Chemoattractants induce tyrosine phosphorylation of ERK2 in *Dictyostelium discoideum* by diverse signalling pathways

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Two homologues of mitogen-activated protein kinases have been identified in *Dictyostelium discoideum* (ERK1 and EKR2). We here demonstrate transient tyrosine phosphorylation of ERK2 in response to the chemoattractants cAMP and folic acid that correlates with activity. To investigate the signalling pathways, we studied the response in strains with altered cAMP-dependent protein kinase (PKA) status. The degree of cAMP-induced ERK2 tyrosine phosphorylation was increased in cells over-expressing PKA activity but no such increase was observed in the response to folic acid. Our observations suggest that cAMP-induced ERK2 tyrosine phosphorylation is positively modulated by a PKA-regulated step which is not involved in the response to folic acid, suggesting the presence of diverse signalling pathways leading to ERK2 activation.

INTRODUCTION

The slime mould *Dictyostelium discoideum* grows vegetatively as single amoebae but, on starvation, a multicellular developmental programme is initiated in which aggregation is co-ordinated by pulsatile emissions of cAMP which acts as a chemoattractant for surrounding cells. Binding of cAMP to cell-surface cAMP receptors (cARs) activates certain effectors including adenylate cyclase (ACA) [1]. Activation of ACA produces cAMP which is rapidly secreted to relay the cAMP signal. Receptor-coupled activation of ACA requires several components such as cAR1 or 3, heterotrimeric G-protein $\beta\gamma$-subunits and a cytosolic regulator of ACA. Surprisingly, it has been reported that ERK2, a homologue of mitogen-activated protein kinase (MAPK) isolated from *Dictyostelium*, is also essential for receptor-coupled activation of ACA [2].

The role of intracellular cAMP during early development is not clearly defined. Strains that lack the ability to activate ACA cannot aggregate, as they cannot generate a chemotactic signal. They can be rescued by pulsing with extracellular cAMP, suggesting that intracellular cAMP is not required for early development [3]. However, a cAMP-dependent protein kinase (PKA) comprised of one regulatory subunit and one catalytic subunit has been characterized in *Dictyostelium* and is present during early development [4]. Expression of a mutated form of the regulatory subunit that cannot bind cAMP acts as a dominant inhibitor of PKA activity, as the two subunits remain associated whatever the cAMP concentration [5]. Expression of such a mutant subunit on a constitutive promoter blocks aggregation and interferes with the regulation of early gene expression [6], suggesting that PKA plays an important regulatory role during these early stages. Cells that overexpress the constitutively active catalytic subunit (KP cells), which have four times more PKA activity than wild-type cells [7], and *rdeC* mutants, which lack a functional regulatory subunit [8], show rapid development at the later stages but no alterations in early processes have been reported.

Two MAPK homologues have been identified in *Dictyostelium* [2,9]. The signals leading to ERK1 activation are not known, but recently it has been reported that a pulse of extracellular cAMP, acting through the cell surface receptor, induces a transient activation of ERK2 measured as phosphorylating activity towards myelin basic protein (MBP) using an in-gel assay system [10]. The sequence of *Dictyostelium* ERK2 is highly homologous to that of MAPKs from other eukaryotes and includes the conserved TEY motif. Phosphorylation of both the threonine and tyrosine residues in this motif has been shown to correlate with activation of MAPKs in other organisms, the phosphorylations being co-ordinately regulated by dual-specificity kinases and phosphatases [11]. A recent report that the activation of *Dictyostelium* ERK2 correlates with an increase in phosphate content is consistent with a conserved mechanism of regulation in *Dictyostelium* [12], although the components of the upstream regulatory systems have not been characterized in this organism.

In the present study we monitored tyrosine phosphorylation of ERK2 on the TEY motif by Western-blot analysis using antiserum specific for the phosphorylated version of the TEY motif in ERK2. We found that the addition of extracellular cAMP elicits tyrosine phosphorylation of ERK2 on its TEY motif with kinetics comparable with those of the cAMP-induced ERK2 activation previously reported [10]. In addition, we demonstrated that another chemoattractant, folic acid, induces tyrosine phosphorylation, correlating with transient activation of ERK2 as measured using an in-gel kinase assay. This is consistent with a role for ERK2 in regulation of the chemotactic response. We also investigated the ability of PKA activity to modulate the phosphorylation of ERK2 in response to these agents to clarify the importance of intracellular cAMP during early development.

MATERIALS AND METHODS

Cell growth and development

Dictyostelium cells were cultivated axenically at 22 °C in defined

Abbreviations used: MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; PMAPK, phosphorylated MAPK; cAR, cell surface cAMP receptor; ACA, adenylate cyclase; MBP, myelin basic protein.

