Accumulation in Marine Sponge Grafts of the mRNA Encoding the Main Proteins of the Cell Adhesion System*

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**Main Proteins of the Cell Adhesion System**

Species-specific cell recognition in sponges is mediated by proteoglycan-like extracellular complexes termed aggregation factors (AFs), unidentified so far in other phyla, although Northern blots suggested the existence, in several human tissues, of proteins related to those found in the AF of the marine sponge _Microciona prolifera_ (1). Cell adhesion in this species is mediated by calcium-dependent carbohydrate interactions of homologous _Microciona_ AF (MAF) molecules via N-linked glycans. A proposed model suggested that species specificity could be at least partially provided by the different spacing of glycosaminoglycan chains on the protein core (2), thus postulating the existence of a different AF core protein in each species. Unexpectedly, an extraordinarily high intraspecific variability was found for the MAF core protein (1), suggesting that AFs might also be involved in individual specificity.

The immune and cell adhesion systems are clearly related, as deduced from phylogenetic relationships between molecules of both groups (3) and as expected from the fact that the cells of the immune system depend on regulated interactions with other cells to be activated (4). An evolutionary connection between cell adhesion and histocompatibility, however, has yet to be demonstrated. Presumably, it was around the time when metazoans appeared that the primitive machinery regulating cell interactions had to evolve and diversify into the whole array of molecules that allowed the far more complex structures leading to multicellularity first and then to tissues and organs. A second possibility is that the cell adhesion system of higher animals appeared _de novo_, and therefore, vertebrate and invertebrate histocompatibility would be the result of convergent evolution rather than of a common ancestry. The finding, in an ever increasing number of invertebrates including sponges, of Ig-like domains (5, 6) and of extracellular matrix components common to all pluricellular animals (7) suggests that the latter view is less likely.

A surprising characteristic of sponges, considering their phylogenetic position as the most primitive extant metazoans, is that they possess a highly evolved histocompatibility system. 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, which include (i) an inflammation-like massive migration of certain cell types toward the region of contact (8), (ii) phagocytosis and/or cytotoxic reactions (9, 10), and (iii) the layering of a collagen barrier separating the apposed tissues (8). Any grafting between two genetically different sponge individuals is almost invariably incompatible in the many species investigated (1, 9, 11, 12), exhibiting a variety of transitive qualitatively and quantitatively different responses, which can only be explained by the existence of an extensive genetic polymorphism at the locus or loci controlling graft acceptance and rejection. In the echinoderm_ Botryllus schlosseri_, contact between two genetically distinct individuals leads to the resorption of one of them by the other through a phenomenon that is controlled by highly polymorphic loci (13). The frequency of resorption in _Botryllus_ is comparable to that of rejection in sponges, suggesting that both might be analogous manifestations of a sophisticated histocompatibility system in invertebrate organisms.
brates that would have been an ideal building block for the vertebrate major histocompatibility complex. And yet, despite the relevant cytological and genetic studies outlined above, there is an absolute lack of sequence and evolutionary data concerning invertebrate molecules involved in allogrecognition.

Individual variability of sequences related to the main protein of MAF (MAFp3) matched the elevated sponge alloimmunity (1), although from Southern blot results, it could not be established whether such variability resided in the AF proteins themselves or if it also represented sequence-related molecules not belonging to the AF. MAFp3 and another protein suspected to belong to the AF seemed to be translated together as a longer polypeptide from the same mRNA (1, 14). Here, we suspected to belong to the AF seemed to be translated together with molecules not belonging to the AF. MAFp3 and another protein which we suspected to belong to the AF seemed to be translated together with molecules not belonging to the AF. MAFp3 and another protein which we suspected to belong to the AF.

