Evidence for a role for the *Dictyostelium* Rap1 in cell viability and the response to osmotic stress

Rujun Kang, Helmut Kae, Hermia Ip, George B. Spiegelman and Gerald Weeks*

Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, BC V6T 1Z3, Canada
*Author for correspondence (e-mail: gweeks@unixg.ubc.ca)

Accepted 27 June 2002
Journal of Cell Science 115, 3675-3682 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00039

Summary

The *Dictyostelium* genome contains a single *rapA* gene, which encodes a Rap1 monomeric G protein. As attempts at generating *rapA*-null *Dictyostelium* cells had been unsuccessful, expression of antisense RNA from the *rapA* gene under control of the folate repressible discoidin promoter was used to reduce cellular levels of the Rap1 protein. As Rap1 levels gradually decreased following antisense *rapA* RNA induction, growth rate and cell viability also decreased, a result consistent with the idea that *rapA* is an essential gene.

The Rap1-depleted cells exhibited reduced viability in response to osmotic shock. The accumulation of cGMP in response to 0.4 M sorbitol was reduced after *rapA* antisense RNA induction and was enhanced in cells expressing the constitutively activated Rap1(G12V) protein, suggesting a role for Rap1 in the generation of cGMP. *Dictyostelium* Rap1 formed a complex with the Ras-binding domain of RalGDS only when it was in a GTP-bound state. This assay was used to demonstrate that activation of Rap1 in response to 0.4 M sorbitol occurred with initial kinetics similar to those observed for the accumulation of cGMP. Furthermore, the addition of 2 mM EDTA to osmotically shocked cells, a treatment that enhances cGMP accumulation, also enhanced Rap1 activation. These results suggest a direct role for Rap1 in the activation of guanylyl cyclase during the response to hyperosmotic conditions. Rap1 was also activated in response to low temperature but not in response to low osmolarity or high temperature.

Key words: Stress responses, Differentiation, Rap1 activation, Antisense, cGMP, *Dictyostelium*, Viability

Introduction

The mammalian Rap proteins are part of the Ras subfamily of proteins, which exhibit ~50% identity to the Ha-Ras protein (Reuther and Der, 2000). The *rap1* gene was discovered originally by its ability to suppress the malignant phenotype of *ras*-transformed cells (Kitayama et al., 1989), and Rap1 is capable of competitively inhibiting Ras signaling pathways by binding to downstream effectors, suggesting a mechanism for suppression (Cook et al., 1993; Boussiotis et al., 1997; Okada et al., 1998; Hu et al., 1999). However, Rap1 can itself act in growth-factor-induced signaling pathways (Yoshida et al., 1992; Altschuler and Rebeiro-Neto, 1998; Zwartkruis et al., 1998). Rap1 is also involved in B and T cell activation (McLeod et al., 1998; Reedquist and Bos, 1998), platelet activation (Franke et al., 1997), neutrophil activation (M'Rabet et al., 1998), neuronal cell differentiation (Vossler et al., 1997; York et al., 1998) and cyclic-AMP-mediated signaling (DeRooij et al., 1998; Kawasaki et al., 1998). More recently, evidence has been presented to indicate that Rap1 is involved in mammalian cell adhesion and in the inflammatory response (Tsukamoto et al., 1999; Reedquist et al., 2000; Katagiri et al., 2000; Caron et al., 2000). Thus, in mammals Rap1 is capable of many roles depending upon the cell type. Dissecting the various functions of Rap in mammalian cells is complicated by the fact that there are at least four *rap* genes (Reuther and Der, 2000), raising the possibility of redundant or overlapping functions.

In *Drosophila*, there is a single *rap* gene, and loss-of-function mutations are lethal at the larval stage (Hariharan et al., 1991). This lethality can be rescued by expressing *rap* under the control of a heat shock promoter, and cell proliferation was unimpaired but morphogenesis and cell movement were abnormal during development in the absence of Rap1 function (Asha et al., 1999).

In *Dictyostelium*, the available evidence suggests that there is also a single *rap* gene, *rapA*, previously designated *rap1*, encoding a protein, Rap1, 75% identical to mammalian Rap1A (Robbins et al., 1990). The overexpression of *rapA* results in cells with a variety of cytoskeletal defects, including a flattened cell morphology and failure to contract in response to contraction stimuli (Rebstein et al., 1993). In addition, cells overexpressing activated and dominant-negative forms of Rap1 exhibited alterations in phagocytosis and fluid phase endocytosis (Seastone et al., 1999). To provide a more definitive assessment of Rap1 function in *Dictyostelium*, we attempted to disrupt the *rapA* gene by standard procedures. However, these attempts failed, suggesting that *rapA* might be an essential gene in *Dictyostelium*. In the present study, we have expressed a *rapA* antisense construct under the control of the folate repressible discoidin promoter (Blusch et al., 1992) and have examined the effects of Rap1 depletion on cell function. We have also demonstrated that Rap1 is activated in response to hyperosmotic stress and low temperature.

Materials and Methods

Growth of *Dictyostelium discoideum* cells

Dictyostelium Ax-2 cells were grown in HL-5 broth culture, either in shake suspension (Watts and Ashworth, 1970) or in 10 cm Petri dishes at 22°C. To determine growth rates, cell numbers were counted in a hemocytometer. To determine cell viability, *Dictyostelium* amoebae were diluted and plated in association with *Klebsiella oxytoca* on nutrient-rich agar plates (Sussman, 1987) and incubated at 22°C. Under these conditions only viable cells form plaques on the bacterial lawn.

Vector construction and transformation

To create the *pVEII-AS5′*construct, the 5′ portion of the *rapA* gene (nucleotides –15 to +218) was synthesized by PCR using the oligonucleotides 5′-TGCTCTAGAGCTCGAATTCATCATGCC-3′ and 5′-TGCTCTAGAGCAGTAAATTGTTCAGTACG-3′ as primers and *rapA* cDNA (Rebstein et al., 1993) as the template. Both primers contained *Kpn*I sites, and the PCR product was inserted into the *Kpn*I-digested *pVEII* vector. The resulting constructs contained the antisense *rapA* DNA linked to the folate repressible discoidin promoter. The orientation and promoter-gene fusions were confirmed by sequencing.

