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Post-translational glycosylation of the contact site A protein of *Dictyostelium discoideum* is important for stability but not for its function in cell adhesion

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The functions of type 1 and 2 carbohydrates of the contact site A (csA) glycoprotein of *Dictyostelium discoideum* have been investigated using mutants lacking type 2 carbohydrate. In two mutant strains, HG220 and HG701, a 68-kd glycoprotein was synthesized as the final product of csA biosynthesis. This glycoprotein accumulated to a much lower extent on the surfaces of mutant cells than the mature 80-kd glycoprotein did in wild-type cells. There was also no accumulation of the 68-kd glycoprotein observed within the mutant cells nor was a precursor of lower molecular mass detected, in accordance with previous findings that indicated cotranslational linkage of type 1 carbohydrate by N-glycosylation. Pulse-chase labelling showed that a 50-kd glycopeptide was cleaved off from the mutant 68-kd glycoprotein and released into the medium, while the fully glycosylated 80-kd glycoprotein of the wild type was stable. These results assign a function to type 2 carbohydrate in protecting the cell-surface-exposed csA glycoprotein against proteolytic cleavage. HG220 cells were still capable of forming EDTA-stable contacts to a reduced extent, consistent with the low amounts of the 68-kd glycoprotein present on their surfaces. Thus type 1 rather than type 2 carbohydrate appears to be directly involved in intercellular adhesion that is mediated by the csA glycoprotein. Tunicamycin-treated wild-type and mutant cells produce a 53-kd protein that lacks both type 1 and 2 carbohydrates. While this protein is stable and not transported to the cell surface in the wild type, it is cleaved in the mutants and fragments of it are released into the extracellular medium. These results suggest that the primary defect in the two mutants studied is relief from a restriction in protein transport to the cell surface, and that the defect in type 2 glycosylation is secondary.

Key words: cell adhesion/contact sites A/*Dictyostelium*/post-translational glycosylation/carbohydrate function

Introduction

The contact site A (csA) glycoprotein, a cell adhesion molecule of aggregating cells of *Dictyostelium discoideum* (Müller and Gerisch, 1978), is modified by two types of oligosaccharide residues. Type 1 carbohydrate is N-linked and added cotranslationally, type 2 carbohydrate is added as a post-translational modification (Hohmann *et al.*, 1987). The type 1 and 2 glycosylation steps can be independently blocked, offering an opportunity to investigate the roles of different carbohydrate residues in transport of the csA protein to the plasma membrane, in protecting the protein against proteases, and in the functioning of the molecule in intercellular adhesion. In a previous study type 1 glycosylation was shown to be inhibited by tunicamycin (Hohmann *et al.*, 1987). The results of this study led us to conclude that type 1 carbohydrate is neither essential for transport of the csA protein to the cell surface nor for its protection against proteases. But it might be important for cell adhesion, since tunicamycin blocks EDTA-stable cell contacts that are mediated by the csA glycoprotein (Ochiai *et al.*, 1982).

The goal of the present paper was to study the functional implications of a block in type 2 glycosylation. In a class of mutants, designated as *modB* mutants (Murray *et al.*, 1984), type 2 glycosylation is blocked but type 1 glycosylation is unaffected (Bertholdt *et al.*, 1985). We have used two independent mutant strains defective in type 2 glycosylation, including an axenically growing derivative of the *modB* mutant strain HL220 of Murray *et al.* (1984). Results obtained with these mutants indicate that

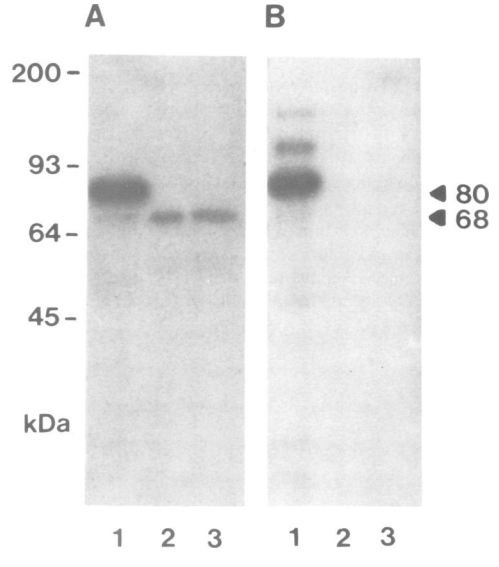


Fig. 1. Synthesis of a 68-kd form of the csA glycoprotein by two mutant strains defective in type 2 glycosylation. Total homogenates of wild-type cells (lanes 1), and of mutant HG220 (lanes 2) or HG701 cells (lanes 3) harvested at 6 h of starvation were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using either [^{125}I]mAb 294 (**A**) or [^{125}I]-mAb 178 (**B**). mAb 294 recognizes the polypeptide of the csA glycoprotein and mAb 178 an epitope on type 2 carbohydrate that is also present on a 95-kd glycoprotein with another polypeptide moiety. The autoradiograph shows in lanes 1 that both antibodies recognized the mature 80-kd csA glycoprotein of wild-type cells and mAb 294 recognized in addition a 68-kd precursor which has not yet received type 2 carbohydrate (Hohmann *et al.*, 1987). Lanes 2 and 3 show that both mutants produced a protein that resembles the precursor of the wild type in its apparent mol. wt and in its non-detectability by mAb 178. The quantity of the mutant 68-kd component was much lower than that of the wild-type 80-kd glycoprotein. Total proteins of 1×10^6 cells were applied per lane. Positions of mol. wt markers are indicated on the left, apparent mol. wts of csA components on the right.

type 2 carbohydrate is not essential for the formation of EDTA-stable cell contacts but is important for preventing the cell-surface-exposed protein from being degraded. We also provide data suggesting that in *modB* mutants the control of routing of the csA protein to the cell surface is disturbed.

Results

Selection and properties of mutant strains defective in type 2 carbohydrate

The *modB* mutant strain HL220 described by Murray *et al.* (1984) does not show any reactivity with a series of monoclonal antibodies that recognize different epitopes on type 2 carbohydrate (Bertholdt *et al.*, 1985). HL220 produces a 68-kd version of the csA glycoprotein that still contains the N-glycosidically linked type 1 carbohydrate (Yoshida *et al.*, 1984; Gerisch *et al.*, 1985b). Because the inability of HL220 to grow axenically is inconvenient for studies on the biosynthesis and function of the incompletely glycosylated csA protein, an axenic *modB*$^-$ strain was constructed by crossing HL220 with HU1766, an axenic strain with a *modB*$^+$ wild-type allele and selecting a segregant, HG220, which carries on linkage group VI the *modB502* mutation of its HL220 parent.

