Mutants of *Polysphondylium pallidum* showing delayed modifications of glycoproteins are altered in a regulatory signal for development

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Summary

Binding of a monoclonal antibody, mAb293, to cell-surface glycoproteins of *Polysphondylium pallidum* is known to be blocked by L-fucose, and Fab of this antibody has been shown to inhibit intercellular adhesion of aggregation-competent cells. Mutants with delayed expression of the carbohydrate epitope, ep293, recognized by the antibody, have been shown to be retarded and altered in cell aggregation. The present study shows that ep293 is a modification of carbohydrate structure that is subject to regulation not only in mutant but also in wild-type cells; ep293 is expressed at an early stage of exponential growth in wild-type and only after 12 h of starvation in mutant PN6002. Proteins are already glycosylated before the epitope is expressed. The developmental regulation of pallidin, a lectin known to be an unglycosylated protein, was investigated in parallel with ep293 using a monoclonal antibody. Pallidin was expressed at about the same time as the carbohydrate epitope in cells of the wild-type as well as the mutant. These results indicate a regulatory signal to which various events are coupled. Induction of ep293 and expression of pallidin are two of these events, and mutants such as PN6002 are altered in the timing of the signal.

Key words: cellular slime mould, *Polysphondylium*, fucosylation, pallidin, cell interactions.

Introduction

The mechanisms of cell aggregation in *Polysphondylium pallidum* are comparable to those of the related organism, *Dictyostelium discoideum*. Development from a single-cell stage to aggregation competence is initiated in both species by nutrient deprivation, and in both cell aggregation is the result of chemotaxis and cell adhesiveness that enables the cells to form a multicellular body. Despite these similarities the actual molecules that mediate cell aggregation are different. The chemoattractant of aggregating *P. pallidum* cells is a peptide rather than cyclic AMP as in *D. discoideum* (Shimomura et al. 1982). Similarly, the adhesion systems of aggregation-competent cells are species-specific, as evidenced by the sorting out of mixed cells of the two species under conditions where chemotaxis cannot play a role because cell motility is inhibited (Gerisch et al. 1980). As an approach to the molecular basis of this specificity, we have investigated cell adhesion in *P. pallidum* in parallel to *D. discoideum* (for review, see Gerisch, 1986). Our previous results obtained with *P. pallidum* can be summarized as follows.

Cell adhesion could be completely blocked by Fab from antisera raised against total membrane antigens (Bozzaro & Gerisch, 1978). From aggregation-competent *P. pallidum* cells a pair of glycoproteins was purified, which after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), forms a double band in the 64×10^3 M_r region. This ‘64 K glycoprotein’, also designated contact site 1 (Bozzaro et al. 1981), proved to be the major but not the only cell membrane glycoprotein that neutralized adhesion-blocking Fab. Antisera raised against purified 64 K glycoprotein cross-reacted with a number of other glycoproteins. This cross-reactivity was neutralized by purified oligosaccharide chains obtained from the 64 K glycoprotein by hydrazinolysis (Toda et al.
1984a). The adhesion-blocking activity of Fab from these antisera was also neutralized by the purified oligosaccharides, indicating that it was Fab bound to carbohydrate epitopes that interfered with intercellular adhesion.

In an attempt to investigate a potential role in cell-to-cell adhesion of specific carbohydrate residues, or of specific glycoproteins that carry them, we have raised monoclonal antibodies and have selected mutants altered in the expression of a certain carbohydrate epitope. One antibody, mAb293, proved to be of particular interest because when used in Fab form it completely blocked cell-to-cell adhesion (Toda et al. 1984a). Binding of the antibody to its proper epitope on glycoproteins, designated ep293, was inhibited by free L-fucose, indicating that the antibody recognizes the non-reducing ends of protein-linked carbohydrate chains where the L-fucose residues are located (Toda et al. 1984b). Since the antibody showed much higher affinity for ep293 than for free L-fucose, it certainly recognizes a larger structure than only a terminal L-fucose residue.

ep293 is present on the 64 K glycoprotein as well as on other glycoproteins, and is expressed already during exponential growth of wild-type cells. By using a fluorescence-activated cell sorter, mutants were selected that expressed ep293 only after an extended period of starvation (Francis et al. 1985). In all these mutants aggregation was delayed and atypical, and the shape of the fruiting bodies was less regular than in the wild-type. Genetic recombination showed that the altered morphogenesis was linked to the late expression of ep293. This result indicated a common step in the control of ep293 expression and aggregation, though it did not provide evidence that ep293 itself is required for the cells to aggregate.

