

Monoclonal antibodies against contact sites A of *Dictyostelium discoideum*: detection of modifications of the glycoprotein in tunicamycin-treated cells

H. Ochiai¹, J. Stadler, M. Westphal, G. Wagle, R. Merkl, and G. Gerisch*

Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

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Tunicamycin acts on cell aggregation in *Dictyostelium discoideum* by changing cell movement and by inhibiting the EDTA-stable type of intercellular adhesion. Tunicamycin-treated cells show unco-ordinated pseudopodial activity such that pseudopods are simultaneously extended from all parts of the cell surface, and the cells are unable to move in straight paths. Concurrent with the inhibition of formation of EDTA-stable contacts, N-glycosylation of a glycoprotein specific for aggregation-competent cells is inhibited. This glycoprotein, previously called contact site A, has an apparent mol. wt. of 80 kilodaltons (kd). In membranes of tunicamycin-treated cells, two components are detected that react with certain monoclonal antibodies against contact sites A: one component of 66 kd, the other of 53 kd apparent mol. wt. Another group of monoclonal antibodies reacts only with the 80-kd glycoprotein and the 66-kd component. These results are in accord with the assumption that the glycoprotein carries two carbohydrate chains, and that the antibodies differ in their requirement for glycosylation of the antigen. Despite the coincidence between blockage of EDTA-stable cell adhesion and inhibited glycosylation of contact sites A, direct involvement of the carbohydrate moieties of this glycoprotein in intercellular adhesion seems questionable. EDTA-stable cell adhesion has not been blocked by Fab fragments from antibodies that specifically react with the glycosylated protein.

Key words: glycoprotein/tunicamycin/cell adhesion/monoclonal antibodies/*Dictyostelium*

Introduction

Aggregating cells of *Dictyostelium discoideum* adhere to each other. They form stream-like aggregates and, under the influence of the chemo-attractant cyclic AMP, they move towards aggregation centers. There, the cells assemble into a multicellular mass, which eventually develops into a fruiting body (for reviews on aggregation, see van Haastert and Konijn, 1982; Gerisch, 1982).

Aggregating cells are distinguishable from those at earlier stages of development by the resistance of their intercellular adhesion to EDTA (Gerisch, 1961). Attempts to identify the cell surface molecules involved in the adhesion of aggregating cells have led to the identification of a glycosylated (Müller *et al.*, 1979) and phosphorylated (Coffman *et al.*, 1981; Schmidt and Loomis, 1982) integral membrane protein (Stadler *et al.*, 1982), called contact site A (Beug *et al.*, 1973b). This glycoprotein has been shown to neutralize univalent antibody fragments (Fab) that block the EDTA-stable type of cell adhesion

(Müller and Gerisch, 1978). It has an apparent mol. wt. of ~800 kd is absent from growth phase cells, and is expressed on the cell surface simultaneously with the acquisition of EDTA-stable contact formation (Beug *et al.*, 1973b; Murray *et al.*, 1981; Ochiai *et al.*, 1982).

Tunicamycin blocks N-glycosylation of proteins by inhibiting the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetyl-glucosamine to polyprenolphosphate (Takatsuki *et al.*, 1975; Tkacz and Lampen, 1975; Lehle and Tanner, 1976). Earlier findings that tunicamycin inhibits cell aggregation of *D. discoideum* (Ochiai *et al.*, 1981) have prompted us to study the inhibition of EDTA-stable cell contact formation in relation to inhibition of contact site A glycosylation. Monoclonal antibodies reacting with different portions of the glycoprotein have been used to identify two incomplete forms or fragments of the glycoprotein.

Results

Inhibition of EDTA-stable cell adhesion by tunicamycin

Tunicamycin inhibited cell aggregation in *D. discoideum* without inhibiting cell motility. Cells were shaken in non-nutrient buffer to which 0.5 or 2.0 µg tunicamycin/ml was added during the third and fourth hour of development. The cells were then washed, and shaking was continued. Samples, taken after 8, 13, and 16 h of development, were diluted and plated onto the Teflon film of Petriperm dishes for video-recording. Recording of the 16-h sample of cells treated with 2 µg tunicamycin/ml was continued until 26 h of development. At both concentrations of tunicamycin, aggregation was inhibited but the cells remained motile. The movement differed, however, from normal movement. The tunicamycin-treated cells actively extended pseudopods in all directions, in contrast to normal cells for which polarized pseudopod formation is typical.