An independent mutant defective in type 2 carbohydrate, HG701, was selected from mutagenized HG592 cells. HG592 is a derivative of the AX2 strain with alterations in the control of development which are of no importance in the context of the present study (Gerisch *et al.*, 1985a).

Figure 1 shows immunoblots labelled with mAb 294, an antibody specific for the polypeptide moiety of the csA glycoprotein, and with mAb 178, an antibody that recognizes an epitope on type 2 carbohydrate. These blots indicate that both mutants are defective in type 2 glycosylation, resulting in the production of a 68-kd component containing the csA polypeptide moiety. A feature common to both mutant strains, and also to the original HL220 strain, is the low abundance of the 68-kd component as compared with the fully glycosylated 80-kd glycoprotein of wild-type cells. The reason for this low abundancy has been elucidated by the following experiments.

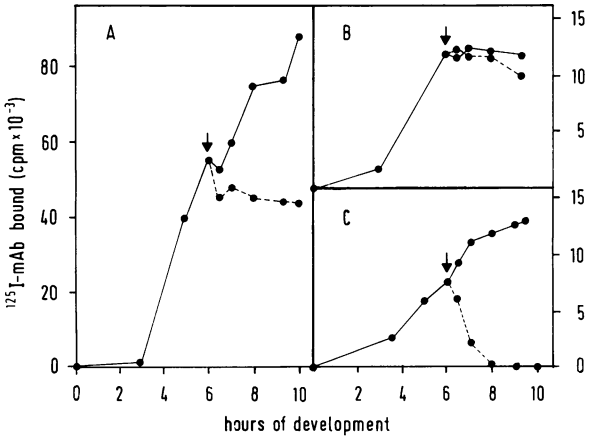


Fig. 2. Slow turnover of the csA glycoprotein (**A**) and of a 66-kd glycoprotein of tunicamycin-treated wild-type cells (**B**), and fast degradation of the 68-kd glycoprotein of HG220 cells (**C**). At 6 h of starvation cycloheximide was added to one aliquot of cell suspensions (arrows) and shaking of the cells was continued (dashed lines). Another aliquot received no additions (solid lines). To quantify csA components, particulate fractions of the washed cells were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with [^{125}I]mAb 294. Sections of the filter that contained bands of csA components were excised and label was quantitated in a γ-counter. Under the conditions of tunicamycin treatment used for (**B**), a csA component of 53 kd was produced in addition to the 66-kd glycoprotein. The 53-kd protein lacks both type 1 and 2 carbohydrate, and in contrast to the 66-kd glycoprotein, continues to accumulate in tunicamycin-treated cells starved for 6 h or longer (Hohmann *et al.*, 1987). The concentration of cycloheximide was 500 μg/ml, the amounts of particulate fractions applied per lane corresponded to 2 × 10^6 cells.

Rapid degradation of the 68-kd glycoprotein in the mutant cells and accumulation of extracellular fragments

In order to determine whether low abundance of the 68-kd glycoprotein is due to its high turnover, we have determined the decay of the 68-kd glycoprotein after blocking its *de novo* synthesis by cycloheximide. For comparison, decay of the 80-kd glycoprotein in AX2 wild-type cells and of a 66-kd glycoprotein produced in tunicamycin-treated wild-type cells was assessed. While the 68-kd glycoprotein lacks type 2 carbohydrate, the 66-kd glycoprotein lacks type 1 carbohydrate (Yoshida *et al.*, 1984). The 66-kd glycoprotein had a long life-time similar to the 80-kd glycoprotein, indicating that absence of type 1 carbohydrate does

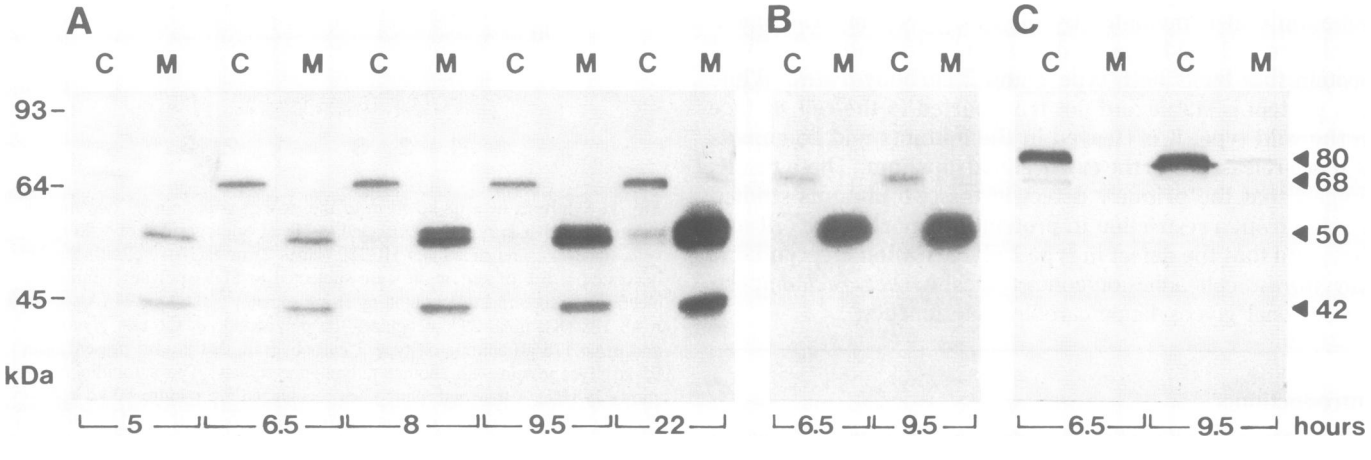


Fig. 3. Accumulation in the extracellular medium of fragments of the 68-kd glycoprotein produced in mutant HG220 (**panel A**) or HG701 (**panel B**) in comparison with wild type (**panel C**). **Lanes C** indicate cellular proteins, **lanes M** extracellular medium. Numbers at the bottom are hours of development at which cells and medium were harvested for SDS–polyacrylamide gel electrophoresis, immunoblotting with mAb 294, and autoradiography. For **panels A** and **C** the medium was evaporated in a speed-vac concentrator. Since this procedure caused partial degradation of extracellular 50-kd fragments into a 42-kd fragment it was omitted for **panel B**. Aliquots of 200 μl cell suspensions were applied per lane for wild type or HG220, and aliquots of 100 μl for HG701. One hundred microlitres contained the proteins produced by 1 × 10^6 cells.