The *pVEII-AS5′* vector was introduced into Ax-2 cells by electroporation, and single cell transformants were selected in 96-well plates in HL-5 medium supplemented with 10 μg/ml G418 and 1 mM folate. Six *pVEII-AS5′* transformants were obtained, and each was plaque purified on a lawn of *K. oxytoca*. The isolated transformants were cultured on 10 cm Petri dishes in HL-5 growth media, supplemented with 100 μg/ml G418 and 1 mM folate for three days and then maintained on HL-5 media containing 10 μg/ml G418 and 1 mM folate. The cultures were frequently divided into fresh growth media to maintain low cell density.

Cell size determination

Exponentially growing cells were centrifuged at 600 ***g***, washed three times with KK_2 and resuspended at 1×10^6 cells/ml. The cell suspensions were left on ice for 15 minutes to produce isolated, spherical cells and then viewed through a microscope. The cells were photographed and their diameters determined from prints.

Shock conditions

Exponentially growing cells were centrifuged at 600 ***g***, washed three times with KK_2 and resuspended in the same buffer at 3.0×10^7 cells/ml and shaken at 160 rpm for 1 hour at 22°C. To induce hyper-osmotic shock, sorbitol was added to a final concentration of 0.4 M. Aliquots were removed periodically and analyzed for either cell viability, cGMP content or the level of activated Rap1.

To induce hypo-osmotic shock, cells that had been shaken at 22°C for 1 hour in KK_2 were centrifuged and resuspended in ddH_2O. Cells were subjected to temperature shock by placing cells at 8°C for cold shock or 30°C for heat shock. Aliquots were removed at various time points and analyzed for the level of activated Rap1.

cGMP assay

Levels of cGMP were determined essentially as described previously (Oyama, 1996). 100 μl aliquots of cells that had been subjected to osmotic shock were added to 100 μl of 3.5% perchloric acid and the mixture incubated on ice for 30-60 minutes with occasional vigorous shaking. The solution was neutralized by the addition of 50 μl of 50% saturated $KHCO_3$ and the mixture incubated for 60 minutes on ice with occasional vigorous shaking. The suspensions were centrifuged at 14,000 ***g*** for 10 minutes at 4°C and 100 μl of the supernatant was assayed for cGMP content using the Amersham Pharmacia Biotech radioisotope dilution assay.

Western blot analysis

Between 5×10^6 and 1×10^7 cells were washed twice in KK_2, resuspended in 1% SDS, and the protein concentration was determined using the Bio-Rad protein assay. A 20 μg protein aliquot of each sample was mixed with an equal volume of SDS sample buffer (0.5% β-mercaptoethanol; 0.5% SDS; 50 mM Tris-Cl, pH 6.8; 12.5% glycerol, and 0.04% bromophenol blue), boiled for 5 minutes and then fractionated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using 12% gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes for 1 hour and probed with either polyclonal Rap1 antibody (Rebstein et al., 1997) or a monoclonal phosphotyrosine antibody (Ingham et al., 1998). The membranes were incubated for 1 hour at room temperature in TBS-Tween (25 mM Tris-Cl, pH 8.0, 1.0% NaCl, 1% Tween 20) containing 5% powdered milk (Carnation) for Rap1 detection or 4% BSA for phosphotyrosine detection. The Rap1 primary antibodies were diluted 1:2,000 in TBS-Tween containing 0.5% powdered milk and incubated with the nitrocellulose membranes overnight at room temperature. The phosphotyrosine antibodies were diluted 1:200 in TBS-Tween and incubated with the membranes overnight at 4°C. The membranes were washed three times for 5 minutes in TBS-Tween and exposed to a secondary antibody (donkey anti-rabbit IgG conjugated to horseradish peroxidase or donkey anti-mouse IgG conjugated to horseradish peroxidase) diluted 1:10,000 in TBS-Tween. The bound antibodies were detected by an enhanced chemiluminescence assay (Amersham). Blots were scanned using a ScanJet-II scanner (Hewlett-Packard, USA). and densitometry was performed using Image Quant (V.1.2) software for MacIntosh.

Binding of bacterially expressed Rap1 to GST-RalGDS

The mammalian Rap-binding domain (RBD) of RalGDS was expressed in *Escherichia coli* as a GST fusion protein as described previously (McLeod et al., 1998). Exponentially growing cultures of bacteria were induced with 0.1 M IPTG for 16 hours at 22°C. The cells were lysed by sonication for two minutes in 50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% TritonX-100; 1 mg/ml lysozyme and 0.1 mg/ml DNase I. The resulting cell lysate (10-50 μl) was incubated with 10 μl of glutathione-Sepharose beads (Pharmacia) at 4°C for one hour, and the beads were then washed three times with wash buffer (20 mM Tris-Cl, pH 7.6; 150 mM NaCl, 0.1% Triton X-100; 10 μg/ml leupeptin; 1 μg/ml aprotinin and 1 mM PMSF).

Rap1 was also expressed as a GST fusion protein in *E. coli*. The original *rapA* cDNA was complete at the 3′ end, but the 5′ end was truncated at an *Eco*RI site, 12 bp downstream of the ATG translation initiation site of the gene (Robbins et al., 1990). The 5′ end was completed through a single oligonucleotide mutagenesis, which eliminated the internal *Eco*RI site through a conservative change at nucleotide +15 and introduced an *Eco*RI site at the 5′ end of the completed cDNA. The *rapA* construct was treated with *Eco*RI, and the 700 bp fragment was ligated to an *Eco*RI-digested pGEX-1 vector. This *gst-rapA* construct was transformed into *E. coli.* Exponentially growing cultures of bacteria were induced with 0.1 M IPTG for 4 hours at 37°C and the bacteria lysed as described above. Cell lysate (0.5 ml) was mixed with 50 μl of packed glutathione-Sepharose beads at 4°C for 1 hour and the beads then washed three times with wash buffer. The bound GST-Rap1 was treated with 10 units/ml thrombin in 50 mM Tris-HCl (pH 7.6); 150 mM NaCl; 2 mM $CaCl_2$ to hydrolyze the peptide bond between Rap1 and GST, and the beads were removed by centrifugation. The supernatant containing the bacterically expressed Rap1 was concentrated using a Centricon 10 (Amicon).

