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Sponge Aggregation Factor: In Situ Localization by Fluorescent Monoclonal Antibody Techniques

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The aggregation factor (AF) from sponges mediates a heterophilic interaction of homologous cells. Applying electron microscopical means, we succeeded only very rarely in identifying the 90 S AF particle in tissue sections from *Geodia cydonium*. By means of a fluorescent antibody technique, we have now localized the cell binding domain of the AF in situ. Previous studies in this laboratory have led to the identification of the 47-kDa cell binding protein of the AF, using the monoclonal antibody (mab) 5D2-D11 [Gramzow M, Bachmann M, Zahn RK, Uhlenbruck G, Dorn A, Müller WEG, J Cell Biol, 102:1344–1349, 1986]. This mab and mab 7D5, directed against a 92-kDa protein in the AF complex, were chosen for the fluorescent studies. By using mab 5D2-D11, the plasma membranes of cells from different regions in the sponge could be brightly stained. However, mab 7D5 reacted only very weakly with the sponge surfaces. By applying the immuno-blotting technique it was furthermore demonstrated that the cell binding protein is present both in the associated form with AF complex and in a free state. Moreover, it was established that the 47-kDa binding protein is not present in (1) homologous glycoconjugates, (2) lectin, or (3) collagen; these components are known to be involved in cell-matrix interaction.

Key words: aggregation factor, monoclonal antibodies, reaggregation, cell recognition, *Geodia cydonium*

Moscona [2] and Humphreys [3] demonstrated that the following three components are essential for the formation of aggregates from dissociated sponge cells: (1) the aggregation factor (AF), (2) Ca^{++}, and (3) cell surface components. The AF has been purified [4,5] and it turned out to be a particle (90 S in the case of *Geodia cydonium*, [6], and 70 S for *Microciona prolifera*, [5]). The particles are composed of a high molecular weight proteinaceous core structure and several lower molecular

Abbreviations used: mab, monoclonal antibodies; AF, aggregation factor; P_i/NaCl, phosphate buffered saline.

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weight proteins covalently attached to it. The cell surface component interacting species-specifically with the AF was later identified, purified, and termed the baseplate [7] or aggregation receptor [8].

In a series of biochemical studies the AF was shown to fulfill a list of criteria to qualify it as a cell adhesion molecule: (1) The AF is involved in forming linkages to and between cells [9], (2) the AF is present prior to and after dissociation of the tissue [10], (3) the purified AF binds to purified aggregation receptor in a model system [7], and (4) the aggregation of cells can be inhibited by polyclonal antibodies raised against purified AF [11]. However, in spite of a large number of publications dealing with the electron microscopical aspects of cell-cell contact in sponges [12], the histochemical proof for existence of the AF—as a particle—is lacking.

With the availability of monoclonal antibodies (mab) directed against the cell binding protein of the AF complex [1], it is now possible to study the distribution of the AF in the tissue. In the present study we describe the cell binding domain of the AF from *Geodia cydonium* as being present in a dense layer on the plasma membrane. On the other hand, by using electron microscopical techniques, the AF could be visualized as a particle only very rarely.

MATERIALS AND METHODS

Materials

The following materials were obtained: Antimouse IgG (whole molecule), tetramethylrhodamine isothiocyanate (TRITC) conjugate (T-5393), and antimouse IgG (whole molecule) peroxidase conjugated (A-2028) from Sigma (St. Louis, MO); nitrocellulose sheets (Ba; 0.45 μm; No. 401180) from Schleicher and Schüll (Dassel, Germany); pathotrol from Dade (Miami, FL).

Live specimens of *Geodia cydonium* (Demospongia) were collected near Rovinj (Yugoslavia).

Preparation of AF and Anti-AF

The AF was isolated and purified from *Geodia cydonium* as described earlier [4,13]. The low molecular weight proteins eluted from the Sepharose 4B column during the separation procedure of the high molecular weight AF [4] were termed "low molecular weight proteins from the extracellular space." Fractions characterized by V_e/V_o values [14] 2.6–2.8 were used for the analyses. Two elution conditions were chosen for isolation: (1) 100 mM Tris-HCl, pH 8.2, 500 mM NaCl, or (2) the same solvent, supplemented with 2 M urea.

