Identification and Further Characterization of the Specific Cell Binding Fragment from Sponge Aggregation Factor

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Abstract. Monoclonal antibodies (McAbs) were raised against the aggregation factor (AF) from the marine sponge *Geodia cydonium*. Two clones were identified that secrete McAbs against the cell binding protein of the AF complex. Fab fragments of McAbs: 5D2-D11 completely abolished the activity of the AF to form secondary aggregates from single cells. The McAbs were determined to react with the AF in vitro; this interaction was prevented by addition of the aggregation receptor, isolated and purified from the same species. After dissociation of the AF by sodium dodecyl sulfate and 2-mercaptoethanol, followed by electrophoretical fractionation, a 47-kD protein was identified by immunoblotting which interacted with the McAbs: 5D2-D11. During this dissociation procedure, the sunburst structure of the AF was destroyed. In a second approach, the 47-kD protein was isolated by immunoprecipitation; 12 molecules of this protein species were calculated to be associated with the intact AF particle. The 47-kD AF fragment bound to dissociated *Geodia* cells with a high affinity (K_a of 7×10^8 M^{-1}) even in the absence of Ca^{++} ions; the number of binding sites was $\sim 4 \times 10^6$/cell. This interaction was prevented by addition of the aggregation receptor to the 47-kD protein in the homologous cell system. Moreover, it was established that this binding occurs species-specifically. The 47-kD fragment of the AF was localized only extracellularly by indirect immunofluorescence staining in cryostat slices. These data suggest that the 47-kD protein is the cell binding molecule of the AF from *Geodia*.

For almost 80 years (35) sponge systems have proven to be a useful model to study the mechanisms of cell–cell adhesion on the molecular level. In Demosponges two types of cell surface recognition processes exist: the initial primary aggregation phase, which is most likely second-order homophilic (16), and the subsequent secondary aggregation phase. The key macromolecules of the latter recognition system are the soluble aggregation factor (AF)[1] (9, 15, 34) and the membrane-bound aggregation receptor (20, 34), hence this adhesion system is third-order heterophilic. Results from detailed biochemical studies with the AF and the aggregation receptor led to the formulation of the modulation theory of sponge cell adhesion (14, 24). The AFs from different sponges are particles of high molecular weights (M_r 2.1×10^7 to 1.3×10^8) (8, 21) and are assembled from a series of proteins that are bound to each other either covalently or noncovalently (12, 21).

Misevic et al. (12) already have succeeded in the partial fragmentation of the AF from *Microciona prolifera* by chemical/biochemical means, which reveals that the cell binding site in AF is highly polyvalent and its structure of high molecular weight ($>1.5 \times 10^7$). In contrast to this result, earlier data (19) from studies with the AF from *Geodia cydonium* showed that the cell binding molecule from this particle has an M_r of ~ only 20,000.

In the approach described here, we used monoclonal antibodies (McAbs), directed against the functional domain of the AF from *Geodia cydonium*, to identify and purify the cell binding protein from the particles. Moreover, we used the McAbs to localize the AF in tissue slices by indirect immunofluorescence staining.

Materials and Methods

Materials

The following materials were used. Class and subclass specific goat anti-mouse antisera were obtained from Tago Inc. (Burlingame, CA); protein A-Sepharose and Ficoll 400 from Deutsche Pharmacia (Freiburg, Germany); nitrocellulose sheets (BA; 0.45 μm; No. 40 1180) from Schleicher & Schüll (Dassel, Germany); ^{125}I-sodium iodine (carrier free) from The Radiochemical Centre (Amersham, England); Dowex AG1-X2 and Bio-Gel P-300 from Bio-Rad Laboratories (Richmond, CA); pathotrol from Dade (Miami, FL); anti-mouse (whole molecule) peroxide conjugate (A-2028) and anti-mouse IgG (whole molecule)

[1] *Abbreviations used in this paper:* AF, aggregation factor; ASW, artificial seawater; CMFSW, Ca^{2+}- and Mg^{2+}-free seawater; CMFSW-E, Ca^{2+}- and Mg^{2+}-free seawater containing EDTA; McAbs, monoclonal antibodies; TRITC, tetramethylrhodamine isothiocyanate.

tetramethylrhodamine isothiocyanate (TRITC) conjugate (T-5393) from Sigma Chemical Co. (St. Louis, MO); Tissue Tek (O.C.T., No. 4583) from Miles Scientific Div. (Naperville, IL).