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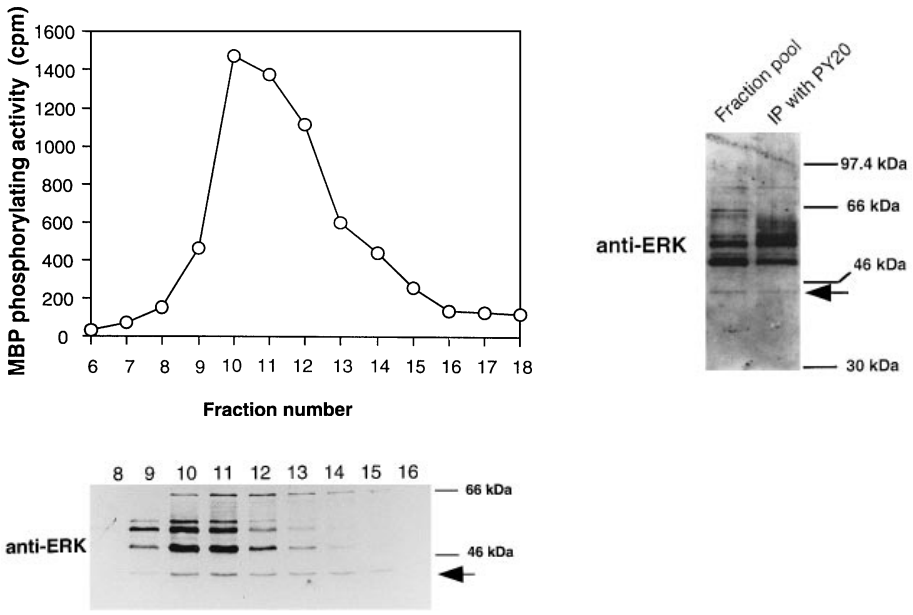

Figure 1 MAPK activity in vegetative *Dictyostelium* cells

Left, an elution profile of MBP-phosphorylating activity. Axenically growing cells (10^9) were harvested and lysed as described in the Materials and methods section. The cell lysate was passed over a phenyl-Sepharose column, and bound proteins were eluted with 50% ethanediol. The column fractions were assayed for MBP-phosphorylating activity and analysed by Western blotting using anti-ERK serum. The positions of the molecular-mass markers are indicated and the position of the 42 kDa band studied in later experiments is marked by an arrow. Right, Western-blot analysis using anti-ERK antibody against proteins immunoprecipitated with agarose-conjugated anti-phosphotyrosine antibody (PY20). Before immunoprecipitation, a pool of three peak fractions (fraction 10–12) obtained from phenyl-Sepharose chromatography was dialysed against EB/NaCl buffer to remove ethanediol. Fraction pool, a sample of the original pool; IP with PY20, proteins immunoprecipitated using PY20.

growth medium [13]. Cells were harvested during exponential growth [(2–6) $\times 10^6$ cells/ml], washed three times with KK_2 buffer (20 mM potassium phosphate, pH 6.1, 2 mM $MgSO_4$) and then resuspended in KK_2 at a density of 2×10^8 cells/ml. For folic acid stimulation (100 μM), the reagent was added immediately to washed vegetative cells. For cAMP stimulation (100 nM), cells were prestarved for 5 h to allow expression of the cARs by shaking in KK_2 at a density of 1×10^7 cells/ml at 22 °C at 100 rev./min before stimulation. The reactions were terminated by freezing the cell suspension in liquid nitrogen. Frozen cells were lysed by thawing in an equal volume of 2 × EB buffer [20 mM β-glycerophosphate, pH 7.4, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine, 25 μg/ml leupeptin, 50 μg/ml PMSF and 0.3% (v/v) 2-mercaptoethanol] containing 0.2 M NaCl. The lysates were cleared by centrifugation and subjected to partial purification.

Partial purification on phenyl-Sepharose

Phenyl-Sepharose (Pharmacia; phenyl-Sepharose high performance) chromatography was carried out as previously described [14] using 10^9 cells. MBP-phosphorylating activity was assayed as in [14]. For time-course experiments, the cell lysates from 10^8 cells were incubated at 4 °C for 30 min with 100 μl of a slurry of phenyl-Sepharose beads, and the adsorbed proteins were eluted by boiling in 50 μl of 4 × Laemmli's sample buffer after multiple washes with EB containing 0.1 M NaCl (EB/NaCl) and subjected to immunoblotting and in-gel kinase assay.

