Development 125, 3343-3352 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV3887

A novel *Dictyostelium* cell surface protein important for both cell adhesion and cell sorting

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Accepted 17 June; published on WWW 6 August 1998

SUMMARY

A mutant of *Dictyostelium* that is aberrant in the process of tip formation (dtfA$^-$: defective in tip formation A) has been isolated by gene tagging. The *dtfA* gene is predicted to encode a protein of 163 kDa. There are no extensive sequence homologies between DTFA and previously identified proteins, but four short N-terminal sequence motifs show partial homology to repeats found in mammalian mucins. Immunofluorescence reveals a lattice-like arrangement of DTFA protein at the cell surface. When developing on a bacterial lawn, cells of the mutant strain (dtfA$^-$ cells) aggregate to form tight mounds, but development then becomes arrested. When developed in the absence of nutrients, a fraction of dtfA$^-$ cells complete development, but there is a long delay at the tight mound stage and the culminants that eventually form are aberrant. In such dtfA$^-$ mounds the prestalk cells fail to move to the apex on cue and so tip formation is delayed. dtfA$^-$ cells also show a conditional defect in early development, in that they are unable to aggregate when plated at low density. In addition dtfA$^-$ cells do not agglomerate efficiently when shaken in suspension. In combination, these results suggest that DTFA may form part of a cell-cell adhesion system that is needed both for optimal aggregation and for efficient cell sorting during multicellular development. The DTFA protein also appears to be important during cell growth, because cytokinesis is defective and the actin cytoskeleton aberrant in growing dtfA$^-$ cells.

Key words: *Dictyostelium discoideum*, *dtfA*, Cell adhesion, Cell sorting, Cell surface protein

INTRODUCTION

Formation of the *Dictyostelium* fruiting body requires the co-ordination of cellular differentiation and movement. Starvation triggers pulsatile cAMP signalling, which leads to aggregation of the cells to form a hemispherical mound in which the cells differentiate into either prestalk or prespore cells. Although initially intermingled with one another, the prestalk and prespore cells separate, with the prestalk cells at the apex of the mound and the prespore cells below them. A nipple shaped tip, composed of prestalk cells, then emerges from the apex of the mound and this initiates a process of ordered cell movement, whereby the mound is transformed into a cylindrical structure known as the first finger or standing slug. At culmination a further set of co-ordinated differentiation events and morphogenetic cell movements leads to the formation of the fruiting body (reviewed by Schaap, 1986). The cell sorting that occurs during slug formation is not at all well understood, but several lines of evidence suggest that chemotaxis to cAMP is one of the major organising forces (Bretschneider et al., 1995).

The cAMP signals that direct aggregation initially emanate from the centre of the aggregation territory, a roughly circular region that can contain up to 100,000 cells. These signals are relayed and amplified by the surrounding cells, which respond to receipt of a pulsatile cAMP signal by themselves synthesising and releasing cAMP. There is considerable evidence (reviewed by Siegert and Weijer, 1995 and Schaap, 1986) that cAMP signalling continues in the multicellular stages, with the tip acting as the signalling centre. Cellular differentiation then provides the heterogeneity in cAMP responsiveness necessary to generate sorting because, relative to the prespore cells, the prestalk cells accumulate elevated amounts of several components of the cAMP sensing system (Coukell, 1975; Ginsburg et al., 1995). These differences may account for the fact that prestalk cells, isolated from first fingers, move towards cAMP at a higher rate than do the prespore cells (Mee et al., 1986; Early et al., 1995).

Direct evidence that differential chemotaxis to cAMP is actually involved in cell sorting comes from a study where experimentally induced over-production of the extracellular form of cAMP phosphodiesterase slowed tip formation appreciably and also from experiments where cAMP artificially applied to the base of aggregates, caused the prestalk cells to reverse their normal polarity of movement and so accumulate in the base (Traynor et al., 1992; Matsukuma and Durston, 1979).

While in total it appears very convincing, the evidence for a role of extracellular cAMP in cell sorting has recently been called into question in a study using a null mutant in *acaA* (the

gene encoding the adenylyl cyclase believed to be responsible for cAMP production prior to spore formation) (Wang and Kuspa, 1997). When PKA is rendered constitutively active in *acaA* null cells, fruiting bodies are formed, even though there is no detectable cAMP accumulation. While a degree of caution needs to be applied when considering this work, most notably over the fact that a positive conclusion was drawn from the failure to detect a labile cellular component, it illustrates the level of uncertainty that exists in this area. Furthermore, an alternative mechanism, selective cell adhesion, has been proposed to play a major role in cell sorting (see review by Loomis, 1988). Here the evidence is less extensive than for cAMP, but the relative dearth of evidence could reflect the difficulty of studying cell adhesion in the later, multicellular developmental stages.

Several cell adhesion systems have been identified that function during multicellular development (Geltosky et al., 1979, 1980; Gao et al., 1992; Steinemann and Parish, 1980; Fontana, 1993). The adhesion systems were initially divided into two types, known as contact sites A (Beug et al., 1973), which is insensitive to EDTA and contact sites B (Garrod, 1972), which is sensitive to EDTA treatment. However, only two molecules involved in adhesion have been characterised in detail, namely gp80 (Muller and Gerisch, 1978), a glycoprotein which mediates contact sites A adhesion during aggregation and gp24 or DdCAD-1, which mediates contact sites B adhesion early in development (Brar and Siu, 1993; Wong et al., 1996). The genes encoding these proteins (*csA* and *cadA* respectively) have been cloned, however mutations that remove the activity of either protein do not greatly disrupt development (Harloff et al., 1989; Siu et al., 1997), suggesting an overlap of function between these two molecules and between them and other adhesion systems.

An alternative, unbiased method of analysing the processes mediating tip formation is to identify mutations that affect the process and to then determine the nature of the mutated gene product. In REMI, Restriction Enzyme Mediated Integration (Kuspa and Loomis, 1992), genes are tagged by insertion of a *Dictyostelium-E. coli* shuttle vector. A restriction enzyme, usually one that cuts *Dictyostelium* genomic DNA very frequently, is mixed with the transforming vector DNA, ensuring that most or all genes are accessible to tagging. After selection of a *Dictyostelium* clone with the desired phenotype, the genomic sequences flanking the vector are isolated by cleavage with an enzyme that does not cut the vector, followed by circularisation and cloning in *E. coli*. Using REMI, we describe the isolation of a novel gene required for tip formation, and show that it has multiple effects on *Dictyostelium* development including a major effect on cellular adhesion.

MATERIALS AND METHODS

Cell culture, transformation and development

The *D. discoideum* strain DH1 (a gift from P. Devreotes), a derivative of KAx3 in which the pyr5-6 gene is disrupted, was grown at 22°C in HL5 medium (Ashworth and Watts, 1970) supplemented with uracil at 200 μg/ml. The strain dtfA$^-$, generated by transformation with a pyr5-6-containing vector, was grown in unsupplemented HL5 medium. The *lacZ* marker constructs ecmAO:*lacZ* (Jermyn and Williams, 1991) and SP60:*lacZ* (Fosnaugh and Loomis, 1989) were introduced by calcium-phosphate transformation and clones were selected and maintained in 10 μg/ml G418 (geneticin; GIBCO, BRL). For development, axenically grown cells (1-5×10^6 cells/ml) were washed twice in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) and resuspended at 5×10^7 cells/ml (unless otherwise stated) and then spotted onto either 2% water agar or nitrocellulose filters (Millipore) supported on pads soaked in KK_2.