Live specimens of *Clathrina coriacea* (Calcispongia), *Geodia cydonium*, *Tethya lyncurium* (both Demospongiae; without spongin fibers), *Mycale massa*, *Spongia officinalis* (both Demospongiae; with spongin fibers) were collected near Rovinj (Yugoslavia).

The aggregation receptor was isolated and purified from *Geodia cydonium* (20).

Buffers

Tris-buffered Ca^{2+}- and Mg^{2+}-free seawater (CMFSW) was made as described (18); Ca^{2+}- and Mg^{2+}-free seawater that contained EDTA (CMFSW-E) was made as CMFSW but with 20 mM EDTA. Ca^{2+}- and Mg^{2+}-containing artificial seawater (ASW) had, in addition to the components in CMFSW, 50 mM $MgCl_2$ and 10 mM $CaCl_2$.

Preparation of Cells

Cells were obtained from the different sponges by dissociation in CMFSW (15), with the exception that the trypsin step was not included in the procedure. The archaeocytes were separated from the cell suspensions by Ficoll discontinuous density gradient centrifugation (25).

Purification and Radioiodination of AF

The AF was isolated and purified from *Geodia cydonium* as described earlier (3, 15); the definition of aggregation units is given previously (15). The purified factor with a protein content of 1.2 mg/ml had a specific aggregation promoting activity of 4.2×10^6 aggregation units/mg. Radioiodination of the AF (50 μg of protein) was done with 5 mCi ^{125}I-sodium iodine using the described chloramine T procedure (4) followed by subsequent purification (16). The specific activity was determined to be 19 μCi/μg of protein.

Aggregation Assay

In the standard assay (volume, 3 ml) (15, 24), a suspension of $25 \pm 5 \times 10^6$ cells/ml ASW was placed into glass tubes and rolled at 35 rpm for 20 min at 10°C. The size of the aggregates was determined optically (24). Preincubation with Fab fragments of McAbs (100 μl) with the AF (100 μl) was performed in CMFSW at 2°C for 20 min without shaking. Subsequent incubation with the cells (in ASW) was done for 90 min (end-point of the reaggregation reaction) at 20°C.

Preparation of McAbs

The general procedure for preparation of specific monoclonal antibodies (McAbs), described by Cuello et al. (4), was used. Briefly, female BALB/c AnHan mice were injected intraperitoneally with 50 μg of purified AF emulsified with complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI). Four boosts were given at 3–4-wk intervals. 3 d after the last boost, spleen cells were prepared and fused with P3X63-Ag 8.653 myeloma cells. The fused suspension was distributed in Linbro plates (96 wells) and fed with HAT medium (Boehringer Mannheim GmbH, Mannheim, FRG) supplemented with 10% fetal calf serum (Boehringer Mannheim GmbH). The clones were inspected for antibody production according to an ELISA assay (see below). The positive hybrid cultures were cloned either in HT-medium (plus 10% fetal calf serum) or in serum-free FEB 100 medium (Seromed, Berlin) under conditions of limiting dilution. The specific antibody secreting clones were recloned five times and then grown in 250-ml tissue culture flasks. The class and subclass specificities of the immunoglobulins produced were determined by Ouchterlony analysis using class- and subclass-specific anti-mouse antisera. Selected IgG containing sera (coded 5D2-D11 and 7B10-D11) were purified by affinity chromatography on protein A-Sepharose (7). The purified preparations were supplemented with Merthiolate (0.02% final conc.) and stored at 4°C.

The Fab fragments of IgGs were prepared by papain digestion (31).

