An activated Ras protein alters cell adhesion by dephosphorylating *Dictyostelium* DdCAD-1

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RasG-regulated signal transduction has been linked to a variety of growth-specific processes and appears to also play a role in the early development of *Dictyostelium discoideum*. In an attempt to uncover some of the molecular components involved in Ras-mediated signalling, several proteins have been described previously, including the cell adhesion molecule DdCAD-1, whose phosphorylation state was affected by the expression of the constitutively activated RasG, RasG(G12T). Here it has been shown that a *cadA* null strain lacks the phosphoproteins that were tentatively identified as DdCAD-1, confirming its previous designation. Further investigation revealed that cells expressing RasG(G12T) exhibited increased cell–cell cohesion, concomitant with reduced levels of DdCAD-1 phosphorylation. This increased cohesion was DdCAD-1-dependent and was correlated with increased localization of DdCAD-1 at the cell surface. DdCAD-1 phosphorylation was also found to decrease during *Dictyostelium* aggregation. These results revealed a possible role for protein phosphorylation in regulating DdCAD-1-mediated cell adhesion during early development. In addition, the levels of DdCAD-1 protein were substantially reduced in a *rasG* null cell line. These results indicate that RasG affects both the expression and dephosphorylation of DdCAD-1 during early development.

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INTRODUCTION

Cell-to-cell adhesion is an important mechanism in multicellular organisms for maintaining cell shape and tissue structure, but it is also a point of integration between cell contact and intercellular signalling (Juliano, 2002; Coates & Harwood, 2001). In mammalian cells, cell adhesion receptors, such as cadherins, integrins and selectins, have been linked to the regulation of intercellular signalling pathways (Juliano, 2002). However, there are still many gaps in our understanding of the relationship between cell signalling and cell adhesion and, in particular, the mechanisms by which signalling molecules, such as Ras, affect and are affected by cell adhesion.

The importance of adhesion during *Dictyostelium* development has been long recognized (Gerisch, 1968). These solitary amoebae feed on bacteria during the vegetative phase but, upon nutrient deprivation, up to 10⁵ cells aggregate, spatially organize and differentiate into multiple cell types (Kessin, 2001). The adhesion protein DdCAD-1 (previously known as contact site B or gp24) is one of the first proteins to be expressed at the cell surface at the onset of starvation (Beug *et al.*, 1973; Yang *et al.*, 1997) and it has been implicated in mediating initial side-to-side contacts

during early aggregation (Beug *et al.*, 1973; Sesaki & Siu, 1996). A second adhesion molecule, termed gp80 or contact site A, is induced by cAMP as development proceeds (Muller & Gerisch, 1978; Faix *et al.*, 1992) and mediates a second type of contact in the developing stream (Beug *et al.*, 1973; Choi & Siu, 1987). gp80 allows tight adhesion between cells following on from the initial adhesive event mediated by DdCAD-1. In addition to the differences in developmental timing, gp80 and DdCAD-1 mediated contacts can also be distinguished by their sensitivity to EDTA, since DdCAD-1 forms EDTA-sensitive contacts, while gp80 forms EDTA-insensitive contacts (Coates & Harwood, 2001).

Dictyostelium has a large number of Ras proteins, of which six (RasG, RasC, RasD, RasB, RasS and Rap1) have been partially characterized (Weeks & Spiegelman, 2003). A role for the RasG signalling pathway in early development has been previously suggested by the findings that the expression of a constitutively activated version of RasG, RasG(G12T), blocks development (Khosla *et al.*, 1996) and the initiation of development of *rasG* null cells is delayed (Tuxworth *et al.*, 1997). This effect appears to be at least partially due to the role of RasG in regulating cAMP production, with both cells expressing RasG(G12T) and lacking *rasG* showing defects in this essential process (Khosla *et al.*, 1996; P. Bolourani &