Immunoblotting

Immunoblot analysis to detect phosphorylated MAPK (PMAPK) was performed using PhosphoPlus MAPK antibody kit (New England Biolabs) according to the manufacturer's instructions. The antibody contained in this kit was raised against synthetic phosphotyrosine peptide corresponding to residues 196–209 of human p44 MAPK and recognizes p42 and p44 MAPK only when catalytically activated by phosphorylation at Tyr-204 in several types of mammalian cells and *Drosophila*. All filters were reblotted with anti-ERK antibody (K-23; Santa Cruz Biotechnology) to evaluate the total amount of MAPK, visualized using an ECL Western-blot detection kit (Amersham).

Quantification of immunoblots

Two or three exposures of autoradiograms were analysed by densitometric scanning to ensure that the results lay within the linear range of detection. Samples to be compared (e.g. inductions of Ax2 and KP cells) were run on the same gel, which was probed with anti-PMAPK and then stripped and reprobed with anti-ERK. This allowed direct comparison of the loading and expression levels of ERK2 between samples so that the relative activation of ERK2 could be assessed. All Western blots shown are a typical example of at least three independent experiments.

Immunoprecipitation

Three peak fractions from phenyl-Sepharose chromatography (fractions 10–12) were pooled and dialysed against 2 × 2 litres of EB/NaCl to remove ethanediol. Proteins containing phosphotyrosine were immunoprecipitated in the presence of 1% Triton X-100 and 0.5% Nonidet P40 using agarose-conjugated anti-phosphotyrosine antibody (PY20; Transduction Laboratories) for 1 h at 4 °C. Proteins were eluted by boiling in 4 × Laemmli's sample buffer and analysed by Western blotting using anti-ERK serum.

In-gel kinase assay for ERK2 activity

In-gel kinase assay was performed basically as described previously [15]. Briefly, the semi-purified samples were separated on SDS/12 % polyacrylamide gel containing 0.5 mg/ml MBP, denatured in 6 M guanidinium chloride (Sigma) and renatured by washing in the buffer containing 0.04 % Tween 40 (Sigma). The phosphorylation reaction was performed in the presence of 50 μM ATP and 50 μCi of [γ-^{32}P]ATP (Amersham) at 30 °C for 60 min. After unbound ATP had been washed out, the gels were exposed to X-ray films. The activity was evaluated by the phosphorimage analyser after normalization to the intensities of non-specific kinase activity. Autophosphorylation of proteins was examined by similar procedures with gels containing BSA instead of MBP, and no autophosphorylation was observed.

RESULTS AND DISCUSSION

MAPK activity in *Dictyostelium* cells

The activity of mammalian MAPKs is characterized by the affinity of the protein for a hydrophobic matrix such as phenyl-Sepharose, preference for basic substrates such as MBP and the dependence of the activity on phosphorylation of both threonine and tyrosine residues [14]. We have identified activities with characteristics consistent with those of mammalian MAPKs in extracts of vegetative *Dictyostelium* amoebae (Figure 1).

When extracts from 10^9 vegetatively growing *Dictyostelium* cells were subjected to phenyl-Sepharose chromatography, a single peak of MBP-phosphorylating activity was eluted by 50 % ethanediol. Western-blot analysis of these fractions using an anti-ERK antibody revealed the presence of bands of 42 and 48 kDa consistent with the predicted sizes of *Dictyostelium* ERK2 and ERK1 respectively, with similar elution profiles to the MBP-phosphorylating activity (Figure 1, left). Immunoprecipitation of a pool of the peak fractions with anti-phosphotyrosine antibody, followed by Western blotting using anti-ERK serum, indicated that these proteins contained phosphorylated tyrosine residues (Figure 1, right). In addition, MBP-phosphorylating activity appeared to depend on the phosphorylation status of the protein, as the activity and intensity of the immunoblot with anti-phosphotyrosine antibody were lost in parallel when cell lysates were prepared in the absence of phosphatase inhibitors (results not shown). This analysis suggested the presence of several proteins with the characteristics expected of MAPKs: hydrophobicity, substrate selectivity and dependence of activity on phosphorylation on tyrosine.

Transient ERK2 tyrosine phosphorylation in response to cAMP

The activity assayed by the column method above is a composite of all MAPKs expressed. As MAPK activity in *Dictyostelium* is expected to be regulated by phosphorylation of the protein on both tyrosine and threonine in a conserved TEY motif (as in other MAPKs characterized), we monitored tyrosine phosphorylation of this motif as a measure of the activity of the signalling pathways leading to activation of a specific MAPK. MAPKs were semi-purified from cell lysates by adsorption on phenyl-Sepharose beads, and the tyrosine-phosphorylation status of the eluted MAPKs was assessed by Western blotting using antisera specific for the phosphorylated version of MAPK (anti-PMAPK).