β-galactosidase staining

Structures developing on filters were fixed and stained with X-gal for 2-3 hours at room temperature (Dingermann et al., 1989).

REMI mutagenesis and redisruption

REMI was performed (Kuspa and Loomis, 1992) using the plasmid pJB1 (Insall et al., 1996). The plasmid was linearized with *Bam*HI and electroporated into DH1 cells with the restriction enzyme *Bgl*II. Transformants were selected for uracil auxotrophy in FM medium (GIBCO, BRL) as described by (Harwood et al., 1995). To re-disrupt the *dtfA* gene, 15 μg of *Bgl*II-rescued plasmid DNA was re-linearised with *Bgl*II and transformed into DH1 cells. The disruption frequency was 3%. The disruption was confirmed by Southern blotting (data not shown) and one of the redisruption mutants (22.7) was used in all subsequent experiments.

Generation of ploclonal antisera to DTFA

To generate polyclonal antisera to DTFA, a C-terminal portion of the protein (Fig. 3a underlined) was expressed as a His-tagged fusion protein in *E. coli*. This was achieved by subcloning a 1.1 kb *Bgl*II-*Pst*I fragment into pQE-31 (Qiagen). Clones containing the *dtfA* sequence were transformed into the *E. coli* strain M15[pREP4] (Qiagen) and grown at 37°C to an A_{600} of 0.6 prior to induction of expression with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 hours. The expressed protein was purified from bacterial lysates using a nickel agarose affinity column and analysed by SDS-PAGE. Because expression levels of the fusion protein were low and it consistently eluted with several contaminating *E. coli* proteins, the fusion protein was further purified prior to inoculation. Nickel agarose purified lysate from 6l of culture was separated on a 12% SDS-PAGE gel and the proteins were visualised by copper staining (Lee et al., 1987). A 47 kDa protein band, corresponding to the predicted size of the fusion protein, was excised from the gel, destained (0.25 M EDTA, 0.25 M Tris pH 9.0) and desiccated. The desiccated gel slice was ground to a fine powder and then resuspended in adjuvant prior to inoculation. Rabbit polyclonal antisera were generated using this fusion protein preparation. The antisera were affinity purified, using fusion protein immobilised on nitrocellulose (Hybond C), in order to reduce the background during immunostaining.

Immunohistochemical staining

Disaggregated cells were obtained by dissociation of tipped mounds or slugs through a 25 gauge needle and washed twice in KK_2. The cells were then resuspended at 1×10^7 cells/ml and allowed to settle on poly-L-lysine-coated slides for 10 minutes prior to fixation. The cells were fixed by one of the following methods: 100% methanol; 1% glutaraldehyde; 4% paraformaldehyde. Permeabilisation of cells after using the latter two fixation methods was achieved with 0.2% Triton X-100. After glutaraldehyde fixation, autofluorescence was quenched with sodium borohydride (0.5 mg/ml) and 50 mM glycine. The slides were washed twice in PBS after these treatments. Whole mounts of slugs were fixed on slides in 100% methanol. Fixed specimens were incubated with primary antisera at 4°C for 16 hours and then washed twice in PBS, prior to incubation with secondary antisera. For F-actin staining, glutaraldehyde fixed, permeabilised and quenched samples were incubated with 100 nM Texas red-conjugated phalloidin (Molecular probes) for 10 minutes. For nuclear staining, fixed cells were incubated for 10 minutes with Hoechst 33258. Samples were visualised with a Leica DMRBE confocal microscope and data was processed using the NIH Image 1.61 package.

Time-lapse videomicroscopy

To observe development of dtfA$^-$ cells, washed axenically grown cells

were plated at 2×10^8 cells/ml on 2% water agar and recorded using a time-lapse video recorder. To observe traction mediated cytofission, phase contrast images were collected from an inverted microscope using a video camera connected to a computer using NIH Image 1.61 software.

Chemotaxis assays

Dissociated, aggregation competent cells were washed, resuspended in KK_2, plated onto 2% water agar and covered with a thin layer of 1.5% water agar. 100 μM cAMP was allowed to diffuse from a fine glass needle tip placed near the cells. The cells were viewed using an inverted phase contrast microscope equipped with an image intensified CCD camera (Prostab Inc., model Hr604-MCP) and their movement before and after release of the cAMP was recorded using a time-lapse video camera. Alternatively, aggregation-competent cells were assessed for chemotaxis using the 2-spot assay (Konijn, 1965; Insall et al., 1996).

Adhesion assay

Cells were developed for varying times on KK_2 filters, disaggregated, washed and resuspended at 5×10^7 cells/ml in KK_2. 1 ml of cell suspension was shaken in a 25 ml flask at 100 rpm for 45 minutes. Using a haemacytometer, the number of cells not in clumps of 3 or more cells was counted. Having counted the number of cells/field before incubation it was possible to calculate the percentage of cells in any field which were present in clumps of >3 cells.

RESULTS

dtfA⁻ cells are conditionally defective in their development

The original *dtfA* null mutant was isolated by screening a bank of REMI mutants for those clones that could not form tipped mounds during development after growth on a bacterial lawn. One such mutant, clone 22, was unable to proceed beyond the loose aggregate stage of development (data not shown). Genomic DNA flanking the site of vector insertion in clone 22 was propagated in *E. coli* and this DNA was used to perform homologous gene disruption. However, the isolates where disruption had occurred showed a less severe phenotype than the original *dtfA* null strain, blocking instead at the tight mound stage, suggesting that there were other, cryptic mutations in clone 22. One such disruptant (clone 22.7, henceforth referred to as dtfA⁻) was used in all subsequent experiments.

When grown on a bacterial lawn, dtfA⁻ plaques display a relatively enlarged clearing zone (the zone adjacent to the bacteria, that contains both growing and aggregating cells). In the central part of the plaque there is an accumulation of tight mounds but mature fruiting bodies are not formed (Fig. 1A).

When they are grown axenically and developed on buffered filters or on water agar, approximately 30% of dtfA⁻ cells pass through the block to tip formation, and we recorded their behaviour using time-lapse video microscopy. Aggregation to form tight mounds seems to occur normally but is followed by the formation of doughnut-like structures (Fig. 1B). Within a period of about one hour these structures undergo disaggregation and, in some cases, yield single cells.

A few hours after disaggregation, the cells quickly re-aggregate to form large irregular mounds (Fig. 1C) that move in an apparently random fashion across the substratum by an unknown mechanism. Approximately 20 hours after starvation, slugs begin to emerge from some of these mounds, leaving behind circular heaps of discarded cells as they migrate away (Fig. 1D). Using pre-stalk and pre-spore cell specific markers we determined that the discarded heaps contain a mixture of cell types (data not shown), that could have differentiated in situ or that might have been left behind as the aggregate moved away. Many of the slugs are multi-headed (Fig. 1E) and go on to produce culminants with twisted stalks and thickened basal discs (Fig. 1F).

