genes codes for a protein module characteristically found in selectins, the family of leukocyte homing and cell adhesion molecules [17]. This region contains amino acid sequences that could mediate Ca^{2+} binding. The combination of structural domains indicates that the hyalectans bind HA through a globular domain (G1) at their amino terminus and carbohydrate structures through their carboxyl terminus G3 domain. The aggregates of aggrecan and HA are stabilized by an *N*-glycosylated protein, link protein (LP), that binds to both HA and aggrecan [18, 19]. The PG aggregate, including LP, is dissociated in the presence of chaotropes such as 4 M guanidine hydrochloride. The interaction between aggrecan and HA is mediated by the amino-terminal G1 domain, whose sequence is homologous to that of LP, which binds to a decasaccharide sequence of the HA molecule [20, 21]. The amino acid sequence of LP can be divided into three domains: an amino-terminal region that falls into the immunoglobulin (Ig) superfamily [22] and two homologous tandem carboxyl-terminal regions, the link modules, that mediate the binding to HA. The LP genomic DNA echoes this structure, in that each domain is

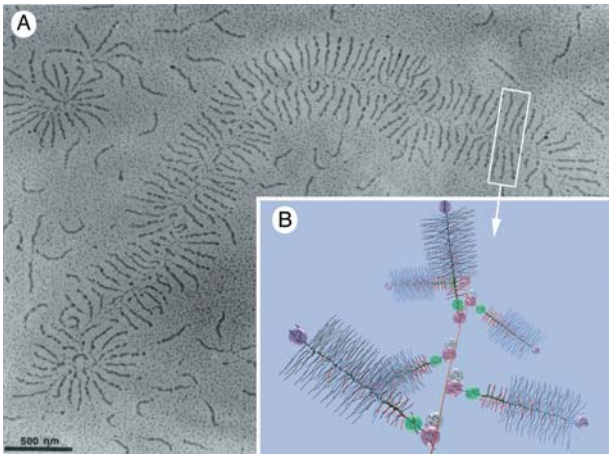

Figure 1. Hyalectan structure. (*A*) Electron micrograph of a PG aggregate from calf cartilage, containing ~180 aggrecan monomers with an overall molecular mass of ~6.5 billion Da. The central HA strand is ~5500 nm in length. The boxed area encloses six monomers, depicted in the model in *B*. (*B*) Model of a portion of the PG aggregate showing six aggrecan monomers (see boxed area in *A*). Each of the six monomers is depicted with a central core protein strand to which the GAGs are covalently linked, giving the appearance of bristles. The core protein (~250,000 Da) contains a midregion with ~100 CS chains (blue) of ~30,000 average molecular mass and a nearly equal number of KS chains (red) of ~10,000 average molecular mass. The monomers are noncovalently anchored to the central HA strand (orange) by (i) an HA-binding site in the *N*-terminal globular-1 (G1) domain (pink sphere) of the core protein, and (ii) a link protein (crystal sphere) that binds to both HA and to the G1 domain of aggrecan. The core protein of aggrecan contains two other globular domains, G2 and the C-terminal G3, shown as green and lavender spheres, respectively. The globular domains of aggrecan also contain *N*-linked oligosaccharides (red Y symbols). From Roseman [65], with permission.

encoded in a separate exon. Proteins and PGs with link modules form a family of hyaladherins (i.e., proteins that interact with HA), such as the cell surface HA-binding protein CD44 that contains a single link module [23]. CD44 can undergo exon shuffling, one of the resulting forms being an HSPG [24]. Other HA receptors exist that do not resemble LP, like the receptor for HA-mediated motility (RHAMM) [25].

Basement membrane PGs

This group contains the modular PGs that do not interact with HA but are designed to interact with various structural components of basement membranes. Like hyalectans, the chimeric structural design based on the repeated use of common protein modules suggests that these PGs may be involved in numerous biological processes [26]. Perlecan is a good representative of this group of molecules. This multidomain PG is one of the most complex gene products because of its enormous dimensions and number of posttranslational modifications [7, 27]. The name derives from its rotary shadowing appearance suggesting a string of pearls. Perlecan comprises five domains that harbor protein modules used by a variety of proteins involved in lipid uptake and metabolism, cell adhesion, basement membrane organization, and cellular growth. Homology scans of the perlecan cDNA sequence reveal significant inner homology between repeated elements [28]. Such is the case for some of the 21 Ig-like repeats of domain IV in human perlecan, which are almost identical, differing by only 2 or 3 nucleotides out of 300. Domain III contains an RGD tripeptide that can promote integrin-mediated cell attachment [29, 30]. Various structural motifs that have been associated with functional characteristics of other proteins are also present in PGs. The core protein of perlecan shows homologies with laminin [31], with adhesion molecules from the Ig superfamily [32], and with the amyloid β protein precursor implicated in Alzheimer's disease [33].

Functions of PGs

The biological roles of PGs are highly diversified, ranging from relatively straightforward mechanical functions to effects on more dynamic processes such as cell adhesion and motility, to complex and still poorly understood roles in cell differentiation and development [5, 34–37]. For example, angiogenesis requires binding of fibroblast growth factor (FGF) to HSPG of the ECM [38]; nerve cell adhesion is mediated by interaction of the neuronal cell adhesion molecule, N-CAM, with cell surface HSPGs [39]; neurogenesis, axon guidance, and synapse development depend on finely tuned interactions of neurons with ECM molecules, including PGs [40, 41], whose enhanced deposition has been directly related to the onset of neurological disorders such as multiple sclerosis [42] and Alzheimer's disease [43, 44]; synaptic vesicles contain abundant PGs [45, 46] that have critical functions in synaptic exocytosis and recovery and in neurotransmitter sequestration; metastasis involves degradation of HSPG in the ECM, catalyzed by tumor cell-derived endoglucuronidases [47, 48]; patterning events dictated by FGF receptors, Wnt, transforming growth factor-β, and Hedgehog families of growth factors are regulated by PGs [49]. FGF action itself seems to be regulated by HSPG isoforms of CD44 [50].

Many PGs can interact with ECM components through their GAG chains. However, specific matrix interactions mediated by core proteins have also been demonstrated [51–54]. Some PG species are attached to the cell membrane in a number of different ways. One group, represented by syndecans, is anchored to the plasma membrane through a transmembrane domain that is intercalated in the lipid bilayer [55]. Other core proteins, represented by glypicans, are bound to the cell surface through linkage to phosphatidyl-inositol, as first described for an HSPG produced by a rat hepatoma cell line [56]. Yet another type of interaction, apparently designed to enable internalization of PGs, is mediated by cell surface receptors that specifically recognize certain core proteins [57].

Interactions of GAG chains

Glycans have immense structural diversity [58], a ubiquitous distribution in vertebrate and invertebrate tissues [59], and are associated with the cell surface [60], as required of cell recognition molecules. Carbohydrate-mediated interactions in cell adhesion and morphogenesis phenomena can be finely modulated by enzymes and divalent cations, representing a highly versatile form of cell recognition and adhesion given the extraordinary plasticity of their structures [61, 62]. Through the combined activity of glycosylating and deglycosylating enzymes, a carbohydrate structure can be made more or less adhesive and its ligand specificity can be altered to meet the particular requirements of changing physiological conditions or of successive developmental steps [63–65]. Differently glycosylated forms of biglycan and decorin are present in newborns and adults [66], suggesting different functional roles. Similarly, syndecan molecules with different levels of HS chain sulfation exhibit dramatic differences in their binding to collagen [67], indicating that the fine structure of HS can differ on identical core proteins, and these differences can control fundamental cellular properties such as cell-matrix adhesion. A peculiar feature of some GAG chains is their ability to self-associate [68, 69], thus directly promoting cell adhesion mediated either by hydrogen bonds between carbohydrates on neighboring cells [65], or by electrostatic interactions with bridging cations

like Ca^{2+} [61, 70]. A number of specific interactions between HA and hyaladherins such as CD44 have pointed to a role for HA in recognition and the regulation of cellular activities [71, 72]. HA, which has an invariant structure, is at least as versatile as the more complex GAGs. Its diversity of functions is directly related to its simplicity in that every cell or tissue makes exactly the same structure but uses the large variety of hyaladherins to dictate functions. Binding of proteins to GAGs can be tight and highly specific, as in the case of antithrombin, which binds with high affinity to a pentasaccharide sequence in heparin/HS [5]. GAG interactions can also be of lower affinity and cooperative. PGs can determine their cellular specificity of binding through a proper spacing between GAG chains attached to the protein core [70]. In such a model of a concerted effect, the function of the protein would be that of a mere scaffold where the active GAGs would be kept at the right positions to favor specific interactions.

The efforts aimed at a better understanding of PG structure and function are often hampered by difficulties in purifying these macromolecules from vertebrate tissues, a process that requires very aggressive extraction methods including extensive use of proteases, detergents, chaotropic agents, and high temperatures for long periods [73–75], yielding very low amounts of often biologically inactive PGs. In the search for new sources of ECM components retaining their in vivo functions, sponges can offer a good alternative: marine sponge PGs can be purified in considerable amounts and in their native conformations by simply soaking the animal for a few minutes in sea water depleted of calcium and spinning down the cells and other debris [76, 77].

Sponge PGs in cell adhesion

Sponges represent the most primitive extant multicellular animals. They lack typical organs, but their tissues are formed by a few classes of highly motile cells that move in a loose and abundant ECM. Fibrillar and nonfibrillar collagens have been cloned from sponges [78–80], including type IV collagen [81], the main component of basement membrane structures. Immunological evidence has been presented for the existence in sponges of fibronectin-like proteins [82, 83]. Lectins have been abundantly isolated from sponges, and studied mainly for their hemagglutinating properties [84–88]. Other molecules involved in cell adhesion have also been identified, including tenascin-like and laminin-like proteins observed in electron microscope images of sponge extracts [89], and cloned integrin α [90] and β subunits [91].

