IDENTIFICATION OF A DEVELOPMENTALLY REGULATED PLASMA MEMBRANE GLYCOPROTEIN INVOLVED IN ADHESION OF *POLYSPHONDYLIUM PALLIDUM* CELLS

Claudia STEINEMANN, Rudolf HINTERMANN and Roger W. PARISH*

Cytology, Plant Biology Institute, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

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1. Introduction

A glycoprotein (contact sites A) is involved in the specific cell adhesion required for cell aggregation during development of the cellular slime mold *Dictyostelium discoideum* [1–3]. The synthesis of contact sites A is developmentally controlled, occurring during aggregation and ceasing when aggregation is complete [4,5]. The sites are subsequently lost from the membrane [4,6]. Contact sites A are antigenic [1–3,6] and cell adhesion can be completely blocked by univalent antibody fragments (Fab) directed against them [2].

Membrane antigens are also involved in the aggregation of another cellular slime mold *Polysphondylium pallidum* [7]. Two target sites of adhesion blocking Fab have been detected, the first being present on growth phase (vegetative) cells, the second appearing during cell differentiation from the growth phase to the aggregation competent stage [7].

We have examined the synthesis of plasma membrane proteins during development of *P. pallidum* and identified three antigens which may be involved in specific cell adhesion [8]. Here we show that Fab directed against membrane antigens of vegetative cells blocks cell adhesion. A butanol extract from the plasma membrane and containing only two antigens (110 kilo daltons (kd) and 71 kd) reversed this blocking effect. We cut the individual antigens from SDS-gels and used them to remove their respective Fab from the total Fab. We could then show that only the 71 kd antigen was responsible for cell adhesion. The 71 kd antigen, although also present on vegetative cells, is strongly synthesized during aggregation [8]. We present evidence that it is lost from the cells when aggregation has been completed.

* To whom correspondance should be addressed

2. Materials and methods

Polysphondylium pallidum (strain Ti-1) amoebae were grown with *Escherichia coli* on agar [8]. Cells were washed and allowed to differentiate on millipore filters [4]. Labelling of cells with D-[1-^{14}C]glucosamine, isolation of plasma membranes with concanavalin A–Triton X-100, electrophoresis on SDS–acrylamide (10%) gels and autoradiography were as described [4–6].

Membrane fractions obtained from vegetative and mid-aggregation cells were used as antigens [8]. The immunization of rabbits and the detection of antigens in gels were carried out as in [6].

Butanol extracts were prepared by shaking a plasma membrane preparation (5 mg protein/ml in 10 mM citrate buffer, pH 5.5) with *n*-butanol (1:0.75, v/v) for 5 min at 4°C. The solution was centrifuged for 5 min at 1900 × *g* and the lower water-phase dialyzed against 500 vol. 0.5% Triton X-100. Following a centrifugation (10 min at 3000 × *g*) the supernatant was concentrated by vacuum dialysis. The resulting 'butanol extract' was used in the experiments examining the effects of Fab on cell adhesion (the vacuum dialysis removed the Triton X-100). The antigens in the 'butanol extract' were detected on SDS–gels using antiserum and anti-IgG coupled to peroxidase [6].

Rabbit immunoglobulins were precipitated from whole serum by adding ammonium sulfate to 40% saturation, stirring overnight (4°C) and centrifuging at 2000 × *g* for 10 min. The pellet was redissolved in 40% saturated ammonium sulfate and again centrifuged. The pellet was dissolved in 20 mM sodium phosphate buffer (pH 8.0) and dialyzed against the same buffer overnight. The IgG was separated from contaminating serum compounds using DEAE-cellulose column chromatography. The dialyzed immunoglobulins (3 ml, 7 mg protein/ml) were applied and IgG eluted with the phosphate buffer.