Aggregation-competent cells of *D. discoideum* are characterized by their ability to form EDTA-stable contacts, in addition to EDTA-sensitive ones which are already apparent during the growth phase (Gerisch, 1961). Normally, aggregation competence is acquired in suspension cultures of *D. discoideum* strain AX2-214 within 6–8 h after the beginning of starvation.

The effect of tunicamycin on cell adhesion was investigated under the same conditions used to study its effect on cell movement. Samples were taken 8, 16, and 24 h after starvation commenced, and cell adhesion was recorded in an agglutinator with or without addition of 10 mM EDTA (Figure 1). The EDTA-sensitive contacts were not affected by tunicamycin, but the EDTA-stable contacts were inhibited at both concentrations.

Inhibited expression of the contact site A glycoprotein

Concurrent with the acquisition of EDTA-stable cell adhesion, the 80-kd glycoprotein, called contact site A, accumulates in the plasma membranes of *D. discoideum* cells. To investigate whether tunicamycin affects either the synthesis or expression of this glycoprotein on the cell surface, fluorescence was monitored after labeling developing cells with the

¹Present address: Department of Botany, Faculty of Science, Hokkaido University, 060 Sapporo, Japan.

*To whom reprint requests should be sent.

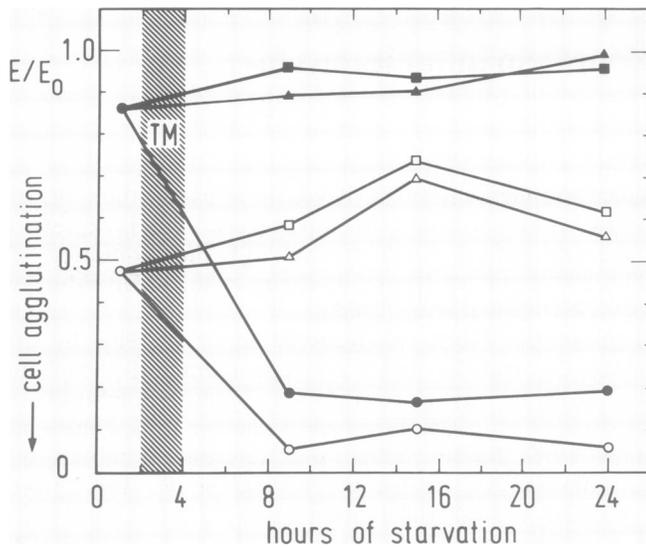


Fig. 1. Cell adhesion in the presence and absence of EDTA. Cells washed free of nutrient medium were shaken in phosphate buffer. At the time points indicated, samples were removed from the suspension, the cells washed and transferred to an agglutinator (Beug and Gerisch, 1972). Open symbols: agglutination in the absence of EDTA; closed symbols: in the presence of 10 mM EDTA. □, ■, cells treated with 2.0 μg of tunicamycin/ml; △, ▲ with 0.5 μg /ml; ○, ●, untreated control cells. Tunicamycin was added 2 h, and removed by washing 4 h, after the beginning of starvation (hatched area). Cell agglutination was determined by recording unscattered light. E is the apparent optical density in the sample, E_0 in an identical sample to which adhesion blocking Fab was added for complete dissociation of the cells. Thus, E/E_0 values close to 1 indicate mostly single cells in the experimental sample, low E/E_0 values indicate large aggregates.

monoclonal antibody 12-120-94, which recognizes the contact site A glycoprotein (Ochiai *et al.*, 1982).

Control cells expressed binding sites for the antibody during the first 6 h of development (Figure 2a and b). In comparison, the intensity of labeling was reduced within cell populations treated during the third and fourth hour of development with 0.1 μg of tunicamycin/ml (Figure 2c). Cells that had been treated with 0.5 μg tunicamycin/ml showed almost no fluorescence (Figure 2d). After 20 h of development the intensity of labeling was about one third of that in 6-h control cells (Figure 2e).