In the present study we have investigated ep293 expression in a wild-type strain, PN600, and in PN6002, one of the mutants obtained from it. We will show that ep293 is not only regulated in the mutant but also in the wild-type, and that the signal for expression of this epitope occurs in the wild-type early during exponential growth. Concerning the mutant, we address two questions. First, is there a delay in synthesis of all the glycoproteins that carry ep293? Alternatively, these proteins may pre-exist without ep293 being expressed either in a non-glycosylated state or as glycoproteins with immature oligosaccharide residues. Second, is the delayed expression of ep293 linked to other developmental events; in particular, is the expression of pallidin, a protein known to be developmentally regulated in the wild-type (Rosen et al. 1974), also delayed in the mutant? Coregulation of ep293 and pallidin would indicate a common regulatory event for various developmental processes.

### Materials and methods

#### Cell culture and development

Wild-type strain WS320 of *P. pallidum* has been used in previous immunochemical studies on cell adhesion (Bozzaro & Gerisch, 1978; Toda et al. 1984a,b), and wild-type strain PN600 was the parent strain for mutants that, like PN6002, had been selected after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Francis et al. 1985). For growth in shaken suspensions, cells were cultivated with 10^10 washed bacteria of *Escherichia coli* B/r per ml in 17 mM-Soerensen phosphate buffer, pH 6.9 (non-nutrient buffer) on a gyratory shaker at 150 revs min^-1 (Bozzaro & Gerisch, 1978). For starvation, cells were harvested from these cultures at the times indicated in Results, washed free of bacteria and resuspended at a density of 10^7 per ml in the non-nutrient buffer for continued shaking. For aggregation on non-nutrient agar, washed cells from shaken suspensions were resuspended at a density of 2x10^7 per ml, and 50 /mu of the suspensions were spread over a circular area of 1 cm diameter on plates containing 2% Difco purified agar in the non-nutrient buffer. All cultures were kept at 23°C under continuous illumination, according to Bozzaro & Gerisch (1978).

#### Purification of the 64 K glycoprotein and its oligosaccharide chains

The 64 K glycoprotein pair was solubilized by extraction with butanol-water of a particulate fraction from homogenized aggregation-competent cells of wild-type WS320, and was purified from the water phase by precipitation with ammonium sulphate, Triton X-114/water separation, and preparative SDS–PAGE in 10% gels (Toda et al. 1984a). For separation of the two components of the pair, gp64j and gp64p, the material enriched in the Triton X-114 phase was subjected to SDS–PAGE in 7.5% gels, the separate bands were cut out, extracted from the gels, and re-electrophoresed in 10% gels as described by Toda et al. (1984a).

For purification of oligosaccharide chains, 64 K glycoprotein purified up to the Triton X-114/water step was digested by Pronase and subtilisin followed by hydrazinolysis. The oligosaccharide was then purified by gel filtration on Sephadex G-50 and G-15 columns (Toda et al. 1984a). Hexoses were determined with phenol–sulphuric acid (Dubois et al. 1956), protein was assayed according to Lowry with bovine serum albumin as standard.

#### Antibody production

Rabbit antisera against the purified 64 K glycoprotein pair were the same as used previously (Toda et al. 1984a). Monoclonal antibodies were obtained by fusing spleen cells from Balb/c mice with either Sp2-01 or NSO/u myeloma cells. The antibodies against ep293 (Toda et al. 1984a,b; Francis et al. 1985) and against pallidin (Toda et al. 1986) have been described previously. The third monoclonal antibody used, mAb311, was obtained by treating 64 K glycoprotein, purified up to Triton X-114/water separation, with anhydrous HF according to Mort & Lamport (1977) before immunization. A 100 /mu sample of the protein in 100 /mu of saline was injected intraperitoneally with 100 /mu of
Freund's complete adjuvant; after 50 days a boost of 50 μg protein with 100 μl of Freund's incomplete adjuvant was given and the spleen cells fused with NS1/1 myeloma cells on the fourth day thereafter. Screening for antibodies that specifically react with the 64 K glycoprotein was performed using intact glycoproteins that had been extracted with butanol–water from a particulate fraction of aggregation-competent WS320 cells. Portions of extract corresponding to 200 μg of protein were separated by SDS–PAGE in 10 % gels of 95 mm length and 35 mm running distance, and blotted onto 85 nitrocellulose filters (Schleicher and Schuell, Dassel, FRG) as described (Stadler et al. 1984). The filters of these 'miniblots' were cut into strips of 4 mm width, and each strip was incubated overnight at room temperature with 100 μl hybridoma supernatant, washed, labelled for 3 h with 10⁶ cts min⁻¹ ml⁻¹ of ¹²⁵I-labelled goat anti-mouse immunoglobulin G (IgG), and autoradiographed.

