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Constitutive overexpression of the contact site A glycoprotein enables growth-phase cells of *Dictyostelium discoideum* to aggregate

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The contact site A (csA) glycoprotein is a developmentally regulated cell adhesion molecule which mediates EDTA-stable cell contacts during the aggregation stage of *Dictyostelium discoideum*. A transformation vector was constructed which allows overexpression of the csA protein during the growth phase. In that stage the csA protein is normally not expressed; in the transformants it was transported to the cell surface and carried all modifications investigated, including a phospholipid anchor and two types of oligosaccharide chain. csA expression enabled the normal non-aggregative growth-phase cells to form EDTA-stable contacts in suspension and to assemble into three-dimensional aggregates when moving on a substratum. After prolonged cultivation of csA overexpressing transformants in nutrient medium the developmental program was found to be turned on, as it normally occurs only in starving cells. During later development of transformed cells, the csA glycoprotein remained present on the cell surface, while it is down-regulated in the wild type. It was detected in both the prestalk and prespore regions of the multicellular slugs made from transformed cells.

Key words: cell adhesion/contact site A glycoprotein/*Dictyostelium*/phospholipid anchor

Introduction

Single cells of *Dictyostelium discoideum* aggregate into a multicellular organism by chemotactic attraction mediated by the release and recognition of cyclic AMP (cAMP), and by cell-to-cell adhesion. By these mechanisms they assemble into aggregation centers that are surrounded by streams of moving cells. Adhesion of aggregating cells is brought about by the combined activities of several adhesion systems. The best characterized adhesion protein of *D.discoideum* is the contact site A (csA) glycoprotein (Gerisch, 1986; Siu *et al.*, 1988). This protein is characteristic of aggregating cells; transcription of the csA gene is induced during the preaggregation phase, as shown by run-off experiments (A.Müller-Taubenberger, personal communication), and one factor controlling expression of the protein is cAMP. The csA protein is modified by a phospholipid anchor at its C-terminus and by two types of oligosaccharide residues which have different functions (Stadler *et al.*, 1989). N-linked, cotranslationally added type 1 carbohydrate residues are required for the activity of the protein in cell adhesion (Hohmann *et al.*, 1987a). Type 2 carbohydrate residues, which are post-translationally added during passage through the Golgi apparatus, are important for protecting the C-terminal half of the protein against a cell-surface located protease (Hohmann *et al.*, 1987b).

The csA glycoprotein mediates an EDTA-stable ('Ca independent') type of cell adhesion. Selective elimination of the csA protein by mutagenesis with nitrosoguanidine (Noegel *et al.*, 1985) or by targeted disruption of the csA gene (Harloff *et al.*, 1989) results in a dramatic reduction in EDTA-stable cell adhesiveness, as measured in a shaken cell suspension, but still enables the cells to aggregate on an agar surface where no shear is applied. Thus, the csA glycoprotein is definitely a cell adhesion molecule but it is dispensable for morphogenesis; in its absence multicellular organization can be established by another adhesion system. In order to determine the role of the csA glycoprotein separately from any other developmentally regulated adhesion system, but in the context of the normal molecular environment of the cell surface, we expressed the protein in growth-phase cells by transforming *D.discoideum* with a vector that contains the csA coding sequence under the influence of a strong, constitutive promoter. Growth-phase cells form only EDTA-sensitive contacts which are insufficient to initiate morphogenesis. We show in this paper that all biosynthetic pathways required for modifying the csA protein are working in undeveloped cells, that in the transformants a stable glycoprotein is transported to the cell surface, and that expression of this single protein is sufficient for the cells to aggregate and to initiate morphogenesis.

Results

Isolation of csA transformants

D.discoideum cells were transformed with the pDCEV vector shown in Figure 1. In this vector the complete coding region for the csA protein is transcribed under the control of the actin 15 promoter. This promoter is active in growth-phase cells where the normal csA gene is not transcribed. For the selection of transformants, the vector contains the phosphotransferase coding unit of Tn*903* providing G418 resistance under the control of a second actin 15 promoter. Since the vector contains no eukaryotic replication origin, stable transformants are only obtained if the vector integrates into the genome.

G418 resistant transformants were cloned and colonies blotted onto nitrocellulose filters for labeling with an antibody recognizing the protein moiety of the csA glycoprotein. CsA overexpressing transformants were recognized by their strong labeling. Production of the csA protein during growth is indicated by labeling of the entire colonies including their boundaries where the cells are still in contact with bacteria (Figure 2). In normal colonies only the aggregates were labeled, and the intensity of their label was much lower than in some of the transformants.

Four of the strongly csA overexpressing transformants were chosen for determining the sizes of csA gene products in immunoblots and for Southern blot analysis. In all of them

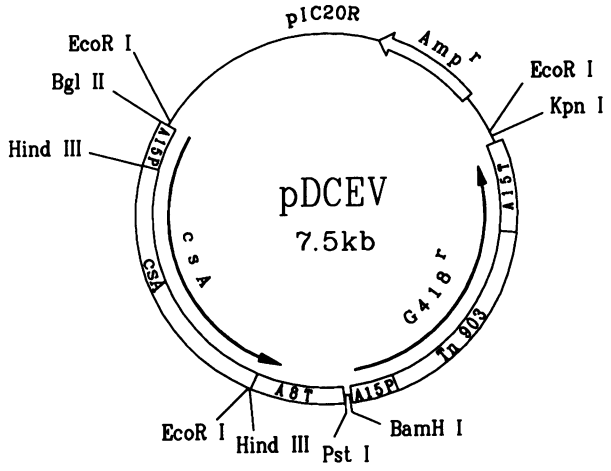

Fig. 1. Contact site A expression vector pDCEV. This shuttle vector is based on the cloning vector pIC20R which includes the β-lactamase gene conferring ampicillin resistance to *Escherichia coli*. For expression in *D.discoideum*, the vector contains two transcription units driven by actin 15 promoters. The Tn*903* phosphotransferase sequence, allowing the selection of transformants with G 418, is terminated by an actin 15 terminator. The complete csA coding unit (Noegel *et al.*, 1986) is terminated by two actin 8 terminators in tandem (Witke *et al.*, 1987).