Incomplete form of the contact site A glycoprotein synthesized by tunicamycin-treated cells

Blotting on nitrocellulose of membrane proteins separated by SDS-polyacrylamide gel electrophoresis has been used to detect an incomplete form of the glycoprotein synthesized under the influence of tunicamycin. Starved cells were cultivated on Millipore filters. Under these conditions and in the absence of tunicamycin, they passed through all stages of development. Experimental groups were transferred together with the filters onto filter pads soaked with a solution of 8 μg tunicamycin/ml and incubated for various periods of time (Figure 3). Crude membrane preparations of the cells were extracted with butanol/water for enrichment of the contact site A glycoprotein in the water phase.

In blots from control cells the 80-kd band of the contact site A glycoprotein was strongly labeled with monoclonal antibody 12-120-94. Two minor bands labeled with the same antibody have been described previously (Ochiai *et al.*, 1982). One of these bands, designated 140 kd, is seen in Figure 3a. This band appears during cell development together with the

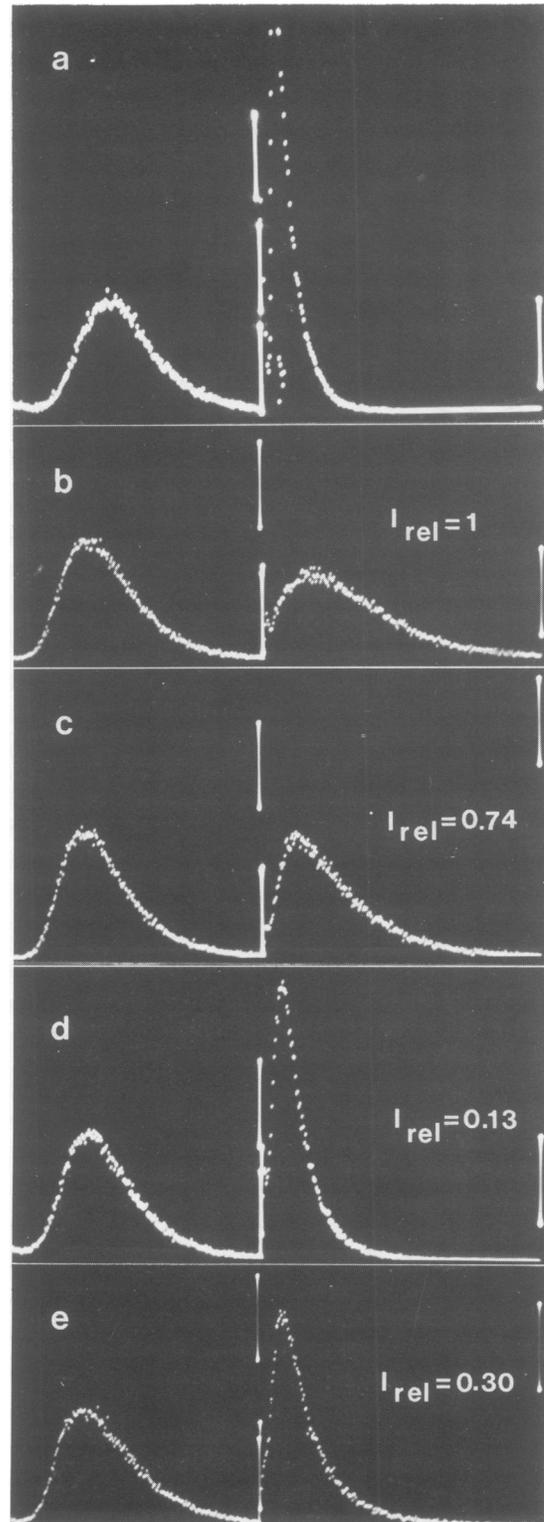


Fig. 2. Light scattering (left) and fluorescence (right) of cells labeled with antibody 12-120-94 and FITC-conjugated anti-mouse-IgG. Cell populations were analyzed using a FACS IV cell sorter. Cell numbers are plotted on the ordinate, relative light scattering or fluorescence intensities on the abscissa. Light scattering is a measure for cell size, which decreases slightly after starvation. a, cells at the beginning of starvation; b, 6-h control cells; c, 6-h cells treated with 0.1 μg tunicamycin/ml from 2 to 4 h of development; d, 6-h cells treated with 0.5 μg tunicamycin/ml from 2 to 4 h of development; e, 20-h cells treated as in d. Average relative fluorescence intensities (I_{rel}) were calculated by subtracting the average fluorescence intensity in panel a (endogenous fluorescence and unspecific antibody binding) from the average intensities calculated for the other samples. The corrected value for b was set as 1.