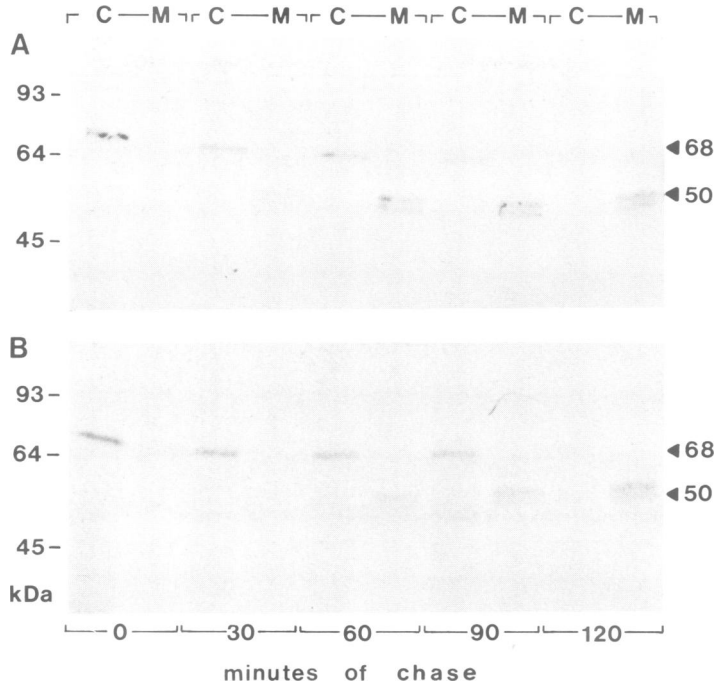


Fig. 4. Pulse-chase labelling with [^{35}S]sulphate of the 68-kd glycoprotein and its extracellular fragments in HG220 cultures. The mutant cells were starved for 5 h, labelled for 5 min with [^{35}S]sulphate and subsequently chased with unlabelled sulphate after centrifugation and resuspension in either fresh phosphate buffer (**A**) or buffer in which cells had been starved for 5 h (**B**). Aliquots of cell suspensions were fractionated into cells (C) and extracellular medium (M) at various times of chase as indicated at the bottom of the figure. Excess mAb 71 was added to detergent extracts of total cells or to extracellular medium, and the immunoprecipitated csA components were subjected to SDS–polyacrylamide gel electrophoresis, blotting onto nitrocellulose, and fluorography. Aliquots of 1 ml cell suspensions were applied per lane which contained the proteins produced by 1×10^7 cells.

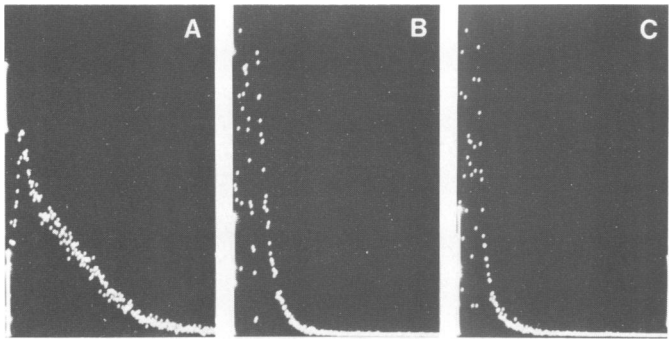


Fig. 5. Cell-surface immunofluorescence labelling of mutant HG220 cells. Living cells were incubated at 14 h of starvation with mAb 71 and subsequently with FITC-conjugated anti-mouse IgG (**A**). For controls, either mAb 178 was used as the first antibody (**B**) or only the fluorescent second antibody was added (**C**). With mAb 178 no specific labelling should be obtained since HG220 cells lack type 2 carbohydrate to which this antibody binds. Immunofluorescence intensities were determined using a FACS IV cell sorter. The abscissas show fluorescence intensities and the ordinates relative cell numbers in the intensity classes. Only in (**A**) a substantial portion of the cells showed significant binding of fluorescent antibody. Examination of the cells in a microscope confirmed that specifically the cell surfaces were labelled in (**A**).