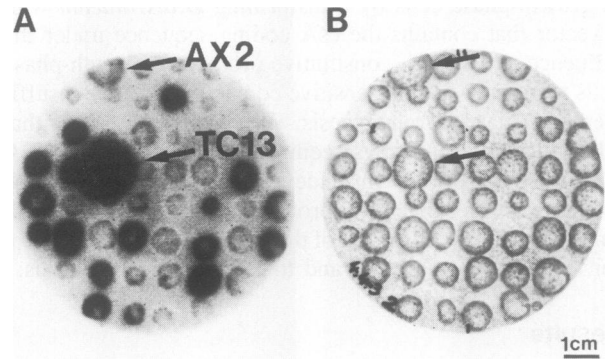

Fig. 2. Blotted colonies of *D.discoideum* AX2 cells transformed with the pDCEV vector. (**A**) Blot labeled with [^{125}I]mAb 294, an antibody specific for the polypeptide moiety of the csA glycoprotein (Bertholdt *et al.*, 1985). (**B**) The same blot stained for total cellular proteins with Ponceau S. The arrows point to TC13, a strongly csA-overexpressing transformant investigated in this paper, and to an untransformed AX2 colony.

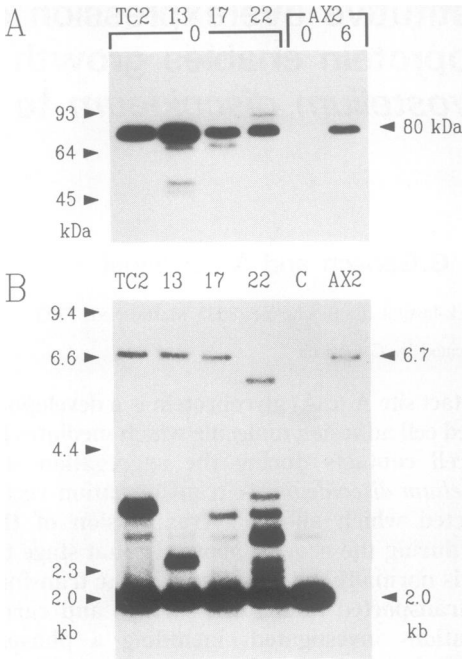

Fig. 3. Immunoblot (A) and Southern blot (B) from four different clones of pDCEV-transformed cells and from the untransformed AX2 parent strain. (**A**) Blot of total cellular proteins separated by SDS−PAGE and labeled for csA using mAb 294. All transformants expressed the csA glycoprotein already during growth (0 h of development), as shown by labeling of a band at the 80 kd position of the csA molecule. In AX2, csA expression was only detected in developing cells. The blot representing total proteins of 1×10^6 cells per lane shows a higher amount of csA in growth-phase cells of the transformants than expressed in the aggregation competent 6 h cells of AX2. (**B**) *Eco*RI digested genomic DNA labeled by the cDNA c512 probe representing the entire csA coding region (Noegel *et al.*, 1986). All four transformants showed strong labeling of the 2.0 kb *Eco*RI band of the vector insert (Figure 1), indicating multiple copies of the vector to be integrated into the genome. Transformants TC2, 13 and 17 showed also labeling of the genomic 6.7 kb *Eco*RI fragment comprising the endogenous csA gene.

a csA molecule of normal size was expressed in growth-phase cells, where the promoter of the endogenous csA gene is inactive (Figure 3A). In addition, a 68 kd protein was clearly detectable in two of the transformants, which corresponds to a normal incompletely glycosylated csA precursor (Hohmann *et al.*, 1987a). In transformant TC13 another labeled protein of ~50 kd was found, which might be identical to a 53 kd product of tunicamycin treated cells (Bertholdt *et al.*, 1985). In these cells the cotranslational addition of N-linked carbohydrate is blocked and subsequent glycosylation in the Golgi compartment is incomplete (Hohmann *et al.*, 1987a). Since TC13 is the most strongly csA overexpressing transformant of the entire set, the rate of csA protein synthesis may exceed in this transformant the capacity of the glycosylation systems. Another atypical band is seen in TC22, representing a 90 kd protein synthesized in addition to the normal 80 kd csA molecule. This protein might be the result of an irregular recombination event which has produced a partial duplication in the csA coding sequence or a fusion with another protein.

Characterization of transformants at the DNA level

*Eco*RI does not cleave in the csA coding region. Therefore, in Southern blots of *Eco*RI digested genomic DNA of AX2 wild-type cells a single fragment of 6.7 kb was labeled by a csA cDNA probe. Since the insert of the pDCEV vector, comprising the csA coding region and the actin 15 promoter, is flanked by *Eco*RI restriction sites, a single fragment of 2.0 kb was obtained when the isolated vector was cleaved (Figure 3B, lanes C and AX2). In three of the transformants analyzed, the endogenous 6.7 kb fragment was seen, indicating that the vector had not integrated by homologous recombination into the csA gene. In the fourth transformant, TC22, the 6.7 kb fragment was missing and a 5.8 kb one was detected, which shows that in this case the vector was inserted into the csA gene (Figure 3B). In all four transformants the 2.0 kb insert of the vector was very strongly labeled, suggesting a copy number of ~10 per haploid genome. This shows that pDCEV, like similar transformation vectors carrying other inserts (Nellen *et al.*, 1984; Nellen and Firtel, 1985; Witke *et al.*, 1987), exists as a

multicopy vector which is integrated into the genome, most likely in the form of tandem repeats.

