A scale associated protein of *Apedinella radians* (Pedinellophyceae) and its possible role in the adhesion of surface components

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SUMMARY

Monoclonal antibodies have been generated against cell surface components of the unicellular phytoflagellate *Apedinella radians* (Pedinellophyceae). One monoclonal antibody, designated *Arg 1E5/1B1*, labels a scale associated protein (SAP) of 145 kDa. Immunofluorescence microscopy of whole cells as well as immunoelectron microscopy of whole cell mounts and thin sections using *Arg 1E5/1B1* have shown that the SAP is located on the proximal surface of body scales and spine-scales. Its specific location suggests that the SAP may play a role in the adhesion of these surface components to the cell membrane and/or to one another. The potential of monoclonal antibody *Arg 1E5/1B1* as a tool to study cell surface morphogenesis and the role of the endomembrane system in *A. radians* is discussed.

Key words: *Apedinella*, cell surface components, cell surface adhesives, monoclonal antibodies

INTRODUCTION

The marine unicellular phytoflagellate *Apedinella radians* (Pedinellophyceae) provides an ideal system for investigating cell surface morphogenesis. The cells produce two types of extracellular surface components, ovoid body scales and elongate spine-scales (Throndsen, 1971; Koutoulis et al., 1988). An imbricate layer of body scales encases the cell, and individual body scales are attached to the cell membrane and/or other body scales by material of unknown composition, while six spine-scales are attached to the cell surface via extracellular microligaments. The spine-scales can undergo a drastic reversible reorientation between posterior and lateral orientations that appears to be coordinated by a complex cytoskeleton. Irrespective of spine-scale orientation, spine-scales maintain a close association with specialized tentacles by a substance also of unknown composition (Koutoulis et al., 1988).

Using detergent extracted cells of *A. radians* as an antigen source, preliminary experiments were conducted to determine the antigenicity of surface components of *A. radians*. Positive results gained from these experiments led to a program to generate monoclonal antibodies, with the aim of characterizing molecules associated with cell surface components (i.e. body scales and/or spine-scales). A monoclonal antibody, designated *Arg 1E5/1B1*, which labelled a specific scale-associated protein was obtained. Studies on cell surface morphogenesis were initiated using this antibody and are described below.

MATERIALS AND METHODS

Maintenance of algal cultures

Cells of *Apedinella radians* were grown in f/2 medium (Guillard and Ryther, 1962) in two different growth cabinets maintained at 16°C and 20°C with 15:9 hour and 12:12 hour light:dark cycles respectively.

Cell lysis and fractionation (ghost preparations)

Cells in logarithmic growth phase were concentrated using a continuous flow centrifuge, pelleted at 350 g for 5 minutes and then lysed in an equal volume of ice-cold stabilizing buffer (15 mM Hepes, 10 mM EGTA, 15 mM KCl, 5 mM MgSO₄·7H₂O, pH 7.2; Höhfeld et al., 1988) containing 2% Nonidet P-40 (Sigma Chemical Co., St Louis, MO, USA) and 1 mM phenylmethylsulfonyl fluoride. After lysis, samples were brought to 10 ml in buffer without detergent, mixed thoroughly and left on ice for 5 minutes. Ghost preparations (ghosts) were obtained by centrifugation at 3000 g for 10 minutes with two washes in stabilizing buffer.

For direct visualization, a drop of ghosts was allowed to settle for 30 minutes onto 50 mesh Formvar-coated copper grids. Excess liquid was removed and preparations were stained with 2% aqueous uranyl acetate and then examined in a Siemens 102 transmission electron microscope. For antibody production, ghosts were stored in liquid nitrogen until use. For SDS-PAGE, ghosts were boiled in the presence of sample buffer (Laemmli, 1970) for 8-10 minutes with intermittent mixing and then stored at −20°C. Preparations were clarified by centrifugation prior to electrophoresis.