In 1907, Wilson [92] pioneered the use of sponges as model animals for the study of cell adhesion, describing the existence of species-specific reaggregation of marine sponge cells that were mechanically dissociated by passing them through a fine cloth. After disaggregation the

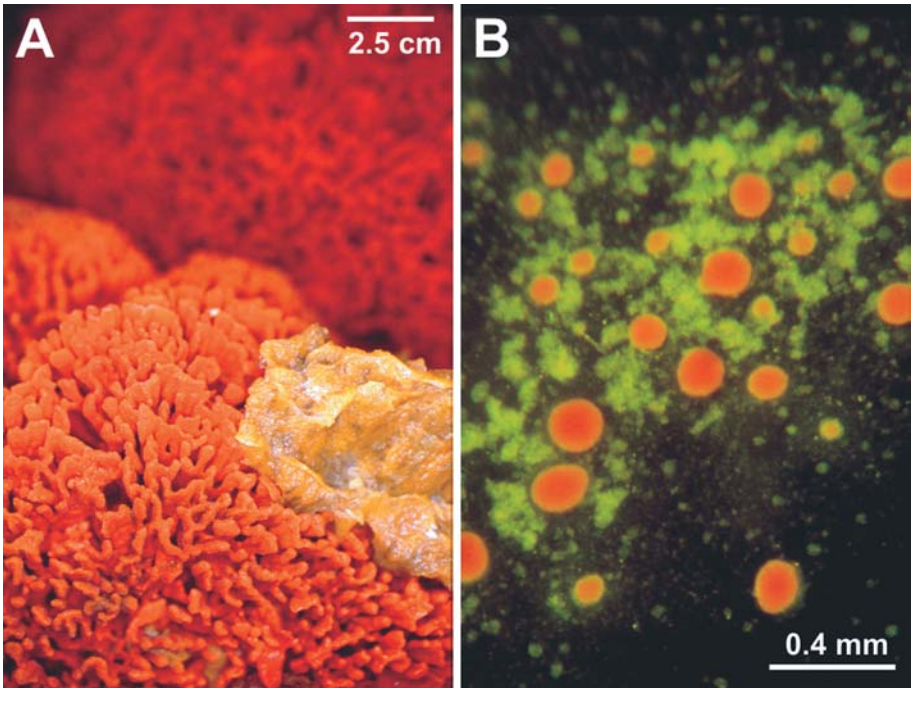

Figure 2. Species specificity of sponge cell adhesion. (*A*) *Microciona prolifera* (orange) and *Halichondria panicea* (brownish) in the wild. (*B*) Reaggregation of a mixture of mechanically dissociated cells from *M. prolifera* (orange) and *H. panicea* (green) after ~30 min of gentle stirring.

cells slowly settled down and started moving. Upon coming in contact, the cells generally adhered, and larger aggregates were formed as new contacts continued to occur, eventually reorganizing perfect miniature sponges in a Ca^{2+}-dependent process [93, 94]. When dissociated cells of differently colored sponge species were mixed and allowed to aggregate (fig. 2), Wilson observed how the cells of one species combined with each other, but not with the cells of a different species. Humphreys [95] and Moscona [96] reported that when sponge cells were washed in cold Ca^{2+}-free sea water they were no longer able to reaggregate, or formed only small clumps. Addition of the collected supernatant in which the cells were washed (referred to as 'aggregation factor', AF) restored species-specific aggregation at low temperatures, when artificial rotation was used to circumvent the inhibition of cell motility and thus promote the cell collisions needed to study adhesion. AF also caused adhesion of fixed cells [96]. Furthermore, enhanced adhesion in the presence of AF was accompanied by a corresponding loss of activity in the supernatant [97], indicating that the AF was associating with the cells.

Sponge AFs have been identified in several marine sponge species [95, 96, 98–100]. Their composition is mainly protein and carbohydrate in variable amounts ranging from 40 to 74% protein [76, 101, 102] with molecular masses in the order of several million daltons. Based on their carbohydrate and protein content, and on their appearance in transmission electron microscope images [99, 103], sponge AFs were soon classified as proteoglycan-like molecules [76]. The cell aggregation activity of marine AFs depends on an extracellular calcium concentration of about 10 mM, similar to that found in sea water. AFs from freshwater sponges have also been described [104] and partially purified [105, 106], although they are still poorly characterized. Freshwater sponge AFs are active at Ca^{2+} concentrations of 1 mM [107], implying major differences when compared to their marine counterparts.

Reliable AF isolations were obtained from the marine sponges *Microciona prolifera* and *Haliclona occulata*, eventually allowing experiments which demonstrated that AFs could cause species-specific cell aggregation [108]. In an elegant approach, AFs purified from three different sponge species were coupled to colored or fluorescent beads that were used for aggregation assays in solution [109, 110] (fig. 3) and for an overlay assay in which the same AFs were bound in patterns to a membrane [109]. Such cell-free systems clearly demonstrate the central role of sponge PGs in the species specificity of cell adhesion.

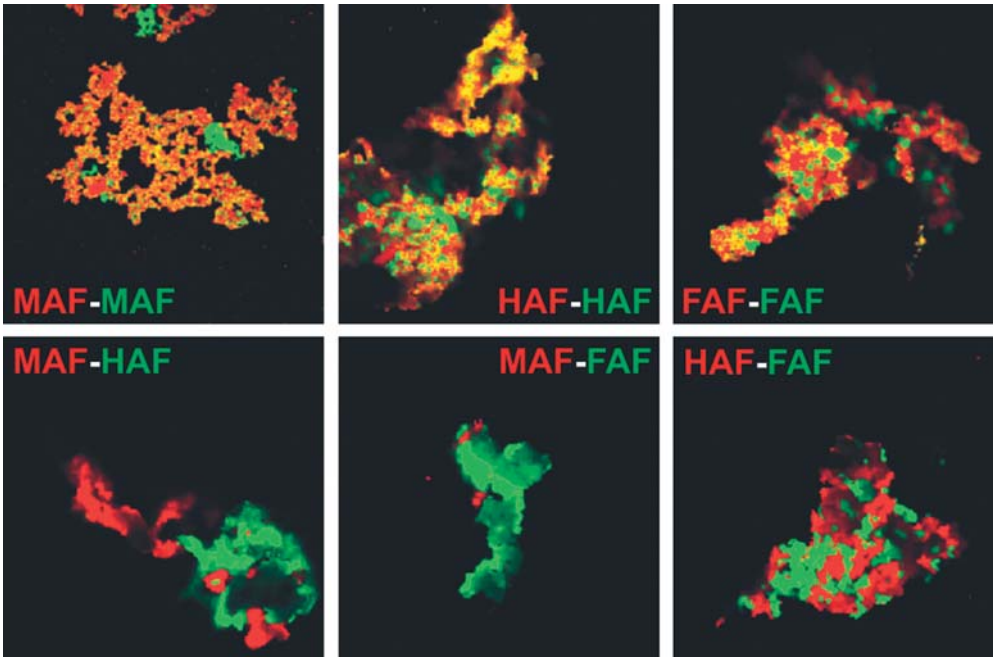

Figure 3. Ca^{2+}-dependent species-specific recognition and adhesion of AFs. Red and green fluorescent beads were coated with AFs from either *M. prolifera* (MAF), *H. panicea* (HAF), or *Ficulina ficus* (FAF). Pairwise combinations of coated beads were mixed, allowed to aggregate for 1 h on a rotary shaker in the presence of 10 mM Ca^{2+}, and finally photographed with a confocal microscope. The three images in the upper row show controls where red and green beads were coupled to the same AF (MAF-MAF, HAF-HAF, FAF-FAF). The dominant yellow color indicates mixed bead populations. The lower row images correspond to the heterologous mixtures (MAF-HAF, MAF-FAF, HAF-FAF), with green beads coupled to AF from one species and red beads to AF from a different species. The green and red areas without any yellow indicate that the beads have sorted out and separated. Magnification × 40. Copyright 1998. Adapted from Jarchow and Burger [110]. Reproduced by permission of Taylor & Francis, Inc. (http://www.tandf.co.uk).

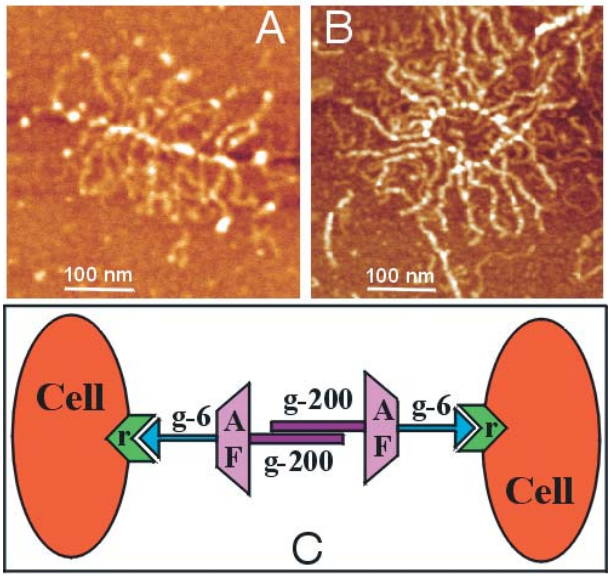

Figure 4. AF-mediated sponge cell adhesion. (*A*) Atomic force microscope (AFM) image of *H. panicea* AF. (*B*) AFM image of *M. prolifera* AF, whose overall molecular mass is ~20 million Da. The color-encoded vertical *z*-scale of both images corresponds to 3 nm. (*C*) Components involved in AF-mediated cell adhesion in *M. prolifera*: g-200, glycan mediating homologous AF-AF interactions; g-6, glycan binding the cell surface receptor (r); AF, core protein. From Jarchow et al. [122], with permission.

Sponge AFs promote adhesion of sponge cells via a mechanism depending on the presence of Ca^{2+} ions [95, 96, 99], through a two-step process involving (i) a Ca^{2+}-dependent self-aggregation of the negatively charged AF complexes [102, 111], and (ii) a Ca^{2+}-independent binding to cell surface receptors [111] (fig. 4). AF receptors have been partially purified from *M. prolifera* [112, 113] and *Geodia cydonium* [114], and found to be nonintegral membrane proteins. A putative aggregation receptor from *G. cydonium* has been cloned [115], which features scavenger receptor cysteine-rich (SRCR) domains. In vertebrates, SRCR repeats are found predominantly in receptors of the immune system [116].

Cadherins are a family of Ca^{2+}-dependent adhesion molecules that bind cells via homophilic interactions [117, 118]. Cells expressing distinct cadherins aggregate separately when mixed in culture. Cadherins are responsible for the cell sorting that is necessary to distribute different cell types to their proper positions during development. During embryogenesis, the expression of different cadherins is spatiotemporally regulated, and correlates with events that involve cell aggregation or disaggregation. Another group of cell adhesion molecules are those related to the Ig superfamily, which generally have Ca^{2+}-independent heterophilic binding [119]. The dual nature of AF-promoted cell adhesion that involves a Ca^{2+}-dependent homophilic binding and a Ca^{2+}-independent heterophilic interaction with cell surface receptors confers on AFs characteristics of both groups of molecules. Besides being the key molecule for sponge cell-cell adhesion, the AF has also been described to participate in cell-matrix interactions such as collagen binding [120, 121].