McAbs directed against the AF were determined by an ELISA (enzyme-linked immunosorbent assay) as follows. 100 μl of purified AF (10 μg/ml) in phosphate-buffered saline (PBS) was pipetted into a well of a microtiter plate (Dynatech, M129B; Plochingen, Germany). After 24 h at 4°C the wells were washed three times with 3% ovalbumin in PBS. 100 μl of a cell fusion supernatant was pipetted into a well. 4 h (at 20°C) later the cells were washed with PBS and 50 μl of peroxidase-labeled anti-mouse IgG solution (5 ng of protein) was added per well. After an incubation at 20°C for 4 h the wells were washed with PBS, the enzyme substrate (2,2′-azino-di-[3-ethylbenzthiazoline] sulfonic acid) was added, and the positive wells were identified.

Binding of McAbs to the AF in Vitro

200 μg of purified AF was incubated overnight in CMFSW (1 ml) with 10 μg of McAbs (5D2-D11) either in the presence (50 μg) or absence of the homologous aggregation receptor. Then 10 μl of TRITC-conjugated anti-mouse IgG (5 μg) was added, and incubation was continued for 2 h at 20°C. The excess of antibodies was removed from the AF by gel filtration, using a P-300 column (15 × 1 cm) that was equilibrated with CMFSW. The elution was recorded with a spectrofluorometer (Zeiss MQ3-PMQ3) with the excitation wavelength set at 556 nm and the emission wavelength set at 700 nm.

Disintegration and Immunoprecipitation of the AF

The radioiodinated AF (0.5 mg) was incubated at 80°C for 2 h in the presence of 0.1% sodium dodecyl sulfate and 50 mM 2-mercaptoethanol. This material was dialyzed against 50 mM Tris-HCl (pH 8.2), and the residual sodium dodecyl sulfate was removed by anion exchange chromatography using Dowex AG1-X2 resin (11). Immunoprecipitation was done exactly as described (2) using 10 μg of purified McAbs (No. 5D2-D11) for 100 μg of radioiodinated and disintegrated AF. Immunoprecipitation was done with 0.3 ml of protein A-Sepharose.

One aliquot was analyzed directly by 12% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (32). After the run the gel was either stained with silver (28) or dried and then autoradiographed using Kodak DEF x-ray film. The second aliquot was treated with a 0.1 M glycine/HCl buffer (pH 2.95; 1.5 M NaCl) to desorb the antigen. After removal of protein A-Sepharose by centrifugation (2,000 *g*; 5 min), the solubilized antigen was dialyzed against CMFSW. Using this procedure 423 ng of protein (47-kD AF fragment) was isolated from 100 μg of intact AF; the specific activity of the protein was 25 nCi/ng.

Binding Studies with Intact Cells

Living cells (2.5×10^7 cells) were incubated in 1-ml assays in the presence of radioiodinated 47-kD AF fragment (0.01–0.1 μg, dissolved in CMFSW, CMFSW-E, or ASW) for 30 min at 20°C. In one series of experiments the assays were additionally supplemented with purified aggregation receptor (from *Geodia cydonium*). After incubation the cell suspensions were layered onto 2-ml 17.5% Ficoll cushions and centrifuged (200 *g*; 5 min; 7°C). The sediments were counted in a gamma counter. The binding data, K_a (association constant), as well as the maximal number of binding sites per cell, were estimated by the mathematical Ligand-program (26).

Blotting Procedure

The purified AF was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (32). The denatured protein sample (30 μg) was applied to a 12% polyacrylamide gel, containing 0.1% sodium dodecyl sulfate. Then the proteins were transferred to nitrocellulose sheets by the blotting procedure described by Towbin et al. (30). After treating the sheets in a blocking solution (1% of bovine serum albumin [BSA] in 10 mM Tris-HCl pH 7.4, 150 mM NaCl) overnight, the blots were incubated for 4 h with a control mouse serum to reduce nonspecific antibody adsorption to hydrophobic proteins. The blots were then further incubated for 24 h at 2°C in the presence of the McAbs (5D2-D11; 1:100 diluted in the blocking solution). The nonbound IgGs were removed by washing three times with 200 μl of blocking solution, supplemented with 0.5% Tween-20, followed by three washing cycles with the blocking solution alone. The formed immunocomplexes were visualized by incubation with anti-mouse IgG (peroxidase conjugated; diluted 1:500 with blocking solution), using the 4-chloro-1-naphthol/hydrogen peroxide procedure (27). To rule out non-specific staining, controls with IgG from non-immunized mice were run in parallel.