Different patterns of additional *Eco*RI fragments carrying csA sequences were found in the transformants (Figure 3B). Some of these fragments might be the outcome of integration of the vector into the genome through its actin 15 promoter sequence, which would eliminate the 5′ flanking *Eco*RI site of the vector. Other fragments, like those observed in TC2 and TC22, must be the result of rearrangements within the vector sequences. Strong labeling of some of the fragments indicates that these events were followed by extensive amplification.

Expression of the csA protein in all developmental stages and its transport to the cell surface

Northern blots of TC13 RNA showed the presence of csA transcripts at high abundancy during growth phase and starvation (Figure 4A). These amounts of transcripts clearly exceeded those of csA mRNA in the full aggregation-competent wild-type cells harvested after 6 or 9 h of starvation. Growth-phase cells of the wild type contained no detectable csA transcript, which shows that the csA RNA in the transformant was entirely transcribed from vector sequences.

Two other developmentally regulated transcripts were monitored. Under the conditions used, these transcripts were undetectable in growing cells of the transformant as they were in growing wild-type cells (Figure 4A). One of these transcripts, the P29F8 gene product, is known to be strictly controlled by cAMP signals similar to the csA transcript (Noegel *et al.*, 1985). The second transcript is the product of the A11H2 gene (Gerisch *et al.*, 1985; Noegel *et al.*, 1985) which seems to encode an α-L-fucosidase (ALF) (Müller-Taubenberger *et al.*, 1989; May *et al.*, 1989). Both the P29F8 and A11H2 transcripts were expressed in starving cells of the TC13 transformant with a time course similar to that of the AX2 wild type (Figure 4A). Transcripts of the α-actinin gene were labeled in a control to show comparable amounts of these constitutively expressed transcripts to be present in wild type and transformant at all stages examined (Witke *et al.*, 1986). It should be emphasized that the results reported here were obtained with cells harvested at an early stage of culture in nutrient medium. As outlined in the last Results section, prolonged culture of transformants up to high cell densities may result in the induction of developmentally regulated proteins even in the presence of nutrient medium.

Immunoblots of total cellular proteins labeled with anti-csA antibody indicate that the transcripts encoded by the vector were efficiently translated (Figure 4B). Not only at the growth phase but also during starvation, the csA protein was more abundant in the transformants than it was in the wild type at the fully aggregation-competent stage.

The csA expression pattern was reflected in fluorescent antibody labeling of living cells (Figure 5). Since in intact cells only cell-surface located csA is accessible to antibody, the strong labeling shows that already during the growth-phase stage the csA protein overexpressed in transformant cells is efficiently transported to the plasma membrane. The csA protein was still present at the multicellular slug stage. Slugs of the transformant were entirely labeled, both in the prestalk area at the tip and the prespore area of the posterior region (Figure 6). In the cells of the slugs, the antibody label

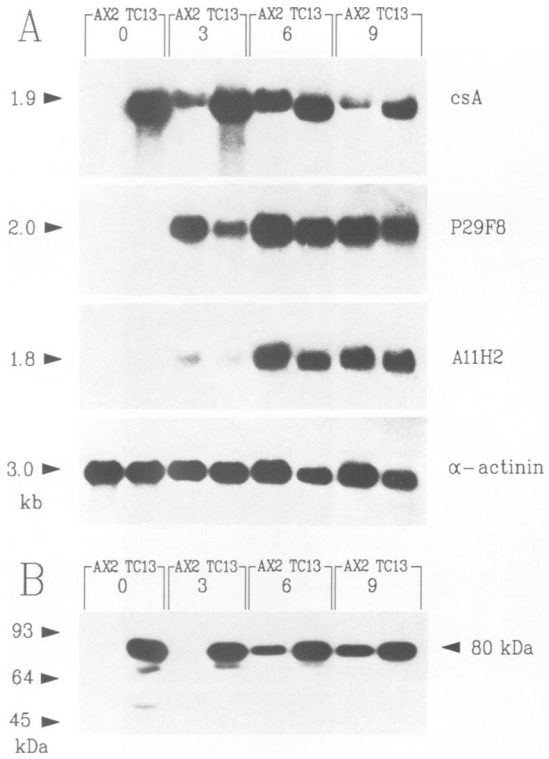

Fig. 4. Comparison of csA transcript and protein expression in transformant TC13 and the parent AX2 strain. Northern and immunoblots of total RNA or of proteins from growth-phase cells (0 h) or developing cells (3, 6, 9 h of starvation) are shown. (**A**) Northern blots probed for csA transcripts with cDNA c512. In wild-type AX2 the csA transcripts showed the typical developmental regulation, in contrast to the transformant where they were strongly expressed at all stages. After removal of the csA probe, the same blot was reprobed with P29F8 cDNA and subsequently with A11H2 cDNA. A parallel blot was probed for α-actinin transcripts. (**B**) Immunoblot from the same experiment labeled with mAb 294.

was concentrated at the plasma membrane (Figure 6D). Since these cells had been permeabilized by methanol fixation, also intracellular accumulation of the csA protein would have been detected. In accord with previous reports on down-regulation of the csA glycoprotein after the aggregation stage (Noegel *et al.*, 1986), slugs of the wild type were almost unlabeled. Only at the very rear end of some slugs a small number of labeled cells, dispersed between unlabeled ones, was found. The presence of csA in these cells may be the result of local re-expression of the protein at late stages of development [Browne *et al.*, 1989; the glycoprotein named antigen 117 in this paper appears to be csA as indicated in a note added in proof in a later report (Da Silva and Klein, 1989)].