IgG was incubated with papain (1 mg papain/100 mg IgG) in 10 mM cysteine, 2 mM EDTA, 0.1 M phosphate buffer (pH 7.0) for 16 h at 37°C. The digest was dialyzed against 0.1 M phosphate buffer, 2 mM EDTA, for 36 h at 4°C. The Fc fragment precipitated out and was pelleted by centrifugation (10 000 × *g*, 20 min). The supernatant was concentrated by vacuum dialysis, applied to a Sephadex G-200 column and eluted with 0.02 M phosphate buffer (pH 7.0), 0.12 M NaCl. Fab was eluted after aggregates and undigested IgG. The purity of the Fab was checked with SDS–gel electrophoresis.

Removal of Fab specific for the 110 kd and 71 kd antigens [8] was as follows: Plasma membranes were isolated from vegetative cells (1.4×10^9) and extracted with butanol. The extract (275 μg protein in 400 μl) was electrophoresed on SDS–slab gels (18 lanes). The regions corresponding to the 110 kd and 71 kd antigens were cut out and SDS removed by shaking the gel pieces in 25% isopropanol/10% acetic acid solution for 20 h, changing the solution once. The gel pieces were then washed with 15 mM Na-phosphate buffer (pH 6.8) for 3 h with one change of buffer. The slices were shaken for 5 h with 175 μg Fab in PDF solution (20 mM KCl, 25 mM $MgSO_4$ in 0.05 M Sørensen phosphate buffer (pH 6.5)). The PDF solution con-