Immunohistochemical Procedures

Fresh sponge cubes (2 × 2 × 2 mm) were fixed in 4% paraformaldehyde (in ASW) for 30 min (4°C) and then washed in PBS. Then the tissue was embedded in Tissue Tek and incubated overnight in a vacuum desiccator under reduced pressure. 2-μm cryostat sections were obtained at −25°C with a Dittes-Duspiva (Dittes, Heidelberg, FRG) cryostat. The sections were washed with PBS and incubated in a 1:200 diluted preparation of the 5D2-D11 McAbs (overnight; 4°C) in a humid chamber. After a further wash with PBS, the slices were incubated with TRITC-conjugated anti-mouse IgG (2 h, 20°C). The excess antiserum was washed off with PBS, and the sections were examined light microscopically. An Osram XBO 140 xenon burner served as light source; an exciter interference filter at 556 nm was used. A heat absorbing filter, Schott BG38, was mounted between light source and exciter filter, a cut off filter,

Leitz K580 was inserted into the microscope tube. For antibody absorption, the McAbs were mixed with 200 μg of purified AF. The mixture was centrifuged (200,000 *g*; 2 h; 2°C) to remove the AF-IgG complex, and the supernatant was used as control antibodies.

Protein was determined by the Fluoram method (33) using pathotrol as a standard. The AF was visualized electron microscopically as described (15).

Results

Antibody Characterization

82 cell hybrids were raised that produced antibodies against the AF (ELISA system). The Fab fragments of the two IgG preparations (coded 5D2-D11 and 7B10-D11) were found to inhibit AF-mediated cell–cell interaction (Table I). Using the Ouchterlony test procedure the 5D2-D11 antibodies were characterized as IgG_{2b} and the 7B10-D11 as IgG_1. The IgGs produced by clone 5D2-D11 displayed a stronger neutralizing effect on the AF activity than the preparation from 7B10-D11 and were therefore used for the subsequent experiments. An amount of 10 μg of IgGs from clone 5D2-D11 abolished the AF activity (30 μg) completely and reduced the size of the aggregates from 2,150 μm (secondary aggregates) to 245 μm (primary aggregates; reference 16) (Table I).

The McAbs were determined to interact with the AF. As shown in Fig. 1, the IgGs from clone 5D2-D11 bound to the high molecular weight AF. This complex was visualized by TRITC-conjugated anti-mouse IgGs; it eluted from the P-300 column within the V_e/V_o (5) range 1.0–1.5. The IgGs that had not reacted with the AF eluted with a V_e/V_o above 2.7. Co-incubation of the AF and the McAbs with the homologous aggregation receptor almost completely suppressed the antibody–antigen reaction, indicating that the IgGs from 5D2-D11 compete with the aggregation receptor for the identical or a spatially adjacent binding site at the AF. The electron microscopical analyses of the fractions 10–14 (Fig. 1) revealed that in both assays (in the presence or absence of the aggregation receptor) 90% of the visible structures had the typical "sunburst" appearance (Fig. 2*a*).

Identification of the Cell Binding Domain of an AF

As known from previous studies (3, 12) the sponge AF is composed of different proteins that are noncovalently or covalently bound to each other (19). The functional domain of the AF was identified after the disintegration of the particle in the presence of sodium dodecyl sulfate and a subsequent separation by polyacrylamide gel electrophoresis (see Materials and Methods; Fig. 3*a*). After the transfer of the proteins to nitrocellulose, the monospecific antibody (5D2-D11), which was found to suppress secondary aggregation (Table I), reacted only with one protein species characterized by a molecular weight of 47,000 (Fig. 3*b*). As a control, the polyacrylamide gel was stained for protein after the blotting procedure. No bands became visible (Fig. 3*c*), indicating that all of the proteins had been transferred on the blot. During the treatment with sodium dodecyl sulfate and 2-mercaptoethanol the sunburst structure of the AF (Fig. 2*a*) appeared to be dissociated into the central ring and the free arms (Fig. 2*b*).