Monoclonal IgGs were purified from hybridoma culture supernatants by precipitation with 55 % saturated ammonium sulphate and protein A–Sepharose chromatography. The IgG was iodinated as described (Noegel et al. 1985) using the chloramine T method. The code numbers of antibodies used in this paper are abbreviations; the complete numbers are mAb25-293-2, mAb55-250-1 and mAb71-311-10, respectively.

**SDS–PAGE and immunoblotting**

SDS–PAGE was performed according to Laemmli (1970), immunoblotting according to Towbin et al. (1979), and two-dimensional electrophoresis according to O'Farrell (1975) as specified by Stadler et al. (1983). When the same nitrocellulose filter was to be labelled with two different iodinated antibodies, the antibody added first was extracted for 1 h at 60°C with 8 M-urea in a solution containing 10 mM-Tris·HCl, pH 7.2, 100 mM-mercaptoethanol, and 5 mg per ml of bovine serum albumin. Subsequently the filter was extensively washed with a solution containing 10 mM-Tris·HCl, pH 7.2, 150 mM-NaCl, and 0.05 % Tween 20, and was autoradiographed before the second antibody was added.

For the experiments shown in Figs 3 and 4, total proteins from 2×10⁷ cells per gel were separated by SDS–PAGE to prepare miniblots as described above. The nitrocellulose strips were incubated overnight at 20°C with 100 μl of incubation buffer containing 10⁶ cts min⁻¹ of the ¹²⁵I-labelled monoclonal antibody and amounts of inhibitor as indicated in Results. The incubation buffer consisted of 10 mM-Tris·HCl, pH 7.2, 150 mM-NaCl, 4 % bovine serum albumin, 0.05 % Tween 20 and 1 % NaN₃. For the competition experiment shown in Fig. 9, 200–210 μg protein in butanol–water extract was applied per lane, and after SDS–PAGE the nitrocellulose strips were incubated for 3 h at 22°C in a total volume of 80 μl incubation buffer containing 0.1 μg of unlabelled IgG from rabbit antisera and amounts of competitor as indicated. After washing the blots were labelled with ¹²⁵I-labelled goat anti-rabbit IgG and autoradiographed.

**Results**

**Early expression of ep293 in exponentially growing wild-type cells**

Two monoclonal antibodies, mAb293 and mAb311, were used to study glycoprotein modification in wild-type cells. Both antibodies were raised against the 64 K glycoprotein pair of wild-type strain WS320. Since the carbohydrate chains to which mAb293 binds dominated the immune response to the glycoprotein, carbohydrate was cleaved off by anhydrous hydrogen fluoride before immunization in order to obtain an antibody against the polypeptide moiety of the glycoprotein; mAb311 was the best one obtained.

Under our conditions of culture in shaken suspensions of E. coli B/r, P. pallidum cells grew up to a density of 10⁷ per ml (Fig. 1A). When cells of WS320 were harvested at various stages of growth, and equivalent amounts of cell homogenates were loaded onto SDS–polyacrylamide gels for electrophoresis and immunoblotting, no labelling with mAb293 was recognizable in cells harvested before a cell density of 10⁶ per ml was reached. At higher cell densities ep293 became coordinately expressed on several proteins, including the 64 K glycoprotein (Fig. 1B). When after almost complete removal of the antibody (Fig. 1C) the same blot was incubated with mAb311, a single protein of about 64 K was labelled, and the band of this protein was already seen at the earliest stages of exponential growth (Fig. 1D). These results indicate that at least one member of the 64 K pair of glycoproteins is constitutively expressed in WS320, and that ep293 represents a modification that is acquired during an early stage of exponential growth. Expression of the epitope is not associated with a significant shift of the apparent molecular weight of the protein.