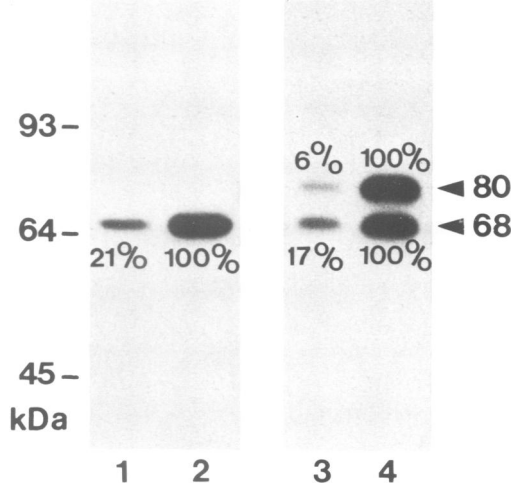
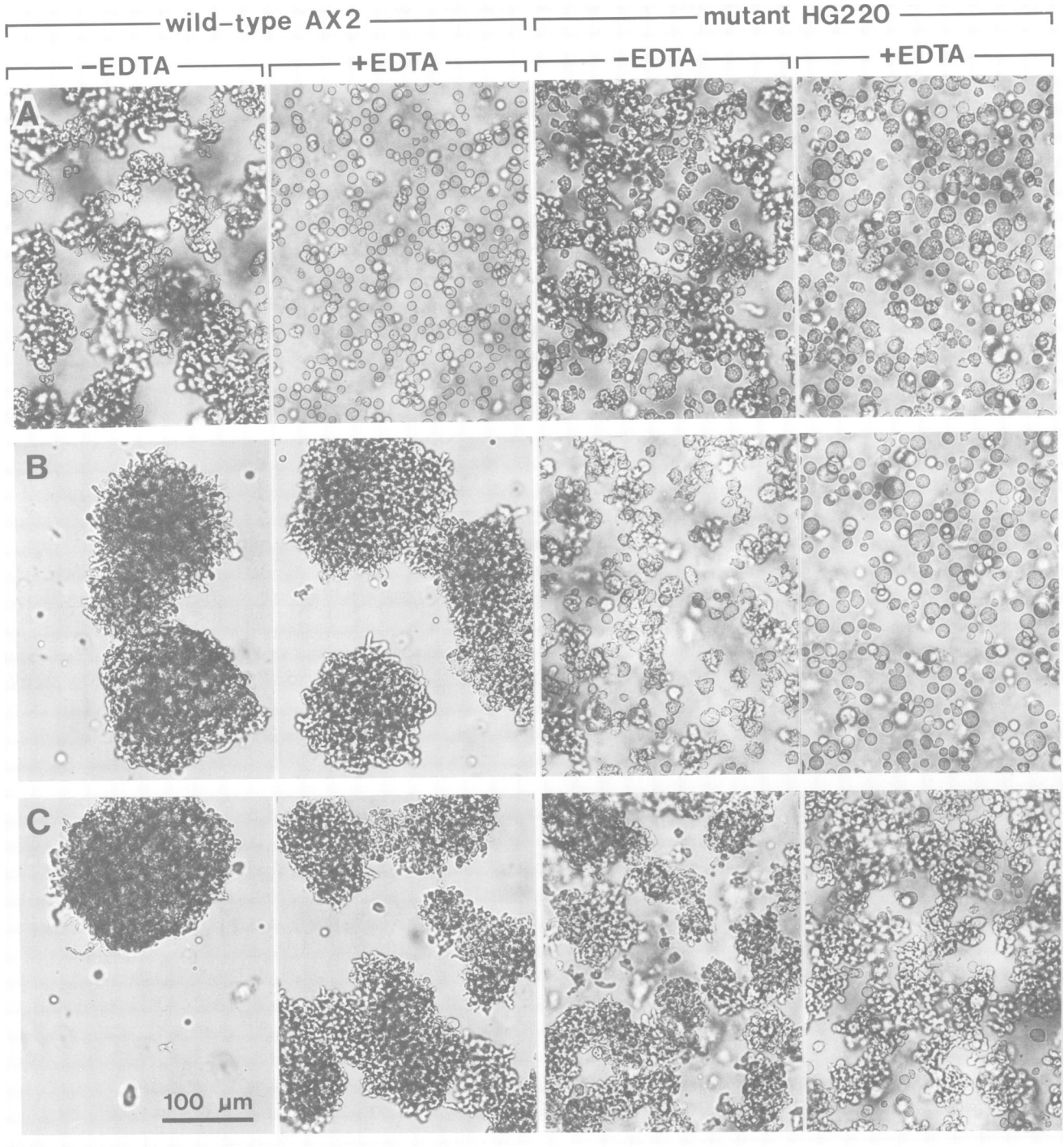
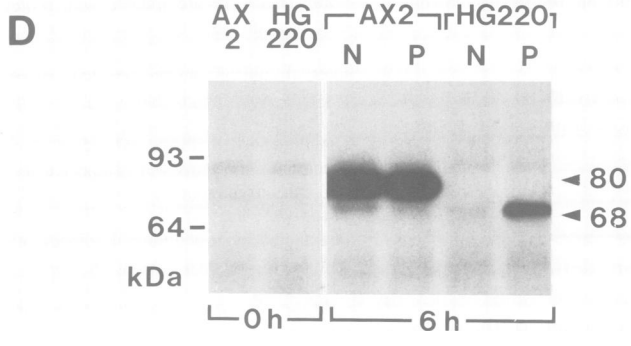


Fig. 6. Ratio of intracellular and cell-surface exposed 68-kd glycoprotein in HG220 cells at 6 h of starvation. For **lanes 1** and **3** living cells of the mutant were labelled with mAb 71. After removal of unbound antibody the cells were extracted with octyloligooxyethylene and the antigen–antibody complexes were precipitated with protein A–Sepharose. For **lanes 2** and **4** csA components were extracted first under lysis of the cells, subsequently incubated with the antibody and precipitated. **Lanes 3** and **4** serve as an internal control for the specificity of cell-surface labelling in that mutant proteins were supplemented by the wild-type 80-kd glycoprotein. An extract of the 10 000 *g* sediment of a wild-type cell homogenate was added simultaneously with the extraction of mutant proteins by detergent. Immunoprecipitated proteins were subjected to SDS–polyacrylamide gel electrophoresis, immunoblotting with mAb 294, and autoradiography. Individual bands were cut out from the filter and antibody bound to them was quantitated by γ-counting. Radioactivity associated with the bands in **lanes 2** and **4** was assigned a value of 100%. Equivalents of 5×10^7 HG220 cells were applied per lane. For **lanes 3** and **4** these were supplemented with wild-type extract to make the 80-kd glycoprotein roughly equimolar to the 68-kd glycoprotein.

not reduce stability of the product (Figure 2A and B). After 4 h of cycloheximide treatment there was not more than a 20% reduction observed in the amounts of these two glycoproteins. In contrast, the 68-kd glycoprotein rapidly disappeared from HG220 cells (Figure 2C); it was no longer detectable after 2 h of cycloheximide treatment. A similar fast disappearance as in HG220 was observed in HG701, the second mutant deficient in type 2 glycosylation.

No significant quantities of break-down products of the 68-kd


wild-type AX2
mutant HG220
-EDTA
+EDTA
-EDTA
+EDTA
A
B
C
100 µm


D
AX 2
HG 220
AX2
HG220
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N
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93-
64-
kDa
80
68
0 h
6 h

glycoprotein were found to be associated with the cells. One reason could be rapid degradation into fragments too small to be detected. Another reason could be the release of the entire glycoprotein, or of fragments, from the cells into the medium. Figure 3 verifies the second possibility. In the culture fluid of HG220 cells two or three fragments accumulated continuously over a period of > 10 h of starvation, while in wild-type cultures almost all material recognized by mAb 294 remained bound to the cells. For the blots shown in Figure 3A the extracellular fluid had been concentrated by evaporation. Under these conditions a pair of fragments of ~50 kd and a fragment of 42 kd were found. In other experiments concentration was avoided and only the 50-kd pair was observed. Thus the small fragment is produced secondarily from the 50-kd pair.