The Rap1 protein (~1 μg) was incubated with either 1 mM GDP or

1 mM GTP in 20 mM Tris-HCl (pH 7.6); 10 mM EDTA; 5 mM $MgCl_2$; 1 mM DTT (Dithiothreitol); 0.1 mM PMSF and 10% (v/v) glycerol in a total volume of 20 μl for 30 minutes at 30°C, and $MgCl_2$ was then added to a final concentration of 20 mM to stabilize the binding. 210 ng Rap1 protein was then incubated with 100 ng of GST-RalGDS(RBD) bound to glutathione-Sepharose beads in 20 μl of binding buffer containing 100 μg BSA, 100 mM NaCl, 0.5 mM GTP or GDP, 6.5 mM EDTA, 12.5 mM $MgCl_2$. After 2 hours at 4°C, the beads were pelleted and the unbound material in the supernatant was removed. The beads were washed five times with 0.5 ml of ice-cold 10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 1 mM DTT; 0.1 mM PMSF; 10% glycerol and 0.05% Triton X-100. An equal volume of SDS sample buffer was added to both the beads and the unbound fraction. Both fractions were subjected to SDS-PAGE and western blotting as described above.

Binding of native Rap1 to GST-RalGDS

Samples of *Dictyostelium* cells were pelleted and resuspended in 30 mM HEPES (pH 7.8), 10 mM KCl, 10% sucrose, 1% TritonX-100 and protease inhibitor (Roche). The protein concentration was determined using the BioRad protein assay. Cell lysate (5-100 μg protein) was incubated for 1 hour at 4°C with 20 μg GST-RalGDS(RBD) that had been pre-bound to glutathione-Sepharose beads. After three washes with 30 mM HEPES (pH 7.8), 200 mM KCl, the beads were incubated with an equal volume of SDS sample buffer and the solubilized material subjected to SDS-PAGE and western blotting.

Results

The effect of reducing Rap1 levels on cell growth and morphology

All six independently isolated transformants that contained the folate repressible *pVEII-AS5′* construct could be maintained in HL-5 media supplemented with 1 mM folate and 10 μg/ml G418 for as long as two months without loss in viability. However, the transformants grew progressively more slowly upon removal of folate. Fig. 1A shows the growth of one of these transformants, *pVEII-AS5′-1*, following folate removal. Growth was initially normal, but after cells were passaged into fresh media their growth rate declined and growth terminated after ~10 days of incubation. Cell viability started to decline after 4 days of incubation in the absence of folate and by 12 days only 30% of the cells were still viable (Fig. 1B). After 15 days in the absence of folate, no viable cells were detected (data not shown). The growth of cells that had been transformed with the *pVEII* vector in HL-5 media containing 10 μg/ml G418 was identical to that of the parental Ax-2.

Rap1 protein levels in the *pVEII-AS5′-1* transformant were found to gradually decline during growth without folate, reaching ~20% of their initial value by 10 days and being barely detectable beyond 12 days (Fig. 2). These results indicate that the reduction in Rap1 protein level correlated with reduced cell growth. A similar decrease in viability and Rap1 protein level was observed in the two other *pVEII-AS5′* transformants that were examined in detail (data not shown). As a control, samples were also assayed for RasG by western blot analysis, as this protein is another member of the *Dictyostelium* Ras subfamily and any non-specific effect of the *rapA* antisense construct would be expected to be manifested as a drop in RasG level. Only a slight decrease in RasG was observed during the course of the experiment.

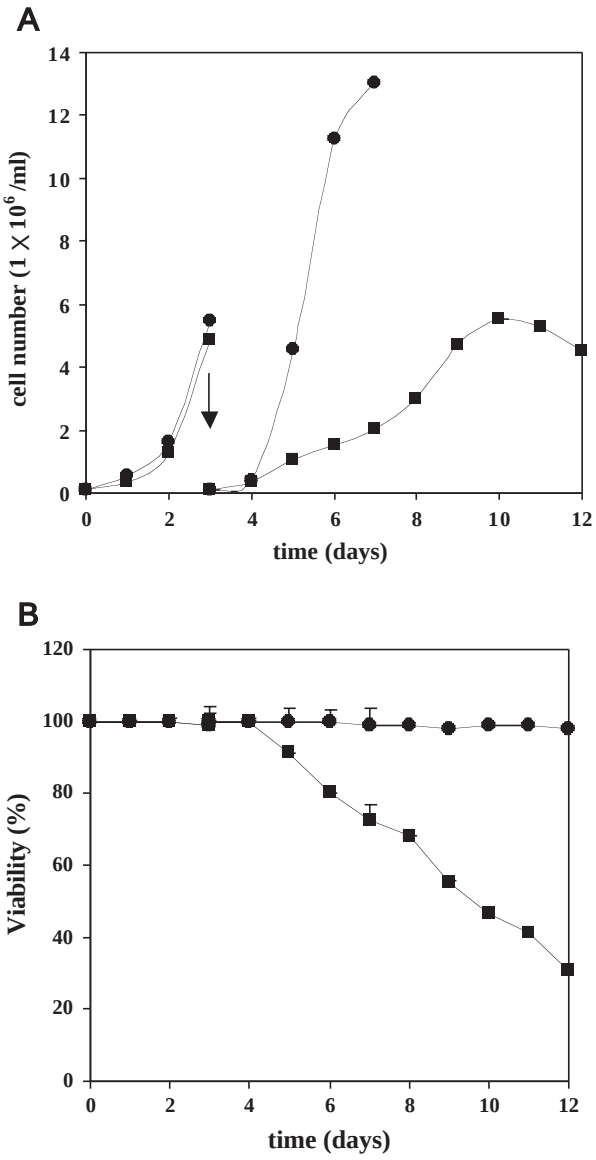

Fig. 1. Growth and viability of the *pVEII-AS5′* transformants. (A) The *pVEII-AS5′*-1 transformant (■) and the Ax-2 parent (●), which were grown in shake suspension HL-5 growth media in the presence of 1 mM folate, were washed and transferred to fresh HL5 media in the absence of folate at a cell density of 1×10^5 cells/ml. After three days of growth in shake suspension, when the population was in late exponential phase at a density of ~5×10^6 cells/ml, it was diluted into fresh HL-5 media to a density of 1×10^5 cells/ml and growth continued. Growth was monitored by hemocytometer counts (●,■). (B) The viability of the *pVEII-AS5′* transformant (■) and Ax-2 (●) was determined by plating on rich nutrient agar plates in association with *Klebsiella oxytoca*. Cell viability is expressed relative to the viability at the start of the experiment and is the mean±s.e. for two experiments.