We confirmed the efficacy of this assay by examining whether the kinetics of tyrosine phosphorylation in response to a pulse of extracellular cAMP is consistent with the cAMP-induced transient activation of ERK2 previously reported [10]. The addition of cAMP rapidly induced the immunoreactivity of a 42 kDa protein in PMAPK immunoblots, consistent with the previously reported molecular mass of ERK2, although the levels of a protein with the same molecular mass detected with the anti-ERK antibody did not change over the time course of the experiment (Figure 2, top). Reblotting with an anti-phosphotyrosine antibody confirmed a similar kinetic increase in phosphotyrosine associated with the 42 kDa protein (results not shown), indicating that the reactivity with PMAPK antibody really did represent an increase in tyrosine phosphorylation of the 42 kDa protein. To confirm that the 42 kDa band corresponds to ERK2, we examined its behaviour in a mutant strain, HS174, which expresses less than 10 % of the wild-type levels of ERK2 because of a plasmid insertion in the 3′ non-coding region of the gene [2]. cAMP induced tyrosine phosphorylation of the 42 kDa protein at significantly lower levels (below 10 %) in HS174 compared with Ax2 cells or a control transformant (Figure 2, bottom). Expression of the 42 kDa protein detected by anti-ERK serum was also substantially reduced in HS174, indicating that the 42 kDa protein detected with both antisera is ERK2. In some immunoblots, (see e.g. Figures 2, bottom, and 5c), the PMAPK antibody recognized proteins that were not at reduced levels in HS174 cells. These proteins were not detected using another batch of the antibody that had been more highly purified by the manufacturer (e.g. Figures 2, top, and 3), indicating that these non-specific bands are due to an impurity of the antibody.

In Ax2 cells cAMP-induced phosphorylation of ERK2 peaked at 30–60 s and then decreased to undetectable levels after 4 min (Figure 2, top). These kinetics of tyrosine phosphorylation were comparable with those of ERK2 activation previously reported

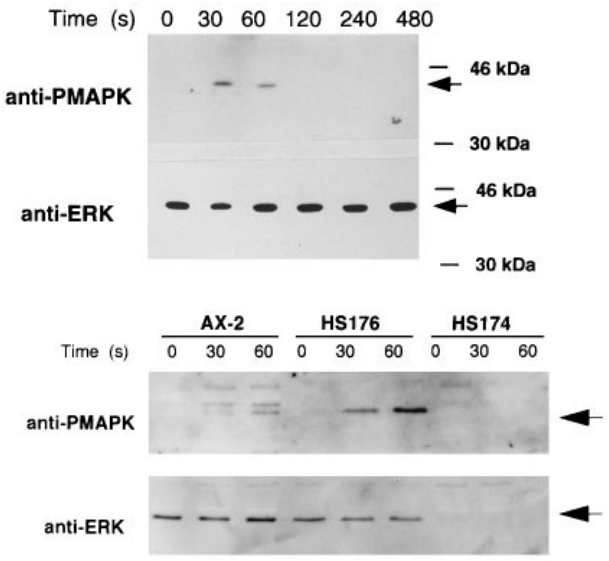

Figure 2 cAMP-induced tyrosine phosphorylation of ERK2 in Ax2 cells

Top, Western-blot analysis of PMAPK and total MAPK in response to cAMP in Ax2 cells. Ax2 cells prestarved for 5 h in KK_2 were stimulated with cAMP (100 nM) and harvested at the indicated time. After cell lysis, proteins were semi-purified on phenyl Sepharose as described in the Materials and methods section. The amounts of PMAPK and total MAPK were evaluated by Western blotting with anti-PMAPK and anti-ERK sera as shown. The positions of the molecular-mass markers are indicated and the 42 kDa band of interest is marked with a solid arrowhead. Bottom, Western-blot analysis of PMAPK and total MAPK in Ax2, HS176 and HS174 cells. Prestarved cells were stimulated with cAMP (100 nM) and harvested at the indicated time after stimulation. The amounts of PMAPK and total MAPK were evaluated as described above. The 42 kDa band of interest is marked with a solid arrowhead. HS176, control transformant that contains ERK2; HS174, cells with a reduced level of ERK2.