General structure of AFs

The supramolecular structure of the *M. prolifera* AF (MAF) was elucidated by using immunochemical and electrophoretical procedures, combined with atomic force microscope (AFM) imaging [122]. Twenty units from each of two *N*-glycosylated proteins, MAFp3 and MAFp4, form the central ring and radiating arms of MAF, respectively (fig. 5), stabilized by a hyaluronidase-sensitive component [122]. The average length of an arm is about 140 nm, and the average circumference of the ring is about 280 nm. Each of the 20 arms is attached to one of the 20 globular structures in the ring in a 1:1 stoichiometry, with an estimated molecular mass for the whole molecule of 2×10^7 Da [76, 77]. The structure of the AF from *G. cydonium* has also been investigated, and found to be equivalent to that of *M. prolifera* in the dimensions and sunburst-like appearance [99], core proteins sequence [123], glycosylation [124], and role in specific sponge cell adhesion [99]. AFs extracted from *Halichondria bowerbankii*, *Terpios zeteki*, *H. occulata*, and *Halichondria panicea* have a very similar size and structure, but with a linear backbone [103, 122] that strongly resembles the classical image of hyalectans (fig. 4A). Although the use of several preparative techniques to isolate AFs indicated that the purified particles were homogeneous [76, 98, 125], several glycoprotein subunits and associated proteins are assembled around the PG core structure that is the AF itself [77]. MAF has been described to contain at least 11 different subunits that can be dissociated upon Ca^{2+} removal [126]. Besides MAFp3 and MAFp4, two other glycoproteins have been identified through amino-terminal sequencing, and multiple protein bands in SDS-polyacrylamide gels suggest the existence of several other components either belonging to the AF complex or copurifying with it [127]. Two cell surface glycoproteins of 68 and 210 kDa have been found to be associated with MAF (fig. 6C), and have been partially chemically characterized [128–130]. The 68-kDa glycoprotein was detected in far-Western analyses of cell membrane extracts from *M. prolifera* and *H. panicea* by biotinylated AFs from several sponge species and by biotinylated glycans obtained from these AFs [122]. A sponge 68-kDa protein also bound to CS and HA [122]. The AF from *G. cydonium* is tightly associated with glycosyltransferases [131, 132], lectins [88, 133, 134], and a series of different glycoproteins [121, 135]. Polyclonal antibodies raised against purified AF from *G. cydonium* inhibited cell aggregation of homologous cells but not of heterologous *Tethya lyncurium* cells

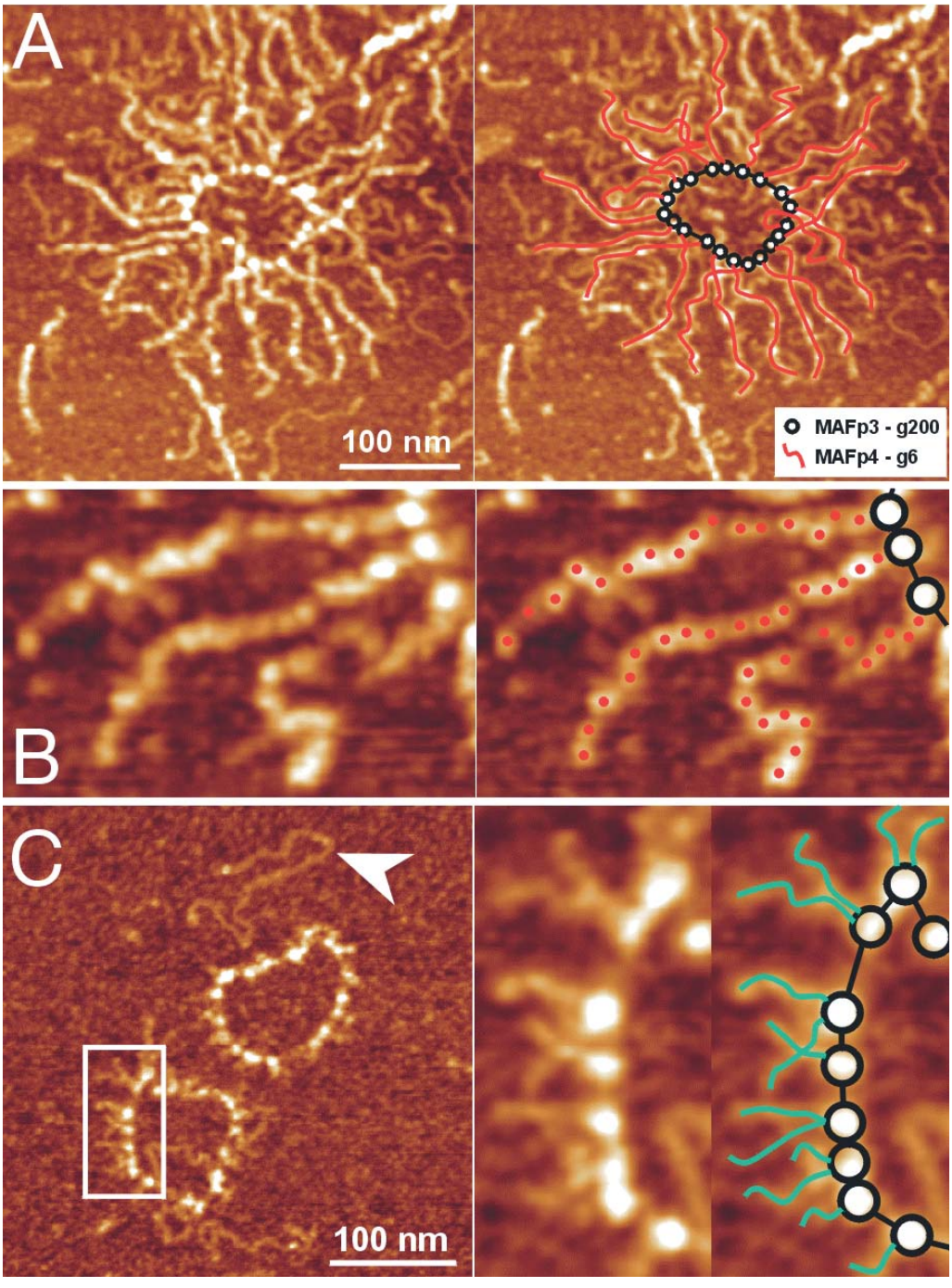

Figure 5. Proposed model for the structure of MAF. (*A*) AFM image of native MAF showing the localization of MAFp3 in the ring (black circumferences) and of MAFp4 in the arms (red lines). MAFp3 carries the g-200 glycan and MAFp4 carries the g-6 glycan. (*B*) Detail of an AFM image of MAF showing the ~15–16 domains (red dots) observed in each arm in the native structure. (*C*) AFM image of MAF rings and of an isolated rod-like chain (arrowhead). We suggest that in the native AF, the rod-like molecule runs along the circumference of the ring, stabilizing its interaction with the arms. The enlarged inset shows a detail of the ring structure with short protruding chains that might represent the g-200 glycan (turquoise lines). The color-encoded vertical *z*-scale of all the images corresponds to 3 nm. From Jarchow et al. [122], with permission.

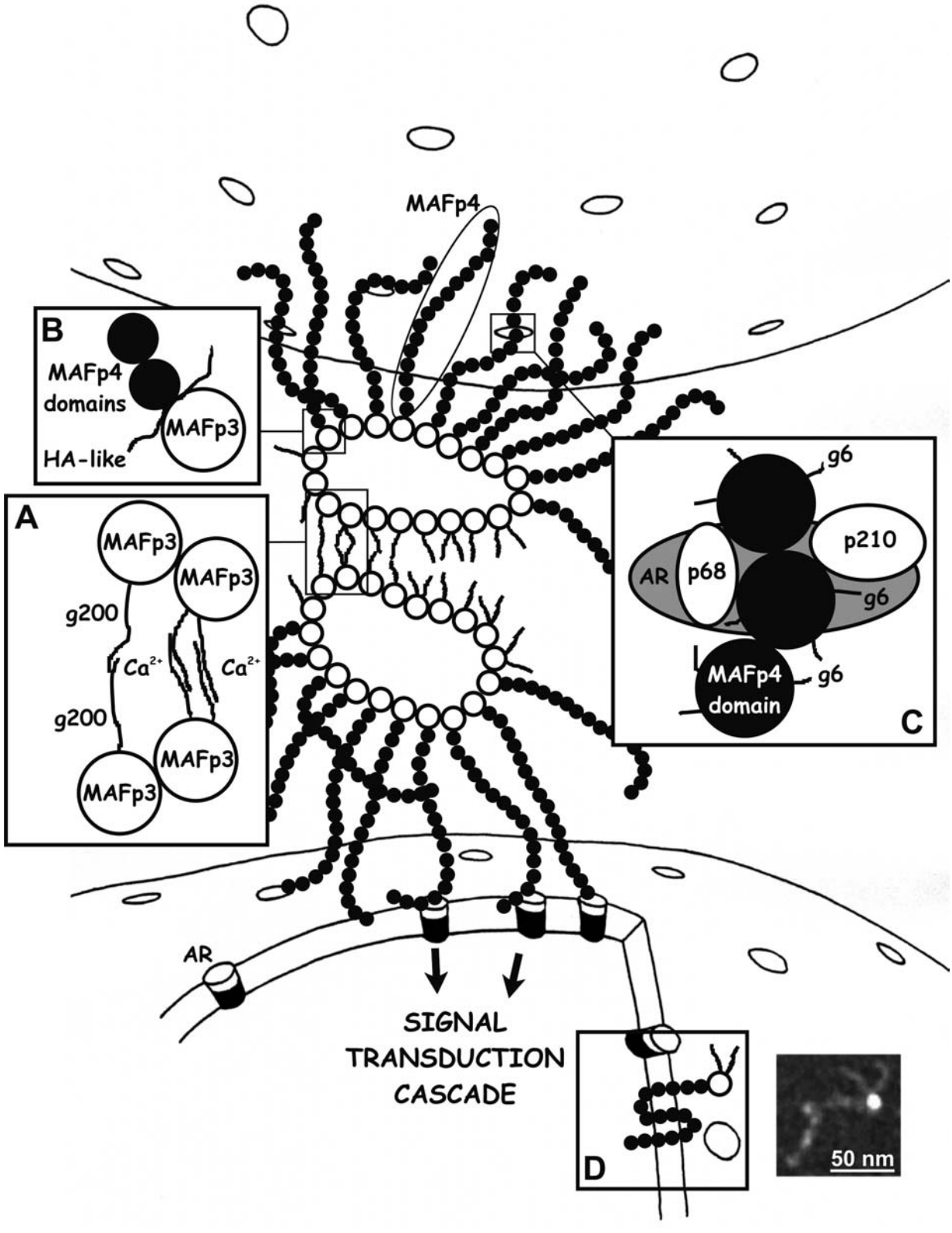

Figure 6. Proposed model for MAF-mediated sponge cell adhesion. To simplify the model, only half of MAFp4 and of g-200 chains are represented in each MAF molecule. (*A*) Ca^{2+}-dependent self-interactions of the MAFp3-bound g-200 glycan mediate self-binding of two AF complexes. (*B*) MAFp3 and MAFp4 interact through an HA-like molecule. (*C*) The binding through the g-6 glycan to cell receptors (AR) probably involves other pericellular proteins like, for *M. prolifera*, p68 and p210. (*D*) To illustrate possible direct interactions of MAFp3 and MAFp4 we have included a putative membrane-linked MAFp3-MAFp4 monomer. The inset shows an AFM image of a MAFp3/MAFp4 unit. Note the two chains protruding from MAFp3, supposed to be the g-200 glycan.

[136], thus confirming that the species-specific activity resides on the AF structures. However, whereas in *Microciona* the dissociation of the AF structure eliminated its activity, in the sponges *G. cydonium* and *Suberites domuncula*, subunits of 47 and 55 kDa were purified and shown to carry the species specificity of binding [135, 137]. Recently, the 47-kDa subunit has been cloned [137] and found to compete with the AF complex for the binding sites at the cell surface. We will center the discussion below on the PG structure that constitutes the bona fide AF, without attempting to enter into details about the constellation of associated proteins, whose undoubtedly important effects on AF function are still not clarified.