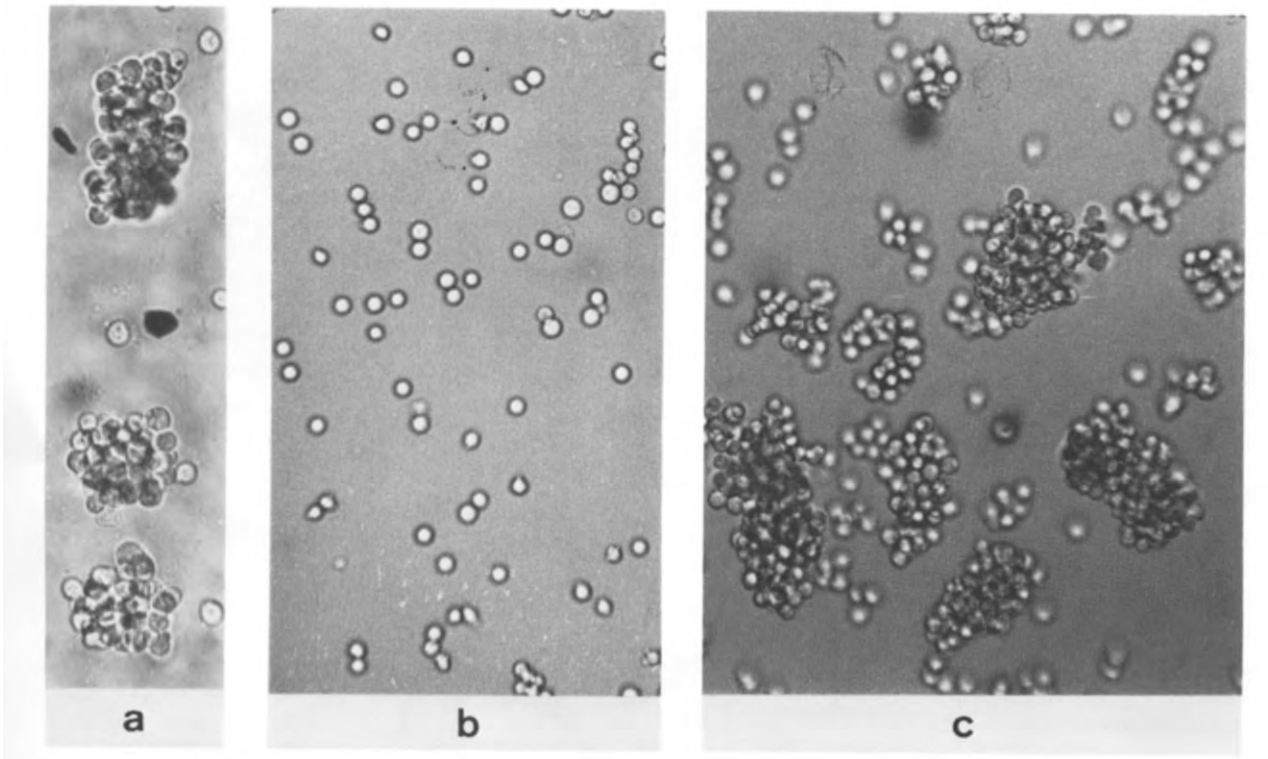

Fig.1. The effects of Fab (vegetative) on the adhesion of *P. pallidum* cells. Cells were photographed after 120 min in the roller culture. (a) Control. (b) Fab (vegetative). (c) Fab (vegetative) and butanol extract (25 μg protein/ml) from vegetative cell plasma membranes.

taining Fab which had not bound to the antigen in the gel was removed and the gel shaken overnight in 1.0 ml PDF solution. The two PDF solutions were combined (2.0 ml) and concentrated by vacuum dialysis (final vol. 0.25 ml). Hence, two Fab fractions were obtained from which the Fab binding to the 110 kd and 71 kd antigens, respectively, had been at least partially removed.

Cell agglutination was assayed by incubating vegetative cells (4×10^7 cells/ml) in a roller culture (40 rev./min Multi-purpose Rotator, Sci. Indust.) in PDF or 0.02 M phosphate buffer (pH 7.0), 0.12 M NaCl. Both solutions also contained 10 mM EDTA [7] and gave identical results. Fab was usually added to give 0.75 mg protein/ml final conc.

3. Results

3.1. *Effects of Fab (vegetative) on agglutination*

When vegetative cells were incubated in the roller cultures they began to show signs of agglutination after 30–60 min. At 120 min quite large aggregates had already formed and very few single cells were present (fig.1a). If Fab against vegetative cell membranes was present, agglutination was inhibited (fig.1b). Some monolayer cell clumping occurred but contacts were readily broken by shaking the suspension. Hence, the Fab was blocking sites on the cell surface involved in adhesion. Fab remained effective for 3.5–4 h by which time it was presumably inactivated (phagocytosed?) and aggregation began.

The IgG (vegetative) included antibodies directed against a number of plasma membrane proteins. These were actin, myosin heavy chains, a carbohydrate-binding protein (pallidin) [9], and 180 kd, 110 kd and 71 kd antigens [8] (fig.2a). If plasma membranes were treated with butanol an extract was obtained containing only the 110 kd and 71 kd antigens [8] (fig.2b). When cells were incubated with Fab in the presence of the butanol extract, agglutination occurred as in the controls (fig.1c). This meant the antigens in the extract were competing with cells for adhesion-blocking Fab.

3.2. *Effects of Fab (mid-aggregation) on agglutination*

When Fab directed against membranes from mid-aggregation stage cells was added to the assay system,

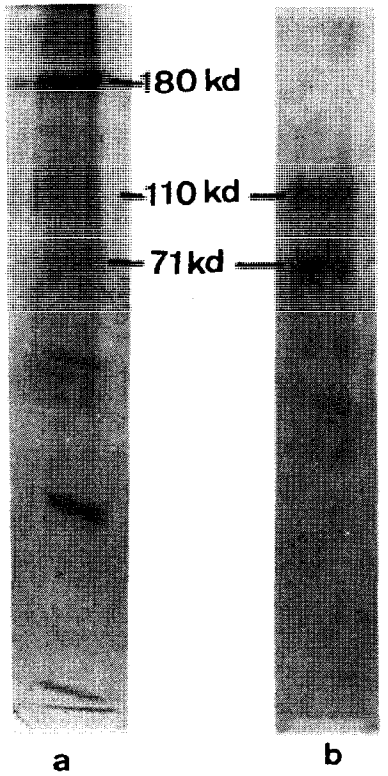

Fig.2. Dodecylsulfate gels of plasma membranes and butanol extract of plasma membranes incubated with antiserum raised against membranes from vegetative cells. Antibodies binding to specific proteins were detected using IgG coupled to peroxidase [6]. (a) Plasma membranes of vegetative cells. (b) Butanol extract of plasma membranes isolated from vegetative cells.