Monoclonal antibodies (mab) that specifically recognize AF were obtained after immunization of mice [1]. The IgGs 5D2-D11 and 7D5 were purified from the culture supernatants of two clones [15] and used for the studies. Mab 5D2-D11 was determined to react specifically with the cell binding domain of the AF [1], while mab 7D5 binds to a protein in the AF complex which is not involved in the binding to the aggregation receptor, localized on the cell surface of *Geodia* cells.

Preparation of Other Extracellular Components

The galactose-specific lectin from *Geodia cydonium* was isolated, purified, and characterized as described earlier [16]. Glycoconjugates were isolated from fresh *Geodia* material by the described method [13]; the preparation was lectin-free. Native

Geodia collagen was isolated in the absence of denaturing agents [17]; two fractions were used: (1) semipure preparation after the second precipitation step and (2) pure preparation after an additional gel filtration step. The collagen preparations were both lectin- and glyco-conjugate-free; no sunburst structures which are typical for the AF [4] were detectable electron microscopically.

Immunoblotting

Proteins were resolved by polyacrylamide gel electrophoresis (15% gels) in the presence of sodium dodecyl sulfate, transferred to nitrocellulose paper, reacted sequentially with mabs and antimouse IgG (peroxidase conjugated), and detected after incubation with 4-chloro-1-naphthol/hydrogen peroxide [1]. As a control, the polyacrylamide gels were stained for protein with Coomassie brilliant blue after each blotting procedure. In all cases reported in this contribution, no bands became visible, indicating that all of the proteins had been transferred to the blot.

Preparation of Specimens

Tissue cubes (2 mm^3) were fixed in 2% osmic acid (in 100 mM Tris-HCl, pH 8.2; 500 mM NaCl) for 24 hr at 20°C, dehydrated with ethanol in a graded series (70% to 100%), and embedded into Histoplast (Shandon, Frankfurt) at 60°C. Sections (3 μm thick) were attached to glass slides.

Labeling of Sections

The Histoplast of the embedded sections was removed with Rotihistol (Roth, Karlsruhe) followed by ethanol and phosphate-buffered saline (P_i/NaCl). Sections were incubated with mab IgG (10 μg/ml in 0.5% bovine serum albumin in P_i/NaCl) for 2 hrs at 20°C. After washes in P_i/NaCl, sections were incubated with TRITC-conjugated antimouse IgG diluted 1:200 in P_i/NaCl containing 0.5% albumin. Sections were then washed in P_i/NaCl [9:1 (v/v)] and examined under epifluorescence with a Leitz microscope. Control sections were incubated with preimmune serum from mice in place of the first antibody.

Miscellaneous

For electron microscopy, small sponge pieces were prefixed in 2% (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4; 0.2 M sucrose) for 3 days. Postfixation was carried out in 1% (v/v) osmium tetroxide in 0.05 M cacodylate buffer (pH 7.4) for 2 days. Then the specimens were embedded in Araldite and sliced; sections were stained with uranyl acetate-lead citrate [18].

Protein was determined by the Fluoram method [19] with "pathotrol" as a standard.

RESULTS

Electron Microscopical Aspect of Plasma Membranes

Considering the fact that the isolated AF—as a proteinaceous particle complex—causes cell-cell adhesion in vitro [20,21], this particle is only very rarely seen in tissue sections as checked at a magnification of 90,000×. Such a rare example is shown in Figure 1a. The *Geodia* AF is embedded in an electron-dense material, surrounding the plasma membranes. Figure 1b shows the normal aspect of cell-cell