The glycan moiety of AFs

The main carbohydrates present in the composition of MAF are fucose, glucuronic acid, mannose, galactose, and GlcNAc, found in variable amounts depending on each preparation [101, 138]. The carbohydrate composition of two main MAF glycans suggested the presence of previously unknown structures [70, 139], as confirmed later with the elucidation of two oligosaccharide epitopes, a pyruvylated trisaccharide and a sulfated disaccharide, recognized by monoclonal antibodies inhibiting sponge cell aggregation [140, 141] (fig. 7A, B). The structure of a third non-reactive oligosaccharide has also been determined [141] (fig. 7C), and confirmed the singularity of sponge glycans, in accordance with results obtained in the analyses of sulfated polysaccharides purified from a variety of marine sponge species [142]. Moreover, the existence of two distinct sites that participate in the aggregative interaction, while a similar charged structure is not involved, suggests that the binding is not just caused by nonspecific electrostatic interactions of the acidic carbohydrate chains. The role of carbohydrates in cellular adhesion of *M. prolifera* has also been studied using surface plasmon resonance detection [143]. The data obtained showed that the sulfated disaccharide interacts with itself not simply through electrostatic interactions, since other sulfated carbohydrates analyzed with the same procedure did not self-associate. These results strongly support the existence of true carbohydrate self-recognition. No information is as yet available on the overall structure of the MAF glycans. Despite containing GlcNAc, galactose, uronic acid, and sulfate, they seem not to represent any of the main classes of GAGs of higher animals, as suggested by the large amount of fucose and their resistance to common GAG-degrading enzymes [70]. Preliminary nuclear magnetic resonance (NMR) analyses of the intact glycan chains indicated such structural diversity that the possible presence of a common backbone with a repeating saccharide unit could not be determined [141].

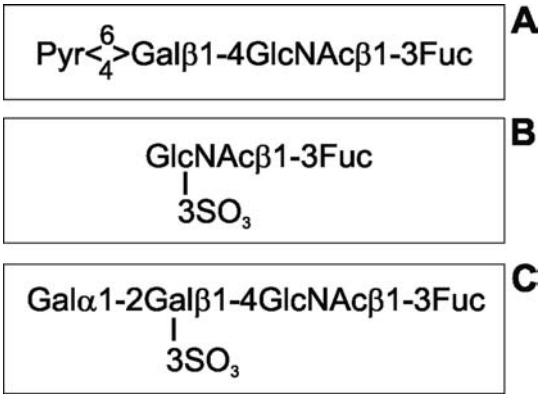

Figure 7. Structures of three carbohydrate units present in the AF of *M. prolifera*. *A* and *B* are directly implicated in species-specific cell adhesion, since monoclonal antibodies directed against them inhibit the aggregation of sponge cells. Structure *A* is present both in the g-200 glycan mediating self-interaction of the AF and in the g-6 glycan binding to cell membrane receptors, while structure *B* is only present in g-200. Structure *C* is a major oligosaccharide recognized by a third monoclonal antibody.

Antibodies raised against RHAMM efficiently blocked the binding of HA to *Microciona* cells, and, reciprocally, preincubation in the presence of HA dramatically reduced the binding of the anti-RHAMM antibodies to the cells [144]. Hyaluronidase digestion of MAF completely abolishes its cell aggregation activity [122], a result consistent with our previous observation that addition of exogenous HA to MAF preparations with low activity raised the aggregation efficiency of the factor to that found in the most active preparations [127]. Finally, the treatment of MAF preparations with the highly HA-specific *Streptomyces hyalurolyticus* hyaluronidase eliminated HA-like structures that could be identified in AFM images of dissociated MAF, with a concurrent modification of the electrophoretic mobility of MAF glycoprotein components [122]. Although all this evidence suggests both the presence of HA in MAF and its functional role in sponge cell adhesion, fluorophore-assisted carbohydrate electrophoresis analysis of limit hyaluronidase digests failed to detect any HA-derived polysaccharide in MAF [122]. Moreover, the ability to synthesize HA has not been detected in insects and lower animals [72]. We therefore hypothesize the existence of a novel GAG structure closely related to HA [122], whose nature is still unknown (fig. 6B). The only GAG shared by sponges and higher animals is chondroitin-4-sulfate [145]. Chondroitin-6-sulfate, HS, DS, heparin, and HA have not been detected in sponges so far.

Glycoprotein subunits of MAF obtained through dissociative denaturing procedures were able to bind *Microciona* cells, but with decreasing affinity, which correlated linearly with the decreasing size of the subunits [146], suggesting polyvalency in the cell-binding site. A small glycopeptide of 10 kDa obtained after trypsin digestion of MAF showed very little affinity for homotypic cells in its

monomeric form, but reconstitution of binding affinity at the same order of magnitude as the native molecule could be obtained by polymerizing the glycopeptide fragment by chemical cross-linking [101]. Indeed, carbohydrate-protein interactions are characterized by weak forces that are greatly augmented upon multimerization [147]. Eventually, both MAF-cell and MAF-MAF binding affinities were reconstituted upon chemical cross-linking of protein-free glycans isolated from the AF complex into large polyvalent polymers [70, 138, 139]. This suggested an active role for the carbohydrate moiety of MAF in the aggregation of sponge cells. For the AF-mediated cell adhesion in *G. cydonium*, the interrelation between glycosyltransferases and glycosidases has been described to control the process [148]. Cell aggregation would occur via a linkage of the AF with the aggregation receptor (AR) after addition of glucuronic acid to the AR by a glucuronosyltransferase. Conversely, cell separation would result from the activation of a cell membrane-bound β-glucuronidase. Interestingly, carbohydrate-modifying enzymatic activities have been found to be associated with the AF [131, 132]. In *G. cydonium*, desialylated cells showed a reduced aggregation potency; during aggregation, however, enzymatic sialylation of the AR has been described to occur in the presence of the AF complex [132]. In *M. prolifera*, MAFp4 has significant sequence homology with a bacterial endoglucanase [149], suggesting that GAG-modulating activity might reside in the AF itself.

Binding of MAF to the cells has been shown to be species specific [111] and the result of the polyvalent interaction with cell surface receptors of a repetitive *N*-linked MAF glycan of about 6 kDa termed g-6 [139, 150] (fig. 4C). The g-6 binding to cells likely requires several accessory proteins besides the actual receptor. In the model proposed for *G. cydonium*, the AF proteoglycan is associated with at least two adhesion-promoting proteins [137], one of which associates with a lectin which, in turn, binds to the cell membrane receptor. In *M. prolifera*, the 68- and 210-kDa proteins described above can be the corresponding counterparts of this cell-binding complex (fig. 6C). Furthermore, MAF-MAF self-interaction has been demonstrated to be based on carbohydrate-carbohydrate interactions mediated by an about 200-kDa glycan termed g-200 [70, 138] (fig. 4C). When protein-free g-200 glycan was cross-linked into polymers of similar valency as the native AF, the Ca^{2+}-dependent self-interaction activity could be reconstituted [70], thus indicating that g-200 self-interaction is also polyvalent and cooperative. Unlike the g-6 binding to cells, however, it can be reproduced in the absence of protein, thus representing a genuine carbohydrate-carbohydrate interaction (fig. 6A).

The binding strength between two individual AFs was measured with AFM (fig. 8) [151, 152]. Under physiological conditions, the average forces of individual interaction sites between two AF molecules have been esti-

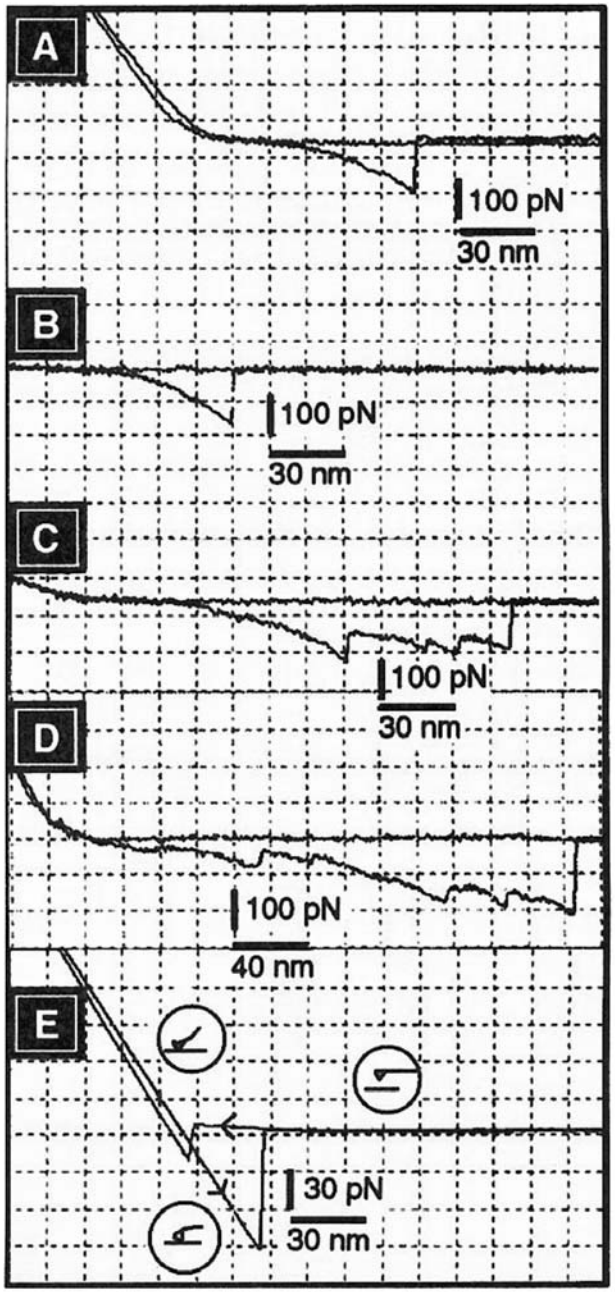

Figure 8. Typical force-distance curves for the interaction between individual MAF molecules immobilized on the AFM cantilever and surface in Ca^{2+}-containing Tris-buffered sea water. The x-axis shows the vertical movement of the cantilever, and the y-axis shows the bending of the cantilever and thus the force acting on it, as depicted by the encircled schemes in *E*. Repulsion forces acting on the cantilever are indicated by the curve sections above the horizontal approach-retract line, whereas those below it represent adhesive forces between the cantilever and the surface. Approach curves go right to left and retract curves go left to right (see arrowheads on curves in *E*). *A–D* represent typical MAF-MAF interactions, whereas *E* is an example of the interaction between two gold surfaces covered with self-assembled monolayers (1-dodecanethiol). Reprinted with permission from Dammer et al. [151]. Copyright 1995 American Association for the Advancement of Science (http://sciencemag.org).

mated to be about 40–50 pN [152]. Often, multiple jump-off events of 40 ± 15 pN were seen in a singular approach-and-retract cycle (fig. 8C, D), while the average forces measured between a single pair of molecules amounted to about 125 pN (fig. 8A, B), with maxima up to 400 pN [151], enough to hold the weight of ~1600 cells. This interaction was specifically blocked by a monoclonal antibody raised against g-200, confirming the carbohydrate nature of at least one of the binding partners. These results seem to indicate the existence of multivalent binding of three to ten binding pairs [151], a result, as we will see below, in accordance with the proposed structure of sponge AFs [122]. The species specificity of the self-interaction of g-200 has been investigated by measuring with AFM the interaction forces between glycans isolated from different sponge species [Bucior et al., unpublished observations]; preliminary results indicate that the interaction forces between g-200 from the same species are significatively higher than those measured between g-200 from two different species.