Southern and Northern Blots and in Situ Hybridization—Sponge genomic DNA was isolated as described (14). Drai-digested, electrophoresed, transferred to a membrane, and hybridized with probes labeled with alkali-labile digoxigenin as specified before (1). Probes I and III (both ~ 1 kb; defined in the legend to Fig. 1) were polymerase chain reaction-amplified from subcloned regions lacking DraiI sites. Probe I was derived from an intronless cDNA sequence, and probe III from a genomic DNA sequence containing at least one intron. Before probing, the membranes were treated twice with 1.5 M NaCl for 10 min each and once with 3 M NaCl for 1 h on ice. After 2 h of hybridization, the membranes were washed twice with 0.1 M NaCl, 1 M Tris-HCl, pH 7.4, and × 2 SSC for 10 min each. To establish grafts, 1-cm-long sponge papillae were pushed together on a 0.5-mm-thick stainless steel insect pin until the contact between the tissues was as intimate as possible. During these operations, the sponge tissue was always kept under seawater. The pins were stuck into the underside of a Styrofoam raft that was placed on a tank. Following overnight seawater at 20 °C for the times indicated. Total RNA from grafted tissues was prepared using standard protocols (19). Briefly, the grafted papillae with a mean volume of 125 mm² were removed from the pins and gently squeezed with flat-tipped forceps for 30 s in 1 ml of Ca²⁺- and Mg²⁺-free artificial seawater at 0 °C in order to release the cells, which were immediately pelleted for 1 min at 200 x g. 0.9 ml of the cleared supernatant was then removed, and the cell pellet was quickly but gently resuspended in the remaining 100 µl before adding 6 volumes of 4 × guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The viscous solution was gently mixed until it became homogeneous, and the RNA was then lowered with 0.1 volume of 2 × sodium acetate, pH 4. The proteins were extracted with 1 volume of phenol equilibrated with 0.2 mM EDTA and isopropyl alcohol and incubated for 1 h on ice. The samples were centrifuged at 20,000 × g for 30 min, and the RNA contained in the water phase was precipitated with 0.2 volume of isopropanol alcohol, washed twice with 80% ethanol, and resuspended in diethyl pyrocarbonate-treated H₂O. For Northern blotting, 5 µg of total RNA were loaded per lane on denaturing 0.8% agarose gels containing formaldehyde. RNA probes were labeled with the digoxigenin RNA labeling kit (Boehringer Mannheim) as described (14). In situ hybridization of 20-µm-thick sections was performed as described (20) without prior desalification of the tissue.

Immunohistochernistry—Grafted tissues were fixed with 3.7% formaldehyde in artificial seawater at the indicated times after being pinned together. Without removing the needle, grafts were immersed in the fixing solution and incubated overnight at 4 °C. To dissolve the spicules, grafted tissues were desalified according to Humason (21). Grafts were washed twice with artificial seawater and then with progressively increasing amounts of ethanol in artificial seawater (from 30 to 70%), with a final wash in 70% ethanol in water. Spicules were then removed by treatment with 1.5 x hydrofluoric acid in 70% ethanol for 4–7 h at room temperature. Grafts were stored in 70% ethanol at 4 °C until processing. The fusion protein was routinely detected with either a polyclonal antibody against MAFp3 and another protein which we suspected to belong to the AF seemed to be translated together with molecules not belonging to the AF. MAFp3 and another protein which we suspected to belong to the AF seemed to be translated together with molecules not belonging to the AF.
at a flow rate of 50 μl/min. An aliquot of 10% of the effluent was directed on-line to an API 300 triple quadrupole mass spectrometer (PE Sciex, Concord, Canada), and 90% was collected for further analysis. The ion spray voltage of the mass spectrometer was set to 5000 V, and a mass range of 300–2400 Da was scanned with a step size of 0.5 Da and a dwell time of 0.75 ms/maSS. Sequence analysis was carried out on a Model 477A protein sequencer (Applied Biosystems, Inc., Foster City, CA) according to the recommendations of the manufacturer.

RESULTS

A New Protein Component of MAF—Of the several cDNA-derived forms described for the main protein of MAF, we decided to elongate, in the 5′-direction, MAFp3C and MAFp3D as representatives of two distinct groups identified by sequence relatedness (1). The sequences shown in Fig. 1a could not be extended further due to mismatches between the existing and newly obtained sequences. We interpret this result as a consequence of the variability of these proteins, which reduces the probability of amplifying the same form used to generate the antisense gene-specific primers.

Chemical deglycosylation of MAF yielded, as the main product, MAFp3, whose first identified cDNA predicted a polypeptide of 35 kDa (14), beginning with Pro-1883 in MAFp3C. Since this residue is immediately preceded by Asp, and the Asp–Pro bond is especially sensitive to acidic conditions (25) such as those of the deglycosylation reaction, the possibility exists that MAFp3 could be the result of chemical cleavage of a bigger protein rather than an independent entity in vivo. However, 36 other Asp–Pro bonds are present in the sequences from Fig. 1a, although we never detected any peptides arising from cleavage at those positions. This supports the view that our deglycosylation conditions do not cause extensive damage to the protein backbone, and therefore, MAFp3 is likely to be translated as part of a single peptide, which is then processed into at least two proteins. Later in this work, we confirm that the cDNA sequences extended in the 5′-direction correspond to a longer mRNA coding for a new protein component of MAF that we will term MAFp4.