There are three possible locations for the cleavage of the 68-kd glycoprotein: (i) intracellular vesicles, particularly lysosomes, (ii) the cell surface, and (iii) the extracellular medium after release of the glycoprotein from the membrane. Pulse-chase labelling was used to distinguish between these possibilities. In the first case, labelled 50-kd fragment should first be detected within the cells. In the second case cell-associated label should be exclusively in the 68-kd glycoprotein and extracellular label in the 50-kd fragment. In the third case the labelled 68-kd glycoprotein should be detected *in toto* as an extracellular component before it is cleaved. The 68-kd glycoprotein is sulphated at its type 1 carbohydrate residues (Hohmann *et al.*, 1985). *In vivo* [^{35}S]sulphate labelling showed that the 50-kd fragment is also labelled, indicating that it is a glycopeptide. After a pulse of 5 min only cellular 68-kd glycoprotein was found to be labelled (Figure 4). During the chase, labelled 50-kd fragment accumulated in the extracellular medium. At no time of the chase was labelled 50 kd fragment associated with the cells or labelled 68-kd glycoprotein discovered in the medium. We conclude from these results that the 68-kd glycoprotein is transported to and cleaved at the cell surface.

According to Figure 4, the half-life of the 68-kd glycoprotein was ~1 h in cells that had been starved for 5 h in phosphate buffer. No difference was found between cells that were resuspended after the pulse in fresh buffer (Figure 4A) and cells that were resuspended in buffer in which cells had been starved for 5 h (Figure 4B). This result makes it unlikely that proteases released into the medium during the early starvation period are responsible for degradation of the 68-kd glycoprotein, but it does not exclude extracellular proteases produced during the chase period to be responsible. Probably cell-surface-bound proteases are involved in degradation of the incompletely glycosylated product.

Cell-surface exposure of the mutant 68-kd glycoprotein

Degradation of the 68-kd glycoprotein on the cell surface rather than in lysosomes would imply that transport of the protein to the surface of mutant cells is possible in the absence of type 2 carbohydrate. In order to test this directly, intact cells were fluorescence labelled with mAb 71 as the first antibody, and binding of the antibody was monitored using a second antibody and a fluorescence-activated cell sorter (Figure 5A). HG220 cells were labelled by mAb 71 on their surfaces. As a control for the specificity of antibody binding, mAb 178 was used which recognizes type 2 carbohydrate on the csA glycoprotein (Bozzaro and Merkl, 1985). No significant increase of fluorescence beyond the intensity observed with control cells that had not received first antibody was found with mAb 178 (Figure 5B and C), in agreement with the defective type 2 glycosylation in *modB* mutants.

To determine the ratio of intracellular and cell-surface-exposed 68-kd glycoprotein at the 6-h stage of development, living cells were incubated with the antibody, washed, and lysed with detergent. The antigen−antibody complexes were precipitated with protein A−Sepharose. In parallel, cells were lysed first and the solubilized, total cellular antigen was precipitated. Figure 6 shows in lanes 1 and 2 that ~21% of the total antigen was precipitated when living cells were incubated with the antibody. Lanes 3 and 4 of Figure 6 show a control in which cells were incubated with antibody as in lane 1 and washed to remove unbound antibody. The detergent was supplemented with an extract containing 80-kd glycoprotein from wild-type cells (Figure 6, lane 4). Only 6% of the 80-kd glycoprotein was precipitated, which gives a measure of internal proteins that are artificially coprecipitated with the cell-surface-exposed ones (Figure 6, lane 3). The same precipitate contained 17% of the total 68-kd glycoprotein from the mutant cells. We conclude, therefore, that at least 10% of the cellular 68-kd content of the HG220 cells were cell-surface exposed. The same result was obtained with HG701, the other mutant defective in type 2 glycosylation.

EDTA-stable cell adhesion in mutant HG220

The finding that the 68-kd glycoprotein is exposed on the cell surface of HG220 cells, although at much lower amounts than the complete 80-kd glycoprotein is expressed on the surface of wild-type cells, prompted us to reinvestigate the effect of the *modB* mutation on the EDTA-stable cell adhesion that is attributed to the csA glycoprotein. In the original strain, HL220, either no (Loomis *et al.*, 1985) or weak (Murray *et al.*, 1984; Gerisch *et al.*, 1985b) EDTA-stable cell adhesion was found. Accordingly, the missing type 2 carbohydrate was either considered to be involved (Loomis *et al.*, 1985; Springer and Barondes, 1985) or not essential (Gerisch *et al.*, 1985b) for this type of cell contact.

In Figure 7 A−C cell adhesion in the absence or presence of EDTA was studied in undeveloped wild-type and HG220 cells with no 80- or 68-kd glycoprotein being present, and in cells starved for 6 h in which either one of these glycoproteins was expressed. One aliquot of the cells was stimulated by pulses of cyclic AMP to enhance the expression of the 68-kd glycoprotein in the mutant (Figure 7D). The stimulated mutant cells clearly acquired EDTA-stable adhesiveness during early development, although the cell agglutinates formed in the presence of EDTA remained smaller than with wild-type cells. We conclude that type 2 carbohydrate is not essential for the EDTA-stable cell adhesion of aggregation competent cells, and suppose that the weak EDTA-stable adhesion of mutant cells reflects the small amount of 68-kd glycoprotein present on their surfaces.

Degradation of the 53-kd protein produced in tunicamycin-treated mutant cells

As previously shown, a 53-kd protein that is produced in tunicamycin-treated wild-type cells and lacks both type 1 and 2

Fig. 7. EDTA-stable cell adhesion in mutant HG220 cells stimulated by cyclic AMP. (**A**−**C**) Agglutination of wild-type and mutant cells in the absence of EDTA (−) or in the presence of 10 mM EDTA (+). The cells were either harvested at the end of growth (**A**), at 6 h of starvation without stimulation by cyclic AMP (**B**), or at 6 h with stimulation during the starvation period by pulses of 20 nM cyclic AMP applied every 6 min (**C**). (**D**) Immunoblot of cells from the same cultures labelled with mAb 294. Total homogenate of 1 × 10^6 cells was applied per lane. N, non-stimulated cells; P, cells stimulated by pulses of cyclic AMP.

