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Dual role of cAMP and involvement of both G-proteins and ras in regulation of ERK2 in *Dictyostelium discoideum*

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***Dictyostelium discoideum* expresses two Extracellular signal Regulated Kinases, ERK1 and ERK2, which are involved in growth, multicellular development and regulation of adenylyl cyclase. Binding of extracellular cAMP to cAMP receptor 1, a G-protein coupled cell surface receptor, transiently stimulates phosphorylation, activation and nuclear translocation of ERK2. Activation of ERK2 by cAMP is dependent on heterotrimeric G-proteins, since activation of ERK2 is absent in cells lacking the Gα4 subunit. The small G-protein rasD also activates ERK2. In cells overexpressing a mutated, constitutively active rasD, ERK2 activity is elevated prior to cAMP stimulation. Intracellular cAMP and cAMP-dependent protein kinase (PKA) are essential for adaptation of the ERK2 response. This report shows that multiple signalling pathways are involved in regulation of ERK2 activity in *D.discoideum*.**

Keywords: cross-talk/*Dictyostelium*/G-protein/MAP kinase/ras

Introduction

In *Dictyostelium discoideum* exhaustion of the food source triggers a developmental program, which results in the aggregation of ~10^5 solitary amoebae that form a multicellular organism (Williams, 1988; Devreotes, 1989). Cyclic AMP functions both as a cell–cell signal and as an intracellular second messenger (Firtel *et al.*, 1989). Extracellular cAMP exerts its effects in a similar fashion to hormones and neurotransmitters by acting through G-protein coupled cell surface cAMP receptors (cARs) (Klein *et al.*, 1988; Devreotes, 1994); ultimately it activates adenylyl cyclase and several other effectors (Snaar-Jagalska *et al.*, 1988b; van Haastert *et al.*, 1991; Devreotes, 1994). Although most of the cAMP produced is secreted to mediate cell–cell signalling, part of the cAMP remains intracellular and activates cAMP-dependent protein kinase (PKA) (de Gunzburg and Veron, 1982; Harwood *et al.*, 1991). *Dictyostelium* cells can also detect and react to extracellular signals like folic acid (Janssens *et al.*, 1987), DIF (differentiation inducing factor), adenosine (Schaap *et al.*, 1993) and two protein factors, PreStarvation Factor (PSF) and Conditioned Medium Factor (CMF; Clarke *et al.*, 1988; Jain *et al.*, 1992). This implies that cAR-independent signal transduction pathways play an important role in cell movement and differentiation also. In addition to genes for eight α subunits and one β subunit of heterotrimeric G-proteins (Devreotes, 1994), five ras genes have been identified in *Dictyostelium*; the functions of the ras proteins in signal transduction remain poorly defined (Reymond *et al.*, 1986; van Haastert *et al.*, 1987; Robbins *et al.*, 1989; Daniel *et al.*, 1994).

In general, Extracellular signal Regulated Kinases (ERKs) or Mitogen Activated Protein (MAP) kinases are serine/threonine protein kinases, which are activated in response to a wide variety of extracellular stimuli. Activation of ERKs has been observed in response to stimulation of G-protein coupled receptors (Faure *et al.*, 1994; Koch *et al.*, 1994) or tyrosine kinase receptors (Boulton *et al.*, 1991; de Vries-Smits *et al.*, 1992; Blumer and Johnson, 1994; Burgering and Bos, 1995). ERKs are activated through phosphorylation on threonine and tyrosine residues by upstream kinases designated MEKs (MAP-Kinase/ERK kinases) (Crews *et al.*, 1992; Zheng *et al.*, 1993). MEKs can be activated upon binding of ligands like EGF, PDGF and insulin to tyrosine kinase receptors. These receptors activate ras by stimulating ras-GTP formation (Satoh *et al.*, 1990; Burgering *et al.*, 1991). The binding of ligands like lysophophatidic acid (LPA) and thrombin to G-protein coupled receptors can also lead to activation of ras (van Corven *et al.*, 1993). Ras can activate both raf and MEKKinase, which activate MEK, which in turn activates ERK (Howe *et al.*, 1992; MacDonald *et al.*, 1993; Avruch *et al.*, 1994). MEKKinase may also activate MEK in a ras-independent way as in *Saccharomyces cerevisiae* pheromone signalling (Lange-Carter *et al.*, 1993). Activated ERK is translocated to the nucleus where it is involved in regulation of gene expression (Chen *et al.*, 1992; Seth *et al.*, 1992).

Intracellular cAMP has been shown to antagonize the activation of ERKs by ras-dependent signalling pathways in mammals (Burgering and Bos, 1995). PKA interferes with the ras–raf interaction by direct phosphorylation of raf (Chuang *et al.*, 1994). Consequently ras-mediated activation of raf and its downstream components MEK and ERK is reduced (Burgering *et al.*, 1993; Cook *et al.*, 1993).

In *D.discoideum*, two developmentally regulated ERK genes have been described. ERK1 has been shown to be necessary for vegetative growth and multicellular development (Gaskins *et al.*, 1994). ERK2 is essential for receptor-mediated activation of adenylyl cyclase and also for differentiation (Segall *et al.*, 1995; Gaskins *et al.*, 1996). Phosphorylation has been shown on ERK1 tyrosine

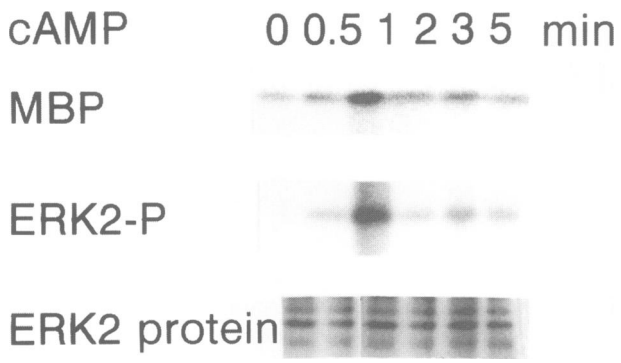

Fig. 1. cAMP-induced activation and phosphorylation of ERK in *Dictyostelium*. Cells were stimulated with 10 μM cAMP for the times indicated. ERK activity (upper panel) was measured by an *in vitro* kinase assay with MBP as a substrate. ^{32}P-phosphorylated MBP was analyzed on 15% polyacrylamide gels and visualized by autoradiography. *In vivo* ^{32}P-phosphorylation of ERK (middle panel): *Dictyostelium* cells, labelled with [^{32}P]orthophosphate, were incubated with 10 μM cAMP for the times indicated. Following solubilization, ERK was immunoprecipitated from the cell lysate, dissolved in sample buffer and subjected to electrophoresis on 12.5% polyacrylamide gels containing 0.0625% bisacrylamide. The lower panel shows a silver staining of part of the immunoprecipitates used in the upper panel.