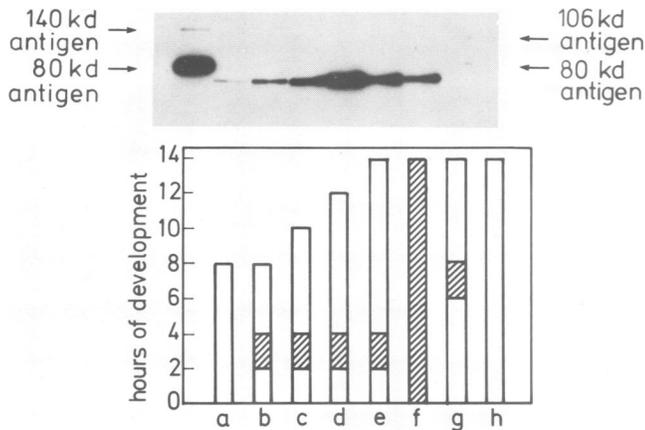


Fig. 3. Slow expression of a lower mol. wt. form of contact sites A in tunicamycin treated cells. **Top:** labeling of antigens blotted from SDS-polyacrylamide gels with antibody 12-120-94. **Bottom:** times of development after removal of nutrient medium (columns), and of treatment with 8 μ g tunicamycin/ml (hatched areas), for the samples shown on top.

80-kd band and might represent a precursor of the contact site A glycoprotein. Another band, designated 106 kd, appears during the slug stage; it appears in Figure 3h as a band labeled faintly. This figure also shows that, in agreement with previous results, the 80-kd glycoprotein disappears almost completely after the aggregation stage.

None of the antigens labeled in control cells were seen in tunicamycin-treated cells. Instead, a new labeled band appeared in the 66 kd position (Figure 3b–g). Figure 3b–e shows that it is sufficient to incubate the cells with tunicamycin during the third and fourth hours of development in order to suppress fully the appearance of the complete glycoprotein. Synthesis of contact sites A begins after 4 h of development. This means that either a precursor glycolipid is synthesized before the commencement of contact site A synthesis, or tunicamycin is stored within the cells. The latter explanation appears to be correct because incubation of cells with tunicamycin during the seventh and eighth hours of development has an even stronger effect (Figure 3g).

Very little of the 66-kd component was present after 8 h of development in membranes of tunicamycin-treated cells, i.e., at a stage when in control cells the 80-kd glycoprotein was fully expressed (compare Figure 3a and b). Substantial amounts of the 66-kd component were not found earlier than 14 h after starvation (Figure 3e). The delay in the appearance of a modification of the glycoprotein detectable with antibody 12-120-94 is in agreement with the data obtained by fluorescent labeling of living cells (Figure 2).

Under all conditions shown in Figure 3b–g, a discrete band appeared in the 66 kd position. No material of intermediate mol. wt. between 66 and 80 kd was labeled. This may be explained in one of two ways: (1) carbohydrate is linked to the protein in large blocks, so that lack of one block already shifts the molecule to the 66 kd position; or (2) carbohydrate is linked to the protein in several smaller units, but in our experiments, the tunicamycin concentration was sufficiently high to fully inhibit N-glycosylation. If (2) were correct, one would expect material of intermediate size to appear with decreasing tunicamycin concentration. Therefore, cells were incubated with either 2.0 or 0.5 μ g tunicamycin/ml, under the same conditions used to determine EDTA-stable contact formation.