Monoclonal antibody preparation

Frozen ghosts were thawed and resuspended in filter-sterilized
(0.22 µm) phosphate buffered saline (PBS: 147 mM NaCl, 16 mM Na₂HPO₄, 4 mM Na₂H₂PO₄·2H₂O). Equal volumes of Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT, USA) and ghosts were mixed and then injected intraperitoneally into 3 Balb/c mice (day 0). Subsequent injections were given on days 22, 43 and 91. Test bleeds were performed on days 54 and 112 and sera were prepared according to the method of Harlow and Lane (1988). Sera from test bleeds were screened using immunofluorescence and immunoblot analysis as described below. A final boost was given on day 246 and on day 251 the spleen cells from an immunized mouse were fused with NS1-A myeloma cells using polyethylene glycol (Thompson et al., 1983). Hybridoma supernatants were screened on days 10-20 post-fusion using immunofluorescence microscopy on intact A. radians cells. Hybridoma cell lines of interest were cloned by limiting dilution (Zola, 1987) and the resulting supernatants were screened for immunopositive monoclonal antibodies using immunofluorescence microscopy. The heavy and light chain types of the monoclonal antibodies were determined following the manufacturer’s instructions of a Mouse Monoclonal Antibody Isotyping Kit (Amersham, UK). Monoclonal antibody producing cell lines were stored in liquid nitrogen and prior to storage at 20°C, supernatants were buffered with 1 M Tris, pH 8.0, (1:20) and sodium azide was added to 0.02%.

**Immunofluorescence microscopy**

Intact A. radians cells were processed following the protocol of Koutoulis et al. (1988) and then allowed to settle overnight on multilw slides (Carlson Scientific Inc., Peotone, IL, USA) coated with 0.05% poly-L-lysine (Sigma Chemical Co., St Louis, MO, USA). Preparations were rehydrated by rinsing slides in PBS containing 1 mM EGTA (PBS/EGTA) and non-specific binding sites were blocked by adding 3% BSA (fraction V) in PBS/EGTA (PBS/EGTA/BSA) to each well for 30 minutes. Slides were then rinsed and preparations incubated in serum or supernatant (at various dilutions in PBS/EGTA/BSA) at 37°C for 2 hours. After rinsing in PBS/EGTA, the preparations were labelled with a 1:32 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (ICN, Lisle, IL, USA) in PBS/EGTA/BSA at 37°C for 0.5% benzoyl peroxide in the PBS/EGTA. The serum or supernatant with PBS/EGTA/BSA, serum and prior to storage at 20°C, supernatants were buffered with 1 M Tris, pH 8.0, (1:20) and sodium azide was added to 0.02%.

**Electrophoretic and immunoblot analysis**

Polypeptides were resolved by SDS-PAGE essentially as described by Laemmli (1970), using a 6% resolving gel with 4% stacking gel. Molecular weight standards were obtained from Pharmacia (Piscataway, NJ, USA).

For immunoblot analysis, resolved polypeptides were transferred from gels to 0.45 µm nitrocellulose filters (Schleicher and Schuell, Germany) essentially according to the procedure of Towbin et al. (1979). Transferred polypeptides were visualized by staining blots with 0.1% India Ink in 12.5 mM Tris, 137 mM NaCl, 2.7 mM KCl (TBS) containing 0.1% Tween-20 (TBST). Immunoreactive polypeptides were detected by blocking blots, immediately following transfer, in 10% non-fat milk powder in TBST for 1 hour. Blots were washed 3 times in TBST, 3 times in TBS and then incubated in the monoclonal antibody diluted in TBS containing 3% BSA (TBS/BSA) overnight at room temperature. Blots were washed as above and incubated in a 1:500 dilution of horseradish peroxidase-conjugated sheep anti-mouse antibody (Silenus, Hawthorn, Victoria, Australia) in TBS/BSA for 1-2 hours at room temperature. Blots were then washed several times in TBS and immunoreactive bands were visualized using 4-chloro-1-naphthol and H₂O₂. Control incubations were performed as described above.

**Glycan detection**

Protein preparations resolved by SDS-PAGE were blotted onto nitrocellulose filters as described above. The detection of glycoconjugates was carried out using a Glycan Detection Kit (Boehringer Mannheim, Germany). Oxidation and digoxigenin labelling were performed on blots according to Method B of the manufacturer’s instructions. Immunolabellings, as described above, were also performed after oxidation with NaIO₄ and after oxidation and subsequent digoxigenin labelling to determine whether antigenicity was retained.