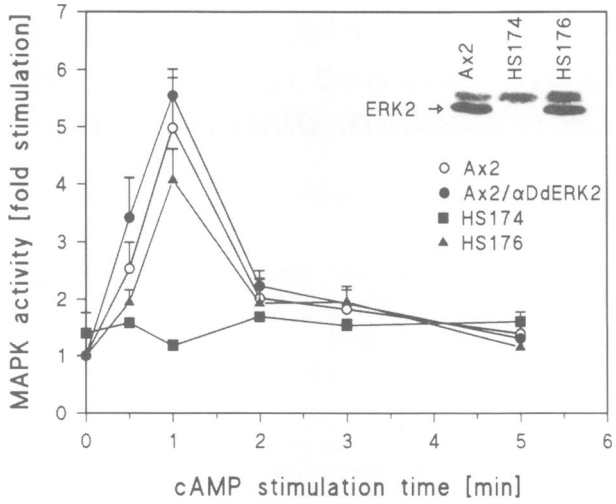

Fig. 2. Time course of cAMP-induced ERK2 activation of *Dictyostelium* Ax2 (wild-type) cells, HS174 (ERK2$^-$) cells and HS176 (control for HS174) cells. Cells were stimulated with 10 μM cAMP for the times indicated and lysed. ERK was immunoprecipitated with the commercial ERK polyclonal antibody (Ax2, HS174, HS176) or the specific DdERK2 antibody (Ax2/αDdERK2). ERK activity was then determined as described in Materials and methods. Results are expressed as fold stimulation of ERK activity when compared with the basal level (t = 0) of ERK activity of the Ax2 wild-type cells. Mean values ± SEM of six experiments for Ax2 and four experiments for HS174, HS176 and Ax2/αDdERK2 are presented. The inset shows a Western blot of total protein preparations of the three cell lines probed with the anti-ERK polyclonal antibody. The arrow indicates *Dictyostelium* ERK2.

and on ERK2 tyrosine and serine residues, however, none of these phosphorylation events has been linked to activation of these ERKs so far. The wide variety of biochemically and genetically well-defined signalling mutants of *Dictyostelium* constitutes a good tool to study the regulation of ERK activity in response to extracellular stimuli.

In this report we demonstrate that, in *Dictyostelium*, extracellular cAMP induces phosphorylation, activation and nuclear translocation of ERK2 in a G-protein-dependent manner. Overexpression of the mutant rasDThr$_{12}$ gene, which encodes a constitutively active form of one of the *Dictyostelium* ras proteins, leads to an increased basal level of ERK2 activity. We also show that intracellular cAMP antagonizes both ERK2 activating pathways via PKA.

Results

Extracellular cAMP induces activation and phosphorylation of ERK2

We measured ERK activity in *Dictyostelium* cells stimulated with cAMP. After various times of stimulation, cells were solubilized, ERK was immunoprecipitated from the cell lysates and the protein kinase activity was measured *in vitro* using myelin basic protein (MBP) as a substrate. *In vivo* stimulation of aggregation competent cells with 10 μM cAMP transiently stimulates ERK activity (Figure 1, upper panel). ERK activity is already significantly increased 30 s after cAMP stimulation and peaks at 1 min, with the maximum activity being five times the MBP phosphorylation observed in unstimulated cells. The ERK activity decreases to basal level after 5 min and remains at basal level for up to 15 min of cAMP stimulation (data not shown). In other eukaryotic systems, ERK activation is caused by phosphorylation on the highly conserved TEY motif (Ray and Sturgill, 1988). Since this motif is conserved in both *Dictyostelium* ERKs, it is expected that phosphorylation of ERK in *Dictyostelium* shows the same transient response to cAMP as ERK activity does. The *in vivo* labelling experiment shown in Figure 1 confirms that ERK phosphorylation correlates strongly with ERK activation. The bottom panel of Figure 1 demonstrates that comparable amounts of ERK protein are immunoprecipitated after different times of stimulation. Since we cannot distinguish between the two cloned *Dictyostelium* ERKs with the commercial ERK antibody used, we made use of cells with a disrupted *erkB* gene (HS174), which lack the ERK2 protein, to address the question as to which of the two *Dictyostelium* ERKs is responsible for the cAMP-induced ERK activation. Absence of the ERK2 protein in the HS174 cells was confirmed by Western blot analysis (Figure 2). The cAMP-induced ERK response is absent in the HS174, ERK2$^-$ cells (Figure 2). The parental control strain HS176 has an ERK response almost identical to the wild-type Ax2 strain. To further positively identify ERK2 as the activated ERK in *Dictyostelium*, we measured the ERK response using the polyclonal antibody αDdERK2 which is raised against bacterially expressed DdERK2 and specifically recognizes ERK2 in *Dictyostelium*. There was no significant difference between the ERK responses, measured with the two different antobodies (Figure 2). These results show that ERK2 activity is stimulated by cAMP treatment. The αDdERK2 antibody was also used to identify ERK2 as the major band in anti-ERK immunoprecipitates (Figure 1, bottom panel). The limited supply of αDdERK2 forced us to use the commercial ERK antibody in most experiments.

To exclude that absence of cAMP receptors on the surface of ERK2$^-$ cells accounts for the absence of the ERK response, cAMP binding to ERK2$^-$ cells was measured. Cell-surface cAMP binding to ERK2$^-$ cells is

