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

David M. Secko,¹ Chi-Hung Siu,² George B. Spiegelman¹ and Gerald Weeks¹

¹Department of Microbiology and Immunology, University of British Columbia, Life Sciences Centre, 2350 Health Sciences Mall, Vancouver, British Columbia V6T 1Z4, Canada
²Banting and Best Department of Medical Research, and Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1L6, Canada

**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 $10^9$ 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 S 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.).

**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., 2002) provided an opportunity to confirm that these three phosphoproteins were in fact DdCAD-1. Total protein detected with the phosphothreonine antibody. This conclusion that the phosphothreonine-reacting spots were RasG(G12T), with a control Ax2 strain (Ax2::MB), were subjected to 2D immunoblot analysis. The three phosphothreonine-reacting spots that had been characterized as DdCAD-1 were all altered phosphorylation, or (ii) changes in the total protein 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
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
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...
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. cadA 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 cadA null cells, was found to migrate in the same position as DdCAD-1 phosphorylation.

---

**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.

**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 Axiosplan fluorescent microscope and photographed. Bar, 5 µm.
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<sup>-</sup>) cells. The level of DdCAD-1 phosphorylation was therefore measured in vegetative rasG<sup>-</sup> cells and compared to the level in the parental cells (Fig. 7). Total protein samples, taken from the rasG<sup>-</sup> 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<sup>-</sup> strain (Fig. 7), rather than the anticipated increase. However, the total amount of DdCAD-1 was greatly decreased in rasG<sup>-</sup> 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
and control cells (Fig. 1B), the changes in DdCAD-1 residues. Since total DdCAD-1 protein levels were not pI shift might be due to modification of other amino acid contain a phosphorylated threonine residue, although the addition, since the separated components were all detected likely that it is phosphorylated at more than one site. In resolved into at least three spots by 2D electrophoresis, it is tion has not been previously described. Since DdCAD-1 was implicated as a major adhesion molecule during early development (Coates & Harwood, 2001), its phosphoryla- tion of DdCAD-1. 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 Ca^{2+} (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 phos- phorylation on DdCAD-1 function came from the observation that cells expressing RasG(G12T) became cohesive during vegetative growth (Fig. 5). Constitutive gp80 over-expression 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 move- ment 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,

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 Ca^{2+} (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 phos- phorylation on DdCAD-1 function came from the observation that cells expressing RasG(G12T) became cohesive during vegetative growth (Fig. 5). Constitutive gp80 over-expression 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 mamma- lian 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,

![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 pl ranges are indicated.](http://mic.sgmjournals.org)
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 phosphorylation of DdCAD-1 regulates its phosphorylation.

We previously found that rasG null cells express lower levels of discoïdin, 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 discoïdin 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 \(\beta\)-catenin by Src or Fcr results in the disruption of cadherin/\(\beta\)-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/\(\beta\)-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.

ACKNOWLEDGEMENTS

This research was supported by a Canadian Institutes of Health Research Operating Grant to G.W.

REFERENCES