Transformation of dtfA⁻ cells with a *dtfA* over-expression construct restored the ability of these cells to develop relatively normally and produce mature fruiting bodies (Fig. 2). Development of the transformed dtfA⁻ cells is not entirely wild-type as the fruiting bodies are malformed, but this is as might be expected because expression of the *dtfA* gene in these cells is directed by the Actin-15 promoter and is therefore not subject to normal temporal regulation. Over-expression of the *dtfA* gene in parental cells had no observable effect on development (data not shown).

The DTFA protein shows a limited homology to mucins

The structure of the gene was deduced by sequencing the rescued DNA and also from a partial length cDNA clone. Analysis of the deduced sequence revealed a single, continuous

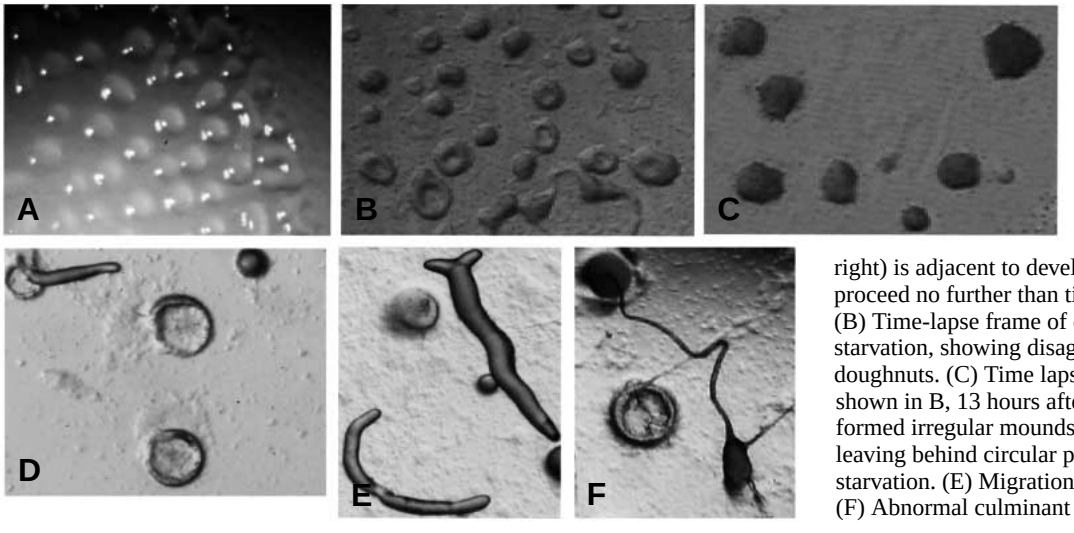

Fig. 1. Developmental morphology of dtfA⁻ cells. Axenically grown dtfA⁻ cells were allowed to develop on a bacterial lawn (A) or 2% water agar (B,C,D,E,F) as described in the Materials and Methods. (A) The clearing zone (top right) is adjacent to developing aggregates which proceed no further than tight mounds (left side). (B) Time-lapse frame of dtfA⁻ aggregates 10 hours post-starvation, showing disaggregating mounds and doughnuts. (C) Time lapse frame of the same field shown in B, 13 hours after starvation, showing re-formed irregular mounds. (D) Emergence of slugs, leaving behind circular piles of cells, 20 hours after starvation. (E) Migration of large multi-tipped slugs. (F) Abnormal culminant after 30 hours starvation.

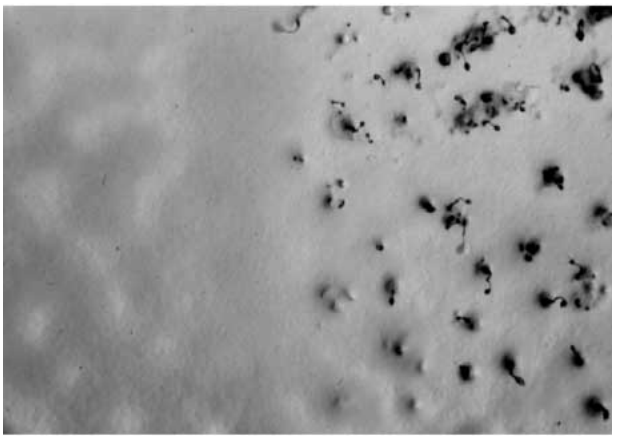

Fig. 2. Complementation of the developmental defect in dtfA⁻ cells. Disruptant cells (22.7) were transformed with a DTFA overexpression construct derived from EXP4(+) (Dynes et al., 1994), containing the entire coding region of the *dtfA* gene. Transformed cells (right) were selected for G418 resistance and grown side-by-side with non-transformed (22.7) cells (left) on a bacterial lawn.

open reading frame encoding a 1402 amino acid protein, DTFA with a predicted molecular mass of 163 kDa (Fig. 3A).

In common with many developmentally regulated *Dictyostelium* genes, the *dtfA* gene contains several tracts of AAC repeat sequence that are, depending on the reading frame, translated into homopolymeric tracts of asparagine, threonine or glutamine residues (Shaw et al., 1989). In addition there are proline and serine repeats and also an asparagine/glycine repeat (underlined), which is similar to that identified in a fungal cell wall protein (Lora et al., 1994). The N terminus of DTFA contains 2 identical copies of the sequence TTTTVQPPQIVSPP and 2 partial copies of the same sequence (underlined). This Thr/Pro rich sequence contains part of a repeated motif found in mammalian mucins (Fig. 3B) that is thought to be responsible for O-linked glycosylation (Gum et al., 1989). The DTFA sequence also includes 14 putative N-glycosylation sites, one of which (NVTT) is located within the most N-terminal of the mucin-like repeats.

Disaggregated mound stage cells have a lattice-like distribution of DTFA protein at their surfaces

In order to localise the DTFA protein, a polyclonal rabbit antiserum was generated to a C-terminal portion of the protein (indicated in bold in Fig. 3A) and was affinity purified using the immunogen. Parental slugs and dtfA⁻ slugs (i.e. slugs after escape from the block) were disaggregated, fixed and incubated with affinity purified anti-DTFA antiserum or with pre-immune antiserum. After staining with a fluorescent second antibody, the cells were viewed by confocal microscopy. Parental cells incubated with the pre-immune serum show no staining, whereas approximately 10% of cells incubated with the immune serum show surface staining (Fig. 4A). This staining is generally restricted to one region of the cell but we have not been able to define this region in any way. Its localisation does not correlate with the intracellular distributions of myosin or actin (data not shown) or with the anterior or posterior of the cell. 3-D reconstructions show a lattice-like distribution of the protein (Fig. 4C) over the cell