The protein moiety of AFs

Each MAFp3 ring unit carries one or two copies of the g-200 glycan involved in homologous self-interactions between AF molecules (fig. 6A), whereas each MAFp4 unit carries about 50 copies of the g-6 glycan that binds cell surface receptors (fig. 6C) [122]. In force interaction experiments performed with AFM, the interaction between a single pair of PG molecules was found to be broken at a distance of about 160 nm [152] (fig. 8C), consistent with a model where the self-interacting units are on the ring structure, whose maximal elongation upon stretching is about 140 nm, i.e., half of its circumference length. Other AFM force measurements gave maximal stretches of up to 260 nm [151] (fig. 8D), which probably correspond to measurements made between open AF rings like those occasionally observed in AFM images [122]. From the multiple jump-offs of the AFM force interaction curves (fig. 8C, D), one can deduce that the MAF-MAF interaction binding sites are not localized in one region of the ring, a result again consistent with the proposed distribution of g-200 throughout the ring (figs. 5, 6).

MAFp3 is a 35-kDa protein containing several putative *N*- and *O*-glycosylation sites (fig. 9) that is translated from a variety of transcripts ranging from 1 to ~12 kb [127, 149]. As seen in AFM images [122], one or two short chains protrude from each of the 20 MAFp3 globular structures forming the ring of MAF (fig. 5C). Whenever rings were observed to form dimers or larger aggregates, they appeared to be interconnected through such chains, thus suggesting that these are the g-200 glycan (fig. 6A). In AFM force measurements, the interaction between purified g-200 was broken at a distance of about 25 nm [Bucior et al., unpublished observations], a figure consistent with the measured length of the chains supposed to be the g-200 glycan, shown in figure 5C. A dibasic motif such as that found in RHAMM [21] is located in MAFp3 [127]. If this motif is positioned to interact with negative charges in analogy with the RHAMM interaction with HA, this site may be crucial for binding an HA-like structure and MAFp3 [144]. The already mentioned HA-like molecule observed in AFM images of dissociated MAF [122] has a length equal to the circumference of the MAF ring (fig. 5C), suggesting that in the native complex it runs along the MAFp3 subunits that form the ring (fig. 6B), exactly as HA, aggrecan, and link protein do in a linear arrangement in hyalectans (fig. 1). MAFp3 has been found to be an extremely polymorphic protein, as deduced from direct cDNA sequencing of several different allelic forms and from restriction fragment length polymorphism analysis of genomic DNA isolated from different individual sponges (fig. 10). Chemical deglycosylation of MAFp3 extracted from different individuals followed by mass spectrometry of its peptidic fragments revealed that the polymorphisms detected at the DNA level represent, at least partially, variability in the actual proteins present in each individual [149].

MAFp3 is translated, in some cases, as a much longer polypeptide together with MAFp4 [149], which has an estimated molecular mass of ~400 kDa. MAFp4 is also polymorphic, contains putative transmembrane regions, and has a stretch sharing 30% homology with the cytoplasmic domain of the Na^+–Ca^{2+} exchanger [127], a membrane protein responsible for the maintenance of low intracellular Ca^{2+} levels. Several laboratories are reporting sequence and structural homologies of Na^+–Ca^{2+} exchangers, and therefore of MAFp4, with a number of apparently unrelated proteins such as integrin β4 [153], the ECM3 protein involved in sea urchin embryo development [154], and VLGR1, a G protein-coupled receptor [155]. Another important feature of MAFp4 is that it contains repeated domains (fig. 9). The deduced partial polypeptide consists of a 16-fold reiterated motif, which shows significant similarity with a repeat in an endoglucanase gene from the symbiont bacterium *Azorhizobium caulinodans* [149]. The modular nature of the sequence of MAFp4 is structurally reflected in its AFM images [122], where it shows as a string of pearls (fig. 5B) similar to the transmission electron microscope images of modular PGs like perlecan. An alternative role for the hydrophobic stretches identified as possible transmembrane regions could be related to the internal folding of MAFp4 in its structural domains. From the estimated size of the full-length cDNA it can be deduced that about two of the ~117 residue-long tandem repeats of MAFp4 (fig. 11) will fold into each of the 16 bead-like domains observed in AFM images (fig. 5B). As in the modular PGs, there is a significant inner homology between the

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   1 YAGVTIGFEDVSISVNEPDGVATLTVVVLDRLLQRPVEVFLSTADNTATSSGVLDFLNLTDLVLEFDEDTLALEINVTIIDGDILENFETFFANLDTDDP 100
 101 QVVLAPDSAEVEIRDINDEITVGFDPTDYEVNEADGTAILFVVLLNGTLERNVTVLFETASDSATQDAPADYEATSGLLTFSPDVDVIPVPVSIINDTIV 200
 201 EGNETFVGLLDAQGQPVIVDPPREQAMVLITEDPADEVVIGFEETVYPSMEGEEVTVCVVLVSGELERDVVVRVSSADGTATGGDDYVPVVGEELTFNAD 300
 301 TARECFNFTTIEDDVLEPEENVSLILMSDDPMVVTEPELSEVIISDTNRVVVGLEETVLFIDEEDGATIQVCVVLVEGTVDRDVTVNIATSDGTATSIAP 400
 401 MDYEALNVDLAFVPGEIPRVCDNVTVLNDDISENPEDFFVTLSTTDPSAEVDPDRDVATATINDLDEIVIGFVLEMDNVLEDDGFIDILVEVKNFGQLER 500
 501 NVTVMFFTVDGTGDRPALDDSDYNSTTASLLFLPGQETTPLSVRVPINTDGDLEGPEIFFADLMTDEERVTLDPNRTTIVIIDADEISVGFVDAPYQGLE 600
 601 ESEFATLTIAVMGAELGTELLVNLTTEDIGEAIAGVDYEPLDIVLTFSEDVRTIQINVTLIDDNFLEGDEDFMGSLEILTTGTNAQLVPGTETAIVTILD 700
 701 NDMVVIGMDETMVNVDEDIGQRELCASIMSGELMREVQVMVVYADRTASDGDDYTSMSHTLTFGPDVDDQQCFNVTIVDDDVYELRGDFFVNLTTSEPLV 800
 801 TLMPTTTVVMIDDEDEVIIGWVLPSYQAAEADGSVEICAELVSGAASVGSVLPLVNIVLTDGTAIVGTDTGMQTSPTFFNFQSTTPTTMCTTVTLIADDI 900
 901 LEDPEHIFANLEIPTTPRVVIDPPQTQVNITDSDTAVIGFDPENYQVFEEDGVATPFVAVLDGTLDTEVEVQFDTLNGDAMDPNDFTATTVLLLFSPTQT 1000
1001 RVPVNVTIIDDNVVERNETFPANLTLISDNDRVTIDPPMATVLIIDNDVPVIGFEQTQHVIVEVENDTAVIQVCVALMPPPSLDREVTVSLSSQNGTAEE 1100
1101 PNDYNSVMADLVFNADNDVQCVNVTANEDDIIEGTENFTLVLDSDDNVLLLPEEAEVQIMDLGSILVMFAEPVYTFNEEDGLGIIELVKSASVSQPFTVR 1200
1201 VFGGPSNQSDIERSADGIDIIVEFDAGLMAPGSIFINFTLTDDEIALEADETYNVQFEILETLGNIVRPGILPQTQVIVLDPDVVNVGFGRPSVTVNESD 1300
1301 GEFMMCVLKDRETVFPVEVTISTSPDTAIEGADYDPANVPSSVTIPADENMTCFTTDTVLDDILALEEDEVFLLDIENVLPDDPRINVEADNVTVTILDD 1400
1401 DVPTIGFEQTQYSVRENDTVGTEVCVSVMAPPSLGSTVTVMLVSMDGTAQAPDDFAAVMEDLVFGADIDLLCVNITATGDDVAEGMENFTLALASDNNDV 1500
1501 ILMPEQADVEILDSGMIFVMFAEPVYTYNEEDGVGTIEIVKTGATSEPFTVRVYGGPSNQTDIERSADGIDIIVEFQAGPSAPDSIFINFTLTDDEVALE 1600
1601 ADETYDVRFEILETLGDIVQPGILLETQVVVLDPDVVSVGFGRPNVTRNESDGQFTMCVVKDRDTAVPVVVTISTADGTATEGQDFDPANVPATVIIDPD 1700
1701 SAMECFDSNAILDDEVALEDEEFFLLLAELLDPDDPRVNLTDPETMVIILDDDVVSVQFEQPTYTYDETDGSAQVCLVKDAETATSFTVDNIVTEDGTAM 1800
1801 DGVDYTGGPYSVMFDAPADDIKCIMINLTVDNVDEDTENFVVSLAELVADEANVLVGVQNVTTVNIVDRGVTIFVNDTVIGDPLFTVPIYVPEDQLDAMN 1900
1901 LSQLFLCYEIHGVSDQWFNLVTDECTSVNARYGMFNQDLNVIDEIGVRAVDTADQCVNIRVDVGNCTADVNDVALDVMGRYSMNGVSVRRYRNRVRISVP 2000
2001 NCNDLTLVMWVFCETRTLQDPFDGSEVTGDMIKFVVMRGLNTGNRPSHGLLGQFWNIPVSIAPYTGLLRDNSVAEGRFVINITTSGCSDPPVQRSYTGWLY 2100
2101 DLTWEFQEGPCLYVGNRQAGPIYEVMDPNDNVIEDRYKNYKVDSAFSEEGFDFGIFMEERCDISVMPTTEAPSAVPTTEAPTSEEPTTGSGLGSGIGDEP 2200
2201 TTSIP*

DXD, DXNDN: calcium binding motifs
N: potential N-glycosylation sites
SG: potential CS attachment sites
RGD: integrin binding motif
RRYRNRVR: potential HA binding motif
[ST]XX[DE], [ST]X[RK], [RK]X(2,3)[DE]X(2,3)Y: Ser, Thr, and Tyr phosphorylation motifs, respectively
*: stop codon
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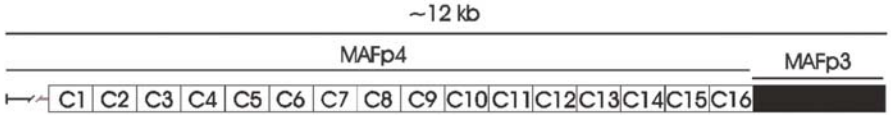

Figure 9. cDNA-deduced sequences of MAFp3 and (partial) MAFp4 isoform C (GenBank accession number AF020902), represented according to the scheme at the bottom of the figure. The MAFp4 domains (C1–C16) have a mean size of 117 residues. According to the estimated full length of the MAFp4 cDNA, the sequence shown in the figure corresponds to about half of the total cDNA length. The underlined carboxyl-terminal region corresponds to MAFp3, and the rest to MAFp4. Double-underlined stretches correspond to putative transmembrane regions. Adapted from Fernàndez-Busquets et al. [149], with permission.