The radioiodinated *Geodia* AF was disintegrated by sodium dodecyl sulfate and 2-mercaptoethanol as described under Materials and Methods. Using the specific McAbs (No. 5D2-D11), that protein, carrying the binding activity of the AF to

Table I. Influence of McAbs against Geodia AF on the Secondary Aggregation of Dissociated Homologous Cells

McAbs added during preincubation with the AF	Size of the secondary aggregates
	μm
None	2,150 ± 470
5D2-D11	
1 μg	1,210 ± 255
3 μg	430 ± 85
10 μg	245 ± 50
7B10-D11	
1 μg	1,840 ± 405
3 μg	1,090 ± 205
10 μg	685 ± 150

Where indicated, Fab fragments of two IgG preparations were preincubated with 30 μg of AF as described in Materials and Methods. Incubation in the presence of cells was performed in the standard assay. The results are from five parallel determinations; the SDs are given.

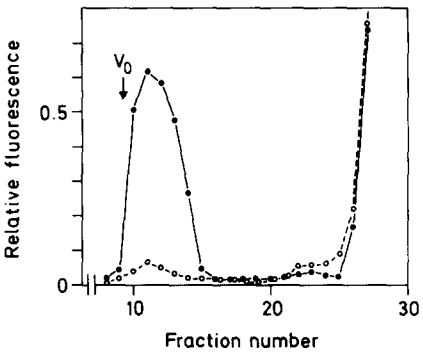

Figure 1. Binding of McAbs (5D2-D11) to the AF in the absence (●) or presence of the aggregation receptor (○). After formation of the AF-IgG complex, the latter was visualized by TRITC-conjugated anti-mouse IgG. The elution was recorded spectrofluorometrically as described under Materials and Methods. In the presented experiment 0.2 ml of the reaction mixture was passed through a P-300 column (15 × 1 cm); fractions of 0.5 ml were collected.

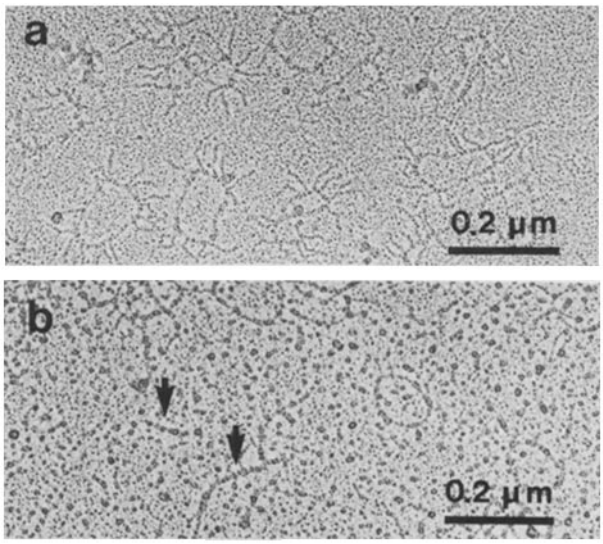

Figure 2. Macromolecular configuration of the purified and biologically active AF from *Geodia cydonium* (*a*). (*b*) Appearance of the AF after its treatment with 0.1% sodium dodecyl sulfate and 50 mM 2-mercaptoethanol (80°C; 2 h) (arrows, liberated arms of the AF).

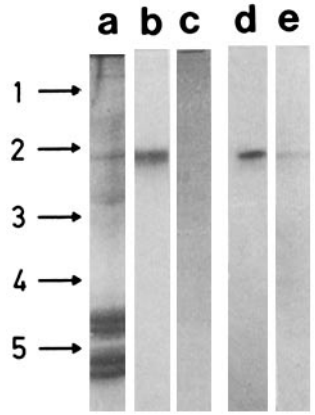

Figure 3. Identification of the binding protein in the aggregation factor complex. (*a*, *b*, and *c*) Protein blot analysis of the functional domain of the AF. Purified AF was electrophoresed on a 12% polyacrylamide gel under denaturing conditions and either directly stained with Coomassie Brilliant Blue (*a*) or the proteins were transferred to a nitrocellulose filter and incubated with McAbs (5D2-D11) to identify the binding protein as described under Materials and Methods (*b*). (*c*) Polyacrylamide gel of AF (as in *a*) after blotting procedure and staining with Coomassie Brilliant Blue. (*d* and *e*) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the radioiodinated binding protein in the complex AF; visualization was performed by autoradiography (*d*) or by silver staining (*e*). The binding protein was purified by immunoprecipitation. Molecular weight standards were (*1*) bovine serum albumin (M_r 66,000), (*2*) ovalbumin (45,000), (*3*) carbonic anhydrase (31,000), (*4*) soybean trypsin inhibitor (21,500), and (*5*) lysozyme (14,400).