Phospholipid and carbohydrate modifications of the csA protein in growth phase cells

Certain biosynthetic systems responsible for the modification of *D.discoideum* proteins depend on the stage of development, as has been reported for the system responsible for trimming of N-linked glycans and the addition of L-fucose to peripheral mannose (Ivatt *et al.*, 1984). Therefore, we have investigated the decoration of the csA protein with three modifications: the phospholipid anchor, N-linked and post-translationally added carbohydrate residues. The phospholipid anchor can be identified by

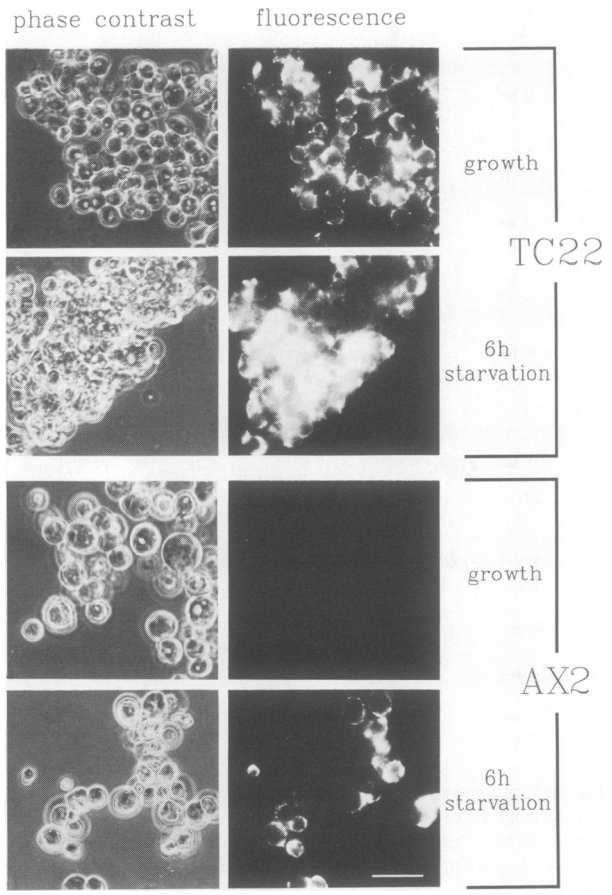

Fig. 5. Fluorescent antibody labeling of TC22 and AX2 cells harvested during growth in suspension and after 6 h of starvation. Living cells were incubated with mAb 71 recognizing the native csA protein, followed by FITC-labeled anti-mouse IgG. The bar indicates 20 μm.

labeling of the csA protein with [^{3}H]palmitic acid (Stadler *et al.*, 1989). Incorporation of palmitic acid into the csA glycoprotein was assayed under two conditions where the endogenous csA gene was not expressed: (i) incubation of growth-phase cells for 2 h in starvation buffer immediately after the removal of nutrient medium, (ii) during 16 h of growth within nutrient medium. As shown in Figure 7, the palmitic acid label was detected at the 80 kd position of the csA molecule under both conditions. Association of the label with the csA glycoprotein was confirmed by two-dimensional gel electrophoresis (data not shown).

For detection of N-linked type 1 carbohydrate residues, mAb 353 was employed. This antibody recognizes specifically type 1 carbohydrate in its fucosylated state (G.Gerisch, unpublished results). In Figure 8 labeling of total cellular proteins from growth-phase (0 h) cells of transformant TC13 and from aggregation-competent (6 h) wild-type AX2 cells is compared. The anti-polypeptide antibody mAB 294 used as a reference labeled csA in the blots with high specificity. MAb 353 resembled this antibody both in the specificity for csA and in the relative intensity of labeling the glycoprotein from the 6 h wild type and the 0 h transformant cells. This result indicates that the fucose-containing carbohydrate epitope recognized by mAb 353 is a modification highly specific for the csA molecule, and that the csA molecules expressed in growth phase cells are already endowed with this modification.

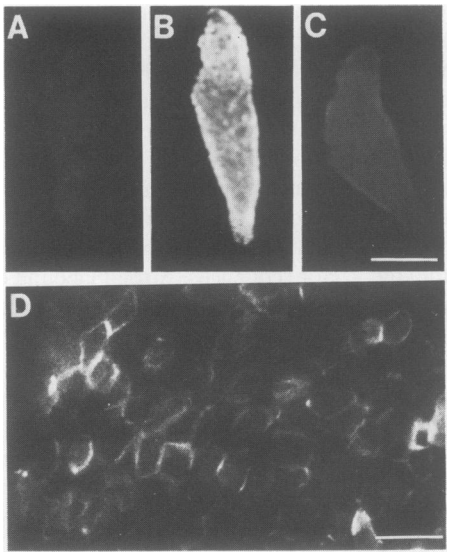

Fig. 6. Persistence of csA in slugs produced by transformed cells. Squeezed slugs were fixed in cold methanol and labeled for A, B and D with mAb 71 and FITC-conjugated anti-mouse IgG. For the control in C, only the fluorescent second antibody was applied. (**A**) Slug of wild-type AX2; (**B** and **C**) slugs of transformant TC13;. (**D**) TC13 slug cells showing cell-surface labeling. Bar for A–C indicates 250 μm, bar for D 10 μm.

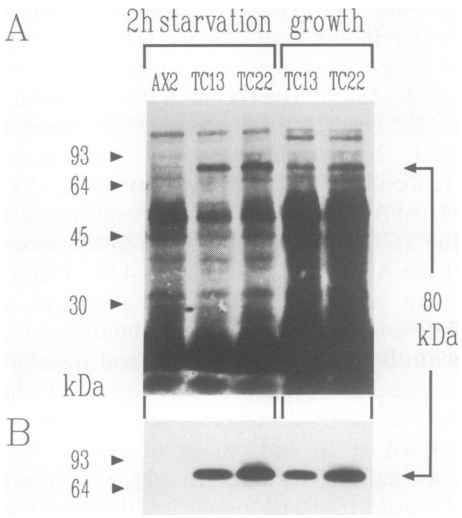

Fig. 7. *In vivo* incorporation of palmitic acid into the csA glycoprotein. (**A**) Cells of AX2 and of two transformants were incubated with [^{3}H]palmitic acid either during the first 2 h of starvation or during growth in nutrient medium. Particulate fractions were subjected to SDS–PAGE, blotted and fluorographed. (**B**) The same blot as in A labeled with csA-specific mAb 294.