agglutination occurred as in the controls (results not shown). This was surprising as the antibodies directed against the 110 kd and 71 kd antigens were present [8]. A further antigen (57 kd) also appeared at this stage [8]. Apparently some additional component of the Fab mixture was interfering with the binding of specific Fab to the 110 kd and/or 71 kd membrane antigens. We confirmed this by adding both Fab (vegetative) and Fab (mid-aggregation) to the cells. Agglutination was no longer inhibited by Fab (vegetative).

3.3. *Identification of the antigen involved in cell adhesion*

We wished to determine which of the two antigens in the butanol extract was involved in cell adhesion.

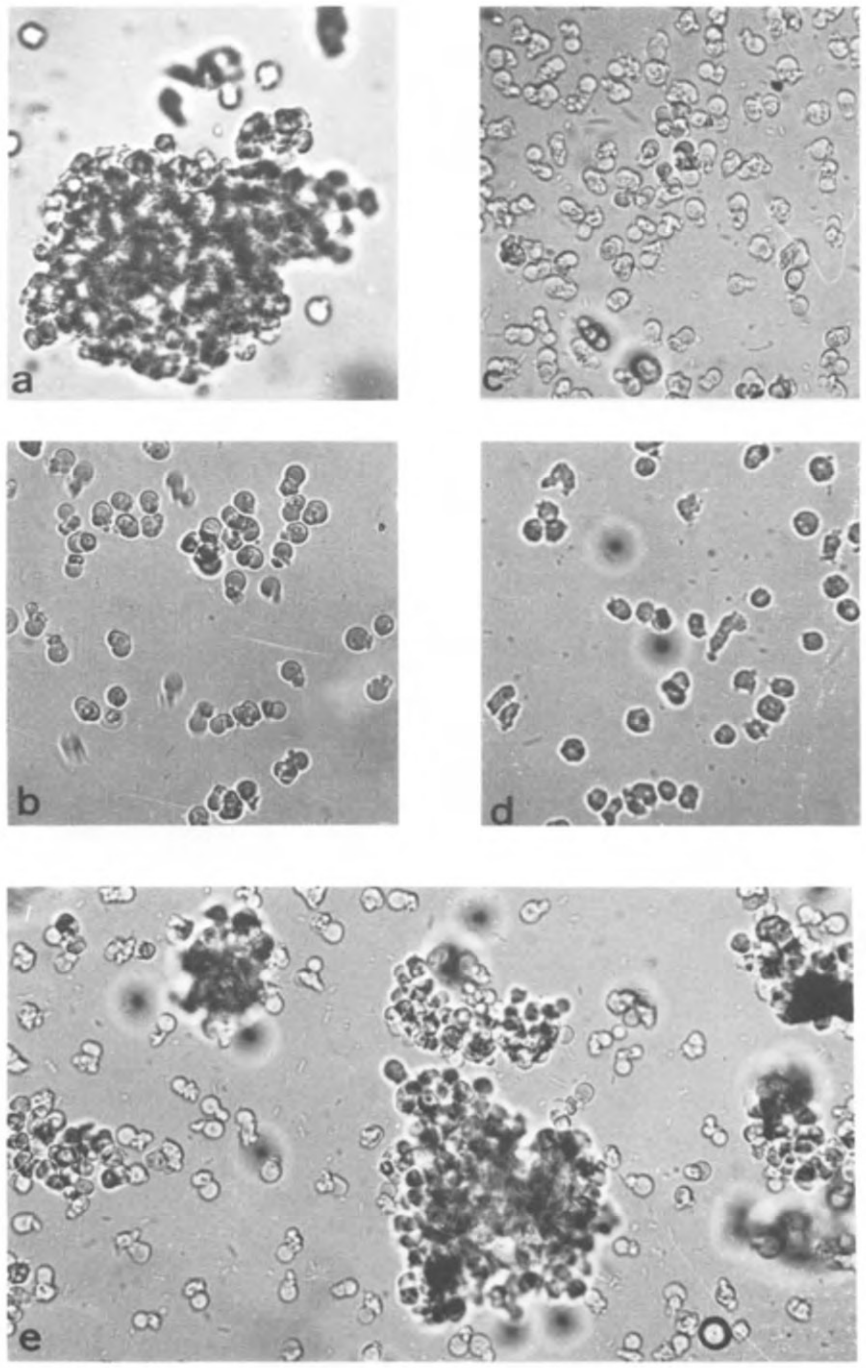