**RESULTS**

**Ghost preparations**

Wholemount studies revealed that surface components

**Wholemounts**

Whole cells, fixed as for immunofluorescence (Koutoulis et al., 1988), were allowed to settle for 3 hours onto 50 mesh Formvar-coated nickel grids. Excess liquid was blotted away with filter paper and the preparations were blocked by floating the grids, cells side down, on a drop of PBS containing 1% BSA (PBS/BSA) for 30 minutes. Cells were incubated in monoclonal antibody diluted in PBS/BSA for 2-3 hours at room temperature. Grids were extensively washed in PBS and then rinsed in distilled water. Controls were performed as described for immunofluorescence microscopy. Preparations were stained with 2% aqueous uranyl acetate.

**Thin sections**

Cells were fixed using 1% glutaraldehyde in 0.2 M medium at room temperature. Nine minutes later, OsO₄ was added (final concentration 0.15% OsO₂) and cells were placed on ice. Cells were fixed for a total of 1 hour, pelleted, washed in 0.2 M medium and then brought into distilled water by sequential transfers. Cells were dehydrated by sequential transfers of ethanol and infiltrated with increasing proportions of LR gold (London Resin Co. Ltd., Surrey, UK) to ethanol over the course of 1-3 days. LR gold polymerization was achieved at 4°C with 0.5% benzoyl peroxide in the dark. Gold sections were cut on a Reichert Ultratcut E ultramicrotome using a diamond knife and collected on 50 mesh Formvar-coated nickel grids. Sections were equilibrated by floating the grids, sections side down, on a drop of PBS for 10 minutes before being blocked and labelled as described above for wholemounts. Negative controls were performed as described above. After labelling, sections were stained with 2% aqueous uranyl acetate for 15 minutes at 60°C and lead citrate for 3-5 minutes at room temperature. Wholemounts and thin sections were examined in either a Siemens 102 or a Jeol 1200 EX transmission electron microscope.
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(body scales and spine-scales) were retained in ghosts of *Apedinella radians* (Fig. 1). Cytoskeletal components were also preserved, demonstrating the potential this procedure possesses for isolating cytoskeletons of *A. radians* (Figs 1, 2A,B).

**Monoclonal antibodies**

Antibodies recognizing *A. radians* components were detected after the first test bleed by both immunofluorescence and immunoblot analysis. A number of antibodies were generated, including those recognizing spine-scales, body scales and cytoskeletal components (not illustrated).

Post-fusion screening of hybridoma supernatants yielded eighteen parental hybridoma lines which labelled intact cells of *A. radians*. Five of these lines were successfully cloned by limiting dilution. Resulting monoclonal antibodies were designated *Arg* (code) to indicate their origin from *Apedinella radians* ghosts. Preliminary immunofluorescence results showed that one monoclonal antibody, *Arg* 1E5/1B1, intensely labelled the cell covering of *A. radians* (Fig. 3). Moreover, labelling of isolated body scales with *Arg* 1E5/1B1 demonstrated that the labelling was localized to individual body scales which comprise the scale case (Fig. 4). Due to these properties, this antibody was further characterized.

**Arg 1E5/1B1**

Antibody isotyping revealed that *Arg* 1E5/1B1 belongs to the IgG1 subclass and possesses a κ light chain (not shown). Immunoblot analysis showed that *Arg* 1E5/1B1 labelled a...
polypeptide band with a molecular mass of 145 kDa (Fig. 5B). Glycan detection experiments demonstrated that several of the polypeptide bands present in the whole cell preparations of A. radians were glycoconjugates (cf. Fig 5A and F). However, these experiments also indicated that the 145 kDa polypeptide recognized by Arg 1E5/1B1 was not labelled with the glycan detection system (cf. Fig 5A and B) and, therefore, is probably not a glycoconjugate. Additionally, the epitope recognized by Arg 1E5/1B1 is not a carbohydrate moiety but is proteinaceous, as oxidation with NaIO₄ (Fig. 5C) or oxidation followed by digoxigenin labelling (Fig. 5D) did not inhibit labelling of the 145 kDa band with Arg 1E5/1B1.

As mentioned above, immunofluorescence studies demonstrated that Arg 1E5/1B1 labelled body scales. At higher magnification, the results of immunogold electron microscopy on wholemounts of A. radians cells and isolated body scales suggested that the surfaces of some body scales were not labelled with Arg 1E5/1B1 (Figs 6, 7), although the edges of all body scales were labelled (Fig. 8). In addition, conspicuous labelling was observed at the bases of spine-scales (Fig. 11).