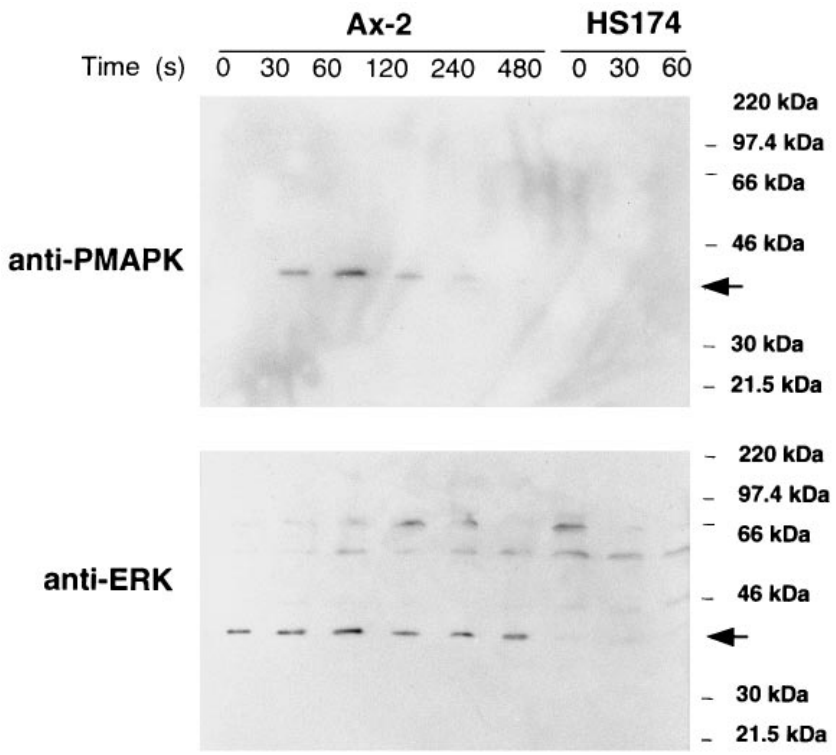

Figure 3 Folic acid-induced tyrosine phosphorylation of ERK2

Western-blot analysis of PMAPK and total MAPK in response to folic acid in Ax2 and HS174 cells. Vegetative cells were stimulated with folic acid (100 μM) and harvested at the indicated time. The amounts of PMAPK and total MAPK were evaluated as described in the legend to Figure 2. The positions of the molecular-mass markers are indicated and the 42 kDa band of interest is marked with a solid arrowhead.

[10] and detected by ourselves using an in-gel kinase assay (results not shown); the fold stimulation in response to cAMP at 30 s obtained in PMAPK immunoblot and in-gel kinase assay was 4.36 ± 0.32 and 4.12 ± 0.56 respectively. The correlation between ERK2 activation and tyrosine phosphorylation is consistent with the mechanism of ERK2 activation characterized in other organisms being conserved in *Dictyostelium*.

ERK2 activation by folic acid

Folic acid acts as a chemoattractant to enable vegetative *Dictyostelium* amoebae to capture bacteria. We observed that folic acid also induced transient tyrosine phosphorylation of the 42 kDa protein with kinetics similar to those induced by cAMP and that the response was significantly reduced in HS174 cells (to below 10 %) (Figure 3). To confirm the correlation between ERK2 activation and tyrosine phosphorylation, we compared the increase in tyrosine phosphorylation of ERK2 induced by folic acid with the increase in the kinase activity detected using in-gel kinase assay. The in-gel kinase assay revealed that a 42 kDa kinase activity was stimulated 4.68 ± 0.66-fold 30 s after the addition of folic acid and returned to undetectable levels 8 min after stimulation (Figure 4a), which is consistent with findings of others (M. Maeda and R. A. Firtel, personal communication). The substantial reduction of this kinase activity in HS174 cells (analysis using the phosphorimage analyser revealed that levels were reduced by 84.5 % at 30 s after the stimulation) is consistent with this activity being due to ERK2. Quantification of the increase in tyrosine phosphorylation (4.34 ± 0.26-fold) and kinase activity (4.68 ± 0.66-fold) for the same samples revealed a similar degree of induction at the peak of 30 s after folic acid addition, and the kinetics of the two responses were indistinguishable (Figures 4b and 4c). This correlation between kinase activity and tyrosine phosphorylation was also observed in the response to folic acid in HS174 cells (Figures 4b and 4c) and validates the PMAPK immunoblot as a direct measure of activation of ERK2. Under the conditions used for the kinase assay, several non-specific kinase activities were detected that were not reduced in HS174 cells (Figure 4a). These kinases were of higher molecular mass than reported for ERK2, and did not alter in activity on treatment with cAMP or folic acid or change in level during the early stages of development (results not shown). The identity of these kinases is unknown but it is possible that they are other members of the MAPK family present in *Dictyostelium* such as ERK1.