As shown in Figure 4, no intermediate band is seen when

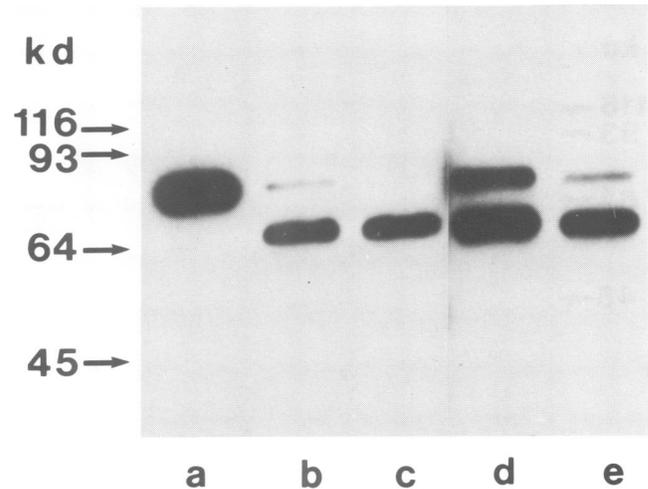


Fig. 4. Appearance of two discrete bands labeled with [125 I]antibody in cells treated with low concentrations of tunicamycin. **a**, purified contact site A glycoprotein as a control; **b**, water phase from butanol/water extracted crude membrane fractions of cells treated from 2 to 4 h of development with 0.5 μ g tunicamycin/ml; **c**, the same, but cells treated with 2.0 μ g of tunicamycin/ml; **d**, the butanol- and interphases of the same extract as in **b**; **e**, butanol- and interphases of the same extract as in **c**. Blots from SDS-polyacrylamide gels were labeled with antibody 12-120-94.

the 80-kd band of the complete glycoprotein is clearly detectable together with the 66-kd band. This result indicates that carbohydrate is linked in large units to the contact site A protein.

Two classes of monoclonal antibodies

For purified contact sites A, a carbohydrate content of 33 mg/100 mg protein had been determined (Müller *et al.*, 1979). The mol. wt. difference between the complete glycoprotein and the 66-kd product of tunicamycin-treated cells seemed to us too small to account for the full carbohydrate content of the glycoprotein. Therefore, we have screened a series of eight monoclonal antibodies for their ability to recognize products of lower mol. wt. that may not be recognized by antibody 12-120-94.

All of these antibodies labeled the 80-kd band of the complete contact site A glycoprotein. With membranes of tunicamycin-treated cells, two different labeling patterns were observed: (1) six antibodies reacted, like 12-120-94, with a 66-kd component; (2) two antibodies reacted with two antigens having apparent mol. wts. of 53 and 66 kd, as shown for 20-6-4 (Figure 5). This antibody faintly labeled a few additional bands, below the 80-kd glycoprotein, whose positions were not shifted in tunicamycin-treated cells. The conclusion is that the 66-kd component synthesized under the influence of tunicamycin has an antigenic determinant in common with the complete 80-kd glycoprotein, and that this determinant is missing in the 53-kd component.

The apparent mol. wts. of 66 and 53 kd were determined using 10% SDS-polyacrylamide gels. In accord with its high carbohydrate content the apparent mol. wts. of the complete contact site A glycoprotein varies with the polyacrylamide concentration (Müller *et al.*, 1979). Under the conditions used here it was 77 kd. Thus, the differences between the apparent mol. wts. of the three components involved are 11 and 13 kd.

The 80-kd glycoprotein is known to contain phosphoserine (Coffman *et al.*, 1981; Schmidt and Loomis, 1982). *In vivo* incorporation of 32 P showed that the 66-kd component syn-

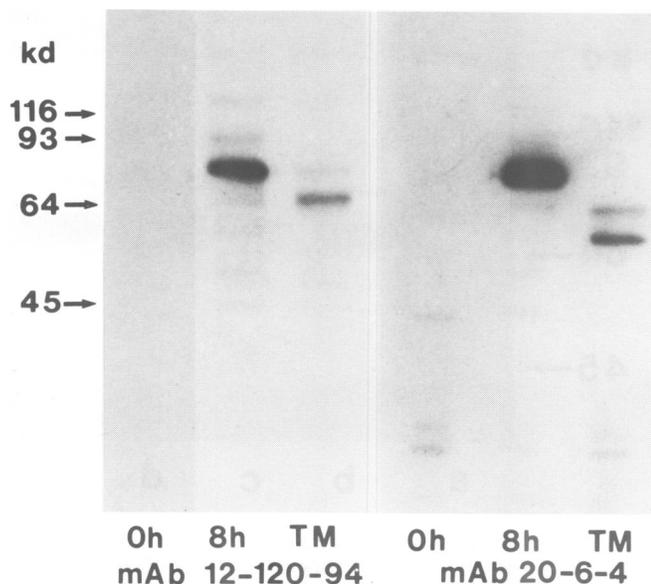


Fig. 5. Blots from SDS-polyacrylamide gels of plasma membrane-enriched fractions, labeled with two different monoclonal antibodies and [125 I]anti-mouse IgG. The panels show cells at the beginning of starvation (0 h), control cells at 8 h after beginning of starvation, cells at 14 h to which 0.5 μ g tunicamycin/ml was added at 3 h of development (TM). The same membrane preparations were used for labeling with the monoclonal antibodies mAb 12-120-94 and 20-6-4. Comparison of the 0 h and 8 h panels shows the stringent developmental regulation of contact sites A.