The third modification, post-translationally added type 2 carbohydrate, is detected by mAb 210. This antibody recognizes an epitope common to type 2 carbohydrate residues of csA and of the P29F8 gene product which, similar to the csA protein, is not expressed in growth-phase cells (Noegel *et al.*, 1985). The P29F8 encoded protein is a plasma membrane component with a trans-membrane domain close to its C-terminus, which in its glycosylated state has an apparent molecular mass of 95 kd (Müller-Taubenberger, 1989). Both the csA and the P29F8 glycoproteins were labeled in aggregation-competent wild-type cells

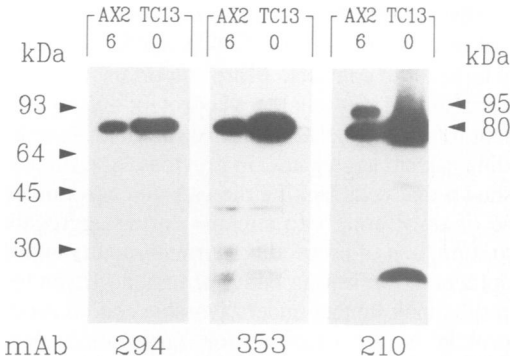

Fig. 8. Modifications of the csA protein by N-linked type 1 and post-translationally added type 2 oligosaccharide residues during growth of transformant TC13. Total proteins from TC13 cells were harvested from suspension culture in nutrient medium, and proteins from 1×10^6 cells/lane were subjected to SDS–PAGE and immunoblotted with the antibodies indicated. Proteins from wild-type AX2 cells harvested at 6 h of starvation were treated in parallel.

(Figure 8). The csA glycoprotein from growth-phase cells of the transformant was also strongly labeled, indicating its normal modification by type 2 carbohydrate. The P29F8 glycoprotein was not detectable in these cells as predicted from the Northern blot analysis showing its developmental regulation (Figure 4A). In addition to the csA band, a band in the 25 kd position was labeled with mAb 210 in the transformant. This band presumably represents a csA degradation product not detected in wild-type cells. This product was neither recognized by mAb 294 nor 353. The epitope recognized by mAb 294 is located in a 50 kd N-terminal fragment of the csA molecule, in which also the N-linked carbohydrate residues recognized by mAb 353 are found (Hohmann *et al.*, 1987b). Therefore, the 25 kd glycopeptide not labeled by these antibodies should be a C-terminal fragment of the csA glycoprotein that is modified by type 2 carbohydrate.

EDTA-stable cell adhesion and aggregation of csA-expressing growth-phase cells

Growth-phase cells of *D.discoideum* are known to form only EDTA-sensitive cell contacts (Beug *et al.*, 1973). In contrast to these normal cells, the csA-expressing growth-phase cells of the transformants adhered strongly to each other in the presence of EDTA, as shown by the formation of large cell clusters in shaken suspension (Figure 9). This result demonstrates that adding the csA glycoprotein to the constitutively expressed cell-surface components of growth-phase cells is sufficient to establish EDTA-stable cell adhesiveness.

Aggregation of transformant cells in the presence of nutrient medium was examined with cells attached to polystyrol surfaces. While even at high density wild-type cells formed monolayers only interspersed with small clusters, transformed cells already began at low density to assemble into three-dimensional aggregates (Figure 10). Time-lapse recording showed no obvious chemotactic orientation of cells during this assembly. Cells getting in contact during their movement adhered to each other and formed irregularly shaped aggregates. Cells usually left the aggregates after a while, indicating a dynamic equilibrium of single and aggregated cells. The aggregates often assumed the structure of hollow mounds, like that shown in Figure 10D. From these results it is concluded that expression of the csA glycoprotein alone enables cells attached to a substratum to organize a three-dimensional multicellular structure.