Fig.3. Identification of the plasma membrane antigen mediating cell adhesion. Cells were photographed after 120 min in the roller culture. (a) Control. (b) Fab (vegetative). (c) Fab (vegetative) remaining after incubation with gel slices lacking antigen. (d) Fab (vegetative) after removal of Fab specifically binding to the 110 kd antigen. (e) Fab (vegetative) after removal of Fab specifically binding to the 71 kd antigen.

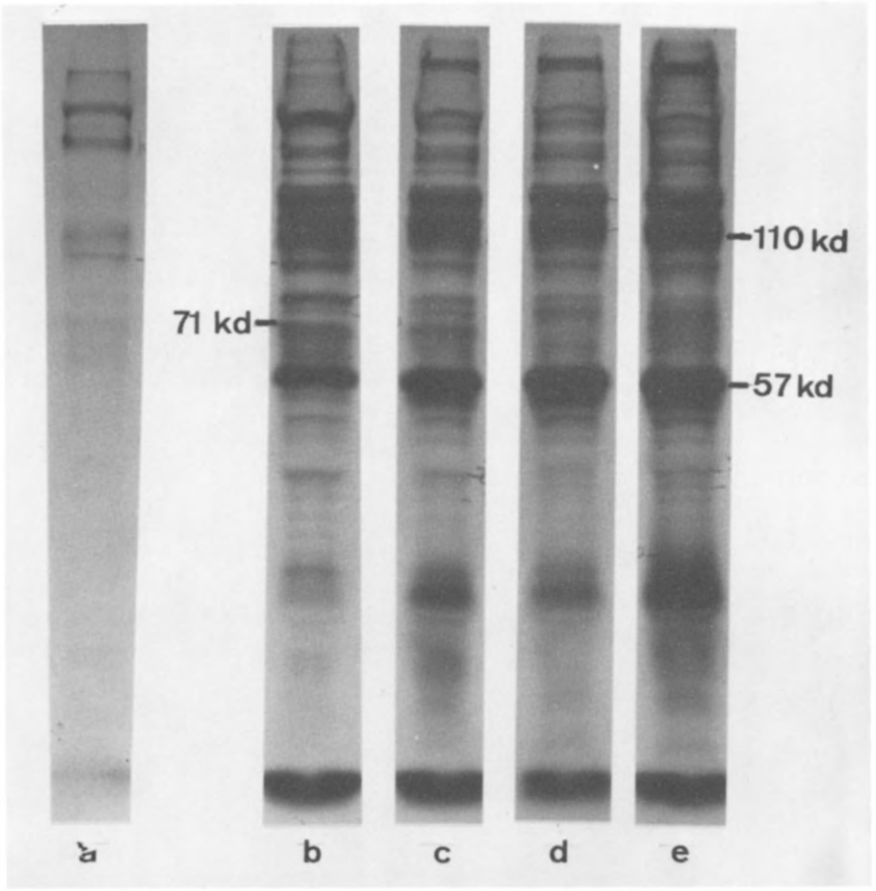

Fig.4. Autoradiograph of dodecylsulfate gel of plasma membrane from 5 developmental stages. The cultures had been continously labelled with [^{14}C]glucosamine throughout development (t = 0, until culmination). The three major developmentally regulated antigens are indicated: (a) Preaggregation; (b) mid-aggregation; (c) late aggregation; (d) tip formation; (e) culmination.