the cells, was isolated by an immunoprecipitation procedure (see under Materials and Methods). The protein was analyzed by polyacrylamide gel electrophoresis and subsequent autoradiography or silver staining. The results showed (Fig. 3, *d* and *e*) that only one protein with a molecular weight of 47,000 D became visible. This value is identical with the molecular weight of that protein in the complex AF, identified by the described blotting procedure using the same McAbs.

Interaction of the Functional Domain of the AF with Cells

A quantitative evaluation revealed that 423 ng of the 47-kD protein was obtained from 100 μg of purified AF (M_r 1.3 × 10^8; reference 21), using the described immunoprecipitation procedure. On a stoichiometric base, ~12 molecules of 47-kD protein were calculated to be associated with the AF complex.

The 47-kD AF fragment bound with a high affinity to homologous cells (Table II). The affinity constants (K_a) as well as the maximal numbers of specific binding sites per cell did not vary remarkably in dependence on the presence (ASW) or absence of Ca^{++} ions (CMFSW or CMFSW-E); the K_a values were determined to be ~7 × 10^8 M^{-1} and the binding sites per cell to be ~4 × 10^6. On the other hand, heterologous cells from sponges of the subclasses Tetractinomorpha (*Tethya lyncurium*) and Ceratinomorpha (*Mycale massa*, *Spongia officinalis*) as well as of the class Calcispongia (*Clathrina coriacea*) did not bind measurable amounts of the AF fragment. As a further control to support the specificity of the binding of the AF fragment to homologous cells, the soluble and purified aggregation receptor from *Geodia cydonium* was added to incubation reaction assay (Table II). The results revealed that in the presence of 2.5 μg of aggregation receptor (M_r 18,000, reference 20; corresponding to 8.4 × 10^{13} molecules), no binding of 47-kD protein to homologous cells could be measured; in this experiment 100 ng of the AF fragment (1.3 × 10^{12} molecules) was added to 2.5 × 10^7 cells. These findings, together with the above-mentioned immunological data (Fig. 1), strongly suggest that the 47-kD fragment of the AF is that protein which interacts with the aggregation receptor, irrespectively of whether the receptor is in the membrane bound or the solubilized state.

Table II. Binding of 47-kD Protein, Which Carries the Biologically Active Domain, to Sponge Cells under Different Incubation Conditions

Cells from	Incubation conditions	K_a	Number of binding sites per cell
		$M^{-1} \times 10^{-7}$	$\times 10^{-6}$
Geodia cydonium	CMFSW	71.3	4.2
	CMFSW-E	68.0	4.1
	ASW	74.7	4.2
	CMFSW + aggregation receptor	0.05	0.08
Clathrina coriacea	CMFSW	<0.05	<0.08
Tethya lyncurium	CMFSW	<0.05	<0.08
Mycale massa	CMFSW	<0.05	<0.08
Spongia officinalis	CMFSW	<0.05	<0.08

Homologous or heterologous cells were incubated in the presence of radioiodinated 47-kD AF fragment as described under Materials and Methods. The binding parameters (association constants and maximal number of specific binding sites per cell) were determined. In one experiment 2.5 μg of aggregation receptor (*Geodia cydonium*) was added to the incubation mixture.