During the period of declining growth in axenic media, the cells were tested for growth on bacterial lawns. The size of the plaques formed on the bacteria were considerably smaller as the time of depression increased, suggesting that growth on bacteria was also impaired. Both pinocytosis and phagocytosis were considerably reduced in the *pVEII-AS5′-1* transformant

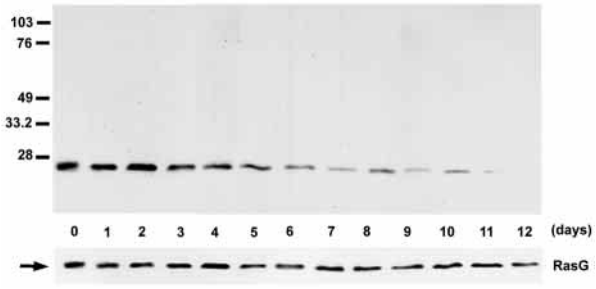

Fig. 2. Rap1 levels during growth of the *pVEII-AS5′-1* transformant in the absence of folate. Cells were harvested at the day indicated following the removal of folate, and the total cellular protein was fractionated by SDS-PAGE. Rap1 levels were determined by western blot analysis using a *Dictyostelium* Rap1-specific antibody. The RasG levels were determined with a RasG-specific antibody.

(data not shown), which may account for the slow growth rate in both axenic media and on bacteria. In addition to the defects in cell growth, the majority of *pVEII-AS5′-1* cells were smaller than the parental Ax-2 cells, although about 5% of the cells were considerably larger (data not shown).

Effect of the reduction in Rap1 level on the response of cells to hyperosmolarity

In view of the effect of Rap1 depletion on cell viability and cell size, we determined the resistance of the Rap1-depleted cells to hyperosmotic conditions. To ensure that the cells used in these tests were viable, we used cells that contained ~40% of the normal level of Rap1. When these partially Rap1-depleted cells were exposed to 0.4 M sorbitol for 120 minutes, only ~15% of the transformant cells survived. By contrast, under identical conditions all wild-type Ax-2 cells remained viable (Fig. 3A).

Dictyostelium cells respond to hyperosmolarity by reducing their cell volume by 50% within 5 minutes (Zischka et al., 1999). This rapid reduction in cell volume correlates with myosin phosphorylation and the activation of guanylyl cyclase (Kuwayama et al., 1996). The importance of the guanylyl cyclase activation in osmoregulation is indicated by the observation that some of the osmosensitive mutants that have been identified are deficient in cGMP production. Furthermore, these mutants become less osmosensitive in the presence of the cell-permeable cGMP analog, 8-Br-cGMP (Kuwayama et al., 1996). We found that cGMP levels were reduced in the *pVEII-AS5′-1* transformant relative to the wildtype following treatment with 0.4 M sorbitol (Fig. 3B), suggesting a possible requirement for Rap1 in the accumulation of cGMP. Furthermore, addition of 8-Br-cGMP to Rap1 depleted cells enhanced their survival following sorbitol treatment (Fig. 3A). These results suggest that Rap1 might be important in regulating the accumulation of cGMP during the response to osmotic shock. Consistent with this idea was the finding that cells overexpressing the constitutively activated Rap1, Rap1(G12V), produced more cGMP in response to sorbitol addition than did wild-type cells (Fig. 3B).

During osmotic shock, there is also a dramatic increase in actin tyrosine phosphorylation (Zischka et al., 1999). The data in Fig. 4 shows that the expected increase in actin tyrosine

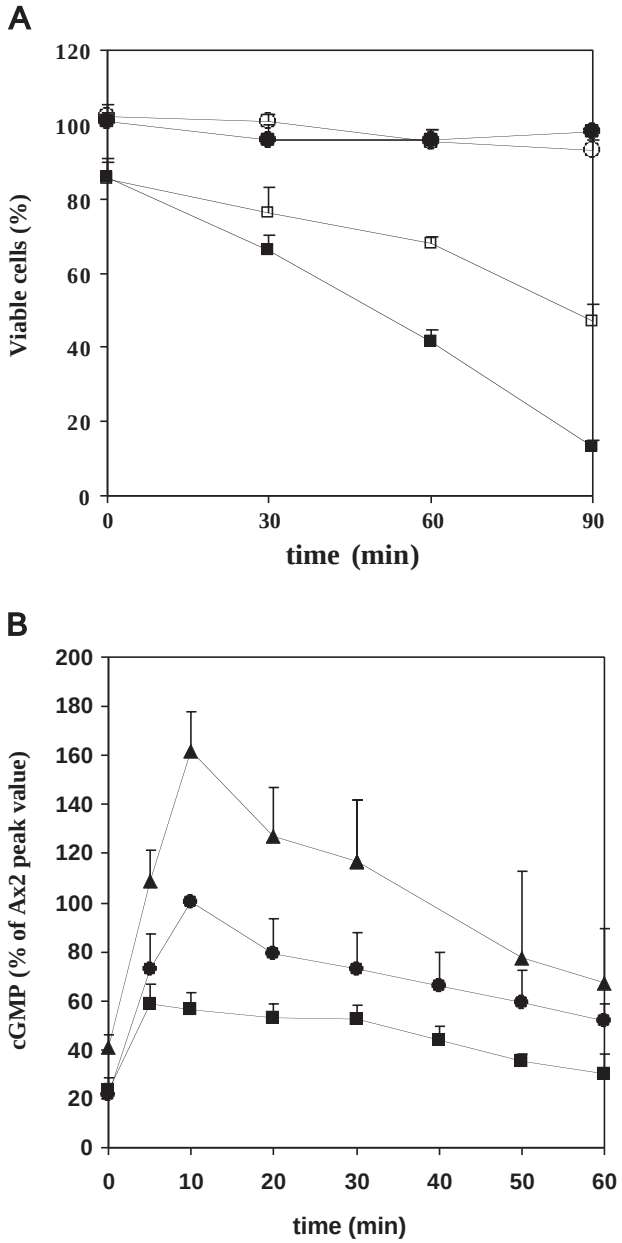

Fig. 3. Response to osmotic shock. (A) Ax-2 (●,○) and *pVEII-AS5′-1* (■,□) cells were shaken in KK_2 buffer containing 0.4 M sorbitol (●,□) or 0.4 M sorbitol and 100 μM 8Br-cAMP (○,■) for the indicated time periods and then diluted and plated in association with *K. oxytoca* to determine cell viability. The number of plaques that were formed on the bacterial lawns were counted are expressed as a percentage of the zero time value and are the means±s.e. for two experiments. (B) Ax-2 (●), *pVEII-AS5′-1* transformant (■) and *pVEII-Rap1 (G12V)* transformant (▲) cells were shaken in KK_2 in the presence of 0.4 M sorbitol for the indicated times. Aliquots were then treated with 3.5% perchloric acid and the amount of cGMP determined. The results are the mean±s.d. of five experiments for Ax-2, three experiments for *pVEII-AS5′* and the mean±s.e. for two experiments for *pVEII-Rap1(G12V)*.

phosphorylation occurred in wild-type cells in response to osmotic shock. Under these conditions, actin is by far the major protein to be tyrosine phosphorylated (Zischka et al., 1999).

