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The *Dictyostelium* Mitogen-activated Protein Kinase ERK2 Is Regulated by Ras and cAMP-dependent Protein Kinase (PKA) and Mediates PKA Function*

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The chemoattractant cAMP, acting through serpentine cAMP receptors, results in a rapid and transient stimulation of the *Dictyostelium* mitogen-activated protein kinase ERK2 activity (1). In this study we show that other pathways required for aggregation, including Ras and cAMP-dependent protein kinase (PKA), are important regulators of ERK2 activation and adaptation. By examining both the level and kinetics of activation and adaptation of ERK2, we show that Ras is a negative regulator of ERK2. Activated Ras or disruption of a Ras GAP gene results in reduced ERK2 activation whereas disruption of putative Ras GEF or expression of dominant negative Ras proteins have a more rapid, higher, and extended activation. CRAC, a PH domain-containing protein required for adenylyl cyclase activation, is also required for proper ERK2 adaptation. PKA overexpression results in a more rapid, higher level of activation, whereas *pka* null cells show a lower level but more extended ERK2 activation. Furthermore, we show that constitutive expression of PKA catalytic subunit bypasses the requirement of ERK2 for aggregation and later development, indicating that PKA lies downstream from ERK2 and that ERK2 may regulate one or more components of the signaling pathway required for mediating PKA function, possibly by directly regulating PKA R or a protein controlling the intracellular level of cAMP.

A cell's ability to respond to an extracellular signal involves both the activation of pathways and subsequent adaptation in which the cells are no longer fully responsive to the extracellular signal. This allows the cells to properly regulate the level and extent of the signaling pathway as well as adapt to changing environmental conditions. Well known examples are the pheromone pathway in yeast and the mammalian cell β-adrenergic receptor (2, 3). In *Dictyostelium*, aggregation is mediated by a periodic activation and adaptation of pathways regulated by G protein-coupled cARs[1] that bind extracellular cAMP as the ligand (4–6). In response to extracellular cAMP, guanylyl and adenylyl cyclase activities are rapidly stimulated and then adapted. If cAMP levels are kept constant the cells remain nonresponsive, whereas removal of the cAMP, which *in vivo* occurs through its hydrolysis by an extracellular phosphodiesterase, allows the pathways to de-adapt within ~5 min.

MAP kinase cascades regulate a variety of intracellular responses through the activation of cell surface receptors (1, 2, 7). During the preaggregation and aggregation stages of *Dictyostelium* development, the MAP kinase ERK2 is activated through cAMP and folate chemotactic receptors (1).[2] Stimulation with extracellular cAMP results in a >40-fold increase in ERK2 activity that peaks at ~50 s and thereupon adapts, reaching its basal level within 5–8 min. ERK2 activation requires the G protein-coupled cAMP receptors that mediate cAMP-stimulated adenylyl and guanylyl cyclase activation, chemotaxis, and gene expression. cAMP-mediated activation of ERK2 is partially independent of Gα2, the Gα subunit required for cAMP stimulation of adenylyl and guanylyl cyclases, all other identified Gα subunits, and the only known Gβ subunit (1). *erk2* null cells are unable to aggregate due to a defect in cAMP stimulation of adenylyl cyclase (8). Adenylyl cyclase (ACA) and other signaling components known to be required for the activation of adenylyl cyclase are found at normal levels in *erk2* null cells. ERK2 is also required for cell type differentiation and morphogenesis during the multicellular stages of development as determined by the analysis of an ERK2 temperature-sensitive mutation (9).