A

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   1  MKDIEASKKPHTTTVAPPQINFIKNNENIFQPKPISNVTTTTVQPPQIVS
  51  PPSPPSPPQTTTIAPPTILPTTKTTTTTTTTTTTTTTVQPPQIVSPPIIN
 101  NLIIQNNLNTPSLSSTPSPLPNNNNSNENDNDINNLKISKEEYQTQIEIQ
 151  QQIQRQQILERQQLLQRRNQEQLDLLERHNDYQELINTHQIFVPPNNRFS
 201  QQIHVSQLKKQSSQSQLQQQLSSQSLQQIQQKSKQPPPQQQQQQQPPPPP
 251  IPLLPQIHQQLKPKQQQEQQQQQEQQQQQTENERINELRRLKRKKEYHND
 301  EYKDDEIYLDNILKGIDIRKLEKLTAVELRKIGKTLGVPMGNNTKGETFQ
 351  RIKSFIENHKKKKQKYREYQSEKNQQQKSNSKKLVNNSTIYDLPIKDIEH
 401  LEQLFWRIFRNIVLFRKIIGNLSRGGFFDQQQQQQQQQQSTMTTTSSSSS
 451  PMTSSDDKFFYCYNYIFSKTYKYDQIIHVSWIVDSNYFGLLKYKVSRGDL
 501  LVFCNHNTINDGDVNDDEDQNTKYEFCKMFKKIFNSIRSIQDKSFYRDLF
 551  TNYSEFIFDRINKRYPIDIYLVGSLAIECNCLVATKLLISEFQFRPVMNH
 601  SLQLAIKSGSYKMVKLIVTTIHRQMSLNPNNKPFDIEMFSKINNPSIKII
 651  NLLIQLRLFTYSNIINLVIADAKLINDNNENDILQFNKNLKNQIFNYSNL
 701  LNSFTFNNNINNNNNNNINNNNNNNNNNNNNNNNNNNNNSNLSNNTIEY
 751  SKIKTKTVFNQFTIKQLINSCKLIVTFDLKNQYQQQYYKRHYEDSDNEEE
 801  EEITQASFTINTMAEIEFIETQITEEEEHCFVKKVIQQQLLKDGEKEDYD
 851  GIKRLAELYVSLNPHLKVYCNFMYKIIYGNENCYDQDLD
 901  VFDSNCFKQSLKYGSPGYWIEYKEVEMKYAFLSNKYRNEITPNLLFKYVS
 951  PNNLNKQLKFIKKIYNSSIENGNSNGNGNGNGGTVIGGILDRLLLFYLII
1001  ENNNLELLSIVIKEFPLISNFCYIAKSNSEIYNLKIPRFIRSIEMLEFCF
1051  SNFRDHFYLPQSNSLTADFYGFQNVELLRKYDQLMVLDDMDKGIIVSNKK
1101  VRASFDDFHFIFEWGSSLKNYNNYLKMLAYIVEDPYGLYTIVTNEILLLS
1151  AILSPTTSSGEPLLNLNIERQLIFQEIISSFSTILEGTELKYYPEQMFQE
1201  STHLKRFFDWIFENRSEDLLIGGRCVITQSVQSHMLYRAGRLDIVLRKGY
1251  YYDDTNEGSKKIMPVGLALVLDDIGKYGDVVALEKYIRSCIPLMKQQSEL
1301  LTLLDKDERESRACFSQCQRHFSSLLSKASIYGRINIFQHIFYNHQFIFD
1351  KKSLLFARKGFLSKISFKKLILECHYHKQHHILDFIQNVIGLDITPKQIK
1401  SN
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B

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Human TM       79 TTTTVTPTP..
Human IM       15 TTTTVTPTP..
Canine TM     246 TTTTVTPTP..
Dd dtfA 2      39 TTTTVQPP...
Dd dtfA 4      85 TTTTVQPP...
Dd dtfA 1      12 .TTTVAPP...
Dd dtfA 3      60 .TTTIAPP...
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Fig. 3. Predicted amino acid sequence of the DTFA protein. (A) The translated ORF of *dtfA*, showing the C-terminal region (bold) against which the polyclonal antiserum was raised. The amino terminal mucin-like repeats and NG repeat are underlined. (B) Alignment of DTFA N-terminal repeats and mammalian mucin repeats. The full, highly conserved, 12 amino acid repeat in all of the mammalian mucins is TTTTVTPTPTPT. Human tracheal mucin (TM) has 10 such repeats (Verma and Davidson, 1993); human intestinal mucin (IM) has 3 such repeats (Gum et al., 1989); Canine tracheal mucin has 4 such repeats (Gerard et al., 1990).

surface. The antiserum yielded this staining pattern on cells fixed using three different fixatives, methanol, glutaraldehyde and paraformaldehyde and it was not necessary to permeabilise cells after glutaraldehyde or paraformaldehyde fixation to obtain such a staining pattern, supporting the notion that the protein is indeed present on the outside of the cell.

The dtfA⁻ cells show no surface staining with either the pre-immune or anti-DTFA antisera (Fig. 4B), confirming that the affinity purified anti-serum is specific to the DTFA protein. There is, however, a low level of punctate intracellular staining in about 10% of the cells. The REMI disruption plasmid inserted only 300 bp from the C terminus of the open reading

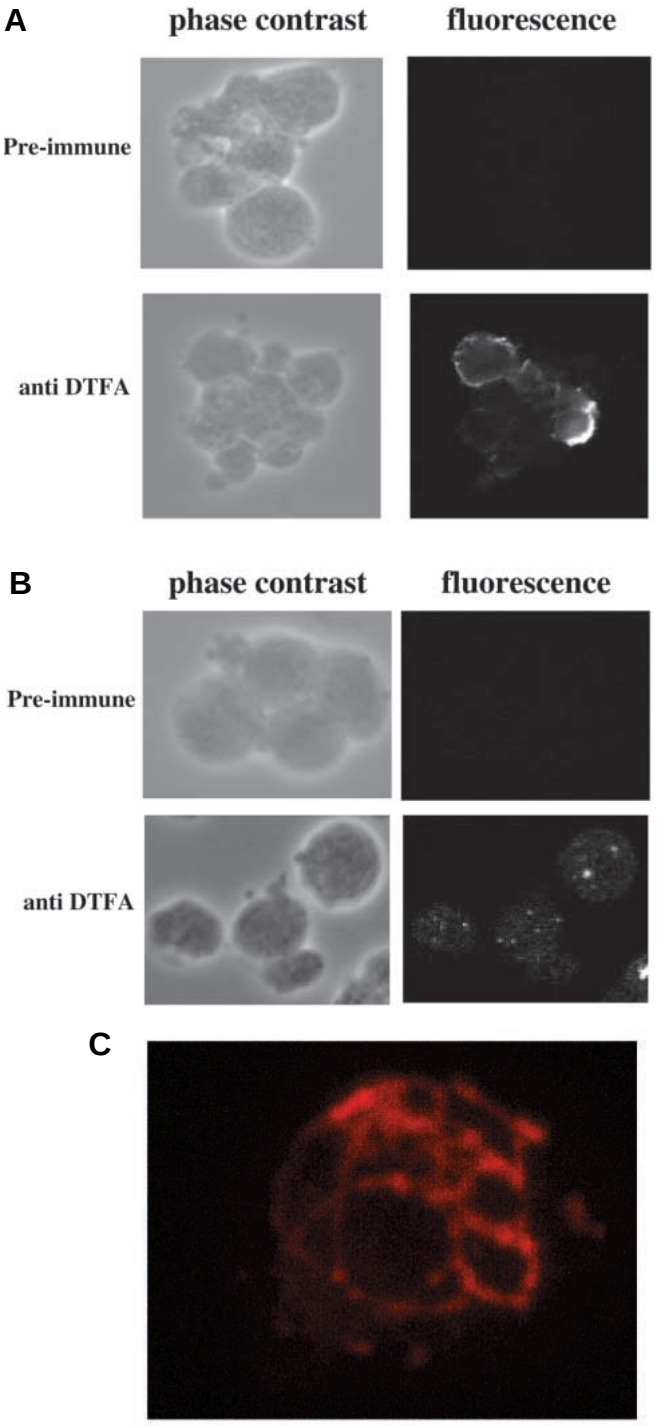

Fig. 4. Detection of DTFA using polyclonal antisera. Parental (A and C) and dtfA⁻ cells (B) were developed to the slug stage on 2% water agar, disaggregated, fixed and probed with antisera as described in the Materials and Methods. Affinity purified pre-immune or anti-DTFA antisera were used as the primary antibody and TRITC-conjugated goat anti-rabbit antiserum as the secondary antibody. A and B are single focal planes; C is a single view of a 3-D reconstruction of 64 optical sections taken through a single cell probed with anti-DTFA antiserum.