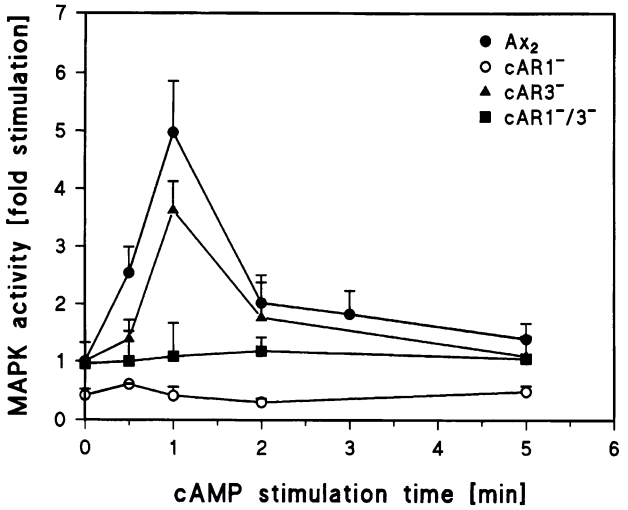

Fig. 3. Time course of cAMP-induced ERK2 activation of Ax2 (wild-type), cAR1⁻, cAR3⁻ and cAR1⁻/3⁻ cells. cAR1⁻ and cAR1⁻/3⁻ cells were pulsed with 100 nM cAMP for 4 h, in order to reach the same developmental stage prior to measurement of the ERK activity. All cell types were treated with 10 μM cAMP for the times indicated. Results are expressed as fold stimulation of ERK activity when compared with the basal level (t = 0) of ERK activity of the Ax2 wild-type cells. Mean values ± SEM of six experiments for Ax2 and three experiments for cAR1⁻, cAR3⁻ and cAR1⁻/3⁻ cells are presented.

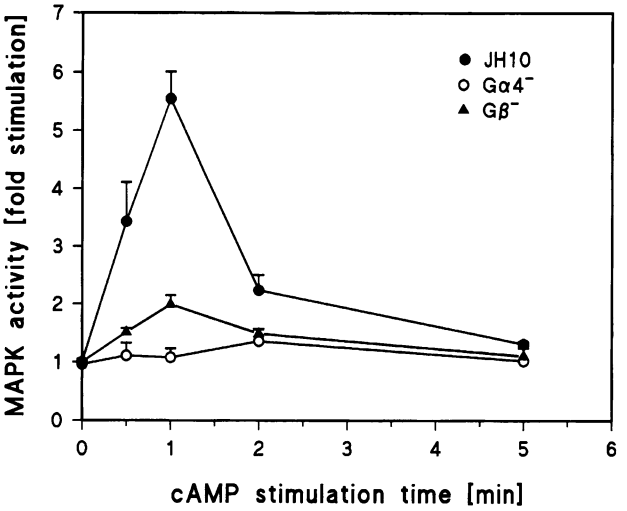

Fig. 4. Time course of cAMP-induced ERK2 activation of JH10 (wild-type/control) cells, Gα4⁻ cells and Gβ⁻/cAR1 (LW14)cells. Gβ⁻/cAR1 cells were pulsed with 100 nM cAMP for 4 h, in order to reach the same developmental stage, prior to measurement of the ERK activity. All cell types were treated with 10 μM cAMP for the times indicated after which ERK activity was determined as described in Materials and methods. Results are expressed as fold stimulation compared with the basal level (t = 0) of ERK activity of the JH10 cells. Mean values ± SEM of three experiments are presented.

60% of binding to Ax2 wild-type or HS176 control cell lines (data not shown). In order to obtain wild-type expression of cAMP receptors, ERK2⁻ cells were pulsed with 100 nM cAMP for 4 h (Soede *et al.*, 1994). cAMP binding was raised to ~80% of the wild-type level, nevertheless no ERK response was detectable after this treatment. From these results it is clear that, at this stage of *Dictyostelium* development, ERK2 is activated and phosphorylated by extracellular cAMP.

cAMP-induced ERK2 activation involves G-protein coupled cell surface receptors and heterotrimeric G-proteins

Most of the cAMP regulated responses in *Dictyostelium* are dependent on cAMP cell surface receptors (cARs) and heterotrimeric G-proteins. To determine which of the four cloned cARs is involved in ERK2 activation, we studied the ERK response in cells lacking cAR1 and/or cAR3. The other two cARs, cAR2 and cAR4, are expressed at extremely low levels at this stage of development and are more prominent during later stages of development (Devreotes, 1994). Figure 3 shows the absence of the ERK response in cAR1⁻ and cAR1⁻/cAR3⁻ cells and a normal response in cAR3⁻ cells, showing that cAR1 is essential for ERK2 activation. The cAMP cell surface receptors are typical seven transmembrane G-protein coupled receptors. Therefore we examined the involvement of heterotrimeric G-proteins in ERK2 activation. For this purpose the ERK response was measured in cells lacking the Gβ subunit (Lilly *et al.*, 1993). Since the Gβ⁻ cells do not enter the developmental program, absence of signalling components might lead to absence of responses. To prevent this problem the strain LW14 (Gβ⁻/cAR1) was used. This strain has a disrupted Gβ gene and over-expresses cAR1 constitutively. The Gβ⁻/cAR1 cells were also pulsed to give the signals necessary to trigger the developmental program. The strong reduction of the ERK response in the Gβ⁻/cAR1 cells (<30% of wild-type) indicates that functional heterotrimeric G-proteins may be involved in ERK2 activation (Figure 4). To study whether and which of the Gα subunits are involved, we measured the ERK2 response in Gα1, Gα2, Gα3 and Gα4 disruption cell lines and found that only cells lacking the Gα4 subunit lack ERK2 activation (data for Gα1, Gα2 and Gα3 not shown). The control strain JH10 shows an ERK2 response identical to wild-type. To conclude we showed that a signalling pathway involving cAR1 and a G-protein containing Gα4 are essential for ERK2 activation.

RasD also activates ERK2 in Dictyostelium

In mammals, ras activates ERK via raf and MEK (Blumer and Johnson, 1994; Burgering and Bos, 1995). Ras in turn is activated by receptor tyrosine kinases and/or G-protein coupled receptors. We studied whether ras can activate ERK2 by using cells overexpressing rasD with an activating mutation at position 12 (Gly→Thr). In rasDThr$_{12}$ cells ERK2 is already activated prior to stimulation of cells with cAMP. The control cell line overexpressing wild-type rasD (rasDGly$_{12}$) behaves in a very similar way to Ax2 wild-type cells (Figure 5). Identical results obtained by using the commercial ERK antibody or the specific DdERK2 antibody, demonstrate that ERK2 activity is elevated and not ERK1. Upon stimulation of rasDThr$_{12}$ cells with extracellular cAMP, only a reduction in ERK activity occurs, showing that the adaptation of the ERK response is independent of rasD.