G. Weeks, unpublished observations). In addition, RasG is activated in response to cAMP stimulation (Kae *et al.*, 2004) and it influences the expression of the *discoidin* gene (Secko *et al.*, 2001), a known molecular marker of the transition from growth to development (Zeng *et al.*, 2000; Primpke *et al.*, 2000). Recently, we reported the identification of proteins whose phosphorylation state was altered by the ectopic expression of RasG(G12T) (Secko *et al.*, 2004). One of the phosphoproteins identified was DdCAD-1. This identification raises an important question: does DdCAD-1 phosphorylation regulate DdCAD-1-mediated cell adhesion? To address this question, we have analysed DdCAD-1-mediated adhesion in a strain expressing RasG(G12T). These studies suggest that the dephosphorylation of DdCAD-1 is affected by the expression of RasG and thereby influences *Dictyostelium* cell adhesion.

METHODS

***Dictyostelium* strains and growth conditions.** *Dictyostelium discoideum* strains were grown in HL5 medium (Watts & Ashworth, 1970) in rotary-agitated suspension (175 r.p.m.) at 22 °C. The generation of the Ax2::MB, Ax2::MB-*rasG*(G12T), *rasG* null (IR17) and *cadA* null strains have been described previously (Secko *et al.*, 2004; Tuxworth *et al.*, 1997; Wong *et al.*, 2002). Ax2::MB and Ax2::MB-*rasG*(G12T) transformants were maintained and grown in 10 µg G418 ml⁻¹, 5 µg blasticidin 5 ml⁻¹ and 5 µg ml tetracycline ml⁻¹. For RasG(G12T) induction, cells were centrifuged at 700 g for 4 min, washed three times in HL5 medium and then resuspended in HL5 medium containing no tetracycline (Secko *et al.*, 2004). Strains were incubated in rotary-agitated suspension (175 r.p.m.) at 22 °C during the induction period.

Electrophoretic and immunoblot analysis. One-dimensional (1D) SDS-PAGE and immunoblot analysis of fractionated proteins was performed as reported previously (Secko *et al.*, 2001), except that 180 × 160 × 1 mm SDS-polyacrylamide gels were used. These gels were run at a constant current of 25 mA per gel for 3 h with cooling. Immunoblots were scanned using a UMAX-II scanner and densitometry was then performed using GeneQuant Analysis software (Molecular Dynamics). Two-dimensional (2D) electrophoresis and immunoblot analysis were performed as reported previously (Secko *et al.*, 2004), except that 7 cm, pH 3–10, ready-to-use Immobiline DryStrips were rehydrated overnight in the presence of 100 µg total protein solution. Antibodies used were as follows: phosphothreonine polyclonal antibody (1:1000 dilution) (Cell Signalling Technology, cat. #9381), DdCAD-1 specific polyclonal antibody (1:3000 dilution) (Sesaki & Siu, 1996) and RasG- and RasC-specific polyclonal antibodies (1:2000 dilution) (Khosla *et al.*, 1994; Lim *et al.*, 2001).

Cell cohesion assays. Cell cohesion assays were performed as described by Wong *et al.* (2002). To determine developmental cell cohesion, vegetative cells were centrifuged at 700 g for 4 min, washed in KK2, resuspended in KK2 at 2 × 10⁷ cells ml⁻¹ and starved in rotary-agitated suspension (175 r.p.m.) at 22 °C for 4 h. The cell suspension was diluted to a density of approximately 2.5 × 10⁶ cells ml⁻¹ and aggregates were dispersed by vigorously vortexing for 15 s. Aggregates were allowed to reform while rotating on a platform shaker at 180 r.p.m. at room temperature. At the indicated times, the number of non-aggregated cells, including singlets and doublets, were scored using a haemocytometer and the number of aggregating cells was determined by subtracting this number from the total number of cells and was expressed as a percentage of the total.