MAF-mediated species-specific cell adhesion depends on polypeptide interactions between N-linked glycans (2, 26). The high number of NX(S/T) consensus sequences found on MAFp4 confirms the expectation of extensive N-glycosylation, a hypothesis backed by the sequencing of several tryptic peptides that yielded a blank instead of a potentially glycosylated Asn (see Figs. 3b and 5c) as result of the inefficient cleavage by trifluoromethanesulfonic acid of protein-bound GlcNAc (23), which accounts for ~20% of the total carbohydrate content of MAF (22). MAF self-interaction is Ca2+-dependent, unlike its binding to the cell receptor (27). The abundant potential Ca2+-binding sites of the type DXD (28) that are present in MAFp4 indicate that self-interaction might not involve only the acidic glycans, but also the protein core. MAFp4 contains a reiterated motif of a mean length of 117 residues (Fig. 1). Data base searches revealed two significant matches (Fig. 1c). As already described by us (14), the MAFp4 repeat shares an ~30% identity with a stretch of the intracellular loop of the Na+–Ca2+-exchanger protein from mammals. In addition to this, we have now identified an 8-fold reiterated motif in a cDNA-deduced endoglucanase gene from the symbiont bacteria Azorhizobium caulinodans ORS571 sharing a similar degree of identity with the MAFp4 repeats. The bacterial repeats are ~115 amino acids long and were also described to show significant similarity to the Na+–Ca2+-exchangers (29).

MAFp4 Is Highly Polymorphic—Allo- and heterogeneic reactions in Micriona a is clearly identified after ~6 h with the naked eye due to a yellowish line originating from the massive migration to the region of contact of gray cells, which have been suggested to be the functional immunocytes of sponges (8). We studied allo-
FIG. 1. Partial cDNA-deduced protein sequence of MAFp4. a, alignment of forms C and D of MAFp3/MAFp4 (GenBank accession numbers AF020902 and AF020903, respectively). The boxed carboxyl-terminal region corresponds to MAFp3, and the rest to MAFp4. Gaps introduced by the alignment program Gap (Genetics Computer Group, Inc., Madison, WI) are represented by dots. Boxed peptides correspond to...
cases (for a review, see Ref. 31), and therefore, it would not be surprising that a small fraction of actin molecules play some extracellular function in sponges. According to Coomassie Blue and Amido Black staining, actin is the most abundant protein in both cell membranes and 70 mm NaCl fractions, a circumstance that favors its apparent cross-reactivity with the purified anti-MAFp3 antibodies.

To investigate the existence of any structural features revealed by antibodies raised against MAF epitopes, 6-μm sections of allogeneic and isogeneic M. prolifera grafts were incubated either with anti-MAFp3 antibody or with the monoclonal antibody Block 2, raised against a carbohydrate epitope of MAF (2). Anti-MAFp3 antibody clearly revealed a thin layer of cells all along the line of contact of allogeneic grafts after several hours of grafting (Fig. 4a). This reaction was not observed in isogeneic grafts (Fig. 4b). Allogeneic grafts at times under 10 h did not show such a pattern (Fig. 4d), indicating that the staining does not come from a mere apposition of epithelial cell layers that might be expressing the epitope before contact. On the contrary, the pinacoderm was not particularly stained by anti-MAFp3 antibody (Fig. 4, b–d). 24 h after grafting, the border line of cells was still easily identified (Fig. 4d), although the time interval during which the signal persisted was found to depend on the individual combination of the grafted tissues.