We cut the antigens from gels and used them to remove their specific Fab from the total Fab. The antigen–antibody gels (fig.2) indicated that the two antigens bound approximately equal amounts of IgG. We also knew that the antigen–antibody complex remained in gels while excess IgG could be removed by washing [6,8].

The Fab that had been preincubated with the 110 kd antigen inhibited cell agglutination as efficiently as untreated Fab (fig.3d). Preincubation of Fab with another region of the gel (containing proteins but no antigens) also had no effect (fig.3c). However, if the Fab specific for the 71 kd antigen was removed (or at least partially removed, we did not quantitate the amount remaining), cell agglutination was no longer inhibited by the remaining Fab (fig.3e). We concluded that the 71 kd antigen was involved in cell adhesion.

3.4. *Evidence that the 71 kd antigen was lost from the plasma membrane during the latter stages of differentiation*

We labelled cells continously with [^{14}C]glucosamine, from first plating out until culmination. Plasma membranes were isolated from cells at 5 developmental stages. The results show that the 71 kd antigen disappeared from the plasma membrane when aggregation was completed (fig.4). The 110 kd antigen was present throughout differentiation (fig.4) [8]. A third antigen (57 kd) which is absent from vegetative cells, was also continously synthesized throughout differentiation (fig.4) [8].

Using antiserum to detect antigens in the SDS–

gels, we were able to confirm the disappearance of the 71 kd antigen from post-aggregative cells (not shown).

4. Discussion

The 71 kd antigen has been shown to be the target of the Fab blocking developmentally regulated cell adhesion of *P. pallidum* cells. The 71 kd antigen is a single glycoprotein which is synthesized until the end of aggregation [8]. Since it is a glycoprotein and strongly incorporates fucose and glucosamine [8], the molecular weight determined on the 10% acrylamide gels may be an overestimate. It is only a minor component of the plasma membrane and cannot be detected on Coomassie blue stained gels.

Unlike Fab against vegetative cell membranes, the Fab against mid-aggregation stage membranes did not inhibit cell adhesion. This was an unexpected and puzzling result since Fab against the 71 kd antigen was included. Some factor (another Fab?) was present that inhibited Fab from blocking the 71 kd antigen. The only major new Fab identified was against the 57 kd antigen which does not occur on vegetative cells [8]. However, the 57 kd antigen is the most likely candidate for the second Fab target site described [7] (see section 1). It will be necessary to isolate the individual Fab species and study their influence on one another to resolve these problems. Fab obtained against membranes of aggregation-competent cells, which carry less 57 kd antigen than do the fully aggregated cells we used, did block adhesion [7].

Since the 71 kd antigen is found in vegetative cells, why do these cells fail to agglutinate? Some possibilities are:

(1) The concentration of the antigen is sub-threshold on the surface of vegetative cells;
(2) It requires a second component which first appears extracellularly when cells are starved (i.e., development is triggered);
(3) The structure of the antigen changes following starvation.

The 71 kd antigen resembles contact sites A of *D. discoideum* [4,6] in being lost from the plasma membrane at the completion of aggregation when its synthesis ceases. Presumably its place is taken by another protein or proteins (e.g., the 57 kd antigen). It is interesting to speculate on why such a switch is necessary. A different type of adhesion may be required, for example, during the post-aggregation phase. On the other hand, all cells may require the 71 kd glycoprotein to participate in aggregate formation. Subsequently, two major cell types differentiate and sort out within the aggregates [10–14]. Sorting out implies specificity in contact sites, one or both cell types carrying their own specific site. Hence, the initial contact sites involved in aggregation would of necessity be discarded. We hope to discover whether all cells in the aggregate synthesize the 57 kd antigen.

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