Vegetative cell cohesion was measured following incubation in HL5 medium alone or HL5 medium supplemented with either 10 mM EDTA or 50 µg DdCAD-1-specific antibodies ml⁻¹ (Sesaki & Siu, 1996). After the indicated times, cells were placed in Nunc tissue culture dishes, viewed using an Olympus CK inverted microscope and photographed with a DAGE-100 CCD camera (Dage Corp.).

Indirect immunofluorescence. Cells were deposited on No. 1 glass cover slips (VWR Scientific) and allowed to adhere for 10 min in HL5 medium. Once adhered, the medium was removed and the cells were gently washed with KK2 to remove the remaining medium. Cells were fixed in 4% formaldehyde in KK2 for 15 min at room temperature. When cells were to be permeabilized, the formaldehyde was removed and cold methanol (−20 °C) containing 1% formaldehyde was added for 5 min (Fukui *et al.*, 1987). Non-specific binding was blocked with 1% (w/v) BSA in PBS for 10 min, prior to incubation with the DdCAD-1 antiserum (1:400 dilution in PBS containing 1% BSA) for 1 h (Sesaki & Siu, 1996). Samples were washed three times in PBS containing 0.05% Tween 20, stained with 1:200 FITC-conjugated goat anti-rabbit IgG (Jackson Laboratories) for 1 h and then washed three times in PBS containing 0.05% Tween 20. The cover slips were mounted in Prolong Antifade mounting medium (Molecular Probes) on Premium microscope slides (Fisher) and images were acquired using a Zeiss Axioplan fluorescent microscope equipped with ×60 objective lenses using a DVC camera (Diagnostic Instruments) and Northern Eclipse imaging software. The specificity of DdCAD-1 antiserum for DdCAD-1 has been confirmed previously (Sesaki & Siu, 1996).

RESULTS

DdCAD-1 is a RasG-regulated phosphoprotein

MS/MS protein sequencing in combination with 2D immunoblot analysis and an anti-phosphothreonine antibody previously identified three proteins with amino acid sequences identical to DdCAD-1 (Secko *et al.*, 2004). The relative levels of these three phosphoproteins was reduced in response to the ectopic expression of constitutively activated RasG, RasG(G12T), from a tetracycline-repressible promoter (Secko *et al.*, 2004). The availability of a strain in which the DdCAD-1-encoding gene (*cadA*) had been disrupted (Wong *et al.*, 2002) provided an opportunity to confirm that the three phosphoproteins were in fact DdCAD-1. Total protein samples from the *cadA* null strain (*cadA*[−]), along with a control Ax2 strain (Ax2::MB) and a strain expressing RasG(G12T) [Ax2::MB-*rasG*(G12T)], were subjected to 2D immunoblot analysis. The three phosphothreonine-reacting spots that had been characterized as DdCAD-1 were all present in Ax2::MB (Secko *et al.*, 2004), but were absent from the *cadA* null strain (Fig. 1A). The corresponding silver-stained protein spots were also absent from the *cadA* null strain (Fig. 1B). Together these results support the conclusion that the phosphothreonine-reacting spots were phosphorylated DdCAD-1.

As previously reported, ectopic expression of RasG(G12T) decreased the overall phosphorylation level of the DdCAD-1 protein detected with the phosphothreonine antibody. This reduction could be interpreted in two ways: (i) as changes in activity of kinases or phosphatases in the cell that result in altered phosphorylation, or (ii) changes in the total protein

Fig. 1. Phosphorylated and total levels of DdCAD-1. (A, B) Total protein (100 µg) from Ax2::MB, Ax2::MB-rasG(G12T) and *cadA* null cells was fractionated by 2D electrophoresis and blotted membranes were probed with anti-phosphothreonine antibody to detect phosphoproteins and silver-stained to detect total protein. The section of the 2D immunoblots and silver-stained 2D gels known to contain the s55, s56 and s57 immunospots, identified previously as DdCAD-1, is shown. Additional silver-stained spots (a, b, c, d) are labelled for reference in (B). Molecular mass (kDa) markers and pI ranges are indicated. (C) Total protein from Ax2::MB (1) and Ax2::MB-rasG(G12T) (2) cells 3 h after removal of tetracycline was fractionated by 1D electrophoresis. DdCAD-1, RasG and RasC proteins levels were detected by immunoblotting with specific antibodies to each of the proteins as described in Methods. Molecular mass markers (kDa) are indicated.

