Nonadaptive Regulation of ERK2 in *Dictyostelium*: Implications for Mechanisms of cAMP Relay

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It is assumed that ERK2 in *Dictyostelium* is subject to adaptive regulation in response to constant extracellular ligand stimulation. We now show, to the contrary, that ERK2 remains active under continuous stimulation, differing from most ligand-activated pathways in chemotactically competent *Dictyostelium* and other cells. We show that the upstream phosphorylation pathway, responsible for ERK2 activation, transiently responds to receptor stimulation, whereas ERK2 dephosphorylation (deactivation) is inhibited by continuous stimulation. We argue that the net result of these two regulatory actions is a persistently active ERK2 pathway when the extracellular ligand (i.e., cAMP) concentration is held constant and that oscillatory production/destruction of secreted cAMP in chemotaxing cells accounts for the observed oscillatory activity of ERK2. We also show that pathways controlling seven-transmembrane receptor (7-TMR) ERK2 activation/deactivation function independently of G proteins and ligand-induced production of intracellular cAMP and the consequent activation of PKA. Finally, we propose that this regulation enables ERK2 to function both in an oscillatory manner, critical for chemotaxis, and in a persistent manner, necessary for gene expression, as secreted ligand concentration increases during later development. This work redefines mechanisms of ERK2 regulation by 7-TMR signaling in *Dictyostelium* and establishes new implications for control of signal relay during chemotaxis.

INTRODUCTION

Seven-transmembrane receptor (7-TMR) signaling regulates multiple intracellular pathways. Intrinsic to many of these signaling cascades is the ability to desensitize or “adapt” to a constant extracellular ligand concentration (Ferguson and Caron, 1998; Kimmel and Parent, 2003; Kimmel *et al.*, 2004; Manahan *et al.*, 2004). In general, desensitization (adaptation) allows for response plasticity toward a variety of signaling conditions and is proposed to be particularly significant for migratory cells to discern the direction of a chemoattractant signal (Manahan *et al.*, 2004; Schneider and Haugh, 2005).

Dictyostelium has proven an excellent model for the study of chemotaxis and adaptive responses in eukaryotes (Kimmel and Parent, 2003; Kimmel *et al.*, 2004; Manahan *et al.*, 2004). During early development, *Dictyostelium* periodically synthesizes and secretes cAMP, which, in addition to its role as an intracellular second messenger, acts as an extracellular chemoattractant. The extracellular cAMP is perceived by the cAMP receptor 1 (CAR1), a cell surface 7-TMR, which activates downstream networks through both G protein-dependent and -independent mechanisms (Brzostowski and Kimmel, 2001; Kimmel and Parent, 2003; Kimmel *et al.*, 2004). One of these cAMP/CAR1 pathways leads to the activation of adenylyl cyclase A (ACA) and the consequent production and relay of the original cAMP stimulus. A key aspect of cAMP signaling is the rapid degradation of the

cAMP ligand by a secreted, extracellular cAMP-phosphodiesterase (PDE). The degradation of cAMP allows cells to reset for a new stimulatory cycle (Kimmel and Parent, 2003; Kimmel *et al.*, 2004; Manahan *et al.*, 2004).

However, loss of cellular response through ligand clearing (i.e., cAMP degradation) is not the only mechanism that cells use to terminate an activated circuit. Many CAR1-regulated pathways are only activated transiently as they adapt (become desensitized) to a persistent cAMP signal (Kimmel and Parent, 2003; Kimmel *et al.*, 2004; Manahan *et al.*, 2004). Such adaptive pathways include the transitory generation of second messengers phosphatidylinositol-3,4,5-triphosphate (PIP₃) and cGMP, as well as cAMP. The activation and deactivation of these cascades generates the oscillating intracellular and extracellular signals that are essential to coordinate chemotactic movement and to organize multicellular development.

The MAP kinase ERK2 of *Dictyostelium* is another pathway that is transiently activated upon CAR1 engagement (Knetsch *et al.*, 1996; Maeda *et al.*, 1996, 2004; Kosaka and Pears, 1997). Although the transient nature of the pathway had been presumed to require an adaptive response, the mechanism for ERK2 deactivation has never been examined directly. MAPK/ERK signaling cascades function broadly in eukaryotes to regulate processes that include cell growth, proliferation, stress response, cell migration, and metastasis and in *Dictyostelium* ERK2 is required for normal chemotactic response, cAMP signal relay, and specification of developmentally regulated gene expression (Segall *et al.*, 1995; Gaskins *et al.*, 1996; Zhang *et al.*, 2003; Maeda *et al.*, 2004; Sawai *et al.*, 2005). Further, it has been recently argued that ERK2 controls cAMP accumulation by negatively regulating the activity of the intracellular cAMP phosphodiesterase

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RegA (Laub and Loomis, 1998; Kimmel and Parent, 2003; Kimmel *et al.*, 2004; Maeda *et al.*, 2004). Thus, it is essential to understand ERK2 activating and deactivating pathways in the context of extracellular signal response. Here, we examine both pathways of ERK2 regulation, demonstrate that ERK2 is nonadaptive, and discuss new implications for ERK2 control of signal relay during chemotaxis and developmentally regulated gene expression in response to persistent ligand stimulation.