Immunogold microscopy using Arg 1E5/1B1 on thin sections of A. radians cells confirmed the labelling pattern observed with the fluorescence microscope. Gold particles densely labelled the cell covering of A. radians (Fig. 12), and sections through the scale case showed that labelling was localized to the surface of the scales (Figs 13, 14). Cross-sections through body scales demonstrated that the antigenic component recognized by Arg 1E5/1B1 was
restricted to the proximal surface of body scales (Fig. 14). This finding explains the “discrepancy” observed in the immunogold wholmount studies: unlabelled body scales correspond to body scales with exposed distal surfaces. In addition, labelling on thin sections of *A. radians* demonstrated that the antigenic component recognized by *Arg 1E5/1B1* is located on the proximal surface of spine-scales (Figs 16, 17). The specific location of this antigenic component, on the rims of body scales and the proximal surface of both body scales and spine-scales, strongly suggests that it may be involved in the adhesion of these surface components to the cell membrane and/or to one another. Due to its location, the antigenic component recognized by *Arg 1E5/1B1* is termed a scale-associated protein (SAP).

Immunogold labelling on thin sections of *A. radians* using *Arg 1E5/1B1* also demonstrated that the SAP is located within the cell in several regions of the cytoplasm. Sections through the posteriorly located dictyosome of *A. radians* showed that *Arg 1E5/1B1* labelled membranes and lumens of Golgi cisternae (Fig. 18). Labelling was also present within the trans-Golgi reticulum and occasionally gold particles were found associated within the cell membrane (Fig. 18). Vesicles with granular inclusions are found throughout the cytoplasm of *A. radians* (Figs 19, 20, 21). These large granular vesicles (LGVs) are often located adja-
cent to the cell membrane (Figs 19, 20, 21) and are prominent in the posterior region of the cell (Fig. 21). Relatively high levels of labelling with Arg 1E5/1B1 were found in LGVs (Figs 19, 20, 21), suggesting a concentration of SAP within these cytoplasmic vesicles.

DISCUSSION

Previous workers have been successful in generating antibodies recognizing surface components of various unicellular organisms. These include: monoclonal antibodies to phaeophyte sperm (Jones et al., 1988), fern spermatozoids (Marc et al., 1988), and oomycete zoospores and/or cysts (Hardham et al., 1986; Gubler and Hardham, 1988; Estrada-Garcia et al., 1990); and polyclonal antibodies to euglenophytes (Rogalski and Bouck, 1980; Bricheux and Brugerolle, 1986), prymnesiophytes (van Emburg et al., 1986) and prasinophytes (Domozych et al., 1991). Cell surfaces contain cell specific components which, in many cases, elicit a strong immune response (Harlow and Lane, 1988). Apparently, cell surface components of A. radians are also highly antigenic since, in this study, five monoclonal antibodies recognizing the cell surface of A. radians were obtained.

One monoclonal antibody, Arg 1E5/1B1, was found to recognize a specific scale-associated protein. Immunoblot analysis demonstrated that this antibody labels a polypeptide with a molecular mass of 145 kDa. From the results of the glycan detection analysis, the epitope recognized by Arg 1E5/1B1 is apparently proteinaceous and the 145 kDa polypeptide is not glycosylated. Ultrastructural studies, in conjunction with immunogold labelling, revealed that this antigenic component is located on the proximal surface of both body scales and spine-scales, as well as the elevated rims of body scales. Due to its location, the antigenic component recognized by Arg 1E5/1B1 is termed a scale associated protein (SAP).