Intracellular cAMP and PKA antagonize ERK2 activation

Intracellular cAMP has been shown to antagonize ras-induced activation of ERK in mammalian cells using membrane permeable cAMP analogues, which are known

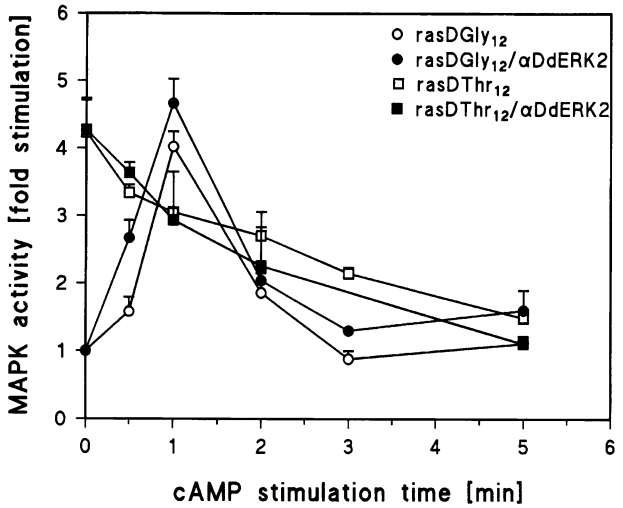

Fig. 5. Time course of the effects of extracellular cAMP on ERK activity in cells overexpressing the wild-type *rasD* gene ($rasDGly_{12}$) and cells overexpressing a mutated form of the *rasD* gene ($rasDThr_{12}$). Cells were stimulated with 10 μM cAMP for the times indicated. ERK was precipitated with the commercial ERK polyclonal antibody ($rasDGly_{12}$, $rasDThr_{12}$) or the specific DdERK2 antibody ($rasDGly_{12}$/αDdERK2, $rasDThr_{12}$/αDdERK2). Results are expressed as fold stimulation compared with the basal ERK activity in $rasDGly_{12}$ cells. ERK activity was determined as described in Materials and methods. Mean values ± SEM of three experiments are presented.

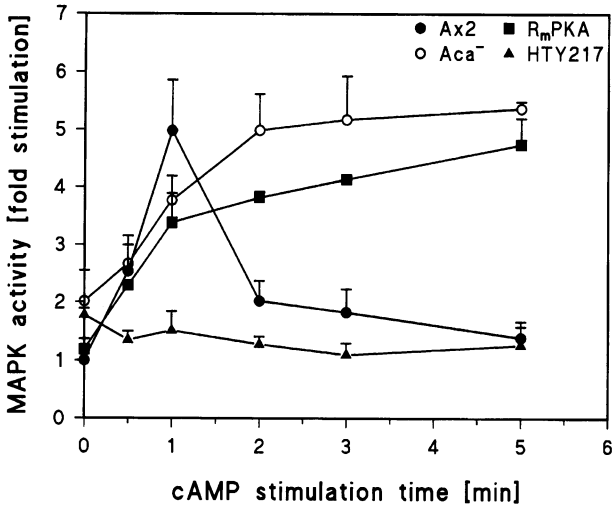

Fig. 6. Time course of cAMP-induced stimulation of ERK activity in Ax2 (wild-type) cells, Aca^- cells (lacking the aggregative adenylate cyclase), R_mPKA cells (overexpressing a dominant negative regulatory subunit of PKA) and HTY217 cells (constitutively active PKA). Aca^- and R_mPKA cells were pulsed with 100 nM cAMP for 4 h, to reach the correct developmental stage, before stimulation with 100 μM 2′H cAMP to induce ERK activity. Results are expressed as fold stimulation compared with basal ERK activity in Ax2 cells. Mean values ± SEM of six experiments for Ax2 and of three experiments for Aca^-, R_mPKA and HTY217 cells are presented.

to activate PKA (Burgering *et al.*, 1993; Cook and McCormick, 1993). To study the effects of intracellular cAMP and PKA in *Dictyostelium*, the ERK response was measured in Aca^-, RmPKA and HTY217 cells. Aca^- cells have the gene encoding the aggregative adenylyl cyclase disrupted (Pitt *et al.*, 1992). RmPKA cells overexpress a dominant negative regulatory subunit of PKA which binds the catalytic subunit but is unable to bind cAMP (Harwood *et al.*, 1991). This leads to permanent inactivation of PKA. HTY217 cells contain a mutated regulatory subunit, which can bind cAMP but cannot interact with the catalytic subunit, resulting in a permanently active PKA (Simon *et al.*, 1992). Cells were stimulated with 2′H-cAMP to prevent any effects of extracellular cAMP leaking into Aca^- cells. This cAMP analogue has a very low affinity for PKA but will bind to the cAMP receptor (van Ments-Cohen and van Haastert, 1989). The adaptation of the ERK response was inhibited in Aca^- and RmPKA cells (Figure 6). No decrease of the response was seen after up to 15 min of stimulation. In contrast, HTY217 cells lack cAMP-induced ERK2 activation (Figure 6). This indicates that intracellular cAMP is essential for turning off ERK2 via an active PKA in *Dictyostelium*.

Activated ERK2 translocates to the cell nucleus

It has been observed that ERK, when activated, can translocate from the cytosol to the cell nucleus. There ERK might be involved in regulating gene expression. Because $ERK2^-$ cells show a defect in differentiation we investigated whether ERK2 translocates to the nucleus upon stimulation. In unstimulated cells, no ERK2 was detected in the nucleus. Stimulation of cells with cAMP gave rise to the appearance of ERK2 in the nucleus (Figure 7). No ERK appeared in the nuclei of $ERK2^-$ (HS174) cells after 1 min of cAMP stimulation. This translocation occurs within 1 min after stimulation, but is not so strongly

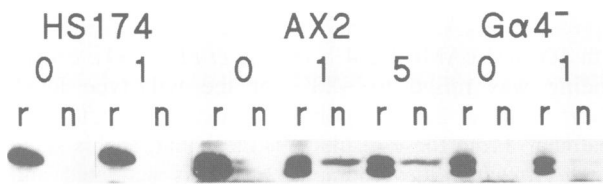

Fig. 7. Nuclear translocation of ERK2 in Ax2 (wild-type), HS174 ($ERK2^-$) and $G\alpha4^-$ cells. Cells were separated into a remainder (R) and a nuclear (N) fraction as described in Materials and methods. Of the R fraction 2×10^6 and of the N fraction 3×10^7 cell equivalents were loaded on a 12.5% PAA gel containing 0.0625% bisacrylamide. Protein was blotted and detected by the polyclonal ERK antibody as described in Materials and methods. The experiment shown is representative for three independent experiments.

transient as the total ERK response. Approximately 1–2% of total ERK2 is translocated towards the cell nucleus and therefore does not significantly affect the total ERK2 response. The ERK2 translocation is absent in $G\alpha4^-$ cells which also lack the total ERK response. Longer stimulation of $ERK2^-$ and $G\alpha4^-$ cells did not cause appearance of ERK in the nucleus (data not shown). These results indicate that part of the activated ERK2 moves to the nucleus. Figure 7 also shows why the frequently used shift analysis for measuring ERK activation does not work in *Dictyostelium*. During PAGE, ERK2 migrates as part of a very hard to separate dimer, with the upper band most likely being ERK1. When ERK2 is phosphorylated, its mobility shifts. The activated ERK2 will co-migrate with ERK1. We can see a different ratio between the two bands of the dimer upon cAMP stimulation (data not shown), but this is not usable to quantify and clearly show ERK2 activation.