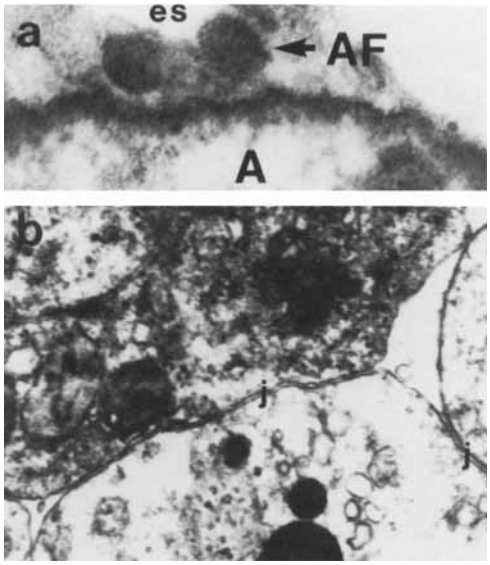

Fig. 1. a) Electron micrograph of aggregating factor (AF) particles present in the electron-dense material surrounding the plasma membrane. A) Archeocyte; es, extracellular space. b) Electron micrograph of a section through *Geodia*. j) Cell junction. Magnification; a: ×90,000; b: ×15,000.

contact (in the absence of the AF complex). The thickness of the fibrillary core structure of the AF, which is not deformable under the conditions used, is 23 nm [22]. Hence, these AF structures should be visible even at a low magnification of 15,000×.

Identification of the Proteins in the AF, Reacting With the mabs 5D2-D11 and 7D5

The following studies were performed with two different mabs: (1) mab 5D2-D11, which reacted specifically with the functional domain of the AF, and (2) mab 7D5, which reacted with a protein in the AF complex which is different from the aggregation receptor-binding protein [1].

As demonstrated in a previous study [1], the AF particle can be desintegrated into a series of protein species in the presence of sodium dodecyl sulfate (Fig. 2A). Mab 5D2-D11 reacted with one protein species of the desintegrated AF complex (Fig. 2Ba). This protein is characterized by a molecular weight of 47 kDa. On the other hand, with mab 7D5 a second protein with an apparent M_r of 92 kDa (Fig. 2Bb) could be visualized.

Extracellular Localization of AF Proteins

Using the same mabs as tools, protein species with identical antigenic properties were identified in the AF-free, low molecular weight protein fractions from the extracellular space. The fraction obtained after gel filtration with a Tris buffer in the absence of urea contained both the 47-kDa protein (with mab 5D2-D11; Fig. 2Ca) and the 32-kDa protein (with mab 7D5; Fig. 2Cb). However, the fractions from gel filtration experiments in the presence of 2 M urea and cut between the same V_e/V_o

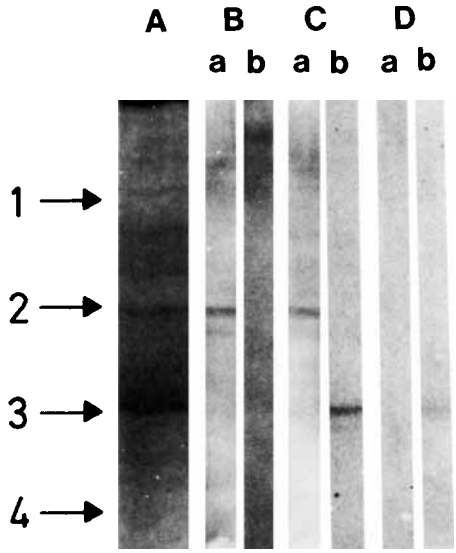

Fig. 2. Immunoblots of purified AF and of low molecular weight proteins from the extracellular space using mabs. A) Purified *Geodia* AF was electrophoresed on 15% polyacrylamide gel under denaturing conditions and stained directly with Coomassie brilliant blue. The proteins of the purified AF (B) low molecular proteins from the extracellular space obtained in the absence (C) or presence of urea (D) were resolved by electrophoresis and immunoblotted with the monoclonal antibodies 5D2-D11 (a) or 7D5 (b). Each lane contained 25 μg of protein. Details are given under Materials and Methods. Molecular weight standards were (1) bovine serum albumin (66 kDa), (2) ovalbumin (45 kDa), (3) carbonic anhydrase (31 kDa), and (4) soybean trypsin inhibitor (21.5 kDa).

values (2.6–2.8) contained only the 32-kDa protein (Fig. 2Db) but not the 47-kDa protein species (Fig. 2Da).

The two mabs chosen did not react with proteins of the *Geodia* lectin or with purified collagen of the same source (Fig. 3B,D). However, the 32-kDa protein was identified in the glycoconjugate fraction (Fig. 3Ab) and the 47-kDa protein in the semipure collagen material (Fig. 3Ca).