Here we examine the regulation of ERK2 activation and adaptation. Using mutants in the Ras pathway, including constitutively active and dominant negative Ras mutations, a Ras GAP and a Ras GEF null mutation, we first show that Ras acts as a negative regulator of ERK2 activity. Mutations in the cytosolic regulator of adenylyl cyclase, CRAC, result in a loss of proper ERK2 adaptation. Examination of cells in which the cAMP-dependent protein kinase PKA catalytic subunit is disrupted or overexpressed indicates that PKA also plays an important role in these processes. Furthermore, we show that overexpression of PKA in *erk2* null cells bypasses the requirement of ERK2 for aggregation and that these cells form normal fruiting bodies. Our results suggest complex pathways involving Ras, adenylyl cyclase and coupled components, and PKA affecting the level and extent of ERK2 activation and that ERK2 functions to control the level of activation of PKA by

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[1] The abbreviations used are: cAR, chemoattractant receptor; MAP, mitogen-activated protein; ACA, adenylyl cyclase; CRAC, cytosolic regulator of adenylyl cyclase; PKA, cAMP-dependent protein kinase; GEF, guanine exchange factor; MBP, myelin basic protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

[2] M. Maeda and R. A. Firtel, unpublished observations.

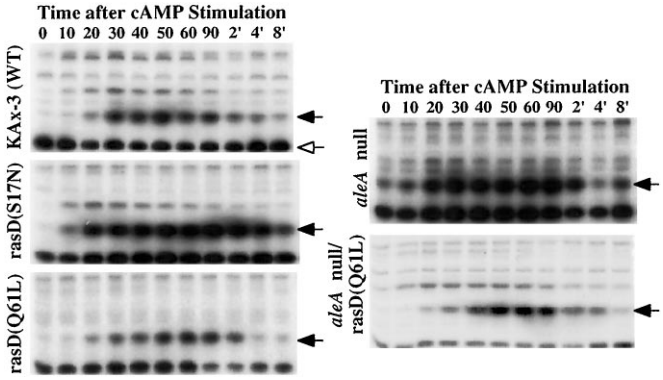

FIG. 1. **Effect of Ras on cAMP-stimulated ERK2 activation.** The results show in-gel assays of cAMP stimulation of ERK2 activity. The activation of ERK2 was measured in aggregation-competent cells (see "Materials and Methods"). "Normalization" controls containing extracts of a time course that allows an internal standardization of the results between gels and between experiments were present in all gels described previously (1), but are not shown. The same amount of total extract protein is loaded in each *lane* and in general between different experiments. The levels of ERK2 were also examined by Western blot and were not detectably different in any samples except *erk2* null cells, which showed no protein (data not shown). The band corresponding to ERK2 (*solid arrowhead*) has been established previously. The 30-kDa kinase is labeled with an *open arrowhead* in the wild-type strain. All exogenous genes are expressed from integrating vectors except Ras(D57Y) described in the legend to Fig. 2. *aleA* null-*aimless* null cells.

regulating the activation of adenylyl cyclase and other components in this pathway.

MATERIALS AND METHODS

"In Gel" Assays for ERK2 Activity—Activation of ERK2 was measured in cells competent to aggregate (aggregation-competent cells), the stage at which the stimulation of adenylyl cyclase is maximal as described previously (1), using in gel assays to quantitate ERK2 activity. Cells were pulsed for 4 h with 30 nM cAMP prior to harvesting, washing, and then activation by cAMP. Samples are normalized for the amount of protein. Because of the possibility that the level of renaturation and activity may not be identical between gels, each gel contains four normalization samples, which allow gels to be compared and quantitated internally. In addition, we quantited the amount of ERK2 protein in samples using the anti-ERK2 antibody (1). The levels of ERK2 were not detectably different in any samples except the ERK2 gene disruption (*erk2* null cells) (data not shown).

Cell Transformation and Cell Culture—Dictyostelium cells were transformed, grown vegetatively, and developed in suspension culture as described previously (1).

RESULTS

Ras Is a Negative Regulator of ERK2—The putative Ras GEF Aimless (*AleA*) is required for proper aggregation of *Dictyostelium* cells (10). *aleA* null cells show normal activation of guanylyl cyclase but have a significantly reduced level of adenylyl cyclase activation in response to cAMP (10), as has been shown for *erk2* null cells (8). To determine whether Aimless, and by implication a Ras protein, was involved in the regulation of ERK2 activation, we examined the kinetics and level of activation of ERK2 in an *aimless* null strain using an in gel assay in which an ERK2 substrate, myelin basic protein (MBP), is added into the SDS gel. After size fractionation, the proteins are denatured and renatured and then assayed for MBP-phosphorylating activity in the presence of [γ-^{32}P]ATP (Ref. 1; see "Materials and Methods"). As shown in Fig. 1 and as described previously (1), wild-type cells show a rapid activation of the 42-kDa ERK2 that peaks at ~50 s and then rapidly decreases to basal levels within 5–8 min. In *aimless* null cells, ERK2 shows a higher basal level of ERK2 activity and a more rapid, higher, and more extended activation of ERK2 than that observed in wild-type cells. Exogenous expression of *Aimless* in these cells, which complements the aggregation defect, also complements the effects of the *Aimless* loss-of-function mutation on the kinetics and level of ERK2 activation (Fig. 2*A*).