MATERIALS AND METHODS

Cell Culturing and Development

Dictyostelium were grown in nutrient-rich D3-T media (KD Medical, Columbia, MD) to log phase ($1-3 \times 10^6$ cells/ml) and harvested for development by low speed centrifugation ($2000 \times g$) for 3 min. Cells were washed once by centrifugation in Development Buffer (DB; 7.4 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 4 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 2 mM MgCl_2 ; 0.2 mM CaCl_2 , pH 6.5) and resuspended in DB to 2×10^7 cells/ml. Cells were shaken at 22°C for ~ 5.5 h at 200 rpm. After the first hour of shaking, cells received exogenous pulses of 75 nM cAMP every 6 min for the next 4.5 h to promote synchronous development. Development on Millipore filter pads were performed as previously described (Kim *et al.*, 1999).

ERK2 Activity Assay

After development with cAMP pulses, cells were washed, resuspended to 1×10^7 cells/ml in phosphate buffer (PB; 7.4 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 4 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.5), and shaken with 2 mM caffeine for 20 min to inhibit endogenous cAMP signaling and to bring intracellular responses to basal levels (Brenner and Thoms, 1984). Cells were then washed from caffeine in ice-cold phosphate buffer, resuspended at 5×10^7 cells/ml, and held at 4°C . Before stimulation, 1.5 ml of cells was shaken at 250 rpm for 1.5 min at 20°C . While shaking continued, cells were stimulated with a 1:100 volume of cAMP (or a nondegradable cAMP analogue Sp-cAMPS, Sigma, St. Louis, MO) to achieve a desired final concentration, and 100 μl aliquots of cells were removed and lysed in $4\times$ LDS sample buffer (Invitrogen, Carlsbad, CA) at selected times. Total protein was separated by electrophoresis using 4–12% NuPage BisTris polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with mammalian α -phospho-threonine/tyrosine ERK2 antibody (Cell Signaling Technology, Beverly, MA, 1:1000 dilution) to assess the level of ERK2 activation. To inhibit the extracellular cAMP PDE, dithiothreitol (DTT) was added 1.5 min before stimulation (as the cell suspension was warming) to a final concentration of 10 mM. Note that as an added level of control for ERK2 response, $-/+$ DTT conditions were performed simultaneously.

Extracellular cAMP PDE Assay

PDE activity was determined as previously described (Manganiello and Vaughan, 1973). Briefly, 1.5 ml of pulse-developed cells was stimulated with 10 μM [^3H]cAMP (50,000 cpm/nM) in the absence and the presence of DTT. Samples, 200 μl , were removed at various times, and cells were briefly pelleted in a microcentrifuge for 15 s. The supernatant was removed and added to a tube containing 100 μl of $4\times$ assay buffer (0.2 M HEPES, 0.4 M EGTA, 33.5 mM MgCl_2) and 100 μl of stop solution (10 mM cAMP, 5 mM ATP, 0.25 M HCl). At the end of the time course, 100 μl of neutralization buffer (0.25 M Tris, pH 8, 0.25 M NaOH) was added to each sample. The endogenous 5'-AMP was converted to adenosine by incubation with 100 μl of *Crotalus atrox* venom 5'-nucleotidase (Sigma; final concentration of 0.3 mg/ml) at 37°C for 30 min. The adenosine product was separated from substrate using ion exchange chromatography (QAE sephadex A25; GE Healthcare Biosciences, Piscataway, NJ). One milliliter of a 50% suspension of swollen and washed sephadex gel in water was placed into a disposable chromatography column, and 0.5 ml of the reaction was added to the column. The adenosine product was eluted with an additional 4 ml of water and quantified by scintillation counting. The amount of adenosine produced directly reflects the cAMP degraded and is proportional to PDE activity in the sample.