frame, so that the disrupted gene still encodes nearly 1300 amino acids of DTFA protein. We believe, therefore, that the

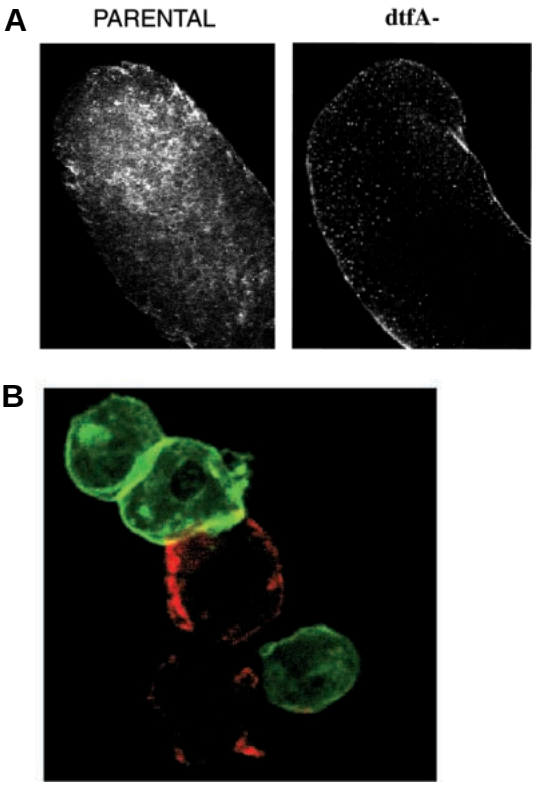

Fig. 5. Cell type specificity of DTFA expression. (A) Parental and dtfA⁻ slugs were fixed and probed with affinity purified anti-DTFA antisera as described in the Materials and Methods. High power (40×) images of slug anteriors are shown. This staining pattern was seen throughout the slugs (data not shown). (B) Parental slugs were disaggregated and fixed before probing with anti DTFA (red) and MUD-1 antisera (Krefft et al., 1983) against prespore cells (green), as described in the Materials and Methods. FITC-conjugated goat anti-mouse antiserum was used as the secondary antibody for the MUD-1 staining. A group of five cells is shown. Regions where both antisera bound appear yellow.

dtfA gene is expressed in the null cells but that the protein is not correctly exported to the cell surface.

The time-course of DTFA expression was also followed, by immunostaining cells disaggregated at different developmental stages (data not shown). DTFA expression is first detected at the tight mound stage and remains detectable in slug cells. Analysis of whole-mount slugs shows cell surface staining in cells scattered throughout the parental slug and punctate intracellular staining in the dtfA⁻ slug (Fig. 5A). This distribution is supported by double staining, using the anti-DTFA antiserum and a monoclonal antibody that specifically recognises prespore cells. In disaggregated slugs both prespore cells and 'non-prespore cells' (presumably the prestalk cells) express DTFA (Fig. 5B) to varying degrees. There is no detectable staining in terminally differentiated stalk and spore cells (data not shown).

Apical sorting of dtfA⁻ prestalk cells is greatly delayed and the defect is cell autonomous

Normal and *dtfA*⁻ cells were transfected with ecmAO:*lacZ* (a prestalk-specific marker) and SP60:*lacZ* (a prespore-specific marker) and their expression was analysed during development

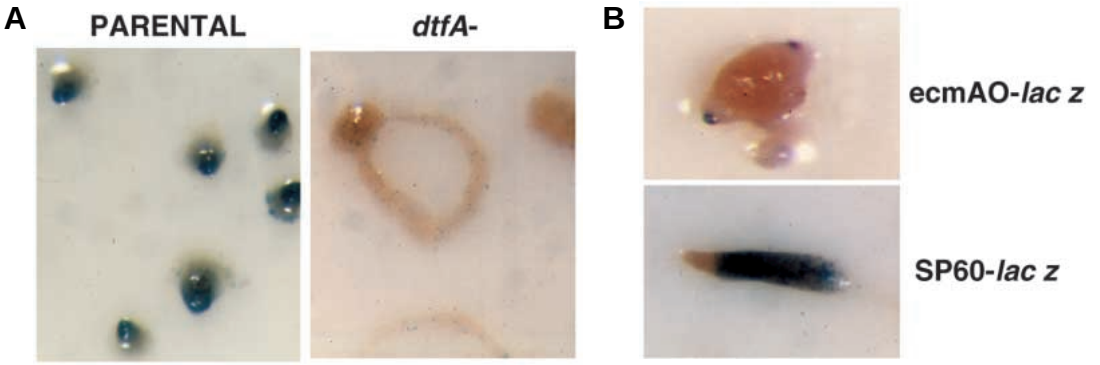

Fig. 6. Prestalk cell distribution in parental and dtfA⁻ aggregates.
(A) Parental and dtfA⁻ cells expressing the ecmAO-*lacZ* reporter construct, developed on KK_2 filters for 12 hours.
(B) dtfA⁻ cells expressing ecmAO or SP60-*lacZ* reporter constructs, developed for 19 hours on KK_2 filters.

on buffered filters. The markers are equivalently expressed in the two strains at the loose mound stage (data not shown), indicating that initial cell-type differentiation is not detectably disrupted in dtfA⁻ cells. However, in the arrested mounds and doughnut structures formed by dtfA⁻ cells, the prestalk cells remain scattered, while in the mounds formed by parental cells the prestalk cells accumulate at the apex (Fig. 6A). Thus dtfA⁻ prestalk cells fail to sort correctly and this presumably explains why they are unable to make a tip. As expected, in those dtfA⁻ aggregates that eventually overcome the block, the prestalk cells do sort correctly (Fig. 6B).

We also followed the fate of dtfA⁻ cells when mixed with a three fold excess of normal cells. The dtfA⁻ prestalk cells were marked by their expression of Actin15:*lacZ*, a construct that expresses β-galactosidase in all cells. In such a synergy experiment the mutant cells are excluded from the upper part of the aggregate (Fig. 7).