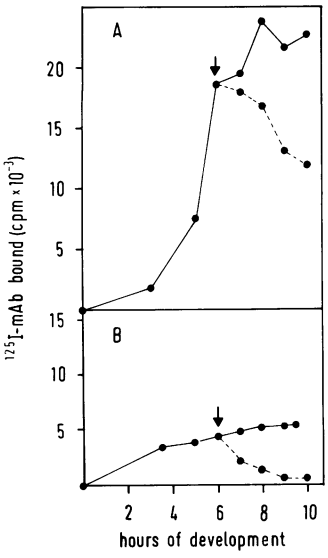

Fig. 8. The 53-kd csA protein of tunicamycin-treated and pretreated cells accumulates in wild type (**A**) to a much higher extent than in mutant HG220 where its disappearance from the cells is accelerated (**B**). To suppress type 1 glycosylation in wild type and mutant, cells were incubated with tunicamycin during starvation. To suppress also type 2 glycosylation in the wild type, its cells were pretreated with tunicamycin during growth. Cycloheximide was added to one aliquot of each cell suspension at 6 h of starvation (arrows) and shaking was continued (dashed lines). The other aliquot received no addition (solid lines). Quantities of cycloheximide and immunoblotting with mAb 294 were as described in **Figure 2.**

carbohydrate accumulates within the cells, while a 66-kd glycoprotein that is produced under the same conditions and lacks only type 1 carbohydrate accumulates on the cell surface (Hohmann *et al.*, 1987). These results combined with those of the present study might suggest that either type 1 or 2 glycosylation is necessary and sufficient for directing the csA protein to the cell surface. If this were true the 53-kd protein produced in tunicamycin-treated HG220 cells should also accumulate within the cells. This 53-kd protein of the mutant is indistinguishable by two-dimensional electrophoresis from the wild-type one (Bertholdt *et al.*, 1985). Figure 8 shows not only that accumulation of the 53-kd protein was drastically reduced in mutant HG220, but that it rapidly disappeared in cycloheximide-treated cells of the mutant. Similar results were obtained with HG701, the other mutant defective in type 2 glycosylation. The half-life of the cellular 53-kd protein was ~1 h in both mutants as compared with >4 h in the wild type.

Figure 9A shows accumulation in the extracellular medium of a pair of degradation products that were produced by tunicamycin-treated HG220 cells and recognized by mAb 294. No traces of these 47- and 45-kd fragments were detected within the cells. The experiment was repeated with the same result using HG220 cells pretreated with tunicamycin during growth (Figure 9B). In the wild type, tunicamycin inhibits preferentially type 1 glycosylation in tunicamycin-treated cells and both type 1 and 2 glycosylation in pretreated cells (Hohmann *et al.*, 1987). The important point is that the 66-kd glycoprotein carrying type 2 carbohydrate, which is synthesized in the tunicamycin-treated wild-type cells, is transported to the cell surface, while the 53-kd protein is under neither condition detected on the cell surface (Hohmann *et al.*, 1987). Figure 9C shows that, in accord with these results, only minute amounts of the fragments were detected together with some undegraded 53-kd protein in the extracellular medium of tunicamycin-pretreated wild-type cells, and that this was only

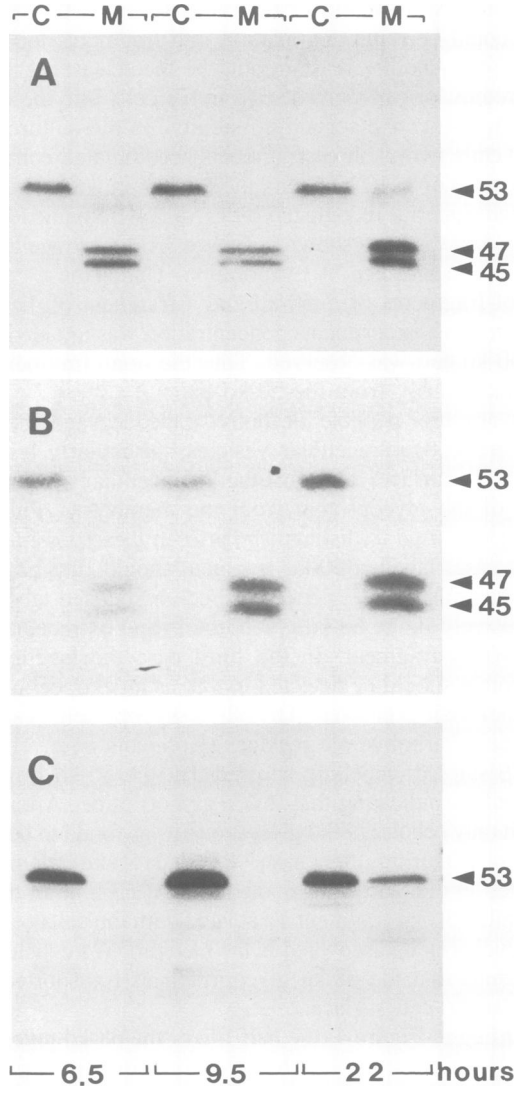

Fig. 9. A pair of fragments of the 53-kd protein accumulates in the extracellular medium of tunicamycin-treated HG220 cells (**panel A**) and also of pretreated HG220 cells (**panel B**), but not in the medium of pretreated wild-type cells (**panel C**). Cells (C on top of the lanes) and extracellular medium (M) were separated after various hours of starvation as indicated at the bottom of the figure, and subjected to SDS–polyacrylamide gel electrophoresis, immunoblotting with mAb 294, and autoradiography. Equivalents of 100 μl cell suspension containing the proteins produced by 1×10^6 cells were applied per lane.

observed after an extremely long starvation period of 22 h. We conclude from these results that in HG220, in contrast to wild type, the 53-kd protein is transported to the cell surface and the fragments are cleaved off thereafter.

Discussion

Type 2 carbohydrate protects the cell-surface-exposed csA protein against degradation

Our primary goal was to elucidate the function of protein-linked carbohydrates in cell-to-cell adhesion. The csA glycoprotein involved in the EDTA-stable cohesion of aggregation competent cells of *D. discoideum* is an excellent subject for such studies since the protein is modified in two steps by different oligosaccharide residues, and glycosylation can be blocked separately for each type of carbohydrate. Tunicamycin treatment of wild-type

cells and also a defect caused by *modB* mutation prevent or reduce EDTA-stable cell adhesiveness (Lam and Siu, 1982; Ochiai *et al.*, 1982; Gerisch *et al.*, 1985b). In the first case predominantly type 1 glycosylation (Yoshida *et al.*, 1984), in the second case specifically type 2 glycosylation is blocked (Bertholdt *et al.*, 1985). The observed deficiencies in cell adhesion do not necessarily mean that the missing carbohydrate is directly involved in cell cohesion. There are at least two other possibilities: (i) the carbohydrate residues are required for transport of the protein to the cell surface, and (ii) they are required to protect the protein against degradation by cell-surface or extracellular proteases.