thesized in tunicamycin-treated cells is also phosphorylatable (Figure 6). 32 P incorporation into the 53-kd band could not be detected, possibly because the background was too high.

Distinction of monoclonal antibodies by binding to living cells

The 80-kd glycoprotein is an integral membrane protein (Stadler *et al.*, 1982). Part of the molecule is exposed to the outer membrane surface; it can be labeled in living cells with antibody 12-120-94 (Figure 2). The six antibodies that failed to react with the 53-kd component showed the same pattern of binding to living cells: they labeled the surfaces of aggregation-competent, but not of growth-phase cells. These results indicate that determinants of the 80-kd glycoprotein which are present on the 66-kd component, and absent from the 53-kd component, are exposed to the outer cell surface. These determinants distinguish contact sites A from other cell surface antigens which are not developmentally regulated.

No surface labeling of aggregation-competent or growth-phase cells was observed with antibody 20-6-4. The same result was obtained with 20-63-1, the other antibody which labeled the 53-kd component of tunicamycin-treated cells. These results indicate a determinant within the membranes or at their inner surface.

Attempts to block cell adhesion by Fab against the glycosylated contact sites A

Contact sites A neutralize polyspecific Fab that blocks EDTA-stable cell adhesion (Müller and Gerisch, 1978; Müller *et al.*, 1979). The polyspecific Fab had been prepared from rabbit antisera against whole membranes of aggregation-competent cells (Beug *et al.*, 1973b) and certainly reacts with more than one determinant on the contact site A molecule. The question is whether the carbohydrate moieties of the glycoprotein are among the target structures to which adhesion blocking Fab molecules bind.

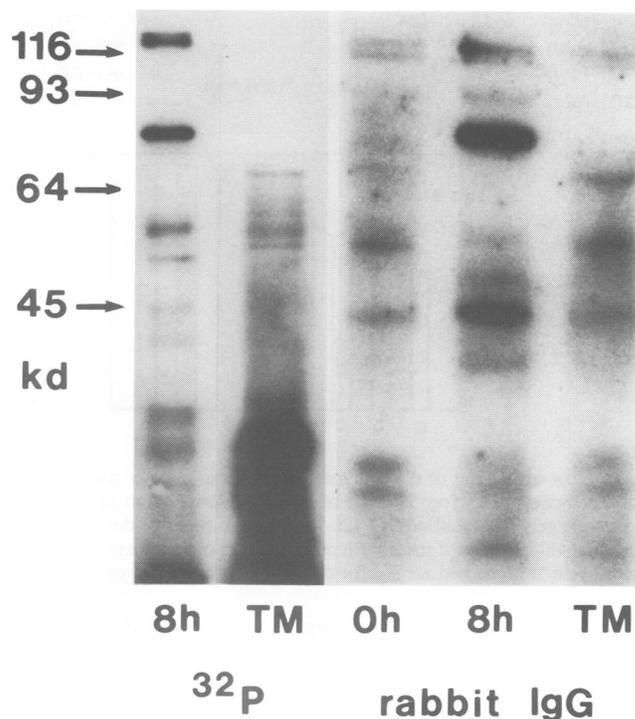


Fig. 6. *In vivo* phosphorylation (left panel), and labeling of membrane proteins with IgG 67/1 from a rabbit that had been immunized with heated, aggregation-competent cells (right panel). [32 P]Phosphate was added to cells 3 h after the beginning of starvation. At the same time 0.5 μ g tunicamycin/ml was added to the experimental samples. Control cells were harvested at 8 h, tunicamycin-treated cells at 14 h after the beginning of starvation (TM). Plasma membrane enriched fractions were extracted with butanol/water, the water phase run on SDS-polyacrylamide gels and subjected to autoradiography. For labeling with rabbit IgG, cells were harvested at the beginning of starvation (0 h); at 8 h for untreated cells; or at 14 h for cells to which 0.5 μ g tunicamycin was added at 3 h (TM). Plasma membrane-enriched fractions were used for SDS-polyacrylamide gel electrophoresis, blotting and labeling with 67/1 IgG and goat [125 I]anti-rabbit IgG.