The DTFA protein is necessary for normal cytokinesis and has a role in aggregation

Even though it is present at a concentration too low to be detectable by immunocytochemistry prior to tip formation, several pieces of evidence indicate that DTFA has a role in growth and in the early stages of development.

dtfA⁻ cells grow poorly (Fig. 8A) and to a large size in shaken culture, features that have been previously associated with a defect in cytokinesis. In support of this notion, shaken cultures contain many multi-nucleate cells (Fig. 8B). Under these growth conditions the average number of nuclei per cell is 2.7 for the dtfA⁻ strain, with a maximum of 17 nuclei counted in a single cell. This is significantly greater than in parental cells, which average 1.9 nuclei per cell, with no more than 7 nuclei seen in a single cell. However, the cytokinetic defect is not as great as some previously characterised mutants (De Lozanne and Spudich 1987; Tuxworth et al., 1997). Cytokinesis defective mutants can often be rescued by growth on a substratum, where they divide by a process termed traction-mediated cytofission, and this is also true of dtfA⁻ cells (Fig. 8C). This mechanism of cell division explains why dtfA⁻ cells grown on plastic dishes are mononucleate (data not shown). To confirm that this defect in cytokinesis was not the cause of the previously observed defects in dtfA⁻ cell aggregation and development, these experiments were repeated using either cells grown in shaken suspension or cells grown on plastic dishes. The results were the same however the cells had been grown (data not shown). Moreover, multinucleate dtfA⁻ cells grown in shaken suspension were found to be mononucleate after 2 hours development on agar (data not shown).

In addition to a defect in cytokinesis, the actin cytoskeleton is also altered in vegetative dtfA⁻ cells. Phalloidin staining of fixed parental cells reveals actin-rich filopodia restricted to the periphery at the site of contact with the substratum, with only the occasional phagocytic crown on the upper surface of the cell. In marked contrast, vegetative dtfA⁻ cells bear filopodia over their entire surface (Fig. 9). Also, patches of polymerised actin are often present within dtfA⁻ cells.

Another difference between dtfA⁻ and parental dtfA⁻ cells emerged when we compared their abilities to aggregate at varying plating densities. The dtfA⁻ cells show a much greater density dependence than parental cells (Fig. 10), so that dtfA⁻ cells are entirely unable to aggregate below a density of 3×10^7 cells/ml, while the parental strain can aggregate efficiently at 1×10^7 cells/ml.

Fig. 7. Distribution of dtfA⁻ prestalk cells in parental/dtfA⁻ chimeras. dtfA⁻ cells expressing the ecm-*lacZ* reporter construct were mixed 1:3 with parental cells and allowed to develop on KK_2 filters for 16 hours.

DTFA is not required for chemotaxis but is required for cell-cell adhesion

One potential explanation for the failure of dtfA⁻ cells to sort to the apex of the mound and for their conditional defect in aggregation is a defect in chemotaxis to cAMP. However, when developing at high cell density, dtfA⁻ cells elongate, polarize, stream and form end-to-end contacts in the same way as parental cells (data not shown). We tested their chemotaxis to cAMP directly, using both a 2-spot assay and micropipette assay. In both assays dtfA⁻ cells respond just as well as their parental counterparts (Fig. 11 and data not shown).

One further observation indicates that dtfA⁻ cells have a defect in cell-cell adhesion that may explain some or all aspects of their aberrant behaviour. Cells were developed on KK_2 filters for various times, disaggregated and then tested for their ability

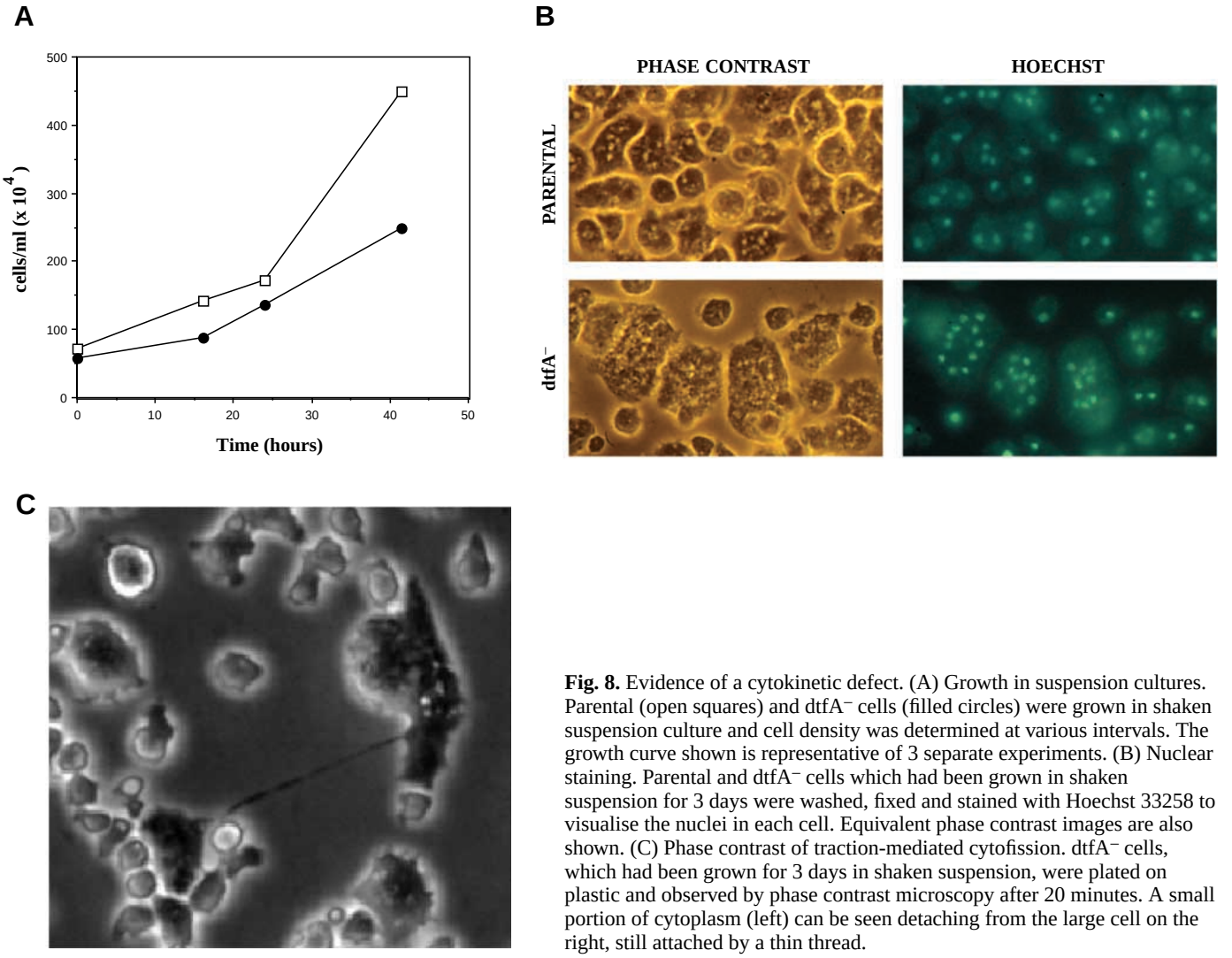

Fig. 8. Evidence of a cytokinetic defect. (A) Growth in suspension cultures. Parental (open squares) and dtfA⁻ cells (filled circles) were grown in shaken suspension culture and cell density was determined at various intervals. The growth curve shown is representative of 3 separate experiments. (B) Nuclear staining. Parental and dtfA⁻ cells which had been grown in shaken suspension for 3 days were washed, fixed and stained with Hoechst 33258 to visualise the nuclei in each cell. Equivalent phase contrast images are also shown. (C) Phase contrast of traction-mediated cytofission. dtfA⁻ cells, which had been grown for 3 days in shaken suspension, were plated on plastic and observed by phase contrast microscopy after 20 minutes. A small portion of cytoplasm (left) can be seen detaching from the large cell on the right, still attached by a thin thread.