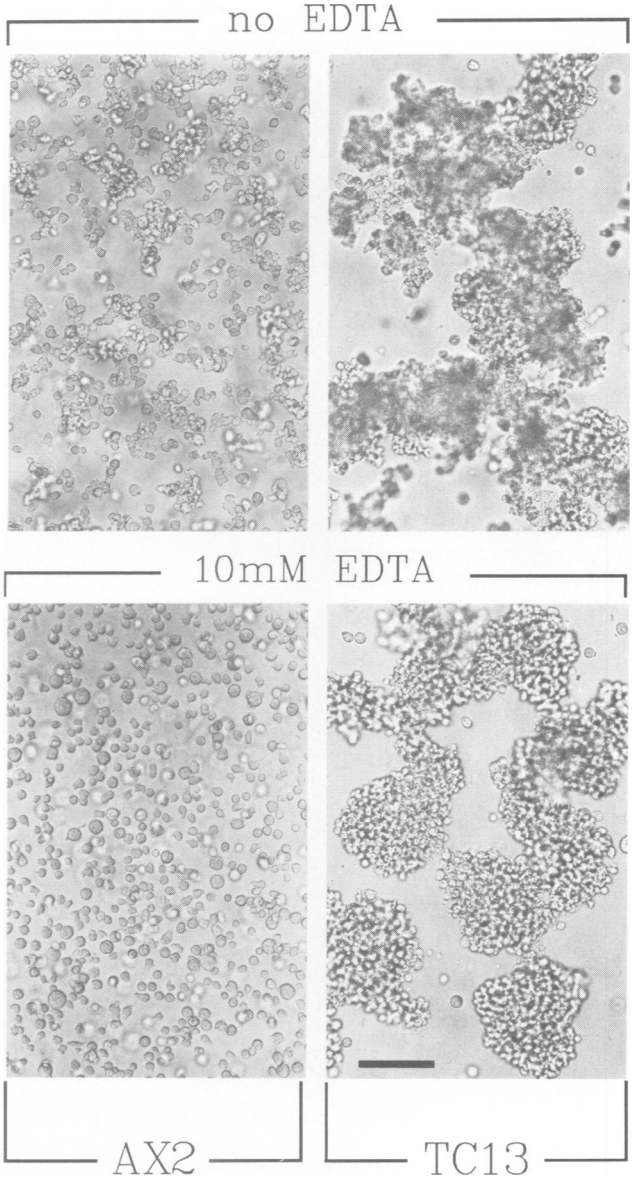

Fig. 9. Photographs showing EDTA-resistant cohesion of suspended TC13 cells. Wild-type and transformant cells grown in plastic flasks were assayed in an agglutinometer immediately after removal of nutrient medium in the absence or presence of EDTA. To make sure that the developmental program was not initiated in these cells, absence of the P29F8 protein, a marker of early development, was verified by immunoblotting (not shown). The bar indicates 100 μm.

When transformant strains were cultivated in nutrient medium up to high cell densities, we often observed that they formed streams of elongated cells as they are typical of wild-type cells that aggregate after a starvation period of 6–8 h in non-nutrient buffer. The transformed cells strongly responded chemotactically within the nutrient medium to cAMP applied through a micropipette, and showed expression of the P29F8 protein known as a strictly regulated marker of development. A detailed analysis is required to clarify how csA-mediated adhesion leads to the induction of developmentally regulated genes and cellular activities.

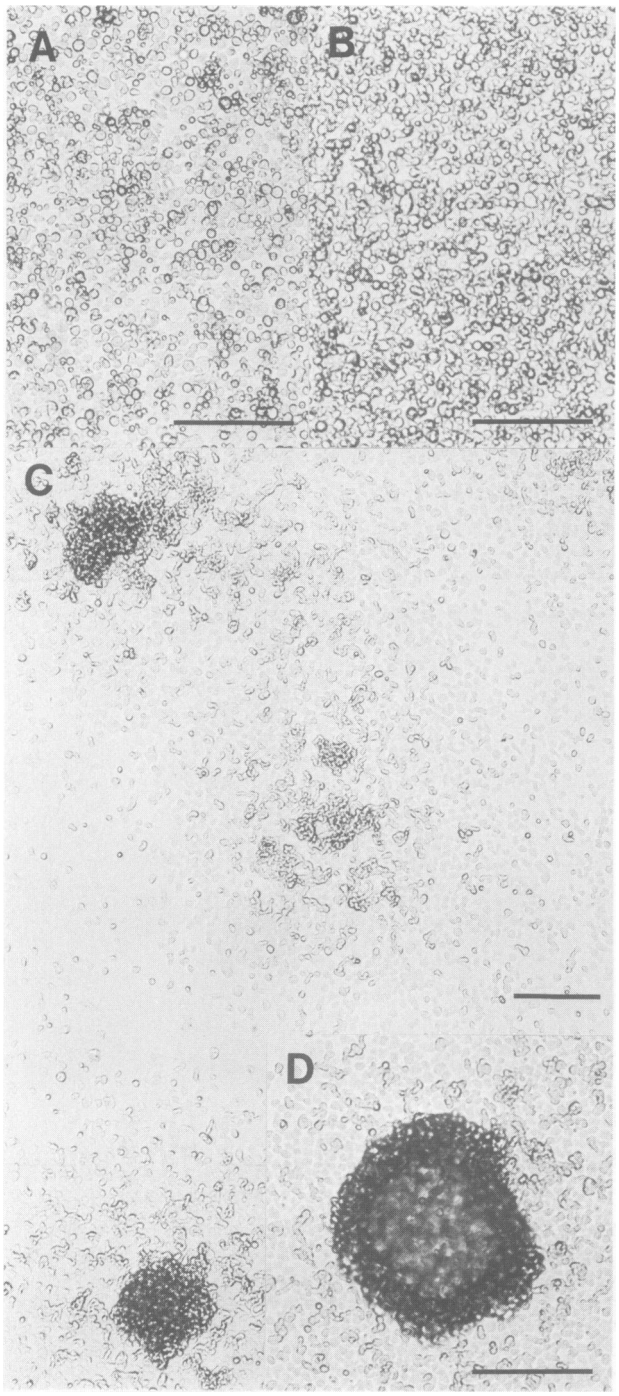

Fig. 10. Behavior of untransformed AX2 cells (**A** and **B**) and transformant TC13 cells (**C** and **D**) attached to plastic surfaces in nutrient medium. An AX2 culture was photographed at high and low cell density, with an interval of 2 days between A and B (again, no aggregation was observed after another 2 days). Cells from a low-density TC13 culture formed aggregates (C) which developed into hollow mounds (D). Bars indicate 150 μm.

Discussion

In this paper we report that expression of the csA glycoprotein enables growth-phase cells of *D.discoideum*, which normally do not synthesize this protein, to agglutinate strongly in suspension by EDTA-stable cell adhesion. This result complements previous reports showing that loss of the csA protein after chemical mutagenesis (Noegel *et al.*, 1985) or gene disruption (Harloff *et al.*, 1989) dramatically reduces the capability of cells to form EDTA-stable ('Ca independent') intercellular contacts. More important is the finding that expression of this single glycoprotein during growth is sufficient for cells attached to a substratum to assemble into three-dimensional aggregates. In previous experiments it has been shown that cells lacking the csA glycoprotein are still capable of associating into streams during aggregation on a substratum, and of proceeding normally with multicellular development up to fruiting body formation. Taken together these results indicate that under favorable conditions the csA glycoprotein is not essential for aggregation; but it is sufficient to convert non-aggregating cells into cells that form three-dimensional assemblies, even when cell-to-substrate adhesion competes with cell-to-cell adhesion.