The staining observed with anti-MAFp3 antibody was not due to the accumulation of MAF, as indicated by routine controls done with monoclonal antibodies raised against carbohydrate epitopes of MAF (Fig. 4e). Additional controls were performed those reported in Figs. 3 and 5, identified by trypsin digestion. Polyclonal anti-MAFp3 IgYs were raised against the amino-terminal half of MAFp3 (anti-MAFp3). An identical gel was stained first with Coomassie Blue and later with Alcian blue to reveal protein- and glycan-containing bands, respectively. The ~400-kDa Alcian blue-stained band was subjected to peptide mass fingerprinting. b, the sequences of the most prominent tryptic peptides were determined either by MS analysis and comparison with the cDNA-deduced sequences (in italics) or by direct sequencing (all others). The residues found in the sequence of MAFp4C (Fig. 1a) are indicated in boldface. Dashes indicate blanks in the sequence probably due to the presence of a modified amino acid. c, Western blots of M. prolifera cell membrane proteins (Membranes) and of peripheral cell membrane proteins (70 μm NaCl extract) fractionated on a 12.5% SDS-polyacrylamide gel and stained with Amido Black or decorated with anti-MAFp3 IgYs (anti-MAFp3) or with rabbit polyclonal antibodies raised against squid actin (anti-actin), d, alignment of the complete actin sequence from the sea urchin Strongylocentrotus purpuratus (GenBankTM accession number P53472) with the main tryptic peptides obtained from M. prolifera actin excised from SDS gels. Nonconserved amino acids are boxed in the sponge sequence.

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FIG. 1. Alignment of the amino-terminal half of MAFp3 (anti-MAFp3) with the main tryptic peptides obtained from M. prolifera actin excised from SDS gels. Nonconserved amino acids are boxed in the sponge sequence.
with sections prepared as those for in situ hybridization, which were not treated with hydrofluoric acid. The results were identical to those obtained with desilicified sections (data not shown), thus indicating that the treatment with diluted hydrofluoric acid, which is a deglycosylating agent, did not significantly remove the carbohydrate epitope recognized by Block 2 from fixed tissue.

Different MAFp3 Forms in AFs of Different Individual Origin—To investigate the possibility that MAFs of different origin might have different core proteins, we proceeded to chemically deglycosylate MAF preparations obtained from different individuals. A series of protein bands between 38 and 52 kDa were detected (Fig. 5, a and b). Samples of different individual origin treated in parallel reproducibly revealed specific combinations of bands, indicating that the observed polydispersity is a reflection of the in vivo situation rather than an artifact of deglycosylation. Anti-MAFp3 antibody decorated all bands with an intensity proportional to that of Coomassie Blue staining, suggesting that all the forms shared a significant degree of homology. To confirm this, we separately excised single bands from SDS gels, digested them with trypsin, and performed LC-MS analysis. The chromatograms shown in Fig. 5c confirm the existence of multiple related proteins. cDNA-deduced MAFp3 forms had been identified whose molecular masses differ between 1 and 5.4 kDa, mainly due to different carboxyl-terminal ends (1), a region for which we could not obtain peptide sequences from our trypsin digests. It is likely that major variability in this region will be responsible for the differences in electrophoretic mobility observed. Amino-terminal analysis of the 39-, 46-, 49-, 51-, and 52-kDa bands yielded, for all of them, the sequence of MAFp3 (PLFTVPI).

Accumulation of MAFp3/MAFp4 mRNA—Northern blots of sponge total RNA showed variable amounts of the MAFp3/MAFp4 mRNA (Fig. 6a). The use of probe I revealed a main transcript of ~12 kb, in accordance with a translated ~400-kDa protein, which might correspond to full-length MAFp4 as suggested by the Alcian blue-stained gel in Fig. 3a. Often, the MAFp3-specific probe recognized abundant RNAs below 12 kb (Fig. 6a, top), suggesting the existence, under still undetermined conditions, of a great variability of MAFp3-related sequences. Occasionally, bands of ~1 kb were also observed, which are thought to represent small transcripts containing only the MAFp3 sequence (14). Accordingly, the use of probes corresponding to MAFp4 revealed the ~12-kb band, but never any signal around 1 kb (data not shown). To study the effect of tissue grafting on the transcription of MAFp3/MAFp4 mRNA, we chose those individuals whose levels of expression in nongrafted controls were constant and low during the duration of
the experiment. A typical result is presented in Fig. 6b. Grafting of isogeneic or allogeneic tissue coincides with an accumulation of the MAFp3/MAFp4 mRNA. As shown by the β-actin control, this does not seem to be the consequence of a generalized enhanced transcription. Occasionally, the control samples removed at the end of the experiment from parts of the animal 10 cm apart showed a dramatic accumulation of the MAFp3/MAFp4 mRNA throughout the body, whereas tissue separated from the animal at the beginning of the experiment and kept under the same conditions as those used for the grafts. Total RNA was separately isolated and analyzed from each papilla of isografts (ISOGENEIC). To observe the transcription levels of individual 69 in allografts (ALLOGENEIC), papillae of individual 62 were used as allogeneic tissue. Total RNA was isolated at the specified times after grafting. As controls, total RNA was isolated from four different parts of the sponge before the experiment started (C0) and from five different parts after the RNA of the last time point had been isolated (C23). d and e, in situ hybridization of 7-h isograft sections with sense and antisense MAFp3-specific RNA probes, respectively. Arrows mark the line of contact. The dark areas in the sense control are due to light refracted by the sponge spicules.