The kinetics and extent of chemoattractant-induced ERK2 activation correlated with tyrosine phosphorylation of the TEY motif in ERK2 as detected by Western blotting. This is consistent with previous reports of a correlation between total phosphate content of ERK2 and its activity [12]. TEY phosphorylation is a prerequisite for MAPK activation in all cases studied to date, being carried out by a dual-specificity kinase which also phosphorylates the threonine residue in this motif [11]. Given the sequence conservation between ERK2 and other MAPKs and the known structural requirements for phosphorylation for activation, and in the light of the correlation described above, it seems highly likely that a similar phosphorylation event is involved in ERK2 activation in *Dictyostelium*. It is possible that other events contribute to the activity and that TEY tyrosine phosphorylation is not the only factor regulating ERK2 activity in *Dictyostelium*, but such events have not been described as yet in this or other systems.

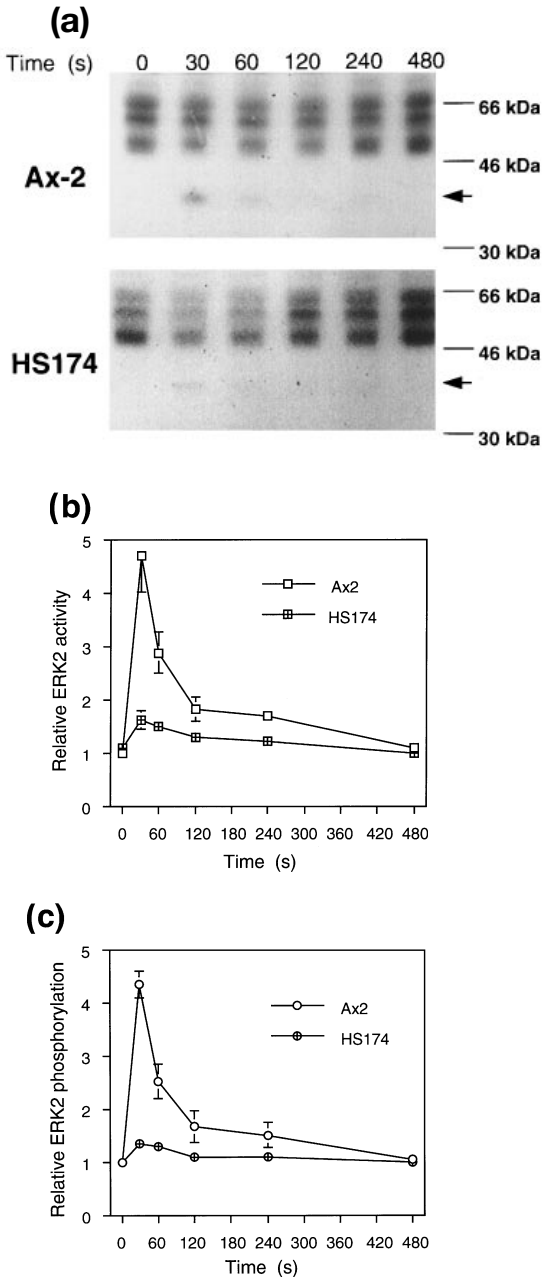

Figure 4 Quantitative comparison between increase in kinase activity and tyrosine phosphorylation of ERK2 in response to folic acid

Vegetative cells were harvested at 0, 30, 60, 120, 240 and 480 s after stimulation with folic acid (100 μM). Cell samples were prepared as described in the Materials and methods section and the same samples were analysed for kinase activity using an in-gel kinase assay and tyrosine phosphorylation using PMAPK immunoblot. The data shown are from one representative experiment of three. (**A**) Stimulation of ERK2 kinase activity in response to folic acid measured by in-gel kinase assay. Semi-purified samples were separated on SDS/12% polyacrylamide gel containing MBP (0.5 mg/ml). The gels were denatured, renatured and incubated in the presence of 50 μCi of [^{32}P]ATP as described in the Materials and methods section and exposed to X-ray film after unbound ATP had been washed out. The positions of the molecular-mass markers are indicated (kDa) and the 42 kDa band of interest is marked with a solid arrowhead. (**b**) Quantification of ERK2 kinase activity. The relative levels of ERK2 kinase activity were measured by the phosphorimage analyser and expressed as means $\pm$ S.D. fold stimulation of the basal level of ERK2 activity. (**c**) Quantification of ERK2 tyrosine phosphorylation. The relative levels of tyrosine phosphorylation of ERK2 of the same samples were measured by densitometric scanning of a Western blot (not shown) with anti-PMAPK antibody. The level is expressed as the mean $\pm$ S.D. fold stimulation of the basal level of ERK2 phosphorylation after normalization to the total ERK2 detected with anti-MAPK antibody.