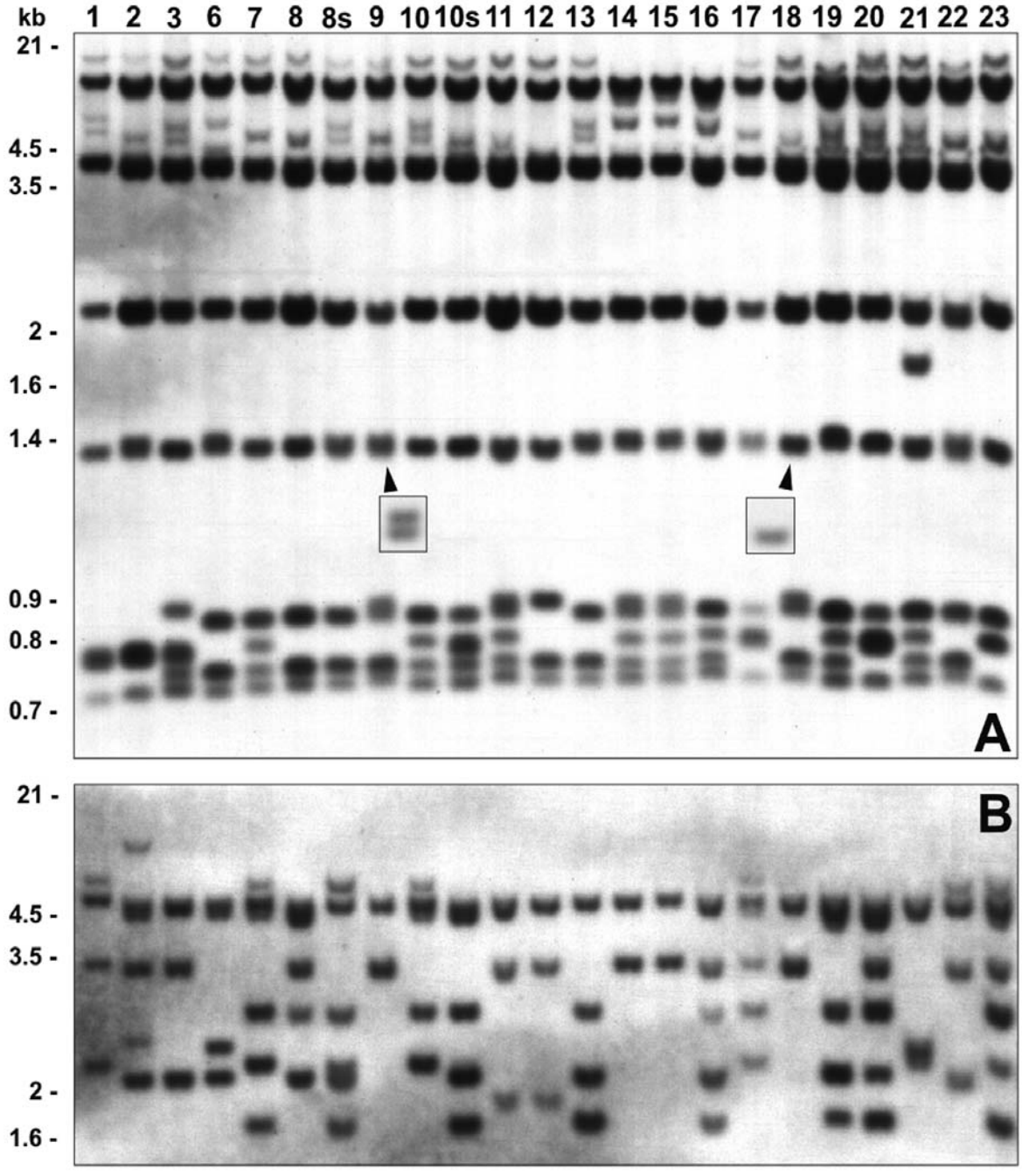

Figure 10. Restriction fragment length polymorphism analysis of *M. prolifera* genomic DNA using MAF-related probes. *Dra*I-digested genomic DNA from each of 23 different individuals was loaded on a 1% agarose gel. The Southern blot was consecutively hybridized to probes corresponding to MAFp3 (*A*) and MAFp4 (*B*). The insets show two examples of conflictive bands that could be resolved in longer runs. From Fernàndez-Busquets et al. [149], with permission.

Figure 11. Intron placement and putative β strands in the 16 sequenced tandem repeats of MAFp4 isoform C. Cysteines are boxed. Stretches of at least five amino acids that form or do not break β strands are shaded. Determined intron positions in repeats 10–16 are marked with arrowheads. From Fernàndez-Busquets et al. [149], with permission.

repeated elements in MAFp4 [149] and the intron placement (fig. 11), suggesting that this protein has also used exon shuffling and duplication during evolution. The use of protein modules and the homologies with disparate proteins suggests that MAFp4, as it has been proposed for most modular PGs, can be involved in numerous biological processes. Such a modular nature is typical of many ECM constituents involved in different aspects of cell binding [156].

Both MAFp3 and MAFp4 contain abundant Ca^{2+}-binding sites and *N*-glycosylation motifs, as well as numerous putative phosphorylation sites (fig. 9) that, as we will see below, might have importance in the event that AFs are directly involved in signal transduction processes. *G. cydonium* cDNA libraries were screened for a sequence related to MAFp3/MAFp4. A positive clone was mentioned to have similarity with P- and E-selectins [123]. Such data will be very important for understanding evolutionary relationships between AFs and other PGs, since hyalectan genes code for a protein module found in selectins [17].

If the MAF circular core were open, the resulting linear structure would be remarkably similar to hyalectans, although the building blocks of both molecules lack sequence homologies. MAFp4 corresponds to the glycosylated core protein of the proteoglycan monomer, carrying the *N*-linked g-6 glycan instead of *O*-linked GAGs. The amino-terminal end of MAFp4 has not been elucidated, and therefore the possibility remains open that, like the core protein of hyalectan PG monomers, it contains a binding domain that interacts directly with the HA-like molecule or indirectly through close contact with MAFp3 which interacts directly with the HA-like component (see fig. 6B). MAFp3 is the sponge counterpart of the LP, both molecules having very similar sizes [127, 157]. Like LP [157], MAFp3 is *N*-glycosylated and contains eight cysteines involved in disulfide bonds [127] that could have a structural role similar to the five intramolecular disulfide bonds of LP. Although translated as a single polypeptide together with MAFp4, in vivo they seem also to exist as independent proteins [122], as the result of a proteolytic processing like that described for some hyalectans [7, 12], thought to be a general feature required for their function.

MAFp3 has an amino terminal domain with a putative binding motif for the HA-like backbone polysaccharide, and a carboxyl-terminal domain with putative *O*-glycosylation sites (fig. 9). In some MAFp3 isoforms, each domain is encoded in a separate exon while in other isoforms, both domains are in a single exon [157]. Unlike LP, though, MAFp3 does not contain an Ig-like domain. However, the amino terminus of MAFp3 is translated as a continuum with the carboxyl terminus of MAFp4, whose tandem repeats strongly resemble Ig-like domains [149] (fig. 11).

The ring of MAF is not linearized by EDTA or reducing conditions [103], and therefore its circularity is not based on calcium-mediated interactions or on disulfide bonds between protein chains. Guanidine hydrochloride and hyaluronidase are also ineffective against the ring structure [122]. This also excludes the stabilization via ordinary noncovalent interactions like those present in hyalectans between proteoglycan monomer, LP, and HA. Chemical deglycosylation, on the other hand, completely disrupts the ring [122], suggesting that covalent interactions between glycans or between glycans and proteins are responsible for its integrity, although such treatment can affect other protein-mediated interactions. However, the ring is unlikely to be stabilized by covalent peptide bonds, because neither MAFp3 nor MAFp4 is internally cleaved by the chemical deglycosylation procedure, and both retain their original molecular masses.

The above-mentioned characteristics clearly place AFs within the PG family of macromolecules. However, the described particularities of their carbohydrate and protein moieties and, as we discuss below, their proposed functions in processes other than cell adhesion, indicate that they should be considered as the first members of a new modular proteoglycan subgroup. Given their so far exclusive distribution in sponges, we propose for them the term spongicans.

Involvement of spongicans in self-nonself recognition

When tissues from different individuals of a given sponge species are brought into contact, they either fuse or reject through cellular events similar to those observed in vertebrate grafts [reviewed in ref. 158]. Molecules from *G. cydonium* have been characterized during the last years that resemble components of the immune system of higher animals, such as Ig-like molecules [145, 159–161], SRCR repeats [115, 162], and short consensus repeats (SCRs) [115]. The prophenoloxidase-activating system, a widely used defense system in animals, has also been shown to be present in sponges [123]. There is, though, a peculiarity of the sponge self-nonself recognition system that so far has not been described in other phyla, which is the possible involvement of PGs in histoincompatibility reactions [158]. In 1970, Van de Vyver [163] described for the freshwater and marine sponges *Ephydatia fluviatilis* and *Crambe Crambe*, respectively, the existence of different strains, or clones, that never merged with each other. Curtis and Van de Vyver [105] prepared AF from two nonmerging strains of *E. fluviatilis* termed *alpha* and *delta*. When the aggregation of *alpha* and *delta* cells by homologous or heterologous factor was investigated, homologous factor increased, whereas heterologous factor decreased the adhesiveness of the cells, suggesting involvement of AFs in allogeneic recognition. In a later report, Curtis [164] studied graft rejection in situ in a population of the marine sponge *Hymeniacidon* sp. 'Interaction modulation factors' were then purified from each individual, and measurements of their adhesion efficiency were made on cells of all the sponges between which grafting had been done. While the adhesiveness of those cells treated with a factor prepared from the same sponge or from another strain whose grafts were accepted was always significant, the use of a factor from those strains that were not graft compatible with the strain type of the cells led to a considerable decrease in cell adhesiveness. The procedure described by Curtis to isolate the 'interaction modulation factors' is equivalent to the method used to extract MAF, suggesting that the molecules are analogous. Indirect involvement of the AF in xenograft rejection in the *Geodia* genus has also been postulated [165].

If the only function of MAF were to determine species specificity, one form of the molecule would probably suffice for each sponge species to be distinguished from the others. Since AF is a supramolecular complex, though, one can argue that different core protein subunits might cooperate to assemble the heteromeric structure. However, each genetically distinct sponge individual was found to possess a different combination of genomic DNA coding sequences for MAFp3 and MAFp4 [149, 157] (fig. 10). Such interindividual variability was also observed when the protein sequences of MAFp3 purified from different sponges were analyzed by gas chromatography-mass spectrometry [149]. Polymorphism has also been detected in the coding region of the human aggrecan gene [166], resulting in individuals with core proteins of different length. One of the MAF-associated glycoproteins described above whose amino terminus was sequenced [127] migrates in SDS-polyacrylamide gel electrophoresis as a ~210-kDa band, and most likely corresponds to the previously described p210 [129, 130]. This glycoprotein carries the MAF pyruvylated epitope also present on g-6 and g-200 (fig. 12A). p210 is variable between individuals in a species, and this intraspecific polymorphism is at least partially due to the glycan moiety [157], since the glycan products resulting from protease digestion of p210 from different individuals had different electrophoretic mobilities (fig. 12B). Besides these species- and individual-specific sponge PG forms, arguments in favor of intraindividual variability have also been provided. AFM images of MAF isolated from a single individual and immunolabeled with monoclonal antibodies specific for the sulfated disaccharide epitope, followed by a gold bead-derivatized secondary antibody, revealed two populations of MAF molecules [152]. While some MAFs were completely covered with gold beads, others did not react (fig. 13), suggesting the presence of a different oligosaccharide structure, although lack of glycosylation of those nonreacting molecules can also be invoked to explain this result.