In earlier experiments, we have immunized rabbits with heated *D. discoideum* cells (Beug *et al.*, 1970). Although Fab from these antisera efficiently bound to the surface of living cells, no inhibition of EDTA-stable cell adhesion was observed (Beug *et al.*, 1973a). IgG from the same rabbit antiserum K67/1 used in these earlier studies has now been applied to label membrane antigens from tunicamycin-treated and control cells.

In control cells, many bands were labeled (Figure 6). Diffuse background labeling was probably due to glycolipid-binding antibodies. Particularly the 80-kd band of aggregation-competent cells was strongly labeled. In tunicamycin-treated cells a band appeared in the same position as the 66-kd band recognized by monoclonal antibodies, whereas the 53-kd band was not seen. Since the antibodies of rabbit serum K67/1 recognize carbohydrate structures on glycolipids and glycoproteins (Beug *et al.*, 1970), it is likely that the 66-kd but not the 53-kd component of tunicamycin-treated cells contains carbohydrate.

The fact that K67/1 Fab does not block EDTA-stable cell adhesion indicates that not all antibodies that bind to the 80-kd glycoprotein also block EDTA-stable cell adhesion, and that carbohydrate moieties of contact sites A are probably not the target sites of adhesion blocking Fab.

Fab from antibody 12-120-94, which showed the same

selectivity for the 66-kd component as the rabbit antibodies, also did not block EDTA-stable cell adhesion. The monoclonal Fab was applied at concentrations up to 1 mg/ml without a detectable effect. For comparison, 0.1 mg poly-specific rabbit Fab per ml inhibited under the same conditions detectably, and 0.6 mg Fab per ml inhibited completely, the EDTA-stable adhesion.

Discussion

The inhibition by tunicamycin of cell aggregation in *D. discoideum* is not due to an overall blockage of cellular functions; this means inhibition of protein synthesis is not the prevailing effect of tunicamycin. Tunicamycin-treated cells remain motile for many hours, in contrast to cycloheximide-treated cells. The defect in co-ordination of pseudopodial activity seen in tunicamycin-treated cells resembles the defects observed in certain mutants (Gerisch, 1980) and suggests the involvement of a glycoprotein in organized cell movement.

It is tempting to explain the tunicamycin effect on EDTA-stable cell adhesion by the inhibition of contact site A glycosylation. However, for three reasons our results do not provide conclusive evidence for a function of the carbohydrate moiety of this particular glycoprotein in cell adhesion. (1) Fab fragments from antibodies directed against the glycosylated protein, and possibly against the carbohydrate moiety itself, did not block EDTA-stable cell adhesion. (2) Not only the glycosylation, but also the rate of synthesis or the lifetime of the protein might be reduced in tunicamycin-treated cells. (3) The effects of tunicamycin on other glycoproteins have not been studied.

The appearance of a 53- and 66-kd component in tunicamycin-treated cells, and the pattern of their labeling with antibodies, can be explained by assuming a contact site A protein with an apparent mol. wt. of 53 kd to which two identical or similar carbohydrate chains, each responsible for an apparent mol. wt. increase of ~12 kd, are attached by N-glycosylation. These assumptions are in good agreement with a carbohydrate content of 33 g/100 g protein (Müller *et al.*, 1979). This content would correspond to a total mol. wt. of the carbohydrate of 17.5 kd, and of ~9 kd per chain. Our unpublished data obtained by two-dimensional electrophoresis indicate that the carbohydrate does not supply additional charges to the molecule. Therefore, the 12 kd value for one carbohydrate chain as indicated by SDS-polyacrylamide gel electrophoresis is likely to be an overestimate.

Although we think that the assumption of two N-glycosidically-linked carbohydrate chains is well-supported, two points need to be considered: (1) part of the carbohydrate might be linked by O-glycosidic bonds; and (2) lack of glycosylation could make the protein highly sensitive to proteases (Olden *et al.*, 1978, 1982). Thus, it is not excluded that the 66-kd component is the protein that lacks N-glycosidically linked carbohydrate, and the 53-kd material is produced from the 66-kd component by proteolytic cleavage.