to reaggregate. At early times there is a marked difference in the ability to reaggregate between parental and dtfA⁻ cells (Fig. 12). After 4 hours of development, dtfA⁻ cells are unable to aggregate, whereas 80% of parental cells can reaggregate. However, after 6 hours development, 40% of dtfA⁻ cells can reaggregate, compared with 90% of parental cells and by 8 hours development both parental and dtfA⁻ cells show almost 100% re-aggregation. Thus the dtfA⁻ mutant lacks the early, EDTA sensitive adhesion system, that is mediated by DdCAD-1, and it appears to be also partially defective in the EDTA resistant mechanism that is mediated by gp80. However, immunostaining shows that both the DdCAD-1 and gp80 proteins are present in developing dtfA⁻ cells at levels similar to those seen in developing parental cells (data not shown). Therefore, dtfA⁻ cells have a defect in the functioning of DdCAD-1 and gp80 rather than in their expression.

As both DdCAD-1- and gp80-mediated adhesion are believed to be achieved through homophilic mechanisms, we examined the ability of dtfA⁻ cells to adhere to parental cells (Fig. 13). GFP-expressing dtfA⁻ cells participate in mixed aggregates, suggesting that DTFA mediates a heterophilic adhesion mechanism.

DISCUSSION

DTFA does not display extensive homology with known proteins but, there are four amino-terminal repeats of the sequence TTTTVxP. Such a sequence is present in mucin proteins, where it forms part of a conserved 23 amino acid tandem repeat (Gum et al., 1989). The mucins are large, highly glycosylated cell surface proteins that contribute to the fluid mechanical properties of mucus (Van Klinken et al., 1995). As DTFA does not share homology with the mucins in any other regions of its protein sequence it cannot be regarded as a mucin homologue, but the presence of common sequence elements suggests that DTFA and the mucins may share some properties. It has, for example, been suggested that these very Thr-rich, repeat regions in mucin are sites of O-linked glycosylation (Gum et al., 1989). The existence of such repeats in DTFA, and of 14 potential sites for N-linked glycosylation suggest that it may also be a glycoprotein, but there is no direct evidence for this. Consistent with a mucin-like role, DTFA is located at the cell surface in a lattice-like structure that has not, to our knowledge, been previously described for any *Dictyostelium* protein.

dtfA⁻ cells have a complex phenotype, suggesting that the

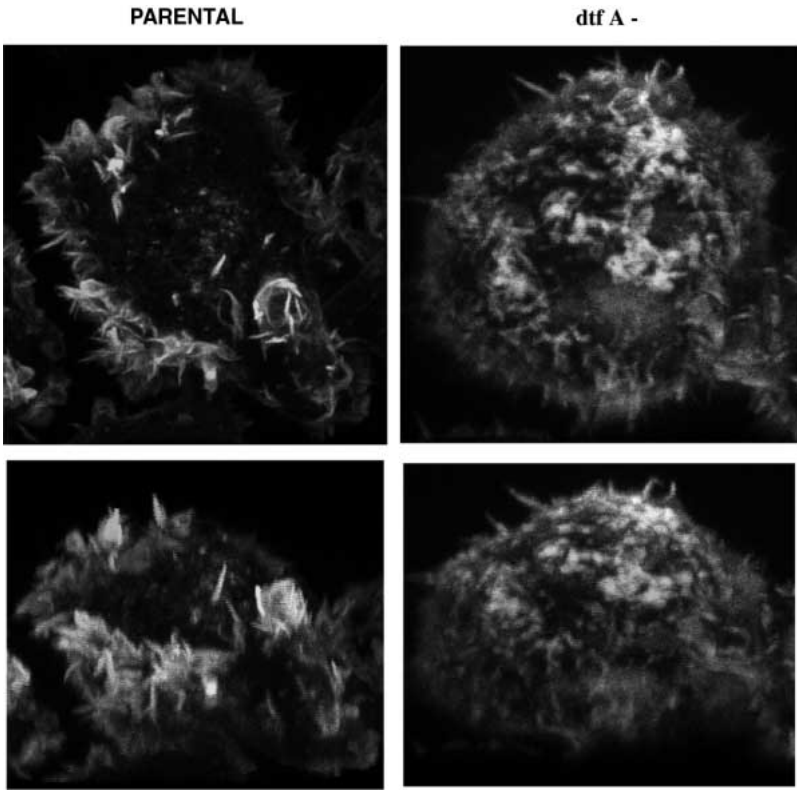

Fig. 9. F-actin staining of vegetative cells. Vegetative parental and dtfA⁻ cells were glutaraldehyde fixed and permeabilised as described in the Materials and Methods. F-actin was visualised with rhodamine-conjugated phalloidin and by confocal microscopy. 3-D images were constructed from 64 optical sections. Two different planes of rotation of the 3-D images are shown.

DTFA protein is important in several different cellular and developmental processes. A defect in cellular adhesion is the earliest detectable developmental phenotypic trait. DdCAD-1/gp24 and gp80, the adhesion molecules that are operative at these times, are both thought to function by homophilic interactions (Siu et al., 1987; Brar and Siu, 1993). Conversely DTFA appears to operate through heterophilic interactions. It may be that the DTFA protein provides some ancillary function that is necessary to augment the effect of both adhesion systems or it may be that DTFA adhesion constitutes another independent system which is required in addition to the DdCAD-1/gp80 systems for proper adhesion. The fact that the *dtfA* null strain shows defective adhesion, while disruptants in either the *cadA* or the *csA* genes develop normally (Harloff et al., 1989; Siu et al., 1997), may reflect the fact that dtfA⁻ cells are defective in both adhesion systems.

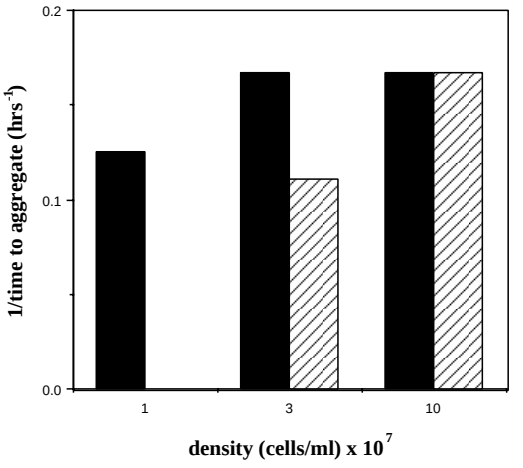

Fig. 10. Comparison of aggregation speeds in parental and dtfA⁻ cells. Axenically grown parental (filled boxes) and dtfA⁻ (striped boxes) cells were washed and resuspended in KK_2 at different densities. The cell suspensions were plated on 2% water agar and allowed to develop at 22°C. The inverse of the time it took to form streams was plotted.