RESULTS

ERK2 Dephosphorylation Is Inhibited by a Persistent cAMP Stimulus

MAPK/ERK activity is regulated by a bipartite process in all eukaryotes (Gutkind, 2000; Hazzalin and Mahadevan, 2002; Roux and Blenis, 2004); activation requires site-specific phosphorylations by upstream kinases on the threonine and tyrosine residues of the TXY motif within the activation loop

Figure 1. ERK2 is transiently phosphorylated upon cAMP stimulation. Differentiated cells were stimulated at 5×10^7 cells/ml with the indicated dose of cAMP, and aliquots were removed and lysed into protein loading buffer at the given times. Total protein was separated by SDS gel electrophoresis and probed on immunoblots with α -phospho-threonine/tyrosine ERK2 antibody to assess the level of ERK2 activation. Experiments at varying cAMP concentrations were performed at least 10 times.

of the enzyme, and deactivation is achieved by dephosphorylation of pTXpY. Generally, multiple MAPK/ERK-kinases (MEKs) and ERK-specific phosphatases are present in eukaryotic cells, which themselves are subject to various modes of regulation, but little is understood of the complexity of ERK2 regulation in *Dictyostelium*.

In chemotactically competent *Dictyostelium* cells, global cAMP stimulation induces the transient phosphorylation/activation of ERK2 (Knetsch *et al.*, 1996; Maeda *et al.*, 1996, 2004; Kosaka and Pears, 1997), but the activation/deactivation mechanisms controlling ERK2 activity remain unclear. Although untested, it has been assumed that the ERK2 pathway responds transiently (i.e., adapts) to a persistent cAMP stimulus. However, the experimental conditions used by previous investigators did not control for the destruction of the extracellular cAMP ligand by the extracellular cAMP-PDE, nor were kinase and phosphatase activities separately analyzed to understand the mechanism of ERK2 activation and deactivation, respectively. Here we carefully dissect these processes.

First, we wanted to analyze the response of ERK2 phosphorylation in *Dictyostelium* to different doses of exogenous cAMP. Cells were differentiated to a chemotactically competent state and stimulated with either subsaturating (<100 nM) or saturating ($>1 \mu\text{M}$) doses of cAMP (Johnson *et al.*, 1992); aliquots of cells were lysed over a time course, and phosphorylated/activated ERK2 was analyzed, as previously demonstrated (Kosaka and Pears, 1997; Maeda *et al.*, 2004), by immunoblot assay using an α -phospho-threonine/tyrosine ERK2 antibody. Regardless of the cAMP concentration, ligand stimulation causes a rapid, but transient, increase in ERK2 phosphorylation that peaks between 30 and 60 s and returns to basal levels by 3 min after stimulation (Figure 1). Historically, micromolar concentrations of cAMP have been used to assay ERK2 activation with the assumption that the cAMP concentration would remain saturating throughout a multiminute time course, and data such as shown in Figure 1 have been used to conclude that the ERK2 pathway transiently responds to a constant cAMP stimulus (Knetsch *et al.*, 1996; Maeda *et al.*, 1996, 2004; Kosaka and Pears, 1997). However, because the extracellular cAMP-PDE is present in a membrane-associated form and is secreted and accumulates as a soluble protein during these "typical" assay conditions (Orlow *et al.*, 1981; Podgorski *et al.*, 1988; Hall *et al.*, 1993), it is unlikely that all of the different cAMP concentrations used would remain unchanged during the time course. In fact, previous analyses of the specific activity of the extracellular cAMP-PDE indicated that even micromolar concentrations of cAMP are subject to rapid degradation (Orlow *et al.*, 1981). Thus despite previous assumptions,

Figure 2. ERK2 remains phosphorylated under conditions that maintain persistent CAR1 stimulation. (A) A saturating stimulus of exogenous cAMP is rapidly degraded by the extracellular cAMP-PDE. Wild-type cells were stimulated with 10 μ M [3 H]cAMP in the absence or presence of DTT. The 5'-AMP degradation product of cAMP was converted to adenosine in vitro, purified by ion exchange chromatography, and measured by scintillation counting. The results are graphed as a relative change in [3 H]cAMP. (B) Wild-type cells were stimulated with 10 μ M cAMP in the absence or presence of 10 mM DTT. (C) Wild-type cells were repetitively stimulated with 10 μ M cAMP at 2-min intervals over the time course in the absence of DTT. (D) Wild-type cells were stimulated with 50 μ M Sp-cAMPS in the absence of DTT. For B–D, pERK2 was assayed by immunoblot as described in Figure 1 and was done at least three times each.

the assay would appear inappropriate to assess ERK2 responsiveness to a persistent cAMP signal.

We therefore decided it was essential to monitor the stability of extracellular cAMP in differentiating *Dictyostelium*. We quantified the input of exogenous [3 H]cAMP through a defined time course and found that >90% of a 10 μ M cAMP stimulus is degraded within 3 min (Figure 2A); 1 μ M cAMP is degraded to <100 nM within the same time frame (unpublished data). These data indicate that even a supersaturating dose of cAMP can be quickly hydrolyzed to subsaturating levels by the extracellular PDE under standard assay conditions. Moreover, these results show that the conditions used in previous studies were not adequate to determine if the ERK2 pathway could transiently respond to persistent stimulation and prompted us to design experiments to reexamine ERK2 activity under true persistent cAMP stimulation.