Discussion

We report here that extracellular cAMP-induced activation of ERK2 in *Dictyostelium*, which is regulated by heterotrimeric G-protein-dependent pathways, can also be activated by rasD. In higher eukaryotes, stimulation of ras by binding of ligands to tyrosine kinase receptors activates a protein kinase cascade, resulting in the stimulation of ERK (Blumer and Johnson, 1994; Burgering and Bos, 1995). Binding of ligands to G-protein coupled receptors can also lead to activation of ERK, probably by stimulation of ras-GTP formation (Hordijk *et al.*, 1994; Koch *et al.*, 1994). It is remarkable that, despite the evolutionary distance of ~1 billion years to mammals, the simple eukaryotic social amoeba *D.discoideum* shows both G-protein and ras-dependent ERK activation in one single cell type.

Stimulation of *Dictyostelium* aggregation competent cells with extracellular cAMP leads to a rapid and transient activation and phosphorylation of ERK2. Studies of cells lacking ERK2 showed that the MBP phosphorylating activity, immunoprecipitated by an ERK polyclonal antibody, results from a protein kinase belonging to the ERK family of protein kinases. This was confirmed by the use of an antibody raised against DdERK2 protein. Furthermore, basal MBP-kinase activity, probably resulting from ERK1, is present in aggregation competent cells but is not regulated by extracellular cAMP.

Using cell lines lacking one of the four known cAMP cell surface receptors showed that binding of cAMP to cAR1 is necessary for ERK2 phosphorylation and activation. cAR1 is the most abundant cAMP receptor at this stage of development. The other cAR present in aggregation-competent cells, cAR3, has been shown to be a 'back-up' receptor for cAR1 (Soede *et al.*, 1994). Treating cells lacking cAR1 with cAMP pulses can partly restore some responses like gene expression, cAMP and cGMP production. Disrupting both cAR1 and cAR3 completely abolishes this possibility to restore these responses. The ERK response absent in cAR1$^-$ cells cannot be restored significantly by treating cells with pulses of cAMP, which leads to the conclusion that cAR1 alone is responsible for ERK2 activation.

The cAMP-induced intracellular responses in *Dictyostelium* are dependent on heterotrimeric G-proteins. Activation of adenylyl cyclase, guanylyl cyclase and phospholipase C is dependent on functional G-proteins (Firtel *et al.*, 1989). The strong reduction of the ERK2 response in cells lacking the Gβ subunit indicated heterotrimeric G-proteins might be involved in ERK2 activation. Consequently we investigated ERK2 activation in Gα1, Gα2, Gα3 and Gα4 knock-out cell lines. Surprisingly, only Gα4$^-$ cells show a defect in ERK2 activation. *Dictyostelium* ERK2 regulation is therefore very similar to MAPK regulation in fission yeast, in which a Gα subunit is also essential for activation (Herskowitz, 1995). The Gα4 subunit was described to be specifically involved in folic acid signalling (Hadwiger and Firtel, 1994), which only occurs during very early development. Also, involvement of Gα4 in gene expression has been shown (Hadwiger and Firtel, 1992). The ERK2 response is therefore the first cAMP-induced response described which is dependent on Gα4. It is, however, not likely that folic acid will induce ERK2 activation in aggregation-competent cells, because folic acid receptors are virtually absent at this stage of development (de Wit and de Wit, 1986). Folic acid can still be responsible for regulating ERK2 at an earlier stage of development, when the folic acid receptors are maximally present.

In addition to heterotrimeric G-protein-dependent ERK2 activation, we describe that rasD can activate ERK2. Cells overexpressing a mutated, constitutively active rasD are not able to aggregate properly and form multiple tipped aggregates that do not proceed to form migrating slugs and mature fruits (Reymond *et al.*, 1986). In these cells ERK2 is constitutively activated. Additional treatment of cells with cAMP induces adaptation of ERK2. This indicates that rasD is upstream of ERK2 and has no direct function in adaptation of the ERK2 response.

Interestingly, the multiple tipped phenotype of the rasDThr$_{12}$ cells can be mimicked by treating wild-type aggregates with high concentrations of cAMP (Nestle and Sussman, 1972). ERK2 has been shown to be involved in cAMP accumulation. ERK2$^-$ cells have a decreased cAMP response *in vivo*. Furthermore there is an impaired GTPγS stimulation of adenylate cyclase *in vitro* (Segall *et al.*, 1995). One could therefore propose that a constitutive activation of ERK2 gives rise to increased cAMP accumulation in rasDThr$_{12}$ cells, which in turn leads to multiple tipped aggregates. The signal responsible for rasD activation remains unidentified in *Dictyostelium*, which makes it impossible to determine the route leading to rasD-GTP formation and consequently to ERK2 activation. The absence of ERK2 activation in Gα4$^-$ cells indicates that cAMP is probably not the signal leading to rasD-GTP formation. It is not very likely that Gα4 activates rasD, because the phenotype of cells overexpressing Gα4 does not show any similarity with the phenotype of rasDThr$_{12}$ cells (Hadwiger and Firtel, 1992).

From mammalian systems it is known that PKA antagonizes the ras-dependent activation of ERK. PKA can phosphorylate raf at Ser43 and thereby decrease the affinity of raf for ras (Chuang *et al.*, 1994). Via this mechanism, intracellular cAMP can antagonize ERK activation by ras. The dual role of cAMP in *Dictyostelium* as a hormone-like substance and an intracellular second messenger, prevented us from using 8Br-cAMP to show adaptation of the ERK2 response. Additionally with *Dictyostelium*, one can use defined mutants, disturbed in intracellular cAMP signalling, as a powerful method to study the role of intracellular cAMP and PKA. We used mutants which either do not produce cAMP (Aca$^-$ cells), are defective in PKA activation (R$_m$PKA cells), or have a constitutively active PKA (HTY217 cells) to show that intracellular cAMP and active PKA is essential for adaptation of the ERK2 response in *Dictyostelium* (Figure 6).