This assay of tyrosine phosphorylation provides a rapid and convenient way of measuring the activity of the signalling pathways leading to ERK2 phosphorylation. It has not been possible to detect changes in ERK2 phosphorylation by measuring increases in apparent molecular mass on SDS/PAGE, as has proven a useful measure of MAPK activation in other organisms [12]. The only means available to study ERK2 activation therefore has involved complex in-gel kinase assays [10] or immunoprecipitation using the commercially available anti-ERK antibody [12], which is not specific for ERK2 (Figure 1) and precipitates other kinase activities in our hands (C. Kosaka and C. Pears, unpublished work). Specific anti-ERK2 serum, which has also been used in immunoprecipitation kinase assays [12], is not generally available. Antiserum that recognizes phosphotyrosine can also be used to demonstrate transient phosphorylation of ERK2 (results not shown) but the presence of many abundant proteins phosphorylated on tyrosine means that this is not a sensitive assay for detecting ERK2 phosphorylation. The assay described here, combining partial purification of ERKs on phenyl-Sepharose and Western-blot analysis using an antiserum specific for tyrosine phosphorylation of a conserved MAPK activation motif, is a sensitive assay of ERK2 phosphorylation and therefore provides a useful tool for monitoring the activity of the pathways leading to ERK2 phosphorylation in *Dictyostelium*.

Effect of PKA activity on ERK2 activation

The role of intracellular cAMP in early development is unclear, and in other systems there have been reports of increased levels of intracellular cAMP having a negative [16] or positive [17] effect on MAPK activation, presumably via PKA activity. To determine the interaction between ERK2 and PKA in *Dictyostelium*, we examined tyrosine phosphorylation of ERK2 in response to chemoattractants in transformants expressing altered PKA activity. The tyrosine-phosphorylation status of ERK2 induced by extracellular cAMP closely correlated with the PKA activity of each cell strain (Figures 5a and 5c). In a mutant strain that has a 4-fold increase in PKA activity because of overexpression of a constitutively active catalytic subunit of PKA (KP cells) [7], ERK2 tyrosine phosphorylation was 4-fold greater than in Ax2 cells, although the level of total ERK2 protein was not significantly different. The extent of increased tyrosine phosphorylation varied slightly between experiments but was always between 3- and 6-fold greater than that seen in Ax2 cells analysed on the same gel. Conversely, in a strain overexpressing a dominant negative regulatory subunit of PKA (Rm cells) in which PKA activity was eliminated [5], ERK2 tyrosine phosphorylation was suppressed. The low level of tyrosine phosphorylation of ERK2 in response to cAMP seen in Rm cells may be explained by the recent finding that expression of the gene encoding cAR1 is significantly reduced in Rm cells [6], as ERK2 activation is dependent on cAR1 expression [10]. Preliminary data revealed that pulsing with cAMP to allow the induction of cAR1 expression does not increase the amount of ERK2 stimulation in Rm cells (C. Kosaka and C. Pears, unpublished work), suggesting that it is not only low levels of cAR1 that limits ERK2 tyrosine phosphorylation in Rm cells. The observations in KP cells clearly demonstrate that ERK2 tyrosine phosphorylation in response to cAMP is positively modulated by a PKA-regulated step.

The close correlation between ERK2 and PKA activities was not observed in response to folic acid stimulation (Figures 5b and 5c). Folic acid induced a comparable level of ERK2 tyrosine