We first tested ERK2 activation under conditions that inhibit the extracellular cAMP-PDE. The extracellular cAMP-PDE of *Dictyostelium* is a type II class PDE and is, thus, resistant to the standard type I PDE inhibitors (e.g., IBMX) used for most intracellular mammalian PDEs. However, 10 mM DTT will effectively inhibit the extracellular *Dictyostelium* cAMP-PDE (Orlow *et al.*, 1981) and is routinely used to measure cAMP accumulation in the absence of degradation (Brzostowski *et al.*, 2004). DTT does not alter the adaptive

response of ACA and other pathways (Brzostowski *et al.*, 2004).

As expected, the addition of DTT 1.5 min before stimulation effectively inhibited PDE activity and maintained constant cAMP levels throughout the assay (Figure 2A). Although the kinetics of cAMP-induced ERK2 phosphorylation (activation) was unaffected by the addition of DTT, dephosphorylation was strikingly inhibited in cells stimulated in the presence of DTT (Figure 2B). In contrast to what had been previously assumed, our data suggest that ERK2 remains persistently phosphorylated in the presence of constant cAMP signal.

To confirm these conclusions and to control for potential nonspecific effects of DTT, we modified assay conditions in ways that maintained the cAMP signal without using DTT to inhibit the extracellular PDE. In one experiment, cells were stimulated with 10 μ M cAMP every 2 min during a 10-min time course, and in a separate test, cells received a single saturating dose of the nondegradable cAMP analogue Sp-cAMPS (Schaap *et al.*, 1993). Under both conditions (Figure 2, C and D), ERK2 remained phosphorylated throughout the time course, indicating that the ERK2 dephosphorylation observed during a standard assay is dependent on ligand-clearing by the extracellular PDE.

Although we suggest that the rapid degradation of extracellular cAMP by PDE and, not receptor adaptation, promotes the deactivation of ERK2, we do not believe our results alter previous conclusions regarding the adaptive regulation of ACA or of other CAR1-activated pathways, such as PIP₃ production, actin polymerization, Ca²⁺-influx, and cGMP accumulation. Adaptive regulation of ACA is well established (Dinauer *et al.*, 1980; Kimmel and Parent, 2003; Kimmel *et al.*, 2004) and the kinetics of activation and deactivation (within ~5 and ~20 s after stimulation, respectively) of the latter pathways occur much faster than does cAMP degradation (Kimmel and Parent, 2003; Kimmel *et al.*, 2004).

Ligand Depletion Promotes ERK2 Dephosphorylation

To test directly the effect of ligand clearing on ERK2 regulation, we sought to rapidly deplete cAMP from the extracellular milieu while monitoring ERK2 phosphorylation levels. We reasoned that if ERK2 dephosphorylation were regulated by a decrease in receptor occupancy, then immediate reduction in cAMP concentration by media dilution should induce a rapid dephosphorylation of ERK2, even in the presence of DTT.

To perform this experiment, we treated cells with caffeine (Brenner and Thoms, 1984), which prevents the synthesis and accumulation of intracellular and secreted cAMP. Caffeine treatment ensured that de novo production of cAMP would not alter the cAMP concentration during the experiment. In a standard ERK2-activation assay, wild-type cells maintained in 2 mM caffeine in the absence and presence of DTT behave identically to untreated cells (Figure 3A).

To assay the effects of ligand dilution, cells were maintained in caffeine when stimulated with 100 nM cAMP in the presence of 10 mM DTT. At 2 min after stimulation, cells were diluted 10-fold with phosphate buffer containing 10 mM DTT. The 100 nM starting concentration of cAMP was chosen so that the diluted ligand concentration, 10 nM, would be brought well below saturating levels for receptor binding (Johnson *et al.*, 1992). Control and diluted samples were taken over a time course, and appropriate volumes were used to ensure equal loading on gels, which was confirmed by Coomassie blue staining (unpublished data). As seen in Figure 3B, phosphorylated ERK2 persists to 8 min

Figure 3. Dilution of the extracellular cAMP concentration induces ERK2 dephosphorylation. (A) Inhibition of intracellular cAMP production by caffeine does not alter ERK2 phosphorylation. Cells were stimulated and maintained in the presence of 2 mM caffeine the absence or presence of 10 mM DTT. (B) Wild-type cells were maintained in 2 mM caffeine for stimulation with 100 nM cAMP in presence of 10 mM DTT. At 2 min after stimulation, an aliquot of cells was removed and diluted 10× into PB containing 10 mM DTT and 2 mM caffeine. (C) *gβ*-null cells were maintained in 2 mM DTT. At 2 min after stimulation, an aliquot of cells was removed and diluted 10× into PB containing 10 mM DTT and 2 mM caffeine. For A–C, pERK2 was assayed by immunoblot as described in Figure 1, and done at least three times each.

after stimulation in the control stimulation, whereas ERK2 is rapidly dephosphorylated in cells diluted 10-fold at 2 min after stimulation. Note that ERK2 remained phosphorylated if cells were diluted with buffer containing the same starting concentration of cAMP (unpublished data). Consistent with our previous interpretation, these results indicate that ERK2 dephosphorylation is initiated by a decrease in receptor occupancy.