An obstacle to determining a hypothetical role for AFs in sponge allorecognition is that histocompatibility molecules are expected to be membrane bound, whereas AFs are extracellular. There is evidence for tight binding of a layer of MAF molecules to the membrane even after chemical dissociation of sponge cells in the absence of Ca^{2+} [111] (fig. 14). This is in agreement with the model proposed by Dunham et al. [167], according to which secreted MAF remains bound to the cell surface where it stays intact and can promote aggregation even in the presence of low concentrations of Ca^{2+}. Considering the dimensions of MAF, ~300 nm across, and the density of the antibody signals revealed in figure 14, sponge cells are covered by an almost continuous sheet of PG. Theoretically, an extracellular molecule binding to cell surface receptors and being highly polymorphic could determine alloincompatibility as effectively as the membrane-bound components of the vertebrate histocompatibility system. Moreover, there are putative transmembrane regions in MAFp4 (fig. 9) that suggest that some of the MAF molecules interacting with the cell membrane can indeed be embedded in it (fig. 6D).

Polyclonal antibodies raised against MAFp3 are specific markers for archaeocytes, the sponge stem cells [168]. Archaeocytes are mobilized upon allogeneic contact (between tissues from two individuals of the same species), and they accumulate in the contact zone (fig. 15). A second type of cell that also migrates to allograft interfaces,

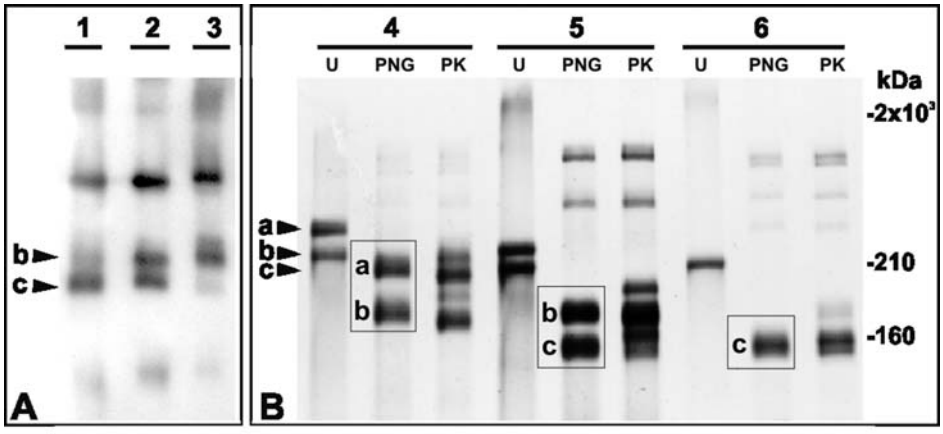

Figure 12. Interindividual variability of MAF glycans. (*A*) Western blot of MAF purified from three different *M. prolifera* individuals (1–3), transferred from 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and decorated with the monoclonal antibody Block 1 that recognizes the pyruvylated carbohydrate epitope, shown in figure 7A, involved in species-specific recognition of sponge cells. The arrowheads indicate two forms of a ~210-kDa glycoprotein (p210) that contains the MAF epitope. The intensities of both bands are similar, suggesting that the different mobilities do not reflect variations in glycosylation but, rather, different sequences either in the carbohydrate or in the protein moiety. (*B*) Dissociative density gradient-purified p210 from three different *M. prolifera* individuals (4–6) was digested either with peptide-*N*-glycosidase (PNG) or with proteinase K (PK) and compared in 5% SDS-PAGE to the undigested samples (U). GAG-containing bands were revealed with Alcian blue/silver stain. The different mobilities of the bands after protein removal indicate that the glycan moiety is responsible for at least part of the variability. PNG digestion reveals that the glycan is *N*-linked. Part *B* from Fernàndez-Busquets and Burger [157], with permission.

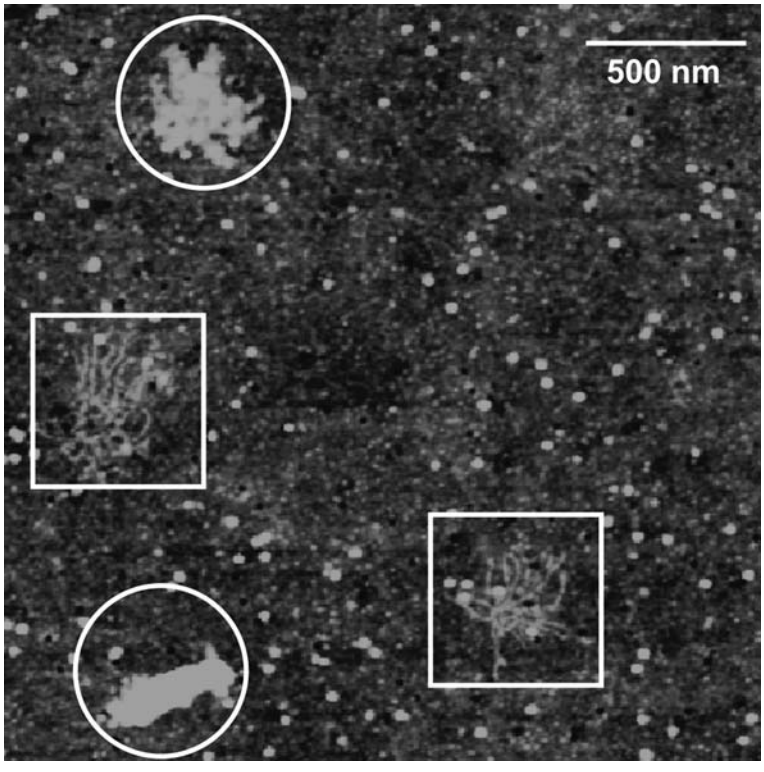

Figure 13. Immunogold labeling of MAF molecules isolated from a single *M. prolifera* individual, visualized in the tapping mode with AFM. Prior to deposition on the AFM surface, the molecules were labeled in solution with the monoclonal antibody Block 2 raised against the sulfated disaccharide epitope shown in figure 7B, followed by a secondary antibody conjugated to 5-nm gold beads (white spots). Two classes of MAF with high (circles) and low (squares) affinity for Block 2 binding can be clearly identified. The black spots are characteristic holes in the glass surface. The vertical brightness scale was set to 5 nm. Adapted from Fritz et al. [152], with permission.

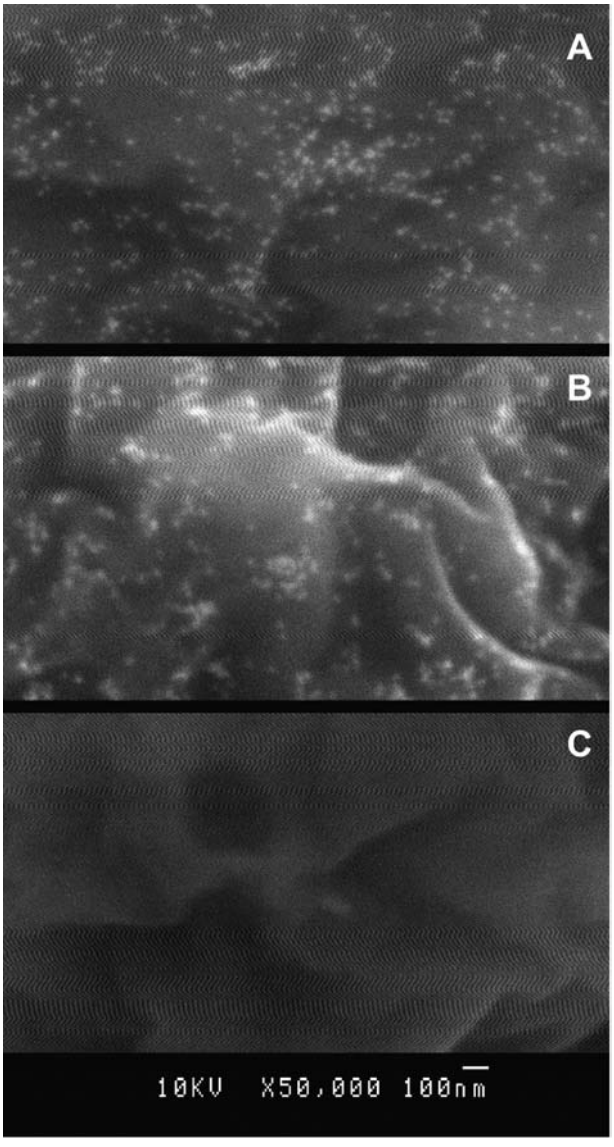

Figure 14. Back-scattered scanning electron microscope image of *M. prolifera* cells revealing cell membrane-bound MAF molecules through the binding of the monoclonal antibodies Block 1 (*A*) and Block 2 (*B*). Chemically dissociated sponge cells were treated as described elsewhere [158]. The binding sites of Block 1 and Block 2 were revealed through incubation in the presence of protein A conjugated to 20-nm gold particles. The white spots originate from electrons back-scattered by gold atoms. Controls made in the absence of primary antibody gave no signal (*C*).

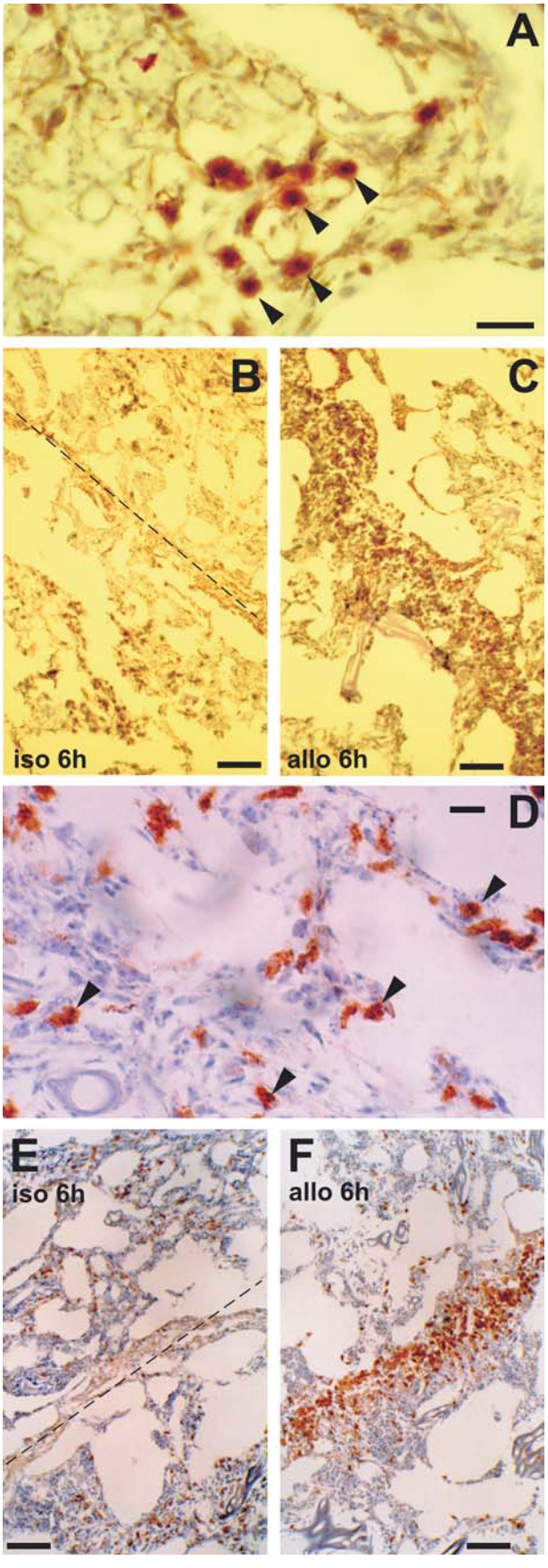

Figure 15. Antibodies against PG molecules as markers of sponge cells involved in allogeneic recognition. (*A*–*C*) Specific archaeocyte staining of *M. prolifera* grafts. Polyclonal anti-MAFp3 immunostaining with slight hematoxylin/eosin counterstain. (*D*–*F*) Specific gray cell staining of *M. prolifera* grafts. Monoclonal anti-CD44 staining with slight hematoxylin counterstain. (*A*, *D*) Nongrafted tissue. (*B*, *E*) Isograft (graft between tissues of a single individual), 6 h after grafting. (*C*, *F*) Allograft (graft between tissues of two different individuals), 6 h after grafting. Arrowheads point at typical archaeocytes (*A*) and gray cells (D) found in nongrafted tissue. The dashed lines indicate isograft contact regions. The bar represents 20 (*A*, *D*) or 100 (*B*, *C*, *E*, *F*) µm. For details see Fernàndez-Busquets et al. [168].