The defect in adhesion in dtfA⁻ cells may explain their other developmental characteristics. A simple hypothesis to explain the defective aggregation of dtfA⁻ cells developing at low density is that cells need to adhere in order to aggregate efficiently. The adhesion defect may also explain the delayed tip formation observed on water agar and the complete inability to form a tip in cells growing clonally on bacteria.

In dtfA⁻ cells prestalk differentiation occurs apparently normally, but the prestalk cells are then delayed or blocked in their movement to the apex of the mound. Several pieces of evidence suggest that movement of prestalk cells to the apex occurs as a result of chemotactic movement towards cAMP signals (Traynor et al., 1992; Matsukuma and Durston, 1979) but there may also be some contribution of differential adhesion. The prespore cells are known to be strongly mutually adhesive (Lam et al., 1981) and this may facilitate separation into discrete prestalk and prespore regions. The DTFA protein may, perhaps, be necessary for specific cell adhesion interactions at late stages so that, in its absence, cellular compartmentalisation may be delayed or abrogated.

During the period when tip formation is delayed, dtfA⁻ cells have a distinctive ‘doughnut’-like appearance and this is also seen in a mutant constitutively active in the G alpha1

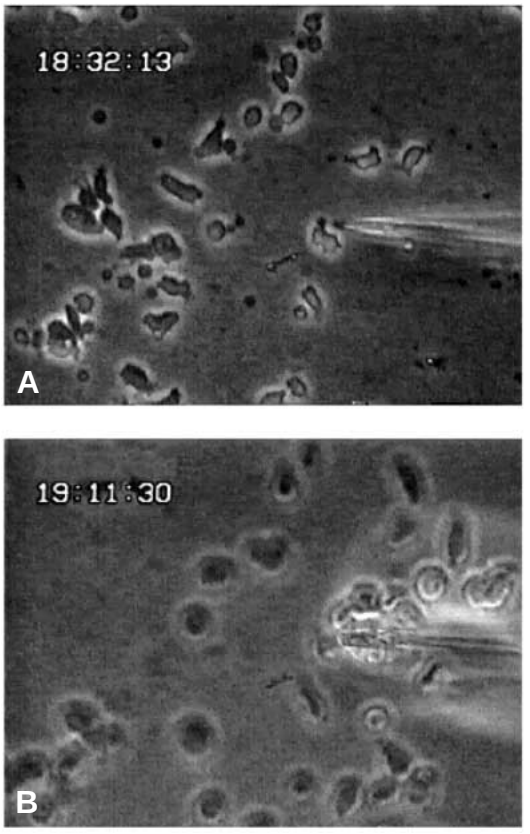

Fig. 11. Chemotaxis to cAMP. dtfA⁻ cells were allowed to develop to the tight mound stage, were disaggregated and then tested for their ability to move towards a needle releasing 100 mM cAMP, as described in the Materials and Methods. Time-lapse video images taken before (A) and 40 minutes after (B) cAMP release are shown.

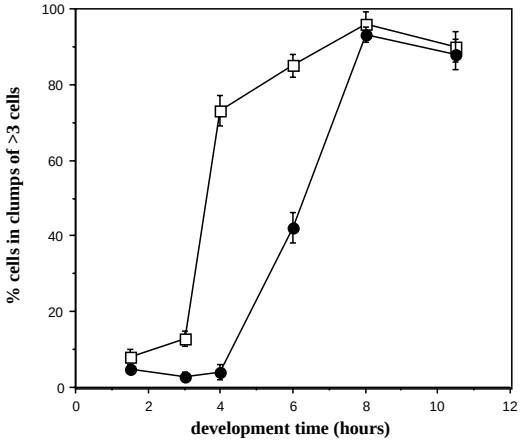

Fig. 12. Comparison of parental and dtfA⁻ cell-cell adhesion during early development. Axenically grown parental (open squares) and dtfA⁻ (filled circles) cells were allowed to develop on KK_2 soaked filters for various times. The cells were disaggregated, washed, resuspended at 5×10^7 cells/ml and shaken at 100 rpm for 45 minutes. The percentage of cells in clumps of >3 cells was determined as described in the Materials and Methods.

protein (Rietdorf et al., 1997). Cells within such structures orbit around the structure, as if locked into a default

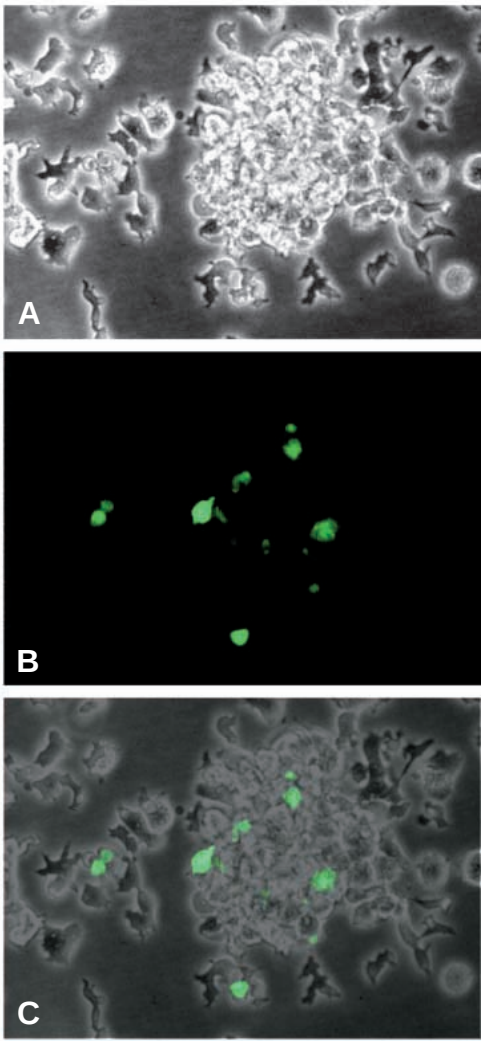

Fig. 13. Demonstration of heterophilic adhesion between dtfA⁻ and parental cells. dtfA⁻ cells transformed with an Actin-15 coupled GFP marker were mixed 1:9 with unmarked parental cells and tested for their ability to reaggregate after 4 hours development (as described in Fig. 12). (A) Phase contrast, (B) fluorescence and (C) merged images of a typical mixed aggregate are shown.

movement pattern because normal movement to the tip is not possible. The ability of a proportion of dtfA⁻ cells to eventually pass through the block in tip formation may reflect the existence of multiple adhesion systems with overlapping redundant functions. The efficient clumping of late aggregation phase dtfA⁻ cells is consistent with this notion, because it shows that some late adhesion systems will function in the absence of DTFA.

While impairment of several of the organism's adhesion systems might have been expected to affect development, the role of a cell adhesion molecule in cytokinesis is not immediately apparent. However, Texas red-phalloidin staining revealed that the actin cytoskeleton is highly abnormal in vegetative dtfA⁻ cells and this disturbance perhaps accounts for their poor cytokinetic abilities. In support of this idea, a similar defect in cytokinesis has been observed in talin null cells (Niewohner et al., 1997). No *Dictyostelium* integrin homologue

has so far been identified but, given the cell surface localisation of DTFA and its apparent link with the cytoskeleton it is possible that it may be a functional homologue of the vertebrate integrin.

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