Immunofluorescence Studies

Sections through different areas of *Geodia* were performed; the areas were identified by phase-contrast microscopy (Fig. 4b,d,g). The mab 5D2-D11 (recognizing the 47-kDa cell binding protein in the AF) brightly stained the plasma membranes of the cells (1) in the central part of the sponge (Fig. 4a), (2) in the choanosome (Fig. 4c), (3) in the outer ectosome (Fig. 4e), and (4) around the silica spicule (Fig. 4f). Adsorption of the mab with AF eliminated the specific antibody staining (not shown).

Parallel studies with mab 7D5, which does not recognize the cell binding protein in the AF complex, gave—if at all—only a weak staining of the plasma membranes. As an example a section through the outer ectosome is shown (Fig. 5).

DISCUSSION

The striking finding in the present study is the low incidence of the AF complex detectable in tissue sections by electron microscopical means. This finding was

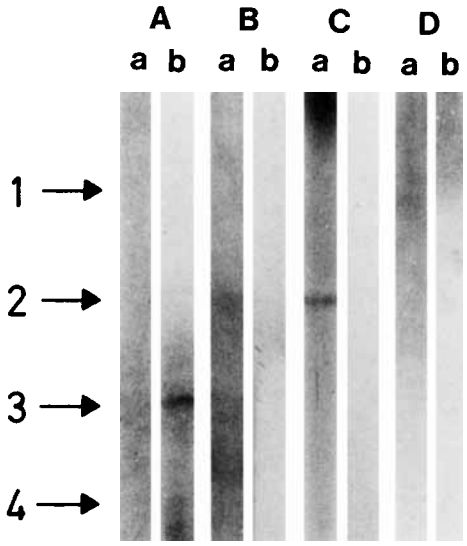

Fig. 3. Immunoblots of glycoconjugates, lectin, and collagen from *Geodia*. Purified glycoconjugates (A), purified *Geodia* lectin (B), semipure (C), as well as purified collagen (D) were electrophoresed, blotted, and incubated with mab 5D2-D11 (a) or with mab 7D5 (b). Each lane contained 25 μg of protein. The arrows mark the positions of the protein standards (see Fig. 2).

unexpected inasmuch 2,000 ball-like AF particles, with the known diameter of 0.07–0.13 μm [23], can bind to an average sponge cell (diameter: 8 μm; [12]) in the *Geodia* system. Jumblatt et al [24] even suggested that in vivo each *Microciona prolifera* cell has on the average some 28,000 molecules of AF associated with it. In order to clarify whether this finding is real, or due to the application of inadequate electron microscopical techniques hitherto used, we applied the method of immunofluorescence staining with mab, directed against the AF.

For this approach, we used one mab (5D2-D11) reacting specifically with the 47-kDa binding protein of the AF and another mab (7D5) binding to a protein (92 kDa) in the AF, which is not involved in the binding to the surface of *Geodia* cells [1]. The indirect immunofluorescence data revealed that mab 5D2-D11 stained the plasma membrane of all cells brightly, while the staining with mab 7D5 was extremely weak. The staining of the plasma membrane of the cells with mab 5D2-D11 was equally bright, irrespective of their localization within the sponge. In general, the quality of the immunofluorescent pictures is not high due to the fact that the *Geodia* tissue is very rich in siliceous spicules.

The immunohistological data suggested that the binding protein of the AF is present not only in the associated form with the AF particle but perhaps also in a free state. This assumption was substantiated by blotting experiments (using mab 5D2-D11) revealing the 47-kDa protein to be present also in the AF-free, low molecular weight fraction from the extracellular space. This finding raises new questions: eg (1) why has there never been detected in previously performed gel filtration experiments [4,5] an aggregation-promoting activity in the low molecular weight range; (2) is the binding protein monovalent; (3) has the AF complex additional functions, which include a role in sorting out or in pattern formation [25,26]? The answers to these questions are expected from ongoing studies.