DISCUSSION

The correlation between the variability of MAF proteins as deduced from restriction fragment length polymorphism analysis and the allogeneic behavior within a sponge population supports a possible involvement of the cell adhesion system in sponge histocompatibility reactions (1). The accumulation in grafts of the mRNA coding for the protein core of the main adhesion molecule of the sponge adds to this hypothesis. The phenomena occurring in M. prolifera grafts can be first identified under the microscope ~1 h after grafting, when the pina-
have shown that Block 2 binds, with very different affinities, individual AF molecules that were purified from a single sponge (32), and this is in agreement with the assumption that different types of AF molecules exist that exhibit distinct specific activities. Peptides containing sequences exclusive of MAFp3D or MAFp4D have never been found in the many AF preparations analyzed. The presence in sponge cell membranes of a 68-kDa protein related to the core protein of a highly glycan-substituted component of the extracellular AF would substantiate the hypothesis of the existence of proteins phylogenetically related to those in the AF, but that might have a function other than promoting species-specific cell adhesion. The appearance in allogeneic grafts of a cell layer turned on to express an epitope recognized by polyclonal antibodies raised against MAFp3 could represent the tissue distribution of the elusive 68-kDa protein. The absence of colocalization with monoclonal antibodies against MAF glycans suggests that the protein being detected does not belong to MAF and might be expressed only under particular physiological conditions such as allogeneic contact.

Mixtures of cells dissociated from different individuals of the freshwater sponge Ephydatia fluviatilis segregated into clumps containing cells of only one individual several days after a mixed aggregate was formed (33). Such individual specificity could be also observed between cell fractions containing only one cell type (34). Different sponge cell types also show selective reaggregation and different adhesive properties (35). Therefore, dissociated sponge cells exhibit species-, individual-, and cell-type specificities in their reaggregation properties. In rejection studies done by grafting in situ a population of the marine sponge Hymeniacidon sp., "interaction modulation factors" were purified from each individual, and measurements of their adhesion efficiency were made on cells of all the sponges among which grafting had been done (36). While the adhesiveness of those cells treated with a factor preparation made from the same sponge or from another strain whose grafts were accepted was always appreciable, factor from those strains that lack the disulfide bond. Finally, despite that most of the superfamily consists of membrane-bound members, non-cell surface proteins like α1-glycoprotein and the basement membrane link protein are also included. Based on simple alignment programs, polymorphic Ig-like featuring genes have been described in the marine sponge Geodia cydonium (41), suggesting that the evolutionary origin of the Ig fold might have been pushed back to the lower contemporary invertebrate phylum, if not beyond. Alternatively, the Ig system may have arisen independently, and the similarity of the Ig domain may be the result of convergent evolution imposed by its functional properties.

The similarities in the domain structure and sequence between the sponge adhesion protein MAFp4 and a bacterial endoglucanase reinforce the hypothesis that the reiterated do-

Molecular Evolution of Sponge Repeated Ig-like Domains

**Fig. 7.** Intron placement and putative β-strands in the tandem repeats of MAFp4C. Cysteines are boxed. Stretches of at least 5 amino acids that form or do not break β-strands are shaded. Intron positions in repeats 10–16 are marked by arrowheads.
mRNA of Novel Sponge Adhesion Protein Accumulates in Grafts

Although the role of AFs in species specificity has been clearly established, we propose here that also individual specificity might be based on AF-related molecules or even on the AF itself. This scenario suggests that modern histocompatibility systems might have evolved from primitive cell adhesion molecules, a hypothesis supported by the occurrence of putative ancestors of typical Ig domains in MAPp4, a protein involved in sponge cell adhesion. If, on the other hand, the histocompatibility system of sponges developed after their split from the vertebrate evolutionary line, it would represent a fascinating alternative to allorecognition strategies.

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