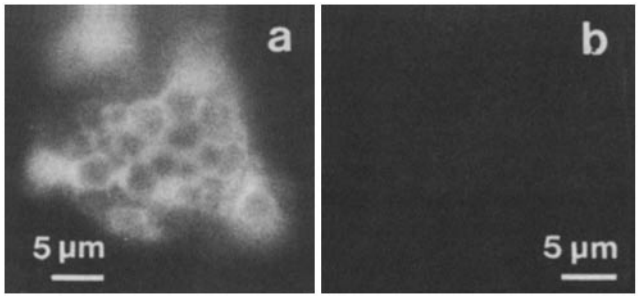

Figure 4. Localization of the AF in sections of *Geodia cydonium*. (*a*) Indirect immunofluorescence staining of a section through a cell cluster in the cortex region with McAbs directed against the functional domain of the AF. (*b*) Staining with the same McAbs adsorbed with AF.

Immunohistochemical Localization of the AF

The AF was localized immunohistochemically in cryostat slices using McAbs specifically directed against the biologically active domain (47-kD protein) of this proteoglycan complex. The antibody–antigen complex was visualized by the indirect fluorescence technique (Fig. 4*a*). With this method the antigen was detectable only extracellularly and was found to be localized on the surfaces of the cells as apparently continuous layers. Adsorption of the McAbs from AF eliminated the specific antibody staining (Fig. 4*b*).

Discussion

The sponge AFs, which are large glycoprotein complexes (M_r 2.1×10^7–1.3×10^8), are provided with a series of functional subunits: (*a*) Ca^{++} binding site(s) (10, 29), which control the stability of the particle as well as its cell binding function; (*b*) the lectin binding site (6), which determines the functional role of the AF in the cell–cell and the cell–substrate interactions; (*c*) two glycosyl transferases (24), which modulate the strength of the interactions between AF and aggregation receptor; and (*d*) the cell binding site. The latter was assumed to interact with the membrane-bound aggregation receptor (20, 34).

Applying immunobiochemical and immunohistochemical techniques, we succeeded in identifying and partially characterizing that fragment of the AF from *Geodia cydonium* that exhibits the cell binding function. After disintegration of the AF in the presence of sodium dodecyl sulfate, a 47-kD protein could be identified by immunoblotting which reacted with a selected McAb. Replacement of sodium dodecyl sulfate by 10% Triton X-100, 50 mM *n*-octyl-βD-glycopyranoside, or 2 mM lithium 3,5-diiodosalicylate during the dissociation procedure of the AF did not result in a quantitative release of the 47-kD protein (data not shown). Subsequent purification of the 47-kD protein by immunoprecipitation and its application for binding studies with homologous cells revealed that ~12, 47-kD protein molecules are associated with one AF particle. Hence the *Geodia* AF is highly polyvalent with respect to its number of cell binding sites. The affinity of the isolated cell binding protein to homologous cells is high and independent of the presence of Ca^{++} ions. This result is a further evidence for the presence of at least one separate Ca^{2+} binding molecular species in the sponge AFs, as already suggested earlier (10). Concerning the *Geodia* AF, 820 mol Ca^{2+} was determined to be bound per 1 mol AF (unpublished observations). It is well established that Ca^{2+} ions play essential roles in AF-mediated cell–cell interactions (35), however, the molecular aspect of this event is not understood. Since our earlier studies (22), which were confirmed later (1), it is well established that macromolecular polycations, but not monovalent cations, can substitute for Ca^{2+}. At least one possibility that appears to be ruled out by our presented data is that Ca^{2+} ions are involved in the species-specific restriction mechanism of the *Geodia* AF, since the binding of the 47-kD protein to cells occurred only in the homologous system.

A further outcome of the binding studies with the 47-kD protein and the homologous *Geodia* cells was the determination of the number of binding sites per cell. Competition experiments revealed that the binding sites on the plasma membrane are identical with the isolated aggregation receptor. Using this approach, 4×10^6 binding sites were determined to be available at one *Geodia* archaeocyte. This figure is close to that determined already earlier (23) using a different, solely biochemical procedure.

On the basis of biochemical (19, 34) and first electron microscopical data (17) it was strongly suggested that (*a*) the sponge AF exists extracellularly and (*b*) it functions there as a bridge-like molecule, linking the cells together. Now, by application of an indirect immunofluorescence staining method using the McAbs, directed against the cell binding protein, the first direct proof for such functions of the AF is given. The staining at the cell–cell contact zones was continuous, which indicates a high density of AF molecules between cells.

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