In *modB* mutants a 68-kd glycoprotein is synthesized as the end product of csA synthesis (Gerisch *et al.*, 1985b). We have shown in this paper that the 68-kd glycoprotein of the HG220 strain is transiently expressed at the cell surface (Figures 5 and 6) and is rapidly degraded such that fragments accumulate in the extracellular medium (Figures 2–4). The largest fragment observed had an apparent mol. wt of 50 kd. It still contained sulphated type 1 carbohydrate residues and had preserved the original N terminus of the csA glycoprotein (J.Stadler and F.Lottspeich, personal communication). The csA protein of HG701, another mutant defective in type 2 glycosylation, was also sensitive to cleavage and fragments of the same size as in HG220 were released into the medium. Thus only a C-terminal fragment containing maximally 18 kd of the polypeptide chain will remain to be associated with the membrane. These results indicate that the major portion of the csA protein is exposed on the cell surface. They are consistent with sequence data showing no strongly hydrophobic region except at the C terminus (Noegel *et al.*, 1986). The csA glycoprotein is probably anchored in the plasma membrane by a phosphatidyl inositol group with its initial C terminus being removed (J.Stadler, personal communication). It might be deduced from the sites of cleavage in the mutants that type 2 carbohydrate residues are linked in the wild type to the polypeptide moiety close to its C-terminal region where they shield the protein against proteases.

Type 2 carbohydrate is not required for EDTA-stable cell adhesion

The short half-life of 1 h of the 68-kd glycoprotein means that the steady state amounts of this glycoprotein present on the cell surface are much smaller than the amounts of the complete 80-kd glycoprotein on the surface of aggregation competent wild-type cells. An estimate based on Figures 1 and 2 indicates that the total amount of the 68-kd glycoprotein in cells of the *modB* strain HG220 corresponded to 10–12% of the amount of 80-kd glycoprotein in the wild type. About 81% of the total 80-kd glycoprotein was exposed on the surface of AX2 wild-type cells (Hohmann *et al.*, 1987), the remaining 19% were apparently on the way from the site of type 2 glycosylation in the Golgi apparatus to the plasma membrane. In HG220 ~15% of the total 68-kd glycoprotein was exposed on the cell surface. The remaining 85% were on the way from the ER to the plasma membrane. Together these results suggest a ratio of 2 to 100 for cell-surface exposure of the 68-kd glycoprotein compared with that of the 80-kd glycoprotein. These data apply to mutant and wild-type cells that have been starved for 6 h.

It is important that in our experiments HG220 cells were stimulated by cAMP pulses during starvation. Wild-type AX2 cells need not be stimulated because they produce their own pulses. This is in contrast to AX3 cells which need to be stimulated in order to fully express the csA glycoprotein (Gerisch *et al.*, 1985b). The *modB*$^-$ mutant strain HL220 is a derivative of AX3 (Murray *et al.*, 1984). In HL220 cells cAMP pulses have been shown to strongly stimulate expression of the 68-kd glycoprotein beyond the marginal levels that are observed without stimulation in starved cell suspensions or in agar plate cultures (Gerisch *et al.*, 1985b). The same was observed for HG220, the HL220-derived *modB* mutant strain used in the present paper (Figure 7D).

The elevated level of expression produced by cAMP stimuli proved to be sufficient for demonstrating a function of the 68-kd glycoprotein in cell adhesion. In a previous paper weak EDTA-stable cell adhesion has been reported in cAMP-stimulated HL220 cells (Gerisch *et al.*, 1985b). In the HG220 strain EDTA-stable cell adhesion was stronger, although still weaker than in wild type and it was acquired, as is typical, between growth and the 6-h stage of development (Figure 7A–C). We conclude from these results that the EDTA-stable adhesiveness of *modB* mutant cells reflects the steady-state amounts of the 68-kd glycoprotein on their surfaces, which means that the lack of type 2 carbohydrate does not abolish the csA-mediated cell adhesion.

Our conclusion differs from that of previous reports in which a direct involvement of type 2 carbohydrate in cell adhesion was suggested (Loomis *et al.*, 1985; Springer and Barondes, 1985; Yoshida, 1987). One argument was that no significant EDTA-stable cell adhesion could be detected in cells of *modB* mutants harvested from agar plate cultures (Loomis *et al.*, 1985). We would prefer to explain this result by the extremely low abundance of cell-surface-exposed 68-kd glycoprotein, the only csA component present in these cells. Another argument was the blockage of EDTA-stable cell adhesion in the wild type by antibodies that are specific for type 2 carbohydrate (Springer and Barondes, 1985; Yoshida, 1987). On the basis of size relationships it appears questionable whether antibodies are reliable probes for functional epitopes on the csA molecule. The external portion of this molecule has a size of ~50 kd. For blockage of cell adhesion either complexes of IgG and Fab with sizes of 200 kd or more (Springer and Barondes, 1980), or Fab fragments with a size of 50 kd have been used (Yoshida, 1987). Thus, in no case was the probe small enough to selectively cover a functionally important site on the target molecule.

The modB mutants studied are proposed to be defective in the selectivity of transport

In tunicamycin-treated wild-type cells a 66-kd glycoprotein that carries type 2 carbohydrate is transported to the cell surface, but not a 53-kd protein that lacks both type 1 and 2 carbohydrate (Hohmann *et al.*, 1987). These results were interpreted to mean that, in the absence of type 1 carbohydrate, targeting of the csA protein to the cell surface requires the presence of type 2 carbohydrate. This is in contrast to results obtained with the *modB* mutant. The 53-kd protein that is produced in tunicamycin-treated HG220 cells is apparently transported to the cell surface and then cleaved (Figure 9). The mutant 53-kd protein is indistinguishable by 2D-electrophoresis from the wild-type one (Bertholdt *et al.*, 1985). Therefore, transport in the mutant appears to be relieved from a restriction that in the wild type causes only the glycosylated protein to be targeted to the cell surface. In wild-type cells this mechanism appears to be responsible for preventing those proteins which are normally modified by type 2 carbohydrate from being passed to the cell surface before that modification has occurred.