It had been previously shown that cAMP-induced activation of ERK2 can occur in the absence of CAR1 interaction with heterotrimeric G protein complexes (Maeda *et al.*, 1996). In accord we found that in *gβ*-null cells, ERK2 is transiently phosphorylated under standard conditions (Knetsch *et al.*, 1996; Maeda *et al.*, 1996; unpublished data), and like wild-type cells, ERK2 remains phosphorylated in the presence of persistent cAMP, but is rapidly dephosphorylated upon a depletion of the ligand (Figure 3B). Therefore, these results indicate that neither ERK2 phosphorylation nor dephosphorylation requires the action of heterotrimeric G proteins.

The Upstream ERK2 Kinase Activity Adapts to the cAMP Ligand

As previously discussed, ERK2 regulation is bipartite, requiring activation through an upstream kinase and deactivation via a phosphatase. Mechanistically, the observed persistent phosphorylation of ERK2 in the presence of continuous ligand (see Figures 2 and 3) could be achieved by various regulatory combinations of ERK2-kinase or -phosphatase activities.

Therefore, we next designed an experiment to analyze whether the upstream kinase activity responsible for phosphorylating ERK2 responds to the cAMP ligand separately from phosphatase activity. For bona fide adaptive pathways, cells that receive an initial, subsaturating cAMP stimulus will respond at the outset but adapt (become insensitive) to that stimulus level; they will, however, remain responsive to a subsequent higher dose (Dinauer *et al.*, 1980). On the basis

Figure 4. ERK2 phosphorylation is regulated by an adaptive pathway. Wild-type cells were stimulated with the indicated initial dose of cAMP in the absence of DTT. At 3 min after stimulation, cells received a second dose of 1000 nM cAMP, and pERK2 was assayed by immunoblot as described in Figure 1. Experiments at varying cAMP concentrations were performed at least twice.

of these criteria, we tested if phosphorylation of ERK2 is adaptive.

Cells were initially incubated with 0, 10, and 1000 nM cAMP and then assayed for secondary responsiveness to a 1000 nM stimulus. Samples were removed and assayed for ERK2 phosphorylation at 1-min intervals. As expected, no ERK2 response is observed in unstimulated cells during the first 3 min of the assay (Figure 4); these cells responded maximally to the 1000 nM stimulation provided at 3 min (Figure 4). As the initial dose of cAMP was increased, the level of ERK2 phosphorylation increased proportionally at 1 min after stimulation, and consistent with an adaptive response, the second, 1000 nM cAMP stimulation produced an inverse (weakened), graded response relative to the initial dose; as the initial cAMP dose increased, the level of secondary ERK phosphorylation decreased (Figure 4). Taken together, our results (Figures 2–4) suggest that ERK2 phosphorylation and activation are mediated by an adaptive pathway, whereas dephosphorylation is inhibited by a persistent cAMP stimulus, and thus ERK2 deactivation requires the destruction of the extracellular ligand.

ERK2 Phosphorylation and Dephosphorylation Are Not Regulated by Production of Intracellular cAMP or by PKA Activity

Recently, a model has been proposed that links activation/deactivation of ERK2 to oscillatory cAMP signal production in *Dictyostelium* (Maeda *et al.*, 2004). Although the model suggests that deactivation of ERK2 is mediated by a PKA-dependent, feedback mechanism (Maeda *et al.*, 2004), our assays performed in the presence of caffeine indicate that that a cAMP-dependent increase in intracellular cAMP and presumably in resultant PKA activation are not required to deactivate ERK2 (see Figure 3A). Therefore, we tested the proposed model for the PKA-dependent, negative-feedback on ERK2 by separately analyzing the activation and deactivation of ERK2 under controlled conditions that manipulate the levels of the extracellular cAMP stimulus, intracellular cAMP, and PKA activity.