Since a raf homologue has not yet been cloned from *Dictyostelium*, it is not clear how PKA is able to antagonize ERK2 activation. PKA can stimulate ERK2 dephosphorylation and/or inhibit ERK2 activation by phosphorylation of a component upstream of ERK2. The exact mechanism of adaptation of the ERK2 response in *Dictyostelium* remains to be determined.

Next to regulating adenylate cyclase, ERK2 has been suggested to be involved in differentiation, pattern forma-

tion and cell type-specific gene expression, since ERK2$^-$ cells are unable to from mature spores and stalk cells (Segall *et al.*, 1995; Gaskins *et al.*, 1996).

Translocation of activated ERK has been shown to occur in mammalian cells. This activation-dependent translocation has been linked to c-myc phosphorylation (Seth *et al.*, 1992) and immediate early gene expression (Chen *et al.*, 1992). Stimulation of *Dictyostelium* cells with cAMP leads not only to activation and phosphorylation of ERK2, but also to translocation of ~1–2% of total ERK2 to the nucleus. Two cell lines that lack the ERK2 response, ERK2$^-$ and Gα4$^-$, show no translocation of ERK2 to the nucleus. Interestingly both cell lines also have in common that they are blocked in prespore gene expression and spore formation (Hadwiger and Firtel, 1992; Segall *et al.*, 1995; Gaskins *et al.*, 1996). It was suggested that ERK2 is indirectly involved in differentiation via regulation of intracellular cAMP levels. This could lead to activation of PKA and gene expression via its targets. A second possibility is involvement of ERK2 in chemotactic responses of cells in multicellular structures, which would explain the aberrant positioning of ERK2$^-$ cells in multicellular stages when mixed with wild-type cells (Segall *et al.*, 1995).

The third possibility is that ERK2 translocates to the cell nucleus and regulates gene expression directly by phosphorylating proteins involved in transcription events. Our data clearly show that ERK2 translocates to the cell nucleus upon activation. The direct involvement of the nuclear-localized activated ERK2 in gene regulation remains to be determined. The fact that lack of ERK2 activation and nuclear translocation in both ERK2$^-$ and Gα4$^-$ cells correlates with inhibition of particularly prespore gene expression and spore formation, indicates that ERK2 could have an important role in gene regulation, differentiation and pattern formation.

Materials and methods

Materials

[^{32}P]orthophosphate and [γ-^{32}P]ATP were from Amersham. The anti-ERK1 rabbit polyclonal antibody (K-23) was purchased from Santa Cruz Biotech. The anti-DdERK2 antibody (αDdERK2) was raised in rabbits against bacterially expressed ERK2 (Y.Wang and Dr J.Segall, unpublished). Protein G–Sepharose 4B fast flow was obtained from Pharmacia. The peroxidase labelled goat anti-rabbit antibody was from Kirkegaard and Perry Labs. Inc.

Culture conditions and cell treatment

D.discoideum cells were grown in HL5 medium (Watts and Ashworth, 1970) supplemented with the antibiotic G418 where necessary. In most experiments the strain Ax2 was used as wild-type. For Gα4$^-$ and Gβ$^-$, JH10 and JH8 respectively were used as parental control strains. Before use, all strains were checked for the correct phenotype. Typically, cells were grown to a density of 5×10^6 cells/ml, collected by centrifugation and washed with 10 mM potassium/sodium phosphate, pH 6.5. Cells were starved by incubating on non-nutrient agar plates at 6°C for 16 h. If necessary, cells were pulsed with 100 nM cAMP for 4 h as described before (Soede *et al.*, 1994). Starvation was followed by shaking the cells in 10 mM potassium/sodium phosphate buffer, pH 6.5 at a density of 1×10^7 cells/ml at 22°C for 1 h. 2.5 mM caffeine was added in order to suppress spontaneous cAMP signalling (Brenner and Thoms, 1984).

ERK activity assay

1.5×10^7 aggregation-competent cells were treated with 10 μM cAMP for various times as indicated in the figures. Each stimulation was stopped by lysing the cells in buffer A (20 mM Tris pH 8.0, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM EDTA, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM Pefabloc). Each separate cell lysate was incubated with the polyclonal ERK antibody or the polyclonal DdERK2 antibody (where indicated) precoupled to protein G–Sepharose in order to immunoprecipitate ERK and in particular ERK2. The washed immunoprecipitate was incubated with 0.5 μCi of [γ^{32}P]ATP, 30 mM Tris pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP and 7.5 μg of MBP as substrate for 30 min (Leevers and Marshall, 1992). The mixture was electrophoresed on a 15% polyacrylamide gel, which was subsequently autoradiographed. Bands were quantified using LKB Ultroscan densitometer.

In vivo ^{32}P-labelling of cells

5×10^7 cells were starved in MES (2-*N* morpholino ethane sulfonic acid) buffer (20 mM MES pH 6.15, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) as described before (Liu and Newell, 1994). Starved cells were resuspended in MES buffer at 1×10^8 cells/ml and labelled with 0.2 mCi/ml of [^{32}P]orthophosphate for 45 min. After extensive washing with MES buffer the labelled cells were incubated with 10 μM cAMP for the times indicated and lysed in buffer A. Subsequently ERK was immunoprecipitated from these cell lysates (Leevers and Marshall, 1992). Washed immunoprecipitates were analyzed on polyacrylamide gels, which were autoradiographed.

Isolation of nuclei from Dictyostelium

Nuclei were isolated by a method similar to that described (Butler and Coukell, 1991). Shortly, washed amoebae were resuspended in ice-cold NL buffer (50 mM HEPES–NaOH pH 7.5, 40 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 5% sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10% Percoll, 2 mM Pefabloc, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μg/ml antipain) and lysed by forced passage through a 3 μm pore size polycarbonate filter (Nuclepore corp.). Nuclei were collected by centrifugation at 3000 *g* for 2 min. The supernatant was kept and called the 'remainder' (R) fraction. The nuclei (N fraction) were washed several times in NL buffer. The purity of the nuclei was checked by microscopy and DAPI staining of the fractions.