level of these targets. To distinguish between these possibilities, Ax2::MB and Ax2::MB-rasG(G12T) cells were sampled after 3 h induction and lysed directly in SDS-PAGE lysis buffer. Ten micrograms of protein was fractionated by 1D SDS-PAGE, transferred to a PVDF membrane and subjected to immunoblot analysis (Fig. 1C). The levels of DdCAD-1, detected with a DdCAD-1-specific antibody (Sesaki & Siu, 1996), remained constant. The level of RasC, a protein that was not expected to be affected by RasG(G12T) induction, also showed no change (Fig. 1C) and, as anticipated from a previous study (Secko *et al.*, 2004), the level of RasG protein increased (Fig. 1C). These results indicate that the total level of DdCAD-1 did not change during RasG(G12T) induction and confirmed that the observed changes in the phosphothreonine immunoblots were due to decreases in protein phosphorylation.

RasG(G12T) expression results in vegetative cell clumping

Vegetative *Dictyostelium* cells are normally not cohesive when grown in shaking suspension in HL5 medium and do not start to adhere to each other until starvation occurs. DdCAD-1 is involved in this initial adhesion (Sesaki & Siu, 1996). When Ax2::MB and Ax2::MB-rasG(G12T) cells were grown in shaking suspension in the presence of 5 µg tetracycline ml⁻¹, they grew as individual cells and were not cohesive (Fig. 2). In contrast, when Ax2::MB-rasG(G12T) cells were grown in the absence of tetracycline to allow the induction of RasG(G12T) expression, some cells formed clumps, whereas, Ax2::MB cells grown in the absence of tetracycline did not clump (Fig. 2). These results showed that RasG(G12T) expression, rather than simply the removal

Fig. 2. Induction of cell cohesion during vegetative growth in response to RasG(G12T) expression. Ax2::MB and Ax2::MB-rasG(G12T) cells, grown in HL5 medium in the presence of 5 µg tetracycline ml⁻¹ (+Tet) or the absence of tetracycline (-Tet) for 3 h, were photographed through an Olympus CK inverted microscope. The arrow indicates an example of the cell-to-cell cohesion observed in the -Tet Ax2::MB-rasG(G12T) cell cultures. Bar, 100 µm.

of tetracycline, induced cell-to-cell adhesion. The finding that RasG(G12T) expression was associated with cell cohesion in vegetative cells suggested the possibility that the activation of RasG signalling prematurely induced cell adhesion through the dephosphorylation of DdCAD-1.

To test whether the RasG(G12T)-induced cell clumping was mediated by DdCAD-1, two properties of the adhesion were examined. DdCAD-1 adhesion is inhibited by calcium chelators like EDTA (Wong *et al.*, 1996) and by DdCAD-1-specific antibodies (Brar & Siu, 1993). Ax2::MB and Ax2::MB-rasG(G12T) cells were grown for 3 h in the absence of tetracycline to allow the induction of RasG(G12T), and in the presence of either 10 mM EDTA or 50 µg DdCAD-1 antibodies ml⁻¹. The addition of EDTA or the DdCAD-1 antibodies prevented Ax2::MB-rasG(G12T) cells from adhering (Fig. 3), whereas cells incubated in the absence of EDTA or antibody formed clumps as before. These results are consistent with the idea that the observed vegetative cell-to-cell adhesion induced by RasG(G12T) expression is mediated by DdCAD-1.