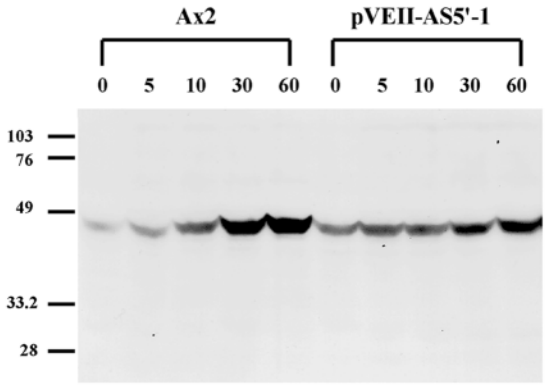

Fig. 4. Tyrosine phosphorylation of actin. Ax-2 and *pVEII-AS5´-1* cells were shaken in KK_2 buffer in the presence or absence of 0.4 M sorbital for the indicated times and lysed in 1% SDS buffer containing 100 mM Na_2VO_4. The cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a phosphotyrosine-specific monoclonal antibody.

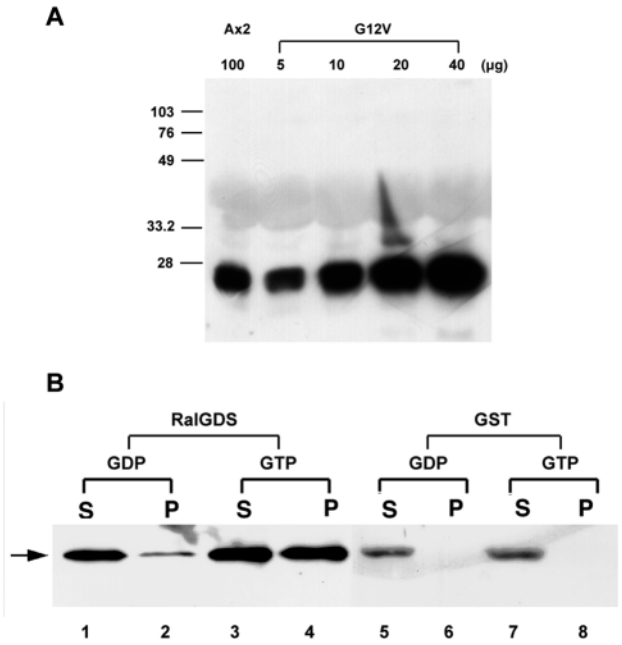

Fig. 5. GST-RalGDS(RBD) binds to the activated form of Rap1. (A) Ax-2 and *pVEII-Rap1 (G12V)* transformant cells were lysed, and the indicated amounts of cell lysate were incubated with 20 µg GST-RalGDS(RBD) that had been precoupled to glutathione-Sepharose beads for 1 hour at 4°C. The beads were pelleted by centrifugation, and the bound Rap1 was released by adding SDS sample buffer. The samples were fractionated by SDS-PAGE, and Rap1 was detected by western blotting using the *Dictyostelium* Rap1-specific antibody. (B) Bacterially expressed Rap1 was pre-equilibrated with 1 mM GDP (lanes 1, 2, 5 and 6) or 1 mM GTP (lanes 3, 4, 7 and 8) and incubated with GST-RalGDS(RBD) bound to glutathione-Sepharose beads (lanes 1-4) or GST bound to glutathione-Sepharose beads (lanes 5-8). The Sepharose beads were pelleted by centrifugation, and both the bound Rap1 in the pellets (lanes 2, 4, 6 and 8) and the unbound Rap1 in the supernatants (lanes 1, 3, 5 and 7) were fractionated by SDS-PAGE and detected by western blotting.

However, in *pVEII-AS5´-1* cells the basal pre-shock level of actin tyrosine phosphorylation was considerably higher than the pre-shock level in wild-type cells, and actin phosphorylation increased only slightly after osmotic shock, suggesting actin tyrosine phosphorylation was deregulated in Rap1-depleted cells.

RalGDS binds specifically to activated Rap1

To ascertain if Rap1 is activated in response to hyperosmolarity, we adopted the GST-RalGDS(RBD) pull-down assay that had been used to determine Rap1 activation in mammalian cells (Franke et al., 1997). The rationale for this approach was the fact that *Dictyostelium* Rap1 and mammalian Rap1 have identical effector domains (Robbins et al., 1990). To demonstrate that RalGDS(RBD) binds specifically to the activated *Dictyostelium* Rap1, we compared the binding of bacterially expressed GST-RalGDS(RBD) fusion protein to Rap1 in lysates of wild type cells with Rap1 in lysates of cells overexpressing the constitutively activated Rap1(G12V). As shown in Fig. 5A, there was appreciably more RalGDS(RBD)-bound Rap1 in the lysates from the *rap1(G12V)* transformant than in the lysate from the wild-type cells relative to the total amounts of Rap1 present in these extracts. These results indicated that RalGDS(RBD) preferentially bound to activated Rap1.

To determine if there was any binding of RalGDS(RBD) to GDP-bound Rap1, bacterially expressed Rap1 was equilibrated with 1 mM GTP or 1 mM GDP prior to binding to RalGDS(RBD). The proportion of Rap1 that binds to RalGDS(RBD) is high in the presence of GTP but low in the presence of GDP (Fig. 5B). Rap1 did not bind to the control GST protein. Hence, interaction with mammalian RalGDS (RBD) is clearly a good measure of the amount of activated Rap1 in the *Dictyostelium* cell.