Immunoblotting and silver staining

Washed whole cells or immunoprecipitates were resuspended in sample buffer (60 mM Tris pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 80 mM SDS) and analyzed on 12.5% polyacrylamide gels containing 0.0625% bisacrylamide (Snaar-Jagalska *et al.*, 1988a). Protein was visualized by silver staining (Harris and Angal, 1989) or blotted to nitrocellulose. Immunoblots were probed with a 1:1500 dilution of rabbit polyclonal anti-ERK1 at 4°C for 16 h. Bands were visualized by peroxidase-labelled goat anti-rabbit antibodies followed by detection with the BM chemoluminescence kit.

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References

Avruch,J., Zhang,X. and Kyriakis,J.M. (1994) Raf meets ras:completing the framework of a signal transduction pathway. *Trends Biochem. Sci.*, **19**, 279–283.

Blumer,K.J. and Johnson,G.L. (1994) Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.*, **19**, 236–240.

Boulton,T.G. *et al.* (1991) ERKs: A Family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65**, 663–675.

Brenner,M. and Thoms,S. (1984) Caffeine blocks activation of cyclic AMP synthesis in *Dictyostelium discoideum*. *Dev. Biol.*, **101**, 136–146.

Burgering,B.M.T. and Bos,J.L. (1995) Regulation of ras-mediated signalling: more than one way to skin a cat. *Trends Biochem. Sci.*, **20**, 18–22.

Burgering,B.M.T., Medema,R.H., Maassen,J.A., van de Wetering,M.L., van der Eb,A.J., McCormick,F. and Bos,J.L. (1991) Insulin stimulation of gene expression mediated by p21 ras activation. *EMBO J.*, **10**, 1103–1109.

Burgering,B.M.T., Pronk,G.J., van Weeren,P.C., Chardin,P. and Bos,J.L. (1993) cAMP antagonizes p21^{ras}-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSOS nucleotide exchange factor. *EMBO J.*, **12**, 4211–4220.

Chen,R.H., Sarnecki,C. and Blenis,J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.*, **12**, 915–927.

Chuang,E., Barnard,D., Hettich,L., Zhang,X., Avruch,J. and Marshall, M.S. (1994) Critical binding and regulatory interactions between ras and raf occur through a small, stable N-terminal domain of raf and specific ras effector residues. *Mol. Cell. Biol.*, **14**, 5318–5325.

Clarke,M., Yang,J. and Kayman,S.C. (1988) Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum*. *Dev. Genet.*, **9**, 315–326.

Cook,S.J. and McCormick,F. (1993) Inhibition by cAMP of ras dependent activation of raf. *Science*, **262**, 1069–1072.

Crespo,P., Xu,N., Simonds,W.F. and Gutkind,J.S. (1994) Ras dependent activation of MAP kinase mediated by G-protein βγ subunits. *Nature*, **369**, 418–420.

Crews,C.M., Alessandrini,A. and Erikson,R.L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science*, **258**, 478–480.

Daniel,J., Bush,J., Cardelli,J., Spiegelman,G.B. and Weeks,G. (1994) Isolation of two novel ras genes in *Dictyostelium discoideum*: evidence for a complex, developmentally regulated ras gene subfamily. *Oncogene*, **9**, 501–508.

de Gunzburg,J. and Veron,M. (1982) A cAMP dependent protein kinase is present in differentiating *Dictyostelium discoideum* cells. *EMBO J.*, **1**, 1063–1068.

Devreotes,P.N. (1989) *Dictyostelium discoideum*: A model system for cell–cell interactions in development. *Science*, **245**, 1054–1058.

Devreotes,P.N. (1994) G-protein linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron*, **12**, 235–241.

de Vries-Smits,A.M.M., Burgering,B.M.T., Leevers,S.J., Marshall,C.J. and Bos,J.L. (1992) Involvement of $p21^{ras}$ in activation of extracellular signal-regulated kinase 2. *Nature*, **357**, 602–604.

de Wit,R.J.W. and Rinke de Wit,T.F. (1986) Developmental regulation of the folic acid chemosensory system in *Dictyostelium discoideum*. *Dev. Biol.*, **118**, 385–391.

Faure,M., Voyno-Yasenetskaya,T.A. and Bourne,H.R. (1994) cAMP and βγ subunits of heterotrimeric G-proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.*, **269**, 7851–7854.

Firtel,R.A., van Haastert,P.J.M., Kimmel,A.R. and Devreotes,P.N. (1989) G-protein linked signal transduction pathways in development: *Dictyostelium* as an experimental system. *Cell*, **58**, 235–239.

Frödin,M., Peraldi,P. and van Obberghen,E. (1994) cAMP activates the mitogen-activated protein cascade in PC12 cells. *J. Biol. Chem.*, **269**, 6207–6214.

Gaskins,C., Maeda,M. and Firtel,R.A. (1994) Identification and functional analysis of a developmentally regulated extracellular-signal-regulated kinase gene in *Dictyostelium discoideum*. *Mol. Cell. Biol.*, **14**, 6996–7012.

Gaskins,C., Clark,A.M., Aubry,L., Segall,J.E. and Firtel,R.A. (1996) The Dictyostelium MAP kinase ERK2 regulates multiple, independent developmental pathways. *Genes Dev.*, **10**, 118–128.

Hadwiger,J.A. and Firtel,R.A. (1992) Analysis of $G_{\alpha}4$, a G-protein subunit required for multicellular development in *Dictyostelium*. *Genes Dev.*, **6**, 38–49.

Hadwiger,J.A., Lee,S. and Firtel,R.A. (1994) The G_{α} subunit $G_{\alpha}4$ couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in *Dictyostelium*. *Proc. Natl Acad. Sci. USA*, **91**, 10566–10570.

Harris,E.L.V. and Angal,S. (1989) *Protein Purification Methods: A Practical Approach*. IRL Press at Oxford University Press, Oxford, UK.

Harwood,A.J., Hopper,N.A., Simon,M.-N., Bouzid,S., Veron,M. and Williams,J.G. (1991) Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. *Dev. Biol.*, **149**, 90–99.

Herskowitz,I. (1995) MAP kinase pathways in yeast: For mating and more. *Cell*, **80**, 187–197.

Hordijk,P.L., Verlaan,I., Jalink,K., van Corven,E.J. and Moolenaar,W.H. (1994) cAMP abbrogates the $p21^{ras}$-mitogen-activated protein kinase pathway in fibroblasts. *J. Biol. Chem.*, **269**, 3534–3538.

Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) Activation of the MAP kinase pathway by the protein kinase raf. *Cell*, **71**, 335–342.

Jain,R., Yuen,I.S., Taphouse,C.R. and Gomer,R.H. (1992) A density sensing factor controls development in *Dictyostelium*. *Genes Dev.*, **6**, 390–400.