DdCAD-1 is localized at the cell surface in response to the expression of RasG(G12T)

During vegetative growth of wild-type *Dictyostelium* cells in axenic medium, DdCAD-1 is localized in the cytoplasm and the absence of DdCAD-1 at the cell surface may explain why vegetative cells are not adhesive (Sesaki & Siu, 1996; Sesaki *et al.*, 1997). During development, as cells become more adhesive, some DdCAD-1 becomes localized at the cell surface (Sesaki & Siu, 1996; Sesaki *et al.*, 1997). The observation that cells expressing RasG(G12T) tended to be adhesive during vegetative growth raised the possibility that DdCAD-1 was localized at the surface upon expression of activated RasG.

Indirect immunofluorescence microscopy was used with DdCAD-1-specific antibodies (Sesaki & Siu, 1996) to

determine if DdCAD-1 was localized at the surface of induced Ax2::MB-rasG(G12T) vegetative cells (Fig. 4). To restrict the staining to DdCAD-1 localized at the cell surface, indirect immunofluorescence microscopy was undertaken using DdCAD-1 antibodies with non-permeabilized cells. Under these conditions, vegetative Ax2::MB cells were largely unstained (Fig. 4), consistent with the finding that DdCAD-1 is localized predominately in the cytoplasm in these cells (Sesaki & Siu, 1996). In contrast, strongly stained immunofluorescence structures that often formed rings (Fig. 4, arrows) were observed for the vegetative Ax2::MB-rasG(G12T) cells, confirming a surface localization of DdCAD-1. Thus, the expression of RasG(G12T) resulted in the movement of some of the DdCAD-1 to the cell surface, providing an explanation as to why the vegetative Ax2::MB-rasG(G12T) cells were more cohesive than the control cells.

Cell adhesion during early development is enhanced in RasG(G12T)-expressing cells

The kinetics of cell cohesion for Ax2::MB-rasG(G12T) and Ax2::MB cells were determined using a standard cell cohesion assay (Lam *et al.*, 1981; Wong *et al.*, 2002). Cells were washed free of tetracycline and starved for 4 h, a time when only EDTA-sensitive DdCAD-1-dependent cell adhesion is occurring (Wong *et al.*, 2002). The cells were dissociated by vigorous vortexing to break up existing aggregates and then allowed to reassociate in rolling tubes (Wong *et al.*, 2002). Some reassociation of the Ax2::MB cells was detected after 20 min (Fig. 5). The reassociation of induced Ax2::MB-rasG(G12T) cells was much more rapid, with significant adhesion occurring within 5 min (Fig. 5). The addition of 10 mM EDTA during the assay completely prevented reassociation of both sets of cells, indicating that the observed reassociation was in fact due to EDTA-sensitive adhesion (Fig. 5). These results indicate that cells expressing

Fig. 3. Disruption of cell cohesion with inhibitors of DdCAD-1-mediated adhesion. Ax2::MB and Ax2::MB-rasG(G12T) cells were washed and incubated for 3 h in tetracycline-free medium with no addition (A), 1 mM EDTA (B) or 50 µg anti-DdCAD-1 antibodies ml⁻¹ (C). The cells were placed on Nunc tissue culture dishes, viewed using an Olympus CK inverted microscope and photographed. Bar, 100 µm.

RasG(G12T) exhibit enhanced DdCAD-1-mediated adhesion during early development.