For the selection of mutants, wild-type strain PN600 was applied because for this strain a complementary mating type is available. This makes possible the genetic analysis of mutants by meiotic recombination (Francis et al. 1985). Expression of ep293 was investigated with a similar result to that with WS320 (Fig. 2A–C), the only difference being that instead of a 64 K glycoprotein a 56 K glycoprotein was labelled by both antibodies (lanes 3 and 4). Comparison with WS320 (lane 5) in the same gel clearly shows the difference in the apparent molecular weights of these two products, a difference that is not seen when other glycoprotein bands of the two wild isolates are compared (Fig. 2B). Hence the molecular-weight heteromorphism appears to be specific for the glycoprotein recognized by mAb311.

In order to find out whether the onset of ep293 expression reflects a more general change in the state of the cells, the expression of pallidin was traced in the

Glycoprotein modification in Polysphondylium
Fig. 1. Growth of *P. pallidum* wild-type strain WS320 (A), expression of ep293 on various glycoproteins (B), and presence of the 64 K glycoprotein (D) during growth of this strain. A. Suspensions of *E. coli* B/r were inoculated with 20 (△), 10 (○), or 5 (□) spores per μl and shaken. Cells were counted at various hours after inoculation. Data indicate that cells grew exponentially up to a density of 7×10⁶ per ml. Numbered arrows indicate stages at which samples were removed for immunoblotting; the numbers correspond to lane numbers 1–9 as indicated at the bottom of D. B. Cells were harvested at various stages of growth and their proteins subjected to SDS–PAGE in 10% gel, immunoblotting with [125I]mAb293, and autoradiography for 4 days. Total cellular proteins corresponding to 5×10⁵ cells were applied to each lane and subjected to SDS–PAGE, immunoblotting with [125I]mAb293, and autoradiography. C. Autoradiogram of the same filter after antibody extraction with 8 M-urea and exposure for 4 days. D. Autoradiogram of the filter after labelling with mAb311 and exposure for 4 days. The apparent increase in labelling of the 64 K glycoprotein at the later stages of growth might be due to remnants of mAb293 that had been left on the filter after extraction as seen in C.

Fig. 2. Growth of wild-type strain PN600 (A), expression of ep293 (B), presence of the 56 K glycoprotein (C), and expression of pallidin (D) during exponential growth of this strain. A. A suspension of *E. coli* B/r was inoculated with 1 spore per μl and shaken. Numbers at arrows correspond to sample numbers 1–4 at the bottom of D. B. Lanes 1–4, PN600 cells were harvested at the stages indicated in A; lane 5, sample from strain WS320 cells harvested during growth at a density of 2×10⁶ per ml. Total cellular proteins corresponding to 5×10⁵ cells were applied to each lane and subjected to SDS–PAGE, immunoblotting with [125I]mAb293, and autoradiography. C. Similar blots as in B, but labelled with [125I]mAb311 for the 56 K or 64 K glycoprotein. D. Similar to B, but labelled with mAb250 for pallidin. Exposure times of the autoradiograms were 3 days for B lanes 1–5 and C lanes 1–4, and 19 h for C lane 5 and D lanes 1–5.
same cells as used for glycoprotein labelling. Pallidin is a soluble lectin; it was of interest in our context not as a lectin but as a developmentally regulated, un-glycosylated protein (Simpson et al. 1975). For the labelling of pallidin a monoclonal antibody, mAb250, was used (Toda et al. 1986). It showed an increase in pallidin from barely detectable levels to substantial amounts concomitantly with ep293 expression (Fig. 2D, lanes 1–4). As in PN600, pallidin was clearly expressed in growth phase cells of strain WS320 (lane 5). This result was unexpected since a previous report indicated that pallidin increased after starvation of the cells at the time when aggregation began (Rosen et al. 1974). The early expression of pallidin cannot be explained by assuming that the cells were partially starved under our growth conditions, since the cells grew optimally on the bacteria with a generation time of less than 3 h (Figs 1, 2).