We examined ERK2 activation/deactivation in *aca*-null cells (Pitt *et al.*, 1992), cells lacking adenylyl cyclase A (ACA), which cannot produce cAMP in response to a cAMP stimulus and are, thus, incapable of cAMP/CAR1-mediated PKA activation. In addition, extracellular cAMP cannot readily diffuse into *Dictyostelium* and, under the conditions used, is unable to elicit the direct, intracellular activation of PKA (Schaap *et al.*, 1993).

First, we show that ERK2 is transiently phosphorylated (activated) in *aca*-null cells when stimulated under conditions where the cAMP ligand can be degraded (i.e., –DTT) by the extracellular cAMP-PDE (Figure 5A). These data are inconsistent with the hypothesis that activated PKA is re-

Figure 5. Ligand-induced production of intracellular cAMP does not regulate ERK2 phosphorylation. (A) ERK2 is persistently phosphorylated in *aca*-null cells stimulated in the presence of DTT (see Figure 2B for details). (B) Dilution of the extracellular cAMP concentration induces ERK2 dephosphorylation in *aca*-null cells (see Figure 3B for details). (C) ERK2 phosphorylation is regulated by an adaptive pathway in *aca*-null cells (see Figure 4 for details). Experiments at varying cAMP concentrations were performed at least three times each.

quired to negatively regulate ERK2. In the presence of a nonvarying ligand concentration (i.e., +DTT), ERK2 phosphorylation persists in *aca*-null cells (Figure 5A). In addition, ERK2 is rapidly dephosphorylated when *aca*-null cells are stimulated with cAMP in the presence of DTT and diluted 10-fold at 2 min after stimulation (Figure 5B). These results indicate that CAR1-induced production of intracellular cAMP does not significantly regulate the ERK2 pathway and that ERK2 deactivation is not dependent on an increase in intracellular cAMP levels. Rather, ERK2 dephosphorylation is mediated by depletion of the extracellular cAMP concentration.

We also examined if the upstream kinase activity responsible for phosphorylation of ERK2 were dependent on an increase in intracellular cAMP. In parallel to previous experiments (see Figure 4), *aca*-null cells were given an initial cAMP stimulus in the absence of DTT, after transient ERK2 phosphorylation, the cells were then challenged with a second saturating dose. As observed in wild-type cells, the second 1000 nM cAMP stimulation produced a graded response in *aca*-null cells that was inversely correlated with the initial dose (Figure 5C), indicating that the production of intracellular cAMP is not required for the regulation of the upstream ERK2-kinase activity.

Our results indicate that cAMP-induced PKA activity does not negatively regulate ERK2. Unfortunately, because cells lacking PKA are unable to develop (Mann *et al.*, 1992), it was not possible to examine the developmental response of ERK2 to cAMP in the complete absence of PKA activity (Kosaka and Pears, 1997). Therefore, we assayed if ERK2 phosphorylation would be persistently inhibited in cells that have constitutive (unregulated) PKA-catalytic activity. We used a strain lacking the PKA regulatory subunit (*pkaR*-null) and *pkaR*-null cells that express a PKA regulatory subunit mutant that is incapable of binding and inhibiting the PKA-catalytic subunit (*pkaR*-/*Rc*). If PKA negatively regulates ERK2, unregulated PKA-catalytic activity should promote constitutive ERK2 dephosphorylation. To the contrary and consistent with our results in experiments with cells that lack ACA (see Figure 5), ERK2 in both *pkaR*-null and *pkaR*-/*Rc* cells is transiently phosphorylated in the absence

Figure 6. Unregulated PKA activity does not alter ERK2 phosphorylation/dephosphorylation in response to cAMP stimulation. (A) ERK2 is persistently phosphorylated in *pkaR*- cells stimulated in the presence of DTT (see Figure 2B for details). (B) ERK2 is persistently phosphorylated in *pkaR*-/*Rc* cells stimulated in the presence of DTT (see Figure 2B for details). Experiments at varying cAMP concentrations were performed at least three times each.

Figure 7. ERK2 phosphorylation increases during development. Cells were developed on filter pads at 1.25×10^6 cells/cm², and total proteins were harvested at the indicated times. pERK2 was assayed by immunoblot as described in Figure 1.

of DTT and remains phosphorylated in the presence of persistent cAMP stimulation (Figure 6).

Levels of Phosphorylated ERK2 Increase during Development

As *Dictyostelium* begin to form multicellular aggregates, there is an increase in levels of extracellular cAMP (Abe and Yanagisawa, 1983). The transition to more continuous cAMP signaling is required to induce aggregation stage and cell-specific gene expression (Kimmel and Firtel, 1991, 2004). Our data predict that levels of ERK2 phosphorylation should increase as extracellular cAMP accumulates during development. We, therefore, tested the simple hypothesis that phosphorylation of ERK2 should rise coincident with the rise in extracellular cAMP during early development. Cells were developed on filter pads and harvested for total proteins at the indicated times, and the level of phosphorylated ERK2 was assessed by immunoblotting (Figure 7). Consistent with the increase of secreted cAMP levels (Abe and Yanagisawa, 1983) and in parallel with the persistent phosphorylation of CAR1 (Johnson *et al.*, 1993), the level of ERK2 phosphorylation rises as cells begin to assemble into aggregates (Figure 7). Note ERK2 expression is constant during chemotaxis and through the rest of development (Segall *et al.*, 1995; Maeda *et al.*, 2004).