DdCAD-1 phosphorylation is decreased during *Dictyostelium* development

Since cells expressing activated RasG were more cohesive and exhibited decreased DdCAD-1 phosphorylation, there could be an inverse relationship between DdCAD-1 phosphorylation and DdCAD-1-mediated cell cohesion. Since wild-type cells exhibit increased DdCAD-1-mediated cell

adhesion as development proceeds (Sesaki & Siu, 1996; Sesaki *et al.*, 1997) and adhesion appeared to be related to phosphorylation, we determined whether DdCAD-1 phosphorylation decreased during development. We used a 1D SDS-PAGE analysis to facilitate the monitoring of multiple samples and to allow a kinetic analysis. *cadaA* null cells were included in the analysis as a control for other phosphothreonine-containing proteins that might co-migrate with DdCAD-1 in the 1D fractionation. A low intensity band, which increased during the development of the *cadaA* null cells, was found to migrate in the same position as

Fig. 4. Immunofluorescent localization of DdCAD-1. Ax2::MB and Ax2::MB-rasG(G12T) cells were grown in the absence of tetracycline for 3 h. Cells were then fixed without permeabilization before being immunostained by indirect immunofluorescence with anti-DdCAD-1 antibodies as described in Methods. The cells were viewed using a Zeiss Axioplan fluorescent microscope and photographed. Bar, 5 µm.

Fig. 5. DdCAD-1 dependent cohesion of developmental cells. Ax2::MB and Ax2::MB-rasG(G12T) cells were washed free of tetracycline and starved for 4 h in KK2 in a shaking suspension. Cells were then dissociated by vortexing, and cell reassociation was monitored over time with or without the addition of 10 mM EDTA. Cells were not in aggregates at time zero and the percentage of cell reassociation was calculated by scoring non-aggregated cells with a haemocytometer [percentage aggregation = (total number of cells – non-aggregating cells)/total number of cells]. Data represent the mean of three independent experiments $\pm$ SD.

DdCAD-1 (Fig. 6A) and the relative densities of the bands were therefore quantified by densitometry so that the contribution of the non-DdCAD-1 signal could be subtracted. There were relatively constant levels of phosphorylated DdCAD-1 during the first 4 h of development of the Ax2::MB cells and then levels decreased about fourfold after 6 h (Fig. 6B).

RasG is required for maximum expression of DdCAD-1

Since the expression of constitutively activated RasG resulted in decreased phosphorylation of DdCAD-1, we postulated that increased phosphorylation of DdCAD-1,

and hence reduced DdCAD-1-mediated cohesion, might occur in *rasG* null (*rasG*[−]) cells. The level of DdCAD-1 phosphorylation was therefore measured in vegetative *rasG*[−] cells and compared to the level in the parental cells (Fig. 7). Total protein samples, taken from the *rasG*[−] strain and the control Ax2 strain, were subjected to 2D immunoblot analysis. All three DdCAD-1 phosphoproteins were found in the Ax2 strain as before, but there was a greatly reduced level of phosphorylation in all three DdCAD-1 species in the *rasG*[−] strain (Fig. 7), rather than the anticipated increase. However, the total amount of DdCAD-1 was greatly decreased in *rasG*[−] cells in comparison to Ax2 control cells (Fig. 7B). These results indicate that the decrease in DdCAD-1 phosphorylation in *rasG* null cells is due to a

Fig. 6. Levels of DdCAD-1 phosphorylation during development. Ax2::MB and *cadA*[−] cells were starved in KK2 in a shaking suspension in the absence of tetracycline. (A) At the indicated times, protein samples were fractionated by 1D electrophoresis and immunoblots were probed with anti-phosphothreonine antibodies to detect phosphorylated DdCAD-1. The filled arrowhead shows the position of phosphorylated DdCAD-1. Blots were then stripped of bound antibody and reprobed with anti-DdCAD-1 antibodies to detect total DdCAD-1 levels (open arrow). Molecular mass (kDa) markers are indicated. (B) The immunoblots probed with anti-phosphothreonine antibodies shown in (A) were quantified by densitometric analysis. The non-DdCAD-1 phosphothreonine-stained material that co-fractionated with DdCAD-1 in the *cadA* null cells was subtracted from the values for Ax2::MB and normalized relative to amount of phosphorylated DdCAD-1 in Ax2::MB cells at 0 h. Data from three independent experiments are shown.