the gray cells, are specifically recognized by monoclonal antibodies raised against the HA receptor CD44 [168]. As a consequence of its high degree of glycosylation, the protein in native MAF is not easily accessible to proteases [70], to classical protein stains [169], or to polyclonal antibodies raised against MAFp3 [122]. The mild hydrofluoric acid treatment used to remove the sponge siliceous spicules during histological processing of *M. prolifera* grafts is unlikely to significantly deglycosylate MAF, given the strong signal obtained in all cell types and all over the ECM with a monoclonal antibody raised against the MAF terminal carbohydrate structure from figure 7B [149]. In addition, MAFp3 mRNA is homogeneously distributed in grafted tissue [149], while positive anti-MAFp3 staining is restricted to only one cell type, thus reinforcing the view that the anti-MAFp3 antibodies target the protein in an endogenous low-glycosylated state. While MAF mRNA is strongly upregulated in both isografts and allografts [149], immunohistochemistry does not reveal a corresponding increase of MAFp3 protein in grafts, but rather a redistribution of its signal following the wanderings of archaeocytes, and this only in allografts [168]. The transcription rate of MAF mRNA likely increases in grafted tissue in response to a higher demand for MAF in its glycosylated form (where the protein is inaccessible to the antibodies) that might be required in cell adhesion phenomena necessary for ECM remodeling of both iso- and allografts. Additional data showed dramatic variations in the appearance of different MAF transcripts between individuals [149], and between sponges collected at different times of the year [127]. The longer transcripts (those containing both MAFp3 and MAFp4 coding sequences) have been suggested to respond to a request of whole MAF, while the shorter transcripts, containing only the MAFp3 coding sequence [149], will end up in the exclusive production of this protein, that in the absence of MAFp4 cannot be assembled into functional MAF [122]. The molecular pathways regulating this switching mechanism are unknown and will surely depend on the particular physiological state of the sponge.

Of interest is that in allograft contact areas, an observed decrease in the intensity of archaeocyte staining is accompanied by an increase in the number of gray cells, which are the most adhesive cell type in *M. prolifera* [170]. Since increased cell adhesion correlates with an elevated concentration of MAF [171], nonglycosylated MAFp3 in archaeocytes could be progressively glycosylated and incorporated into MAF during the process of gray cell differentiation [168]. In favor of this scenario are the observations in allograft interfaces of cells with archaeocyte characteristics that stain positive for CD44 [168]. Accordingly, in mixtures of cells dissociated from two different individuals of the marine sponge *Callyspongia diffusa*, there is an inhibition of aggregation [172]. The abundant gray cells present in *C. diffusa* graft zones could confer higher adhesion, thus slowing cell movement and suppressing aggregation.

The existence in sponges of a cell adhesion-related, highly polymorphic gene system that matches the high level of sponge alloincompatibility might represent an early form of self-recognition. Such an elevated polymorphism is present in the high variability of MAF proteins and glycans that has been described above [149, 157]. An evolutionary relation between cell adhesion and histocompatibility systems, however, has yet to be demonstrated. A prominent domain implicated in cell adhesion, the Ig domain, exists in several copies in the large basement membrane PG [173]. Similar domains are functionally important structures in virtually all Ca^{2+}-independent adhesion molecules such as the neural cell adhesion molecule (NCAM) [174], the intercellular adhesion molecules (ICAMs) [175, 176], and other neural or immune system-associated adhesion molecules. The similarity in folding topology between cadherin and Ig domains has led to discussions about a possible common evolutionary origin [177], although sequence homologies between the two types of domains are small, ranging from 6 to 11% identity. Moreover, while Ig domains are commonly separated by introns, in cadherin genes, all introns are within rather than between domains [177, 178]. No obvious cadherin or Ig-like domains have been identified in the cDNA-deduced MAF proteins, although the repeats in MAFp4 exhibit interesting resemblances [149]. From their size, β strand content, presence of two centrally placed cysteines, and intron placement, some of the MAFp4 repeats are not far from Ig domains (fig. 11). In the rapidly evolving Ig superfamily, present-day members will have diverged considerably from their ancestors of 600 million years ago. However, introns in MAFp3 and MAFp4 are in phase 0 (between two codons), unlike Ig domains which are separated by introns in phase I [179]. Polymorphic Ig-like domains have been found in a receptor tyrosine kinase (RTK) [159–161] and in two adhesion molecules from *G. cydonium* [180], although their involvement in sponge immunity has not been clarified so far. Besides the RTK, other elements participating in signal transduction pathways such as G proteins and Ser/Thr protein kinases have been isolated from sponges [181, 182].

Involvement of spongicans in signal transduction processes

Since sponge AFs can aggregate in a species-specific way formaldehyde-fixed cells [111, 183] and cells kept at low temperatures where metabolism is inhibited [95, 96], the action mechanism of AFs was generally assumed to be exclusively extracellular, without the need for sending any signal beyond the cell membrane. Species specificity has even been demonstrated in cell-free systems (fig. 3)

[109, 110], suggesting that sponge cell aggregation might be the result of the passive interaction of chemical structures, without any participation of active cellular processes. However, sponge cells are not indifferent to the binding of their AFs.

Müller et al. [184] observed that after the addition of purified AF to primary *G. cydonium* cell aggregates, DNA, RNA, and protein synthesis increased, and that the cells exhibited a high mitotic activity. Transcription of polyubiquitin mRNA was greatly increased when the dissociated cells were incubated with AF [185, 186]. Binding of AF to the cells triggered protein phosphorylation [187] and *ras* gene expression [188, 189]. Aggregation between sponge cells promotes production of inositol triphosphate and diacylglycerol from inositol phospholipids with an associated rise in the concentration of cytosolic calcium [123, 190]. These events all eventually lead to the phosphorylation of nuclear proteins and the induction of DNA polymerase activity [191]. Interestingly, the MAF proteins have abundant phosphorylation sites, and MAFp4 contains several putative transmembrane regions (fig. 9). This opens the possibility for the existence of membrane-bound, phosphorylable MAF forms that could directly participate in the activation of second messenger signaling pathways. Worth noting here is that MAFp4 has significant similarity to VLGR1, a G protein-coupled receptor [155].

The RGD peptide and AF also promote DNA synthesis in the sponge *S. domuncula* [189]. Binding of AF to the cells was inhibited by RGD, suggesting that an integrin-mediated interaction is involved. Indeed, MAFp4 contains at least one RGD binding sequence (fig. 9), which could potentially ligate MAF to integrins to initiate a transcellular motility reduction signal such as that described for the integrin-fibronectin association [192]. This strategy would be distinct from the carbohydrate-carbohydrate binding thought to mediate aggregation. Under usual conditions, the high sugar content of MAF might preclude effective integrin-RGD peptide binding, but this situation can be changed under certain circumstances, as when cells are exposed to low levels of environmental sulfate, known to up-regulate α_3 and β_1 integrins in *M. prolifera* [193]. Sulfate restriction has also been shown to reduce MAF secretion [194]. Intriguingly, increased expression of integrin has been reported during the process of autograft fusion in *G. cydonium* [195], a situation where decreased cell adhesion is likely to be required at the gap between the grafted tissues to allow the increased cell movement necessary for tissue remodeling.

The existence of connections between intracellular processes and sponge cell aggregation was also provided by the discovery that calcium played a role as an intracellular messenger in the aggregation of *M. prolifera* cells [196]; addition of Ca^{2+} ionophores caused aggregation in live cells that far exceeded that provoked by Ca^{2+} alone, while agents that block the entry of Ca^{2+}, or which modulate its action within cells, inhibited aggregation. Fixed cells did not respond to ionophores with or without Ca^{2+}, indicating that extensive sponge cell aggregation requires more than simply passive agglutination by AFs. Addition of the protein kinase C activator phorbol myristate acetate dramatically increased the ionophore aggregative effect [197], suggesting a synergistic effect of intracellular Ca^{2+} and protein kinase C to trigger the cellular response of MAF secretion to the external ligand calcium. The observation that *M. prolifera* cells isolated from sponges living in areas with high cadmium levels failed to aggregate in the presence of Ca^{2+} [198] led to studies showing that low levels of salinity and pH facilitate the accumulation of Cd^{2+}, that in turn disrupts normal aggregation responses of the cell, most likely due to Ca^{2+} channel blocking by Cd^{2+} [199].

Perspectives and concluding remarks

Aggregating sponge cells and human blood platelets respond similarly to gaseous anesthetics like nitrous oxide (aggregation inhibitor) and xenon (aggregation potentiator), in a process depending on an extracellular source of calcium [200]. Sponge cell aggregation has also been proposed as a model to study the process of inflammation, as illustrated by the finding that antiinflammatory compounds (aspirin, diclofenac) and inflammatory substances (leukotriene B_4, poison ivy's urushiol) exert opposing effects on sponge cell aggregation [167].

In recent years, spongicans have revealed themselves as potential tools to study human illnesses like AIDS and Alzheimer's disease. A partially purified MAF derivative has been shown to inhibit the replication of the human immunodeficiency virus by specific binding of the viral gp120 envelope protein and interfering with the binding of gp120 to the lymphocyte CD4 antigen [201]. This binding is necessary for viral entry and multiplication in target cells [202]. One of the major pathological features of Alzheimer's disease is the presence of plaques composed of β-structured fibrils made up of amyloid-β peptide (AβP) [reviewed in ref. 203]. Nucleation of AβP can occur by self-assembly or by heterogeneous nucleation resulting from seeding AβP onto non-AβP elements present in the ECM, such as PGs [43, 44]. MAF induces a structural transition in AβP from a random to β-structure [204]. Such a transition correlated with a stabilization of globular aggregated and truncated fibrils. MAF also increased AβP-induced toxicity of nerve growth factor-differentiated PC-12 cells in the absence of Ca^{2+}. These results indicate that AβP is generally susceptible to PG-mediated aggregation, and that sponge PGs represent a simple and reproducible method that can be used to examine potential drugs for the treatment of amyloid-related disorders.

Despite their lack of clear sequence homologies with other known PGs, the protein and carbohydrate components of MAF assemble to form a supramolecular complex remarkably similar in structure to modular PGs and particularly to hyalectans. This seems to represent an example of molecular evolutionary convergence between spongicans and hyalectans. The ease of purification of large amounts of spongicans with a very mild method that preserves their integrity predicts an increasing use of these sponge-derived molecules in the study of PG functions and of the involvement of PGs in the onset and evolution of numerous human pathologies.

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