DISCUSSION

Aggregation in *Dictyostelium* is a complex series of processes that regulate chemoattractant production and secretion (signal relay) and chemotactic response. Individual *Dictyostelium* produce and secrete a cAMP signal that is dispersed through the population. Other cells respond by relaying the signal outwardly, creating a natural wave, but the cells also adapt to the original signal and cease signal production, awaiting a refractory period before regaining responsiveness. Thus, cells become initially exposed to an increasing

Figure 8. A model for the activation and deactivation of ERK2 during cAMP signal relay. Extracellular cAMP stimulates that activation of CAR1, which in turn activates an unidentified ERK2 kinase but also inhibits an unidentified ERK2 phosphatase (PPase). The ERK2 kinase pathway becomes maximally active at ~ 1 min after stimulation by cAMP, but then adapts within ~ 3 min, by an unknown mechanism. Inhibition of the ERK2 PPase is nonadaptive and is constitutively inhibited by continuous cAMP stimulation. In the presence of a persistent cAMP stimulus, ERK2 is constitutively phosphorylated. On removal of extracellular cAMP through action of the secreted phosphodiesterase (PDE), the PPase is reactivated and ERK2 is dephosphorylated; the ERK2 kinase is not reactivated upon removal of the cAMP stimulus. It is suggested that pERK2 phosphorylates and consequently inhibits the intracellular cAMP-PDE RegA (unpublished data), thus allowing intracellular levels of cAMP to accumulate (Maeda *et al.*, 2004). The rise in intracellular cAMP activates PKA, which is genetically and mathematically predicted to promote dephosphorylation of pERK2, reactivate RegA, and, thus, reduce intracellular levels of cAMP (Maeda *et al.*, 2004). However, our data indicate that PKA does not regulate ERK2 phosphorylation and activity. Potentially, the direct phosphorylation of RegA by PKA within the interdomain region at S413 may relieve ERK2-mediated inhibition of RegA.

temporal gradient, as well as a positive spatial gradient of cAMP; subsequently they experience decreasing temporal and negative spatial gradients of cAMP (Soll *et al.*, 2002). Although it is not yet feasible to analyze ERK2 regulation in a chemotaxing cell, our data, which derive from stimulations using both subsaturating and saturating concentrations of cAMP and exposures to increasing and decreasing stimuli, are predictive for how ERK2 activity is regulated by receptor activation.

Indeed, our results establish a new paradigm for ERK2 signaling in *Dictyostelium*, demonstrating that ERK2 is active in the presence of a constant extracellular cAMP signal. This fundamental conclusion contrasts the previous assumptions that the ERK2 pathway is deactivated in response to persistent stimulation (Knetsch *et al.*, 1996; Maeda *et al.*, 1996, 2004; Kosaka and Pears, 1997). Still, ERK2 regulation is complex. We suggest that an upstream ERK2-kinase is rapidly activated in response to cAMP, but then is desensitized to that signal level (see Figure 8); however, because ERK2-phosphatase activity is suppressed if the cAMP concentration is held constant, ERK2 activity is persistent and nonadaptive. Under conditions where the production of the cAMP ligand is destroyed by the extracellular cAMP-PDE, CAR1-mediated inhibition of the ERK2-phosphatase would cease, thus, explaining the observed oscillatory activation/deactivation of ERK2 as cells chemotax into multicellular aggregates (Maeda *et al.*, 2004).

Two recent studies have examined the connection between ERK2 and the periodic production of cAMP during the chemotactic phase of *Dictyostelium* development (Maeda *et al.*, 2004; Sawai *et al.*, 2005). Maeda *et al.* (2004) suggest that ERK2 functions to negatively regulate the intracellular cAMP-PDE RegA, thus modulating intracellular cAMP accumulation and ultimately the level of secreted cAMP,

whereas, Sawai *et al.* (2005) propose that cAMP oscillations were primarily dependent on the action of the extracellular cAMP-PDE, and not via an intracellular circuit.