A number of fixation protocols and embedding resins were tested in order to locate the SAP in thin sections at the ultrastructural level. According to Hayat (1986), “the selection of a fixative for immunocytochemistry must necessarily be a compromise between reliable demonstration of antigens in situ and satisfactory preservation of ultrastructure”. Ultrastructural details in A. radians are best seen using the protocol described by Koutoulis et al. (1988). Immunogold labelling on Spurr-embedded sections, however, is not often successful as the resin impedes immunoreactions (Bendayan et al., 1987). Spurr-embedded sections of A. radians were etched with 10% hydrogen peroxide or
0.58 M NaIO₄ (Bricheux, Mahoney and Gibbs, unpublished observations) to allow antibody-antigen binding, but these methods proved to be unsuccessful. Cells were therefore embedded in LR Gold after being fixed using a variety of protocols (e.g. 2.5% paraformaldehyde/0.3% glutaraldehyde; 1% glutaraldehyde). As expected (see Hayat, 1986), the greatest labelling was observed with the lowest glutaraldehyde concentration but preservation was extremely poor. Recent findings indicate that glutaraldehyde and osmium tetroxide are not as destructive to some antigens as once thought (Hayat, 1986). Good preservation of ultrastructure was observed and substantial gold-labelling was obtained in cells fixed with 1% glutaraldehyde and subsequently with 0.15% osmium tetroxide. Using this protocol, labelling of the SAP was found in various sites within the endomembrane system of the cell, including the Golgi apparatus, trans-Golgi reticulum and the cell membrane as well as LGVs.

Through these studies it became apparent that the SAP traverses the endomembrane system. Since it is destined to be located on extracellular body scales or spine-scales, this protein has all the characteristics of a secretory protein (Farquhar, 1985). As the SAP appears to accumulate in LGVs, it is conceivable that LGVs are indeed storage granules (Farquhar, 1985). The formation and secretion of body scales and spine-scales of A. radians appears to be restricted to the posterior region of the cell (Koutoulis and Wetherbee, unpublished observations). As LGVs have been observed in all regions of the cell (posterior, equatorial and anterior), it is possible that LGVs may secrete their contents from these regions. If this is the case, then at least some of the SAP may become associated with surface components extracellularly. A different interpretation of these results is that LGVs are not secretory granules. Instead, they may be involved in endocytosis by retrieving and storing excessive amounts of the SAP. During immunogold labelling studies, sections of cells undergoing the process of body scale and/or spine-scale formation were not obtained. Therefore, the precise nature of how the SAP becomes associated with body scales and spine-scales during cell surface morphogenesis is not yet known. Work currently in progress, using Arg 1E5/1B1 to label cells in the process of body scale and/or spine-scale formation, aims to clarify when the SAP becomes associated with both these surface components.

It is not known how secreted body scales and spine-scales that have yet to be deployed remain attached to the cell proper. Additionally, it is unknown how spine-scales maintain a close association with specialized tentacles (Koutoulis et al., 1988). The precise location of the SAP strongly suggests that it may be involved in the adhesion of these surface components to the cell membrane (i.e. to the cell proper in the case of body scales and newly secreted spine-scales; and to tentacles in the case of deployed spine-scales). Monoclonal antibodies have been successfully generated against adhesive material of zoospore cell surfaces in two different Oomycetes: Phytophthora cinnamomi (Guhler and Hardham, 1988) and Pythium aphanidermatum (Estrada-Garcia et al., 1990). In both these studies, the adhesive material was localized intracellularly to vesicles situated adjacent to the cell membrane. Ultrastructural studies (Barr and Désauniers, 1989) and molecular comparisons (Gunderson et al., 1987; Ariztia et al., 1990), suggest a close relationship between the Oomycetes and chromophyte algae. Whether LGVs are analogous (or even homologous) structures to the above-mentioned adhesive-containing vesicles remains to be investigated.

Similar studies on Mallomonas splendens (Synurophyceae) in our laboratory have also led to generation of monoclonal antibodies against surface components (Wetherbee et al., 1992), and, as mentioned above, antibodies raised against surface components of other unicellular organisms have been obtained. Since cell surface components typically elicit a strong immune response (Harlow and Lane, 1988) it is not surprising that components of A. radians cell coverings are highly antigenic. Hardham et al. (1986) generated a number of monoclonal antibodies to isolate-, species- and genus-specific components of Phytophthora cinnamomi zoospores and cysts, and suggested that they could use these to identify and monitor this destructive plant pathogen. These principles could be modified to the identification and monitoring of toxic, or potentially toxic, phytoplankton and could be extended to determine relationships between taxa. The production of family-, order- and class-specific monoclonal antibodies may provide the necessary tools in establishing a more natural classification of organisms. Therefore, in addition to obtaining a probe to examine cell surface morphogenesis, this study has generated monoclonal antibodies which may prove to be useful in resolving taxonomic relationships.

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