Expression of the csA protein in growth-phase cells makes it possible to examine biosynthetic pathways in these undeveloped cells that are normally required for protein modification during development. The modifications present on the csA protein synthesized during the growth phase comprise a phospholipid anchor, N-linked type 1 oligosaccharide chains and post-translationally added type 2 carbohydrate residues. The systems responsible for these modifications appear not to be rate-limiting since, except in cases of extreme csA overproduction, only mature, fully modified csA glycoprotein was found to be accumulating. The labeling of csA glycoprotein made in growth-phase cells by mAb 353 is of particular interest because it shows a highly csA-specific modification to occur in these cells. The antibody recognizes a structure on N-linked glycans which contain L-fucose as a component essential for its binding.

The observed EDTA-stable cell adhesion of growth-phase cells as a consequence of csA expression is in accord with previous findings indicating that this type of cell adhesion is mediated by homophilic interaction of csA molecules (Kamboj *et al.*, 1988, 1989), similar to N-CAM mediated cell adhesion of neural cells (Hoffman and Edelman, 1983). Nevertheless, our results do not exclude the possibility that the csA glycoprotein binds also to cell-surface components that are normally present on growth-phase cells. According to Kamboj *et al.* (1989) EDTA-stable cell adhesion is brought about by a fit between the protein moieties of two csA molecules. This would argue against a heterophilic interaction. On the other hand, N-linked carbohydrate residues have been shown to be essential for adhesion (Hohmann *et al.*, 1987a). It is not yet clear whether these oligosaccharide residues are only keeping the protein in an active conformation or represent sites of intermolecular contact. In the latter case, the specificity of carbohydrate structures would determine whether or not other glycoconjugates are recognized by the csA molcule.

Our results are in line with reports showing that in transfected vertebrate cells the expression of single cell-adhesion molecules induces cells to aggregate (John *et al.*, 1990) and to undergo tissue-specific morphogenesis (Dickson *et al.*, 1990). Two observations made on transformed *D.discoideum* cells need to be mentioned in this context. First, the csA glycoprotein appears to favor, in a direct or indirect manner, the expression of other developmentally regulated proteins that are normally suppressed in the presence of nutrients. This point requires identification of steps leading to and factors involved in the pre-early induction. Second, continued presence of the csA glyco-

protein on cell surfaces throughout the multicellular slug stage has no severe consequences for cell differentiation and for patterning into spores and stalk cells. Sorting out of predifferentiated cells is considered to be the basis for the generation of a prespore/prestalk pattern in the slug (MacWilliams *et al.*, 1985), and cell type specific differences in the strength of cell adhesion might play a decisive role in this process (Feinberg *et al.*, 1979; Lam *et al.*, 1981). Quantitative work on cell type proportioning and pattern formation in csA-containing slugs will help to evaluate the importance of differential cell adhesion in sorting out.

Materials and methods

Vector construction and transformation

The *D.discoideum* contact site A expression vectors pDCEV4 and pDCEV20 were constructed in pIC20R (Marsh *et al.*, 1984) by sequentially introducing four different fragments into this vector. The Neo^R resistance cassette was obtained as a 2.2 kb *Bam*HI−*Kpn*I fragment from plasmid pnIb (Leiting *et al.*, 1990). In this cassette the Neo^R gene is transcribed in *D.discoideum* under the control of actin 15 promoter sequences and terminated by the actin 15 terminator (Knecht *et al.*, 1986). For transcription of csA cDNA sequences, the actin 15 promoter was derived from the plasmid BHK (Pears, 1987) and modified by the PCR reaction (Saiki *et al.*, 1985). Using the oligonucleotides 5′-GATCAGATCTAAAAAATTTTTATTTATTTTTAT-TTATTTT-3′ and 5′-GATCAAGCTTATTTAATTTAATTTATTGTT-TTAAGAAAT-3′ as primers, a 280 bp actin 15 promoter fragment (−290 to −9) was amplified. The primers carried a *Bgl*II or *Hin*dIII restriction site respectively. For transcription termination of csA cDNA, actin 8 terminator sequences from the *D.discoideum* transformation vector pDNeoII, which carries two copies of this terminator in a tandem array (Witke *et al.*, 1987), were introduced. The vector was digested with *Bgl*II and *Hin*dIII, blunt ended with Klenow enzyme and religated in order to remove the multiple cloning site (MCS) of pDNeoII. From the resulting plasmid pDNeoII/ΔMCS, the tandem terminator was recovered as a 600 bp *Pst*I−*Hin*dIII fragment. CsA sequences were derived from plasmid p512-6, provided by B.Leiting, carrying the full length 1.8 kb *Eco*RI csA cDNA fragment c512 (Noegel *et al.*, 1986). Non-coding 5′ sequences of c512 were deleted with *Exo*III nuclease employing the Erase-a-base system (Promega) up to positions −20 and −4 of the published sequence, resulting in p5.7 and p5.2, respectively. After partial *Hin*dIII digestion, 1.7 kb csA fragments were recovered from these plasmids.

The actin 15 promoter fragment obtained by PCR reaction was ligated into the *Bgl*II−*Hin*dIII sites of pIC20R. The actin 8 tandem terminator fragment of pDNeoII/ΔMCS was inserted into the *Hin*dIII−*Pst*I sites of this construct. Subsequently the 1.7 kb *Hin*dIII csA subfragments of p5.7 and p5.2 were inserted in sense orientation into the *Hin*dIII site. The resulting plasmids were called p1.T5.7 and p1.T5.2. In the final step of PDCEV vector construction, the 2.2 kb *Bam*HI−*Kpn*I fragment carrying the Neo^R gene of pnIb was cloned into the corresponding sites of p1.T5.7 and p1.T5.2. These constructs were designated as pDCEV4 and pDCEV20. All cloning procedures were performed according to Maniatis *et al.* (1982).