Stimulation of CAR1 activates both ERK2 and the aggregation specific adenylyl cyclase ACA (Kimmel and Parent, 2003; Kimmel *et al.*, 2004). In the model proposed by Maeda *et al.* (2004), activated ERK2 phosphorylates and inhibits the intracellular phosphodiesterase RegA, thereby, increasing intracellular cAMP levels, which, in turn, activate PKA. As cAMP accumulates, it is also secreted, thus recruiting neighboring cells to participate in cAMP signal/relay. Central to the oscillating circuit is the attenuation of cAMP accumulation and production. Because cAMP accumulation is impaired in cells with unregulated PKA activity (*pkaR*⁻) and further impaired in *pkaR*⁻/*erk2*⁻ double mutants (Maeda *et al.*, 2004), PKA was proposed to negatively regulate both ERK2 and ACA (Maeda *et al.*, 2004). The negative feedback via the ERK2/RegA/PKA loop would cause an increase in the activity of the intracellular PDE RegA and a decrease in ACA activity. As a consequence, intracellular cAMP levels would decline, thus reducing PKA activity. The last key component of this proposed oscillatory loop is the destruction of the secreted cAMP ligand by the extracellular PDE, which allows the system to reset for the next round of signaling.

If PKA inhibited pERK2, cells with compromised PKA activity should exhibit persistent ERK2 phosphorylation (i.e., loss of the ERK2 inactivation pathway) irrespective of a transient or continuous extracellular cAMP stimulation; conversely, cells with constitutively active PKA should have suppressed ERK2 activity. Yet, we find that ERK2 phosphorylation persists in *aca*-null cells that are unable to produce intracellular cAMP in response to CAR1 stimulation and are thus unable to activate PKA. Because cells lacking the PKA catalytic activity are unable to develop, they could not be used in our analyses, but ERK2 is regulated identically to that of wild-type cells and in mutants with constitutive PKA activity (*pkaR*⁻ or *pkaR*⁻/*Rc*); ERK2 is phosphorylated transiently under conditions that permit ligand degradation, and ERK2 phosphorylation persists in the presence of constant ligand in all cell lines. In addition, overexpression of PKA does not reduce ERK2 activation (Kosaka and Pears, 1997). Collectively, these results are incompatible with the simple negative regulation of ERK2 by PKA; we do not exclude more complex interactions such as the direct phosphorylation of RegA by PKA for relief of ERK2-mediated inhibition of RegA. In addition, we argue that adaptation of the ACA pathway coupled with the degradation of extracellular cAMP are critical for signaling, conclusions more in agreement with data that indicate cAMP oscillations can be maintained during development in cells lacking either RegA or the regulatory subunit of PKA (Sawai *et al.*, 2005). On the basis of these biological behaviors, Sawai and coworkers concluded that periodic cAMP signaling required the activity of the secreted cAMP phosphodiesterase for ligand clearing and not an intracellular feedback loop regulated by PKA. Nonetheless, the data do not address the potential role of ERK2 and oscillations in intracellular cAMP for chemotactic response to cAMP waves (Wessels *et al.*, 2000; Zhang *et al.*, 2003; Stepanovic *et al.*, 2005).

As *Dictyostelium* chemotax and coalesce into tight aggregates, the extracellular levels of cAMP rise (Abe and Yanagisawa, 1983), and significant changes in gene transcription ensue (Kimmel and Firtel, 1991, 2004). These transcriptional responses can be mimicked in shaking culture by addition of 300 μ M cAMP, which remains stable during the time course, and have been considered nonadaptive (Kimmel and Firtel,

1991, 2004). Like other G protein-coupled 7-TMRs, CAR1 is phosphorylated at key cytoplasmic residues in response to ligand stimulation (Hereld *et al.*, 1994); like ERK2, phosphorylation of CAR1 oscillates during cAMP signaling but persists when the cAMP stimulus is continuous (Klein *et al.*, 1985). Experiments using a temperature-sensitive ERK2 mutant have proven that ERK2 is required to regulate gene expression after chemotaxis (Gaskins *et al.*, 1996). ERK2 signaling may thus be constitutively activated during this stage of development. Our data suggest that the rise in extracellular cAMP during aggregation attributes to the coincident increase in the phosphorylation of ERK2 and of CAR1 (Johnson *et al.*, 1993). The changes in ERK2 phosphorylation may suggest a functional switch as cells transition from an actively crawling, undifferentiated state to a state of differentiating cell types.

Although it remains to be determined if similar control of ERK activity is present in mammalian cells, the broader implication of our observations is that ERK2 activation/deactivation (Gutkind, 2000; Hazzalin and Mahadevan, 2002; Roux and Blenis, 2004) can be controlled in a quick-response manner through a single receptor-type. Potentially, MAPK/ERK phosphatase activity in other cells is also regulated at the level of the ligand-bound receptor. The rapid modulation of ERK activity may allow cells to maintain response plasticity in environments where chemoattractant and other signaling gradients can hastily change or remain persistent.

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