Rap1 activation in response to hyperosmotic stress

Vegetative Ax-2 cells were exposed to 0.4 M sorbitol, and cell lysates were incubated with GST-RalGDS(RBD) bound to glutathione-Sepharose beads. As shown in Fig. 6, the amount of Rap1 bound to RalGDS(RBD) increased within 5 minutes of exposure to 0.4 M sorbitol. The extent of Rap1 activation was reproducibly three-to-four-fold, and there was a slight but consistent decrease in bound Rap1 during the next 5 minutes (Figs 6 and 7). Levels of bound Rap1 increased again as the sorbitol shock continued (Figs 6 and 7). The initial kinetics of Rap1 activation correlated reasonably well with the increase in the cGMP level (Fig. 3B), a result consistent with the possibility that Rap1 regulates the pathway that leads to the activation of guanylyl cyclase. Treatment of cells with 2 mM EDTA enhances the cGMP response to osmotic shock (Oyama, 1996), and we found that treatment of wild-type cells with 2 mM EDTA prior to osmotic shock also produced an enhanced activation of Rap1 (Fig. 7). The addition of EDTA alone had no effect on Rap1 activation (data not shown).

Rap1 is activated by cold stress but not by heat or hypo-osmotic stress

To determine whether Rap1 activation in response to hyper-osmotic conditions was a general or specific response to shock,

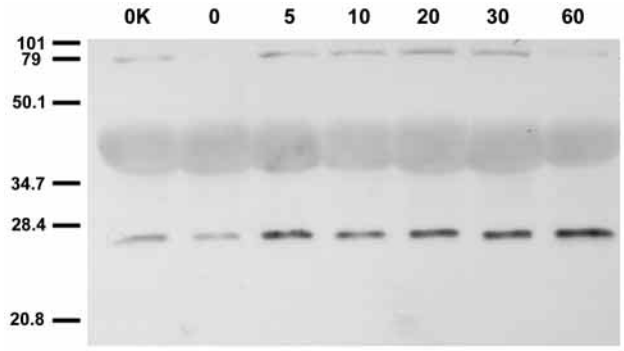

Fig. 6. Rap1 activation in response to hyperosmolarity. Ax-2 cells were withdrawn at the indicated times (lanes 0, 5, 10, 20, 30 and 60 minutes) after the addition of 0.4 M sorbitol and lysed. Cell lysate (100 μg) was incubated with GST-RalGDS(RBD)-glutathione-Sepharose beads, and the amounts of bound Rap1 were determined by western blotting. A sample was also taken immediately following the wash procedure, that is, immediately before the 1 hour adaptation in KK_2 prior to sorbitol addition (lane 0K).

vegetative Ax-2 cells were subjected to three additional stress conditions: low temperature, high temperature and hypo-osmotic conditions. When cells were switched from 22°C to 8°C, Rap1 activation increased after approximately 5 minutes, and the level of activation remained high for 20 minutes (Fig. 8). However, neither a switch to 30°C or resuspension in H_2O had a noticeable effect on the level of activated Rap1 (Fig. 8), indicating that activation of Rap1 is not part of a general stress response.

Discussion

Since previous attempts at isolating *Dictyostelium* cells with a disrupted *rapA* gene were unsuccessful (P. J. Rebstein, PhD thesis, University of British Columbia, 1996) (X. Insall, personal communication), we expressed the antisense RNA from the *rapA* gene to reduce the cell content of Rap1. The data presented here are consistent with the idea that Rap1 is essential. As levels of Rap1 in a *pVEII-AS5′* transformant were gradually reduced, there was a decrease in growth rate and cell

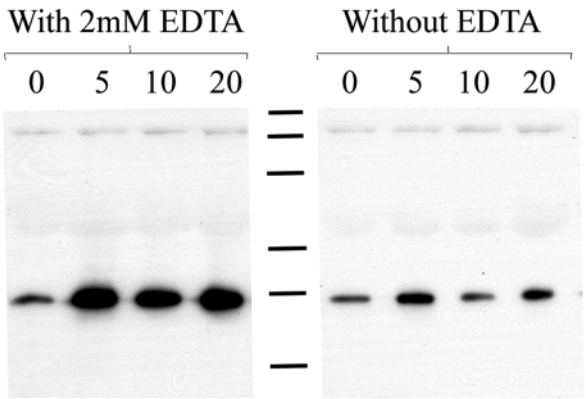

Fig. 7. Effect of EDTA on Rap1 activation. Ax-2 cells were incubated in the presence or absence of 2 mM EDTA immediately prior to the addition of sorbitol and then treated as described in the legend for Fig. 6.

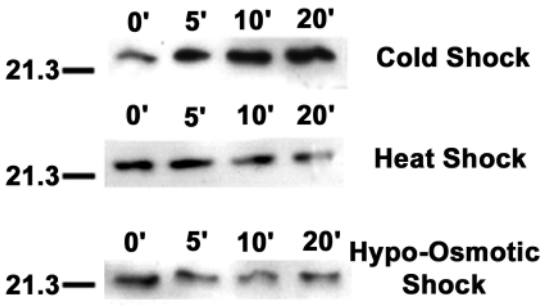

Fig. 8. Rap1 activation in response to different stress conditions. After a 1 hour adaptation at 22°C in KK_2 buffer, vegetative Ax-2 cells were subjected to cold shock, heat shock or hypo-osmotic shock, and samples were taken at the time points indicated. Cell lysates were incubated with GST-RalGDS(RBD) as described in the Methods and Materials, and levels of Rap1 bound to GST-RalGDS(RBD) were determined by western blotting.

viability. It is difficult to pinpoint the actual cause of cell death, but two distinct possibilities are consistent with our data. It had been shown that Rap1 is involved in endocytosis (Seastone et al., 1999), and since the rates of endocytosis in the *pVEII-AS5′* transformant are low (data not shown), the reduced uptake of nutrients could account for the reduced rates of growth and might ultimately lead to cell death. Alternatively, since the overexpression of Rap1 or Rap1-G12V generates alterations in cell shape and contractile responses (Rebstein et al., 1993; Rebstein et al., 1997; Seastone et al., 1999), the lack of Rap1 could impair cytoskeletal function.