It is unlikely that the alteration in transport is due to a second mutation in the cells that carry the *modB* mutation. Parasexual

recombination employed to generate the HG220 strain did not separate the two defects (E.Wallraff, unpublished). In addition, a second mutant defective in type 2 glycosylation, HG701, showed the same rapid loss of the 53-kd protein as was observed in HG220. If the *modB* mutation directly affected type 2 glycosylation, transport of a protein that does not carry type 2 carbohydrate should be the same in wild type and mutant. However, the 53-kd protein is transported differently. It is therefore possible that the alteration of transport is the primary defect in the mutants studied, and that the defect in glycosylation is secondary. Two basic assumptions would accommodate our results: (i) the csA protein is transported with the bulk membrane flow to the cell surface if no specific retardation occurs (Wieland *et al.*, 1987) and (ii) the protein is normally retarded until type 2 glycosylation allows it to pass. *modB* mutants are thought to be defective in the retardation mechanism. Two versions of this hypothesis are conceivable. (i) The mechanism responsible for retardation is also responsible for guiding the protein into the Golgi compartment where type 2 glycosylation occurs. If only the glycosylation allows transport, the 53-kd protein should not be targeted to the cell surface but accumulate within the cells in mutants which lack type 2 glycosylation because of a defective glycosyltransferase. (ii) The glycosyltransferases themselves are responsible for retardation of their substrate proteins. An acceptor protein may remain bound to a glycosyltransferase until it becomes glycosylated. In this case, mutants defective in the acceptor-binding site of a glycosyltransferase or in the entire enzyme would transport proteins to the cell surface that in the wild type are only transported after type 2 glycosylation has occurred.

If the results obtained with the csA glycoprotein are applicable to other type 2 glycosylated proteins, one would expect that their transport to the cell surface is also due to the relief from a restriction in the transport system of *modB* mutants. The transport of three glycoproteins other than csA has been studied in *modB* mutants (West and Loomis, 1985). These proteins were found to be cell-surface exposed in the mutants as they are in the wild type. In the light of our results it appears questionable whether *modB* mutants reflect the normal requirement for a protein to be transported. We believe that mutants defective in type 2 glycosylation, the first of which were the *modB* mutants isolated by Murray *et al.* (1984), are valuable tools for investigating defects in the coupling of transport to post-translational glycosylation.

Materials and methods

Cell culture, treatment of cells with tunicamycin, and measurement of cell adhesion

Cells of the *D. discoideum* strain AX2-214, here referred to as wild type, and of the mutant strain HG220 were cultivated in liquid nutrient medium (Malchow *et al.*, 1972) and harvested at a density of not more than 5×10^6 cells/ml. Washed cells were starved in 17 mM Soerensen phosphate buffer, pH 6.0, at a density of 1×10^7 cells/ml and shaken on a gyratory shaker at 150 r.p.m. (Malchow *et al.*, 1972). Mutant HG701 was cultivated with *Klebsiella aerogenes* on SM agar (Sussman, 1966) and starved as the other cells. Aliquots of 0.4 μg/ml of tunicamycin (Boehringer, Mannheim) were added to cell suspensions either at 1 h of starvation or at 4 h before the end of growth in liquid medium. In the latter case tunicamycin was resupplied after washing of the cells and remained present during the entire period of starvation. Cells supplemented with tunicamycin at 1 h of starvation are designated as 'TM-treated cells', cells to which tunicamycin was added during growth as 'TM-pretreated cells'. If not stated otherwise, development of HG220 cells and of tunicamycin-treated and pretreated wild-type cells was stimulated by pulses of 2×10^{-8} M cyclic AMP applied every 6 min during the entire period of starvation (Ochiai *et al.*, 1982).

Cell adhesion was determined using an agglutinometer as described by Bozzaro *et al.* (1987). Cells were exposed for 1 h to constant shear in 17 mM Soerensen phosphate buffer, pH 6.0, with or without EDTA, by operating the agglutinometer at 40 r.p.m.

Construction of the HG220 strain and selection of the HG701 mutant

The haploid HG220 strain was selected from a diploid (Williams and Newell, 1976; Newell *et al.*, 1977) obtained by parasexual combination of strain HL220 (Murray *et al.*, 1984) and HU1766. HU1766 is a haploid descendant of DU2413 (Wallraff *et al.*, 1984). HG220 carries the *modB502* mutation on linkage group VI, the mutations responsible for axenic growth on linkage groups II and III, and in addition *acrA* on linkage group II and *bwnA* on linkage group IV. HG701 was selected from HG592 (Gerisch *et al.*, 1985a) by the use of mAb 12-120-94 (Ochiai *et al.*, 1982) and of a FACS IV cell sorter essentially as described by Francis *et al.* (1985).

Pulse-chase labelling of mutant HG220 cells with [^{35}S]sulphate

At 5 h of starvation 1 mCi/ml of [^{35}S]sulphate was added to mutant HG220 cells. After 5 min of labelling, the cells were centrifuged for 40 s at 2000 *g*, resuspended at a density of 1×10^7 cells/ml and incubated with 500 μM of unlabelled sulphate either in fresh phosphate buffer or in buffer in which HG220 cells had been starved for 5 h. After various periods of chase samples were centrifuged and cells and supernatant quickly frozen. To make aliquots of cells and supernatant comparable, the pellet was brought to the original volume of the cell suspension and extracted wtih octyloligooxyethylene (Rosenbusch *et al.*, 1982) purchased from Bachem (4416 Bubendorf, Switzerland) as described (Hohmann *et al.*, 1987). CsA forms that were present in the cells or had been released into the extracellular fluid were precipitated with 5 μg IgG/ml of mAb 71 and protein A–Sepharose (Hohmann *et al.*, 1987).

Immunofluorescence studies and cell-surface immunolabelling

Living cells were labelled with monoclonal antibody and FITC-conjugated sheep anti-mouse IgG as described by Bozzaro and Merkl (1985). Binding of antibody to the surfaces of living cells followed by extraction of antigen–antibody complexes with octyloligooxyethylene and immunoprecipitation with protein A–Sepharose was performed as reported by Hohmann *et al.* (1987). Monoclonal antibodies 41-71-21 and 40-178-3 (Bertholdt *et al.*, 1985) are referred to as mAb 71 and mAb 178 respectively.

Gel electrophoresis, fluorography and immunoblotting

Particulate fractions of the cells were obtained by freezing and thawing and centrifugation for 10 min in an Eppendorf centrifuge. SDS–polyacrylamide gel electrophoresis, transfer of proteins to nitrocellulose and incubation of blots with ^{125}I-labelled monoclonal IgG were done as described (Hohmann *et al.*, 1987). Monoclonal antibody 33-294-17 (Bertholdt *et al.*, 1985) is referred to as mAb 294.

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