Evidence that mAb311 recognizes an epitope on the protein moiety

The fact that mAb311 was obtained using HF-treated glycoprotein for immunization as well as its high specificity for a single protein makes it unlikely that this antibody is directed against a carbohydrate structure. However, HF treatment of glycoproteins does not always degrade the carbohydrate chains completely, and in D. discoideum some of the carbohydrate epitopes are relatively specific for a given glycoprotein (Bertholdt et al. 1985). Therefore we have tested the specificity of mAb311 by competition with carbohydrate. First, mAb311 binding to the glycoproteins was assayed in the presence of nine simple sugars. None of these sugars blocked the binding (Fig. 3), while the binding of mAb293 was efficiently blocked by L-fucose, in accordance with previous results (Toda et al. 1984b). Because only some anti-carbohydrate antibodies react strongly enough with simple sugars for blockage to occur, this result does not exclude binding of mAb311 to a carbohydrate epitope. Therefore oligosaccharide chains isolated by hydrazinolysis from the 64 K glycoprotein were used for competition. After purification they blocked the binding of mAb293 to the intact glycoprotein (Toda et al. 1984a). As shown in Fig. 4, an amount of oligosaccharide corresponding to 6 μg of hexose did not affect binding of mAb311, while an amount of the intact 64 K glycoprotein equivalent to 5 μg of protein was sufficient for complete neutralization of the antibody. The 64 K glycoprotein contains about 13% of hexoses relative to protein (Toda et al. 1984a). This means that the oligosaccharide chains that had no effect were in more than 9-fold excess relative to the antibody.

![Fig. 3. Assay for blockage of antibody binding to glycoproteins by simple sugars. Total cellular proteins from PN600 cells harvested after 6 h of starvation were subjected to SDS–PAGE in minigels, immunoblotting, and autoradiography. Blots were incubated for 14 h at 20°C with [125I]mAb311 or [125I]mAb293 in the presence of 100 mM-sugars or in controls without sugar.](image-url)

Proof that the glycoproteins labelled with mAb311 are modified by ep293

The relationship between the two components of the 64 K pair of glycoproteins in the WS320 wild-type strain is unclear. These glycoproteins remained together during all steps of purification and were separated only by cutting them out from SDS–polyacrylamide gels. A qualitative difference between the two components was indicated with an antibody, mAb445, which precipitated only gp64α, the component with the higher apparent molecular weight (Toda et al. 1984a). In contrast to this antibody, mAb311 recognized both components, gp64α and gp64β (Fig. 5, lanes 1 and 2). Neither of these components was identical with the 56 K product of PN600, the second wild-type strain used (lane 3). The reactivity of both components of the 64 K pair with mAb311 indicates homologies in their protein moieties in addition to those in the carbohydrate residues.

To establish that ep293 is linked to the 64 K and 56 K proteins recognized by mAb311, rather than to other proteins of the same apparent molecular weights, particulate fractions of wild-type strains WS320 and PN600 were subjected to two-dimensional electrophoresis and immunoblotting. In the top panel of Fig. 6 selective labelling of the 64 K and 56 K Glycoprotein modification in Polysphondylium
glycoproteins with mAb311 is shown. After removal of most of the antibody, as seen in the middle panel, the glycoproteins were relabelled with mAb293. As shown in the bottom panel, mAb293 intensely labelled the same proteins as mAb311. In addition, some other glycoproteins were recognized by mAb293.

**Coordinated delay of developmental changes in mutant PN6002**

PN6002 is used in this study as the prototype of a number of independent mutants whose common feature is a considerable delay of ep293 expression. Fig. 7A shows this delay for suspension cultures of PN6002; ep293 was not detectable earlier than after 12 h of starvation, and its expression substantially increased through the next 12 h. The 56 K protein was present at all stages tested (Fig. 7B). In the mutant as in the wild-type, acquisition of ep293 was not accompanied by a detectable change in the apparent molecular weight of the 56 K protein.