AX2-214 cells were transformed with pDCEV4 and pDCEV20 essentially according to Nellen *et al.* (1984). Transformants were selected for G418 resistance using 20 μg/l of Geneticin (Sigma) and cloned by spreading dilutions onto SM agar plates containing *Klebsiella aerogenes*. Cells from single colonies were picked and transferred onto new plates in an ordered array and allowed to grow until the colonies had reached a diameter of ~1 cm (Wallraff *et al.*, 1986). Transformants TpDCEV4/AX2-2; TpDCEV4/AX2-13; TpDCEV20/AX2-17 and TpDCEV20/AX2-22 were obtained from four independent transformations. They are designated as TC2, TC13, TC17 and TC22 in this paper.

D.discoideum culture conditions, measurement of cell adhesion, and in vivo labeling

Cells of the *D.discoideum* strain AX2-214 and of the transformants were cultivated at 21−23°C. For colony blotting SM agar plates with *K.aerogenes* (Williams and Newell, 1976) were used. For axenic growth in liquid nutrient medium containing 1.8% maltose (Watts and Ashworth, 1970) cells were cultivated in polystyrol flasks (Flacon 3028) or Petri dishes (Falcon 3003), or in shaken suspension. From the shaken cultures cells were harvested at a density of not more than 5×10^6 cells/ml. Cell density was determined by measuring the volume of the cell pellet in a hematocrit tube (Gerisch, 1960). For starvation, cells were washed and adjusted to 1×10^7 cells/ml in 17 mM Soerensen phosphate buffer pH 6.0, and shaken on a gyratory shaker at 150 r.p.m. Cell-to-cell adhesion was assayed using an agglutinometer as described by Beug *et al.* (1973) and Bozzaro *et al.* (1987). For the assay, the cells were washed and resuspended at a density of 1×10^7/ml in 17 mM phosphate buffer, pH 6.0, and incubated with or without 10 mM EDTA for 1 h at 23°C in the agglutinometer.

For labeling with [^{3}H]palmitic acid (New England Nuclear, NET 043, 30 Ci/mmol), growth-phase cells were either washed twice and resuspended in the phosphate buffer at a density of 1×10^7 cells/ml, or suspended at 1.2×10^6 cells/ml in nutrient medium. The cells were incubated with 1 mCi of palmitic acid per 5 ml for 2 h in phosphate buffer or for 16 h during growth in nutrient medium. Crude particulate fractions were prepared by freeze−thawing of washed cells and centrifugation for 30 min at 10 000 *g*.

Colony blotting, gel electrophoresis and immunoblotting

Colonies derived from cloned cells were blotted onto BA85 nitrocellulose filters (Schleicher & Schuell, 3354 Dassel, FRG), the cells lysed by freezing and thawing, and washed in a Tris/Tween/NaCl buffer according to Wallraff *et al.* (1986). The blots were boiled in water containing 1% SDS for 2 min to denature the csA protein, and incubated for 4 h with ^{125}I-labeled mAb 294 (Bertholdt *et al.*, 1985), followed by autoradiography. SDS−PAGE was performed in 10% gels according to Laemmli *et al.* (1970). If not stated otherwise, total cellular proteins equivalent to 1×10^6 cells were applied per lane, blotted from the gel onto nitrocellulose, and labeled with ^{125}I-labeled mAb 33-294-17, mAb 24-210-2 (Bertholdt *et al.*, 1985) or mAb 123-353-2, in this paper designated as mAb 294, 210 and 353. The antibodies were ^{125}I-labeled by the chloramine-T method.

For determining [^{3}H]palmitic acid incorporation, proteins of the particulate fraction from 1×10^6 of growing and 2×10^6 of aggregating cells per lane were separated by SDS−PAGE, blots dipped into 20% (w/v) PPO in toluene and exposed at −70°C on Kodak XAR-5 film. For subsequent immunolabeling the PPO was removed with toluene.

DNA and RNA isolation and hybridization analysis

DNA was isolated from the purified nuclei of *D.discoideum* AX2-214 strain and digested with restriction endonucleases according to the instructions of the manufacturer. Fragments of nuclear and plasmid DNA were separated on 0.7% agarose gels in Tris-phosphate buffer, pH 7.8 (Maniatis *et al.*, 1982), transferred to nitrocellulose BA85 (Schleicher & Schuell, 3354 Dassel, FRG) and probed with nick translated DNA for 16 h at 37°C in 50% formamide, 2 × SSC, 1% Sarkosyl, 4 mM EDTA, 0.1% SDS, 4 × Denhardt's solution, and 0.12 M sodium phosphate buffer, pH 6.8. The filters were washed twice in 2 × SSC and 0.01% SDS at room temperature and 1 h at 37°C under hybridization conditions.

For the isolation of RNA, cells were lysed with 1% SDS in the presence of 0.05% diethylpyrocarbonate and the RNA purified by repeated phenol−chloroform extractions as described by Noegel *et al.* (1985). For Northern blot analysis 10 μg of total RNA per lane were resolved on a 1.2% agarose gel containing 2.2 M formaldehyde (Maniatis *et al.*, 1982). The RNA was transferred to nitrocellulose filters and hybridized with nick translated DNA probes under the conditions used for Southern blots.

Fluorescent antibody labeling

5×10^6 growth-phase cells or cells starved for 6 h were washed and suspended in phosphate buffered saline, pH 7.2, and incubated for 15 min on ice with 50 μg mAb 41-71-21 (Bertholdt *et al.*, 1985), here designated as mAb 71, in 500 μl PBS. After washing twice in PBS, the cells were incubated for 15 min with FITC-coupled sheep anti-mouse IgG (Diagnostics Pasteur, 92430 Marnes-la-Coquette, France), diluted 1:50 and washed again for fluorescence microscopy. Migrating slugs were collected from water agar, compressed between slide and coverslip, frozen on dry ice and fixed, after removal of the coverslip, for 10 min at −30°C in methanol. After air-drying the specimens were labeled at room temperature with mAb 71 essentially as described for living cells.

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