Fig. 7. Levels of total and phosphorylated DdCAD-1 in a *rasG* null and Ax2 control cells. (A) Total protein from a *rasG*⁻ strain and Ax2 was fractionated by 2D electrophoresis. Blotted membranes were probed with anti-phosphothreonine antibody to detect phosphoproteins. The section of the 2D immunoblot blot known to contain DdCAD-1 is shown (a=DdCAD-1a, b=DdCAD-1b, c=DdCAD-1c). (B) Total protein from induced Ax2::MB (lane 1), induced Ax2::MB-*rasG*(G12T) (lane 2), *rasG*⁻ (lane 3) and *cadA*⁻ (lane 4) cells were fractionated by 1D electrophoresis and blotted with a DdCAD-1-specific antibody. Molecular mass (kDa) markers and pI ranges are indicated.

decrease in DdCAD-1 protein level, suggesting that RasG is an important regulator of DdCAD-1 expression during vegetative growth.

DISCUSSION

In this study, we have confirmed that the phosphorylated molecules, tentatively identified as DdCAD-1 species (Secko *et al.*, 2004), are in fact DdCAD-1 since they are absent in a *cadA* null cell line. Although DdCAD-1 has long been implicated as a major adhesion molecule during early development (Coates & Harwood, 2001), its phosphorylation has not been previously described. Since DdCAD-1 was resolved into at least three spots by 2D electrophoresis, it is likely that it is phosphorylated at more than one site. In addition, since the separated components were all detected with a phosphothreonine-specific antibody, each must contain a phosphorylated threonine residue, although the pI shift might be due to modification of other amino acid residues. Since total DdCAD-1 protein levels were not significantly different between cells expressing RasG(G12T) and control cells (Fig. 1B), the changes in DdCAD-1

phosphorylation were not a result of changes in protein level. These results suggest the existence of phosphatases and/or kinases that act on DdCAD-1, which are also among the downstream effectors of RasG.

DdCAD-1-mediated adhesion is known to be homophilic (i.e. binding is through DdCAD-1/DdCAD-1 interactions) and requires Ca²⁺ (Brar & Siu, 1993; Wong *et al.*, 1996). The work reported here suggests that DdCAD-1 is also regulated by phosphorylation. Initial insight into the effect of phosphorylation on DdCAD-1 function came from the observation that cells expressing RasG(G12T) became cohesive during vegetative growth (Fig. 5). Constitutive gp80 overexpression in vegetative cells has been found to induce EDTA-stable contacts in suspension (Faix *et al.*, 1992). However, the RasG(G12T)-induced cohesion was EDTA-sensitive and was blocked with DdCAD-1 antibodies, indicating that it was mediated by DdCAD-1. The fact that the RasG(G12T)-expressing cells were more cohesive and contained less phosphorylated DdCAD-1 suggested the possibility that dephosphorylation of DdCAD-1 was important in facilitating DdCAD-1-mediated adherence. An important regulatory role for phosphorylation in mammalian cell adhesion has been documented (Lickert *et al.*, 2000; Lilien *et al.*, 2002; Nelson & Nusse, 2004), but this is the first time such a role has been implicated in *Dictyostelium* cell adhesion.

Bacterially grown cells do not express DdCAD-1 until the onset of starvation (Knecht *et al.*, 1987), but axenically grown vegetative cells express high levels of DdCAD-1. These latter cells are not adhesive, because the protein is sequestered in the cytoplasm (Brar & Siu, 1993; Sesaki & Siu, 1996). Axenically grown cells only became adhesive after the onset of starvation and this correlated with the movement of DdCAD-1 to the cell surface. In contrast, axenically grown, RasG(G12T)-expressing vegetative cells were already cohesive and this correlated with the presence of DdCAD-1 at the cell surface in ring-like structures (Fig. 4), suggesting that a RasG(G12T)-dependent signal regulated the movement of DdCAD-1.