Pallidin, which was expressed in the wild-type early during exponential growth at about the same time as ep293, was also coexpressed with ep293 in the mutant (Fig. 7C). It appeared there after an extended period of starvation, while in the wild-type the amount of pallidin remained constant over the whole period of early development (Fig. 7D). These results show that the mutant suffers from a general defect in the timing of development rather than from a specific delay of ep293 expression. This defect in timing also affects the time course of aggregate formation. When transferred onto non-nutrient agar plates at the beginning of starvation, wild-type cells formed fully developed aggregates within 7 h and reached the culmination stage within 12 h (Fig. 8A, B). Under the same conditions, mutant cells required 12 h to form the first, still rudimentary, aggregates (Fig. 8C, D). Thus aggregate formation began in the mutant at about the same time as ep293 expression.

**Protein glycosylation occurs prior to ep293 expression**

The finding that the apparent molecular weights of the 64 K and 56 K proteins did not significantly change when they acquired ep293 suggests that a modification of carbohydrate moieties rather than the addition of entire oligosaccharide chains underlies expression of the epitope. This was supported by labelling of
glycoproteins before and after ep293 expression with antibodies from polyclonal antisera. Although these antisera were obtained by immunizing rabbits with the entire 64 K glycoprotein, they turned out to be almost specific for carbohydrate structures. This was indicated by the strong cross-reactivity of the antibodies raised against highly purified 64 K glycoprotein (Fig. 9). Furthermore, binding of these antibodies to blotted antigens was almost completely blocked by purified oligosaccharide chains. This was not only true for antigens of wild-type PN600 but also for those of mutant PN6002 (Fig. 9). When antibodies from the same rabbit antisera were applied to blots of different developmental stages of wild-type and mutant cells, all stages were labelled. The pattern of labelled bands was similar in the mutant and wild-type, although labelling of wild-type antigens was slightly more intense (Fig. 10). These results indicate that membrane proteins in the mutant were already glycosylated before ep293 was expressed.

Discussion

Developmentally regulated modification of glycoproteins

In this paper we have shown that ep293, an L-fucose-containing epitope on protein-linked oligosaccharide residues, is expressed early during exponential growth in wild-type *P. pallidum* cells, while it is not expressed before 12 h of starvation in cells of a mutant, PN6002. Cells of this mutant did not aggregate before the expression of ep293 began. Even then the aggregation patterns were atypical, as were the structures of fruiting bodies formed thereafter. The possibility that
Fig. 7. Expression of ep293 (A), presence of the 56 K glycoprotein (B), and expression of pallidin (C) during starvation of mutant PN6002. For A–C, growing cells of the mutant were harvested at a density of 5×10^6 per ml, washed and shaken in non-nutrient buffer at a density of 1×10^7 per ml. For D, growing cells of PN600 were harvested at a density of 8×10^6 per ml and starved, in order to compare pallidin expression in the mutant with that in the wild-type. A 30 µg sample of total cellular proteins was applied per lane. After SDS-PAGE, blots were labelled in parallel with [125I]mAb293 for A, [125I]mAb311 for B, or [125I]mAb250 for C and D.

the glycoproteins carrying ep293 are regulated in toto was excluded by using an antibody, mAb311, which recognizes the protein moiety of one of them. Labelling with the antibody indicated that the protein is always present in the wild-type as well as the mutant, independently of whether or not ep293 is expressed (Figs 1, 2, 7). Furthermore, polyclonal antibodies directed against carbohydrate epitopes other than ep293 indicated that proteins were glycosylated before ep293 was expressed (Fig. 10). The conclusion from these results is that ep293 expression is due to a modification of pre-existing glycoproteins.

No significant change in the apparent molecular weight was detected when ep293 became expressed on one of the glycoproteins. This result is in accordance with a modification of carbohydrate residues rather than the addition of new, additional oligosaccharide chains to the glycoprotein. Quite different results have been obtained with modB mutants in D. discoideum (Murray et al. 1984). In these mutants one type of oligosaccharide chain is no longer attached to glycoproteins. In consequence, synthesis of the 80 K contact site A glycoprotein is blocked at the stage of a 68 K precursor that carries only one of the two types of carbohydrate chains normally linked to this glycoprotein (Yoshida et al. 1984; Hohmann et al. 1985). Similarly, the apparent molecular weights of three other glycoproteins are reduced by 3-5 to 14 (×10^3) in modB mutants (West & Loomis, 1985).