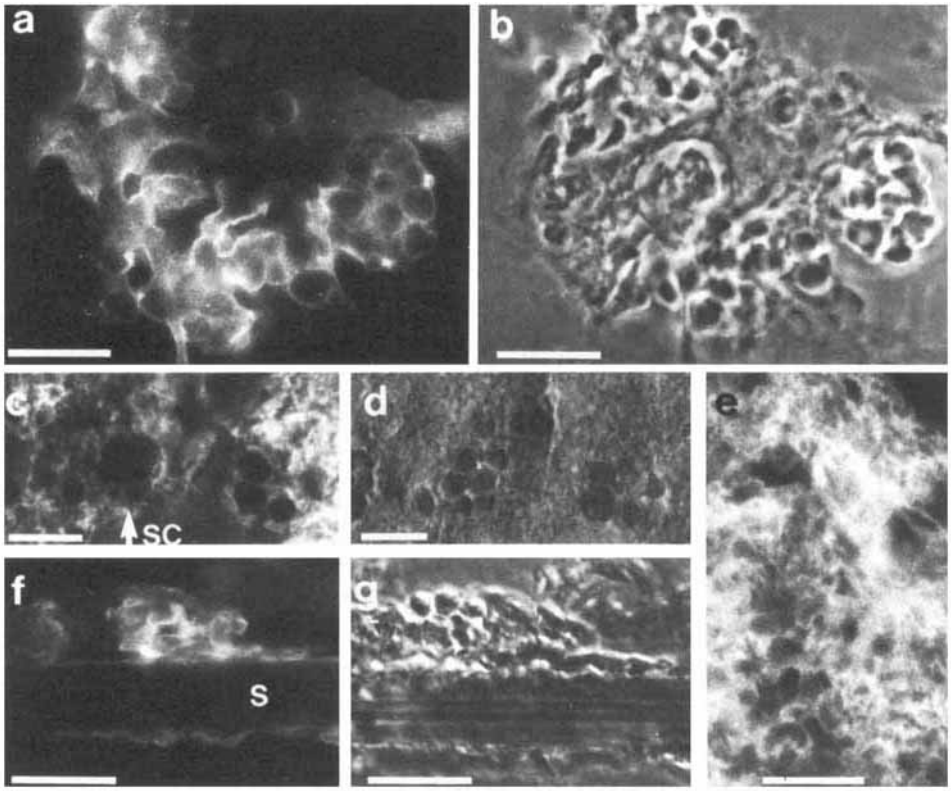

Fig. 4. Localization of the 47-kDa binding protein of the AF in tissue slices by indirect immunofluorescence staining. Section through central part (a) and (b) choanosome, showing spherulous cells [SC] (c), (d), outer ectosome (e), and an area around a silica spicule [S] (f) and (g). Fluorescence staining was performed with mab 5D2-D11 in (a), (c), (e), and (f); phase-contrast micrographs of the corresponding areas (b), (d), and (g). Bars: 20 μm.

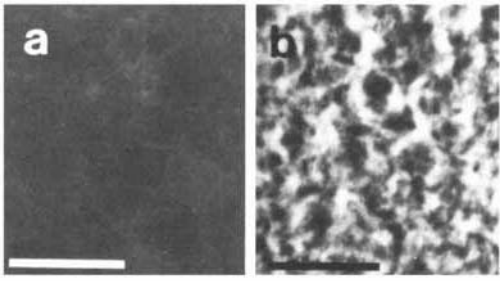

Fig. 5. Section through the outer ectosome region of *Geodia*. The specimen was treated with mab 7D5 and analyzed by indirect immunofluorescence (a); (b) same area, visualized by phase-contrast microscopy. Bars: 20 μm.

A further outcome of the present investigation is the proof that the 47-kDa binding protein is not present in the glycoconjugates, in the galactose-specific lectin and in the collagen fraction from the same source. These three extracellular components were determined previously to be involved in cell matrix interaction [13,16,17]. On the other hand, the 92-kDa protein is present as a 32-kDa fragment not only in the lower molecular weight protein fraction, obtained after gel filtration; it exists also in the glycoconjugates fraction. Because of the fact that it is not yet known which epitope on the proteins (or glycoproteins) is recognized by mab 7D5, it can not be decided if these two macromolecules are distinct species or not.

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