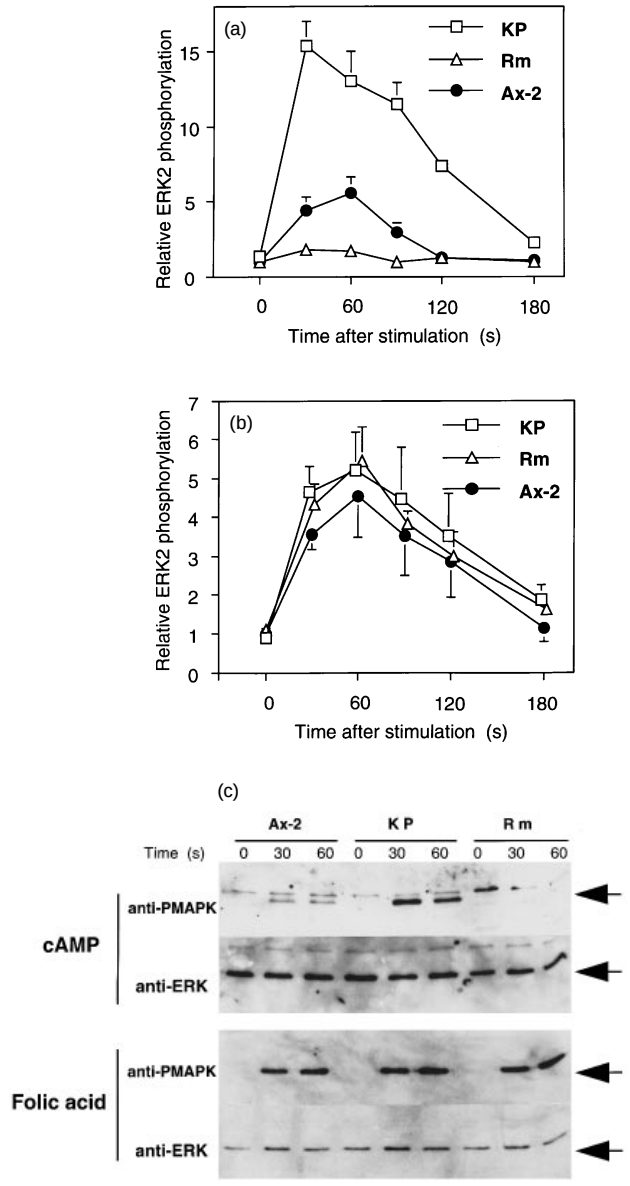

Figure 5 Chemoattractant-induced changes of the ERK2 tyrosine phosphorylation in strains with altered PKA activity

(**a**) cAMP-induced ERK2 tyrosine phosphorylation in PKA mutants. (**b**) Folic acid-induced ERK2 tyrosine phosphorylation in PKA mutants. Cells were harvested at 0, 30, 60, 90, 120 and 180 s after stimulation and analysed for the amounts of PMAPK and total MAPK as described in the legend to Figure 2. The relative levels of tyrosine phosphorylation of ERK2 were measured by densitometric scanning and expressed as means $\pm$ S.D. fold stimulation of the basal level of ERK2 phosphorylation in Ax2 cells ($n = 3$). Ax2, cells with normal PKA activity; KP, mutant expressing constitutively active catalytic subunit of PKA; Rm, mutant expressing a dominant negative regulatory subunit of PKA. (**c**) Typical example of Western blotting for chemoattractant-induced changes of ERK2 tyrosine phosphorylation in PKA. Cells were harvested at the indicated time after stimulation and analysed for the amounts of PMAPK and total MAPK as described in the legend to Figure 2. The 42 kDa band of interest is marked with a solid arrowhead.

phosphorylation, with comparable kinetics, in KP and Rm cells to that in Ax2 cells, indicating that PKA does not modulate the signalling pathways leading to ERK2 tyrosine phosphorylation in response to folic acid. Therefore there must be different pathways leading from the cAMP and folic acid receptors, which converge into a common pathway for ERK2 tyrosine phosphorylation downstream of a step modulated by PKA for cAMP.

Folic acid and cAMP bind to different receptors and these receptors couple to different G-proteins. Cells lacking Gα4 are selectively defective in folic acid-induced responses [18], and those lacking Gα2 lose chemoattraction to a source of cAMP [19]. However, both signals are ineffectual in cells deficient in the only G-protein β-subunit identified in *Dictyostelium* [20], suggesting that the $\beta\gamma$-subunits are responsible for propagating the signal and that the pathways from the two receptors may converge at this point. The activation of ERK2 in response to cAMP has been reported to be both dependent [12] and independent [10] of these $\beta\gamma$-subunits. If independent, then PKA must be able to modulate the alternative pathway stimulated by the cAR1 receptor. If dependent, then PKA may influence ERK2 activation by modulating the coupling of cAR1 to G-protein and so influencing the generation of free $\beta\gamma$-subunits in response to cAMP but not modulating the same step in the folic acid response. An alternative explanation is that PKA can modulate the affinity of the receptor for ligand.

The signalling pathways directly leading to ERK2 activation in *Dictyostelium* are not known. The correlation between ERK2 activity and tyrosine phosphorylation and this rapid assay for tyrosine phosphorylation will facilitate analysis of the signalling pathways leading to ERK2 activation and the downstream events involved in the regulation of aggregation.

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