It is conceivable that ep293 expression reflects conversion of the oligosaccharides from a high-mannose to a complex type, although the analogy of oligosaccharide structure in P. pallidum glycoproteins with that of mammalian and other cells remains to be proven. In D. discoideum, the average l-fucose content of protein-linked oligosaccharide chains increases during development (Ivatt et al. 1984). This increase occurs at the tipped aggregate stage, which is much later than in P. pallidum wild-type cells, where it occurs early during the growth phase. It has not been reported whether proteins of D. discoideum that are specifically expressed at later stages of development acquire new types of oligosaccharides, or whether constitutively expressed glycoproteins undergo changes in the structure of their carbohydrate residues during development.

Coregulation of glycoprotein modification and pallidin indicates a common regulatory mechanism

The regulation of pallidin in mutant PN6002 was investigated because it is the best known developmentally regulated protein of P. pallidum (Simpson et al. 1975). The function of pallidin as a lectin was of no relevance in this context. Pallidin expression proved to be delayed in the mutant, suggesting that ep293 and pallidin are regulated by the same mechanism.

According to previous results pallidin expression coincides with the onset of cell aggregation (Rosen et al. 1974). Together with other results, this coincidence indicated a role for pallidin in cell-to-cell adhesion (Rosen et al. 1976). Under our conditions pallidin was already expressed during exponential growth, long before the cells acquired the ability to aggregate. Since WS320, one of the wild-type strains used by us, was the same as was used by Rosen and coworkers, culture conditions rather than strain differences seem to be responsible for these differences in the timing of pallidin expression.

Transitions in the properties of cells during early exponential growth, as they are observed in P.
Fig. 8. Timing of development in wild-type PN600 (A,B) and mutant PN6002 (C,D) after deposition of cells on non-nutrient agar. Growing cells were harvested from shaken suspension cultures at densities of $6 \times 10^6$ per ml for PN600 or $8 \times 10^6$ per ml for PN6002, washed and transferred onto the agar surface. Photographs were taken after the times indicated.
Fig. 9. Binding of polyclonal antibodies against the 64 K glycoprotein and blockage of their binding by purified oligosaccharide chains from the same glycoprotein. Cells of wild-type PN600 (left panel) or mutant PN6002 (right panel) were harvested at 6 h of starvation and butanol–water extracts of particulate fractions were subjected to SDS–PAGE, immunoblotting, and autoradiography. Lanes 1, control blots with only $^{125}$I-labelled goat anti-rabbit IgG antibody added. Lanes 2, blots incubated with rabbit immune IgG and subsequently labelled with $^{125}$I-labelled goat anti-rabbit IgG. Lanes 3, similar to lanes 2, but purified oligosaccharide chains corresponding to 20 $\mu$g of hexose were added to the rabbit IgG.

*P. pallidum* wild-type strains, indicate a change in the growth conditions two to three generations before nutrients are exhausted. It is conceivable that ep293 and palladin are induced by a factor that is released from the growing cells and accumulates in the medium. However, attempts to induce ep293 with medium conditioned by growing *P. pallidum* cells have not led to consistent results.

Is glycoprotein modification a requirement for normal development?

The adhesion-blocking activity of Fab from mAb293 suggested a function in cell adhesion of glycoproteins recognized by this antibody, but it did not provide conclusive evidence, since steric effects of the Fab on cell adhesion could not be ruled out (Toda et al. 1984a,b). In an attempt to provide independent evidence, mutants defective in ep293 expression were selected. In the 19 stable mutants obtained, substantial delays in expression of the carbohydrate epitope were observed (Francis et al. 1985). The finding that delayed aggregation and reduced stream formation of aggregating cells, together with atypical structure of the fruiting bodies, were genetically linked to delayed and reduced ep293 expression was consistent with a function of this epitope in cell aggregation and/or fruiting body formation. However, the possibility was taken into account that the mutants carried a general defect in the regulation of development rather than a specific defect in glycosylation (Francis et al. 1985). Here we have shown for one of these mutants that indeed a defect in a developmental control mechanism is responsible for the retarded expression of the carbohydrate epitope. Palladin, probably together with other unknown proteins, was coregulated with ep293 expression not only in the wild-type but also in the mutant. Regulatory mutants are of interest in their own right. But they are not useful in attributing a specific function to a certain developmental change, in our case to the expression of ep293. To elucidate the function of the carbohydrate change underlying the expression of ep293, a mutant with a selective loss of this epitope will be required.
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