Transport of DdCAD-1 to the cell surface has been shown to occur via a non-classical pathway involving contractile vacuoles (Sesaki *et al.*, 1997). Sesaki *et al.* (1997) have suggested that this occurs by at least four steps, including (1) DdCAD-1 accumulation into contractile vacuoles, (2) the binding of DdCAD-1 to the contractile vacuole membrane, (3) the association of DdCAD-1 with an 'anchoring' protein on the luminal surface of the vacuoles, and (4) the fusion of a contractile vacuole with the plasma membrane followed by the subsequent dispersal of DdCAD-1 across the surface of the cell. Our data suggest that RasG(G12T) may affect this transport pathway. This possibility is supported by the fact that, after 3 h of development, DdCAD-1-filled contractile vacuoles appear as ring-like structures when merging with the plasma membrane (Sesaki *et al.*, 1997). These ring-like structures bear a distinct similarity to the immunofluorescence staining in RasG(G12T)-expressing vegetative cells,

which is often concentrated into rings (Fig. 4, arrows). Since these structures are not seen until 3 h of development in wild-type cells (Sesaki *et al.*, 1997), this further suggests that RasG(G12T) is prematurely activating this pathway. However, it has yet to be established what role phosphorylation may play in this process and, as such, it is not yet clear whether the phosphorylation of DdCAD-1 regulates its localization or, conversely, whether the localization of DdCAD-1 regulates its phosphorylation.

We previously found that *rasG* null cells express lower levels of discoidin, a protein normally induced at the onset of development (Secko *et al.*, 2001). We have now demonstrated that *rasG* null cells have considerably lower levels of the cell adhesion protein DdCAD-1 (Fig. 7). This observation came as something of a surprise based on the fact that the expression of RasG(G12T) did not affect DdCAD-1 protein levels under the short-term induction conditions used here. However, the observed decrease in total levels of DdCAD-1 was much larger than the decrease observed for DdCAD-1 phosphorylation in these cells (see Fig. 7A versus 7B), which argues that, at least qualitatively, DdCAD-1 is more phosphorylated in the *rasG* null cells. The decrease in levels of both discoidin and DdCAD-1 in *rasG* null cells also suggests that RasG signalling may play a role in protein expression during early development.

DdCAD-1 shares limited homology with the extracellular domain of metazoan cadherins; in particular, the two middle domains of DdCAD-1 (aa 49–146) share 28% identity with the first extracellular repeat of E-cadherin, as well as similarities in their Ca^{2+} -binding site (Wong *et al.*, 1996). Cadherins are involved in cell–cell adhesion and the formation of adherence junctions (Nelson & Nusse, 2004). Metazoan cadherins form complexes with catenins to allow their linkage to the actin cytoskeleton (Jamora & Fuchs, 2002) and the stability of these complexes is regulated by phosphorylation. Tyrosine phosphorylation of β -catenin by Src or Fer results in the disruption of cadherin/ β -catenin complexes and decreased adhesion (Lilien *et al.*, 2002; Nelson & Nusse, 2004). An unidentified transmembrane linker has been postulated to bind to DdCAD-1 (Sesaki *et al.*, 1997); however, the proteins that interact with DdCAD-1 are currently not known. It is possible that DdCAD-1 phosphorylation performs a similar function. It should be noted that cadherin/ β -catenin complexes are phosphorylated on serine/threonine residues, which results in increased stabilization of the complexes (Lickert *et al.*, 2000). Since DdCAD-1 also appears to be phosphorylated on several residues (Fig. 1), its regulation by phosphorylation could be equally complex. It must also be pointed out that DdCAD-1 is not a transmembrane protein and thus its mode of adhesion could therefore be distinct from systems described in mammalian cells. Nonetheless, it will be of interest to determine the pathway leading from RasG activation to DdCAD-1 dephosphorylation, as this will not only help in our understanding of *Dictyostelium* development, but could produce insights into the regulation of metazoan cell–cell adhesion.

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