Janssens,P.M.W. and van Haastert,P.J.M. (1987) Molecular basis of transmembrane signal transduction in *Dictyostelium discoideum*. *Microbiol. Rev.*, **51**, 396–418.

Klein,P.S., Tzeli,J.S., Saxe III,C.L., Kimmel,A.R., Johnson,R.L. and Devreotes,P.N. (1988) A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science*, **241**, 1467–1472.

Koch,W.J., Hawes,B.E., Allen,L.F. and Lefkowitz,R.J. (1994) Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc. Natl Acad. Sci. USA*, **91**, 12706–12710.

Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnson,G.L. (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and raf. *Science*, **260**, 315–319.

Leevers,S.J. and Marshall,C.J. (1992) Activation of extracellular signal-regulated kinase, ERK2, by p21 ras oncoprotein. *EMBO J.*, **11**, 569–574.

Lilly,P., Wu,L., Welker,D.L. and Devreotes,P.N. (1993) A G-protein β-subunit is essential for *Dictyostelium* development. *Genes Dev.*, **7**, 986–995.

Liu,G. and Newell,P.C. (1994) Regulation of myosin regulatory light chain phosphorylation via cyclic GMP during chemotaxis of *Dictyostelium*. *J. Cell Sci.*, **107**, 1737–1743.

MacDonald,S.G., Crews,C.M., Wu,L., Driller,J., Clark,R., Erikson,R.L. and McCormick,F. (1993) Reconstruction of the raf-1-MEK-ERK signal transduction pathway *in vitro*. *Mol. Cell. Biol.*, **13**, 6615–6620.

Nestle,M. and Sussman,M. (1972) The effect of cyclic AMP on morphogenesis and enzyme accumulation in *Dictyostelium discoideum*. *Dev. Biol.*, **28**, 545–554.

Pitt,G.S., Milona,N., Borleis,J., Lin,K.C., Reed,R.R. and Devreotes,P.N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell*, **69**, 305–315.

Ray,L.B. and Sturgill,T.W. (1988) Insulin-stimulated microtubule associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*. *Proc. Natl Acad. Sci. USA*, **85**, 3753–3757.

Reymond,C.D., Gomer,R.H., Mehdy,M.C. and Firtel,R.A. (1984) Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian ras protein. *Cell*, **39**, 141–148.

Reymond,C.D., Gomer,R.H., Nellen,W., Theibert,A., Devreotes,P. and Firtel,R.A. (1986) Phenotypic changes induced by a mutated ras gene during the development of *Dictyostelium* transformants. *Nature*, **323**, 340–343.

Robbins,S.M., Williams,J.G., Jermyn,K.A., Spiegelman,G.B. and Weeks,G. (1989) Growing and developing *Dictyostelium* cells express different ras genes. *Proc. Natl Acad. Sci. USA*, **86**, 938–942.

Satoh,T., Endo,M., Nakafuku,M., Nakamura,S. and Kaziro,Y. (1990a) Platelet derived growth factor stimulates formation of active $p21^{ras}$-GTP complex in Swiss mouse 3T3 cells. *Proc. Natl Acad. Sci. USA*, **87**, 5993–5997.

Schaap,P., Peters,D.J.M., Haribabu,B. and Dottin,R.P. (1993) Gene regulation by hormone-like signals in *Dictyostelium discoideum*. In *Signal Transduction: Prokaryotic and Simple Organisms*. Academic Press, Inc., New York, pp. 353–376.

Segall,J.E., Kuspa,A., Shaulsky,G., Ecke,M., Maeda,M., Gaskins,C., Firtel,R.A. and Loomis,W.F. (1995) A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.*, **128**, 405–413.

Seth,A., Gonzalez,F., Gupta,S., Raden,D.L. and Davis,R.J. (1992) Signal transduction within the nucleus by mitogen-activated protein kinase. *J. Biol. Chem.*, **267**, 24796–24804.

Simon,M.N., Pelegrini,O., Veron,M. and Kay,R.R. (1992) Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. *Nature*, **356**, 171–172.

Snaar-Jagalska,B.E., Devreotes,P.N. and van Haastert,P.J.M. (1988a) Ligand induced modification of surface cAMP receptor of *Dictyostelium discoideum* does not require its occupancy. *J. Biol. Chem.*, **263**, 897–901.

Snaar-Jagalska,B.E., Kesbeke,F. and van Haastert,P.J.M. (1988b) G-proteins in the signal-transduction pathways of *Dictyostelium discoideum*. *Dev. Genet.*, **9**, 215–226.

Soede,R.D.M., Insall,R.H., Devreotes,P.N. and Schaap,P. (1994) Extracellular cAMP can restore development in *Dictyostelium* cells lacking one, but not two types of early cAMP receptors (cARs). *Development*, **120**, 1997–2002.

van Corven,E.J., Hordijk,P.L., Medema,R.H., Bos,J.L. and Moolenaar, W.H. (1993) Pertussis toxin-sensitive activation of $p21^{ras}$ by G-protein-coupled receptor agonists in fibroblasts. *Proc. Natl Acad. Sci. USA*, **90**, 1257–1261.

van Haastert,P.J.M., Kesbeke,F., Reymond,C.D., Firtel,R.A., Luderus,E. and van Driel,R. (1987) Aberrant transmembrane signal transduction in *Dictyostelium* cells expressing a mutated ras gene. *Proc. Natl Acad. Sci. USA*, **84**, 4905–4909.

van Haastert,P.J.M., Janssens,P.M.W. and Ernaux,C. (1991) Sensory transduction in eukaryotes. A comparison between *Dictyostelium* and vertebrate cells. *Eur. J. Biochem.*, **195**, 289–303.

van Ments-Cohen,M. and van Haastert,P.J.M. (1989) The cyclic nucleotide specificity of eight cAMP-binding proteins in *Dictyostelium discoideum* is correlated into three groups. *J. Biol. Chem.*, **264**, 8717–8722.

Watts,P.J. and Ashworth,J.M. (1970) Growth of myxamoebae of the cellular slime mold *Dictyostelium discoideum* in axenic culture. *Biochem. J.*, **119**, 171–174.

Williams,J.G. (1988) The role of diffusable molecules in regulating the cellular differentiation of *Dictyostelium discoideum*. *Development*, **103**, 1–16.

Zheng,C.F. and Guan,K.L. (1993) Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. *J. Biol. Chem.*, **268**, 23933–23939.

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