The properties of the *pVEII-AS5′-1* Rap1-depleted cells resemble those of a strain that lacks both α-actinin and gelation factor, two F-actin crosslinking proteins (Rivero et al., 1996). This double mutant grows slowly, exhibits reduced phagocytosis and is more sensitive to osmotic shock. In addition, most of the double mutant cells are smaller and more rounded than wild-type cells, although approximately 5% of the population are larger (Rivero et al., 1996). All of these characteristics are shared by the Rap1-depleted cells. In view of this similarity, we determined the amounts of α-actinin and gelation factor in depleted cells. Gelation factor levels were normal, but α-actinin levels were reduced (data not shown). However, cells disrupted only in the gene encoding α-actinin exhibit normal properties (Rivero et al., 1996). Thus, either essential regulatory pathways controlled by Rap1 and by α-actinin and gelation factor intersect at some point or there are multiple means of generating the phenotype seen in Rap1-depleted cells. In any case, it is unlikely the deleterious effects resulting from Rap1 depletion are solely caused by the regulation of α-actinin.

The activation of guanylyl cyclase is important for the response to hyperosmolarity (Kuwayama et al., 1996; Oyama, 1996), and the data we have provided indicate that Rap1 plays a role in the activation of guanylyl cyclase. In particular, there was a reduced accumulation of cGMP in cells expressing *rapA* antisense RNA in response to osmotic shock, and the osmosensitivity of these cells was partially reduced by the addition of the cGMP analog 8Br-cGMP. In addition, we showed that Rap1 was activated in response to osmotic shock during cGMP accumulation. These results suggest that Rap1 acts upstream in the pathway that transmits the signal

responsible for guanylyl cyclase activation. Consistent with this interpretation, EDTA treatment, which stimulates cGMP production in response to osmotic stress (Oyama, 1996), also stimulated the activation of Rap1. The activation of guanylyl cyclase in response to osmotic shock does not appear to involve the heterotrimeric G protein complex, as *gβ*-null cells have no deficiency in cGMP accumulation (Kuwayama and van Haastert, 1998). However, GTPγS stimulates guanylyl cyclase in vitro (Janssens et al., 1988; Janssens et al., 1989) and in electro-permeabilized cells (Schoen et al., 1996), suggesting an interaction between guanylyl cyclase and a GTP-binding protein. Rap1 could be this presumptive GTP-binding protein.

A putative intracellular histidine kinase, encoded by the *dokA* gene is important in osmoregulation, demonstrated by the fact that a *dokA*-null strain exhibits increased sensitivity to osmotic shock (Schuster et al., 1996). However, the *dokA*-null strain exhibits normal cGMP accumulation in response to osmotic shock (Schuster et al., 1996), and there is now evidence that DokA acts in a signaling pathway parallel to the cGMP pathway (Ott et al., 2000). We found that Rap1-activation in the *dokA*-null mutant was similar to that in the parental Ax-2 strain during the osmotic shock (data not shown), consistent with the idea that Rap1 acts upstream of guanylyl cyclase. Although Rap1 depletion clearly affected actin tyrosine phosphorylation, there is no evidence as yet to indicate that this phosphorylation is dependent on guanylyl cyclase activation.

Rap1 activation does not occur under all stress conditions, as shown by the fact that there was no activation in response to low osmolarity or to high temperature (30°C). However, Rap1 was activated in response to low temperatures (8°C). These results indicate that Rap1 is not activated as part of a general stress response, signal transduction pathway.

An intriguing question is whether Rap1 has a function during *Dictyostelium* differentiation. It had been shown previously that the overexpression of Rap1 during development was capable of partially reversing the developmental defects produced by activated RasD, suggesting a possible role for the protein during development (Louis et al., 1997). However, other than the fact that the Rap1 protein levels remain constant during development (S. M. Robbins, PhD thesis, University of British Columbia, 1991), nothing more is known about a possible developmental function. Rap1-depleted cells do exhibit delayed differentiation (data not shown), but this may simply be caused by the reduced viability of these cells.

This work was supported by grants from the Medical Research Council of Canada to G.B.S. and CIHR to G.W. We thank Richard Pachal for constructing the *gst-rap1* plasmid.

References

Altschuler, D. L. and Ribeiro-Neto, F. (1998). Mitogenic and oncogenic properties of the small G protein Rap1b. *Proc. Natl. Acad. Sci. USA* **95**, 7475-7479.

Asha, H., de Ruiter, N. D., Wang, M. G. and Hariharan, I. K. (1999). The Rap1 GTPase functions as a regulator of morphogenesis *in vivo*. *EMBO J.* **18**, 605-615.

Blusch, J., Morandini, P. and Nellen, W. (1992). Transcriptional regulation by folate inducible gene expression in *Dictyostelium* transformants during growth and early development. *Nucleic Acid Res.* **20**, 6235-6238.

Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L. and Nadler, L. M. (1997). Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* **278**, 124-128.

Caron, E., Self, A. J. and Hall, A. (2000). The GTPase Rap1 controls functional activation of macrophage integrin $\alpha m\beta_2$ by LPS and other inflammatory mediators. *Curr. Biol.* **10**, 974-978.

Cook, S. J., Rubinfeld, B., Albert, I. and McCormick, F. (1993). RapV12 antagonizes Ras-dependant activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* **12**, 3475-3485.

De Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* **396**, 474-477.

Franke, G., Akkerman, J. N. and Bos, J. L. (1997). Rapid Ca^{2+} mediated activation of Rap1 in human platelets. *EMBO J.* **16**, 252-259.

Hariharan, I. K., Carthew, R. W. and Rubin, G. M. (1991). The *Drosophila Roughened* mutation: activation of a rap homolog disrupts eye development and interferes with cell determination. *Cell* **67**, 717-722.

Hu, C. D., Kariya, K., Okada, T., Qi, X., Song, C. and Kataoka, T. (1999). Effect of phosphorylation on activities of Rap1A to interact with Raf-1 and to suppress Ras-dependent Raf-1 activation. *J. Biol. Chem.* **274**, 48-51.

Ingham, R. J., Holgado-Madruga, M., Siu, C., Wong, A. J. and Gold, M. R. (1998). The Gab1 protein is a docking site for multiple proteins involved in signalling by the 8 cell antigen receptor. *J. Biol. Chem.* **273**, 30630-30637.

Janssens, P. M. W. and de Jong, C. C. C. (1988). A magnesium-dependent guanylate cyclase in cell-free preparations of *Dictyostelium discoideum*. *Biochem. Biophys. Res. Commun.* **150**, 405-411.

Janssens, P. M. W., de Jong, C. C. C., Vink, A. A. and van Haastert, P. J. M. (1989). Regulatory properties of magnesium-dependent guanylate cyclase in *Dictyostelium discoideum* membrane. *J. Biol. Chem.* **264**, 4329-4335.