If, nevertheless, the assumption is correct that not only the 80-kd but also the 66-kd component carries a carbohydrate chain, antibodies like 12-120-94 can be considered as specific for the N-glycosylated contact sites A. Such antibodies bind either to the carbohydrate itself, or they recognize a conformation of the protein which is only achieved after glycosylation, as suggested for an HLA histocompatibility antigen (Wilson *et al.*, 1981), and for a murine retrovirus antigen

(Pierotti *et al.*, 1981). Because our antibodies react with antigens heated with SDS, it is unlikely to be a specific conformation of the protein that is recognized.

Previous results have suggested that in *D. discoideum* more than one type of carbohydrate is transferred from lipid precursors to proteins. Rössler *et al.* (1978) have identified three different precursor mannolipids. Ivatt *et al.* (1981) discovered a group of protein-linked oligosaccharides that remained the same throughout development up to the completion of cell aggregation. These oligosaccharides differ by their smaller sizes from the carbohydrate linked to the contact site A protein. If antibody 12-120-94 binds directly to this carbohydrate, it should be a unique carbohydrate that distinguishes contact sites A from many other glycoproteins in *D. discoideum* membranes, which are not labeled by the antibody (Ochiai *et al.*, 1982).

Materials and methods

Cells of *D. discoideum* strain AX2-214 were cultivated axenically at $23 \pm 1^\circ\text{C}$ and starved in 17 mM Soerensen phosphate buffer pH 6.0 as described (Malchow *et al.*, 1972). Except for the experiment shown in Figure 3, which was performed with cells spread on Millipore filters (HAWP, 0.45 μm pore size), starved cells were shaken. For *in vivo* ^{32}P labeling, cells were washed and resuspended in 10 mM imidazole/HCl buffer pH 6.5, and 30 mCi [^{32}P]phosphate was added to 400 ml of a suspension of 1×10^7 cells/ml. The measurement of cell adhesion and its inhibition by Fab has been described by Beug *et al.* (1973b).

Monoclonal antibodies were collected from hybridomas obtained from three different BALB/c mice immunized with partially purified contact sites A, using Al-hydroxide and *Bordetella pertussis* antigen as adjuvants. The code numbers of monoclonal antibodies, e.g., 12-120-94, denote the number of the mouse from which the spleen was obtained, the well from which the hybridoma was cloned, and the number of the clone picked up from the well. Cloning, purification of monoclonal antibodies from the culture fluid, their labeling with ^{125}I , fluorescent labeling of living cells with antibodies and fluoresceine isothiocyanate (FITC)-conjugated anti-mouse IgG sheep immunoglobulins, SDS-polyacrylamide gel electrophoresis, and blotting were performed as before (Ochiai *et al.*, 1982). Fab was prepared from a monoclonal IgG₁ antibody by incubating 5 mg IgG for 3 h at 37°C with 0.4–0.6 units of papain, bound to CM-cellulose, in 20 ml phosphate buffered saline containing 2 mM EDTA and 0.1 M mercaptoethanol.

Tunicamycin was fractionated by h.p.l.c. and a fraction corresponding to peak B of Keenan *et al.* (1981) was used for all experiments. The purified peak B tunicamycin was lyophilized, dissolved in ethanol, and kept in a stock solution of 0.5 mg tunicamycin per ml of 20% ethanol.

Crude membrane preparations were obtained from frozen cells by centrifugation for 20 min at 13 000 g. Either crude or plasma membrane-enriched fractions were extracted with butanol/water according to Müller *et al.* (1979). Plasma membrane-enriched fractions were prepared from crude membrane preparations using the polyethylene glycol/dextran method of Brunette and Till (1971), where the plasma membranes are collected from the interphase.

Goat anti-rabbit IgG was a gift from Dr. Heinz Schwarz, Tübingen. Tunicamycin was purchased from Calbiochem (La Jolla, CA), FITC-anti-mouse IgG immunoglobulin from the Institut Pasteur production (Paris), papain bound to CM-cellulose from E. Merck (Darmstadt, FRG), Petriperm dishes from Heraeus (Hanau, FRG).

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