Katagiri, K., Hattori, M., Minato, N., Irie, S.-K., Takatsu, K. and Kinashi, T. (2000). Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol. Cell. Biol.* **20**, 1956-1969.

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, A. M. (1998). A family of cAMP-binding proteins that directly activate Rap1. *Science* **282**, 2275-2279.

Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M. (1989). A ras related gene with transformation suppressor activity. *Cell* **56**, 77-84.

Kuwayama, H., Ecke, M., Gerisch, G. and van Haastert, P. J. M. (1996). Protection against osmotic stress by cGMP-mediated myosin phosphorylation. *Science* **271**, 207-209.

Kuwayama, H. and van Haastert, P. J. M. (1998). Chemotactic and osmotic signals share a cGMP transduction pathway in *Dictyostelium discoideum*. *FEBS lett.* **424**, 248-252.

Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature* **229**, 592-596.

Louis, S. A., Weeks, G. and Spiegelman, G. B. (1997). Rap1 overexpression reveals that activated RasD induced separable defects during *Dictyostelium* development. *Dev. Biol.* **190**, 273-283.

McLeod, S. J., Ingham, R. J., Bos, J. L., Kurosaki, T. and Gold, M. R. (1998). Activation of the Rap1 GTPase by the B cell antigen receptor. *J. Biol. Chem.* **273**, 29218-29223.

M'Rabet, L., Coffer, P., Zwartkruis, F., Franke, B., Segal, A. W., Koendermana, L. and Bos, J. L. (1998). Activation of the small GTPase rap1 in human neutrophils. *Blood* **92**, 2133-2140.

Okada, S., Matsuda, M., Anafi, M., Pawson, T. and Pessin, J. E. (1998). Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and Crkll-C3G complexes. *EMBO J.* **17**, 2554-2565.

Ott, A., Oehme, F., Keller, H. and Schuster, S. C. (2000). Osmotic stress response in *Dictyostelium* is mediated by cAMP. *EMBO J.* **19**, 5782-5792.

Oyama, M. (1996). cGMP accumulation induced by hypertonic stress in *Dictyostelium discoideum*. *J. Biol. Chem.* **271**, 5574-5579.

Rebstein, P. J., Weeks, G. and Spiegelman, G. B. (1993). Altered morphology of vegetative amoebae induced by increased expression of the *Dictyostelium discoideum ras*-related gene *rap1*. *Dev. Genet.* **14**, 347-355.

Rebstein, P. J., Cardelli, J., Weeks, G. and Spiegelman, G. B. (1997). Mutational analysis of the role of Rap1 in regulating cytoskeletal function in *Dictyostelium*. *Exp. Cell. Res.* **231**, 276-283.

Reedquist, K. A. and Bos, J. L. (1998). Costimulation through CD28 suppresses T cell receptor-dependent activation of the Ras-like small GTPase Rap1 in human T lymphocytes. *J. Biol. Chem.* **273**, 4944-4949.

Reedquist, K. A., Ross, E., Koop, E. A., Wolthuis, R. M. F., Zwartkruis,

F. J. T., van Kooyk, Y., Salmon, M., Buckley, C. D. and Bos, J. L. (2000). The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J. Cell. Biol.* **148**, 1151-1158.

Reuther, G. W. and Der, C. J. (2000). The Ras branch of small GTPases: Ras family members don't fall far from the tree. *Curr. Opin. Cell Biol.* **12**, 157-165.

Rivero, F., Koppel, B., Peracino, B., Bozzaro, S., Siegert, F., Weijer, C. J., Schleicher, M., Albrecht, R. and Noegel, A. A. (1996). The role of the cortical cytoskeleton: F-actin cross-linking proteins protect against osmotic stress, ensure cell size, cell shape and motility and contribute to phagocytosis and development. *J. Cell Sci.* **109**, 2679-2691.

Robbins, S. M., Suttorp, V. V., Weeks, G. and Spiegelman, G. B. (1990). A *ras*-related gene from the lower eukaryote *Dictyostelium* that is highly conserved relative to the human *rap* genes. *Nucleic Acids. Res.* **18**, 5265-5269.

Schoen, C. D., Schulkes, C. C. G. M., Arents, J. C. and van Driel, R. (1996). Guanylate cyclase activity in permeabilized *Dictyostelium discoideum* cells. *J. Cell. Biochem.* **60**, 411-423.

Schuster, S. C., Noegel, A. A., Oehme, F., Gerisch, G. and Simon, M. I. (1996). The hybrid histidine kinase DokA is part of the osmotic response system of *Dictyostelium*. *EMBO J.* **15**, 3880-3889.

Seastone, D. J., Zhang, L., Buczynski, G., Rebstein, P., Weeks, G., Spiegelman, G. B. and Cardelli, J. (1999). The small M_r Ras-like GTPase Rap1 and the phospholipase C pathway act to regulate phagocytosis in *Dictyostelium discoideum*. *Mol. Biol. Cell.* **10**, 393-406.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

Tsukamoto, N., Masakazu, H., Yang, H., Bos, J. L. and Minato, N. (1999). Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion. *J. Biol. Chem.* **274**, 18463-18469.

Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rum, C. S. and Stork, P. J. S. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf and Rap1-dependant pathway. *Cell* **89**, 73-82.

Watts, D. and Ashworth, J. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119**, 171-174.

York, R. D., Yao, H., Dillon, T., Elig, C. L., Eckert, S. P., McCleskey, E. W. and Stork, P. J. S. (1998). Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622-626.

Yoshida, Y., Kawsata, M., Miura, Y., Musha, T., Sasaki, T., Kikuchi, A. and Takai, Y. (1992). Microinjection of *smg/rap1/krev-1* p21 into Swiss 373 cells induces DNA synthesis and morphological changes. *Mol. Cell. Biol.* **12**, 3407-3414.

Zischka, H., Oehme, F., Pintsch, T., Ott, A., Kellermann, J. and Schuster, S. C. (1999). Rearrangement of cortex proteins constitutes an osmo-protective mechanism in *Dictyostelium*. *EMBO J.* **18**, 4241-4249.

Zwartkruis, F. J., Wolthuis, R. M., Nabben, N. M., Franke, B. and Bos, J. L. (1998). Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signaling. *EMBO J.* **17**, 5905-5912.