To further investigate the possible role of Ras in regulating the activity of ERK2, we examined the effect of overexpressing dominant interfering or dominant active forms of the RasD protein, one of five known *Dictyostelium* Ras proteins (see "Discussion"), in stably transformed strains from the actin (*Act15*) promoter, which is expressed throughout growth and development. Expression of the dominant negative RasD-(S17N) on an integrating vector, which gives a very high level of expression, results in a more rapid and extended level of activation of ERK2 than observed in wild-type cells (Fig. 1). This is qualitatively similar to that observed in the *aimless* null strain, except that the profile is even more extended, with a dramatic effect on adaptation. These observations are strengthened by experiments in which expression of RasD(D57Y) protein (11) from an extrachromosomally replicating vector, which gives a lower level of expression, also results in a more rapid and higher level of activation of ERK2 (Fig. 2*B*; legend to Fig. 2). These results are similar to those of the *aimless* null cells and suggest that Ras negatively regulates ERK2 activity. As would be expected from this conclusion, overexpressing the dominant active RasD(Q61L) results in a substantially reduced and slightly delayed activation of ERK2 compared with wild-type cells (Fig. 1). Disruption of the gene encoding a Ras GAP, which should result in an increase in the level of Ras·GTP in these cells (*ddrasgap1* null cells) (12), also yields a reduced activation of ERK2 when compared with wild-type cells (Fig. 2*B*), consistent with the effect of RasD(Q61L) on ERK2 activation. These results suggest that the Ras signaling pathway is a negative regulator of ERK2 MAP kinase activity (see "Discussion"). Overexpression of RasD(Q61L) in the *aimless* null strain results in a suppression of the high level of ERK2 activation in *aimless* null cells and a delay in the kinetics of activation as observed in wild-type cells expressing RasD(Q61L) (Fig. 1).

Components of the Adenylyl Cyclase/PKA Pathway Are Required for Activation and Adaptation of ERK2—Because ERK2 activity is required for cAMP receptor-mediated activation of adenylyl cyclase, we examined components of the adenylyl cyclase pathway for possible involvement in a regulatory role in modulating ERK2 activity. The PH domain-containing protein CRAC is required for the activation of adenylyl cyclase ACA and is thought to interact with G$\beta\gamma$ (which activates adenylyl cyclase in *Dictyostelium*) and adenylyl cyclase (13, 14). In *crac* null cells, the initial kinetics of ERK2 activation in response to cAMP were more rapid than seen in wild-type cells and the activity remained at an elevated level for >8 min, a time at which the wild-type level of ERK2 activity had returned to the basal level (Fig. 2, *C* and *D*). Complementation of the *crac* null mutation with an *Act15/CRAC* expression vector restored the ERK2 activation profile to that of wild-type cells (Fig. 2*C*). Interestingly, in *aca* null cells, the kinetics of ERK2 activation were indistinguishable from those observed for wild-type cells (Fig. 2*B*), suggesting that the ability of cells to properly stimulate adenylyl cyclase activity is not essential for the normal adaptation of ERK2 but that CRAC and/or a CRAC-associated protein may be essential for the adaptation.

PKA is also known to be important in regulating different aspects of aggregation. *pka* null cells (cells in which the PKA catalytic subunit has been disrupted) (15) are aggregation-deficient due to an inability to relay cAMP (16–19). Previous analyses showed that *pka* null cells or cells expressing a dominant negative PKA regulatory subunit do not express the required aggregation-stage adenylyl cyclase ACA (19, 20); how-

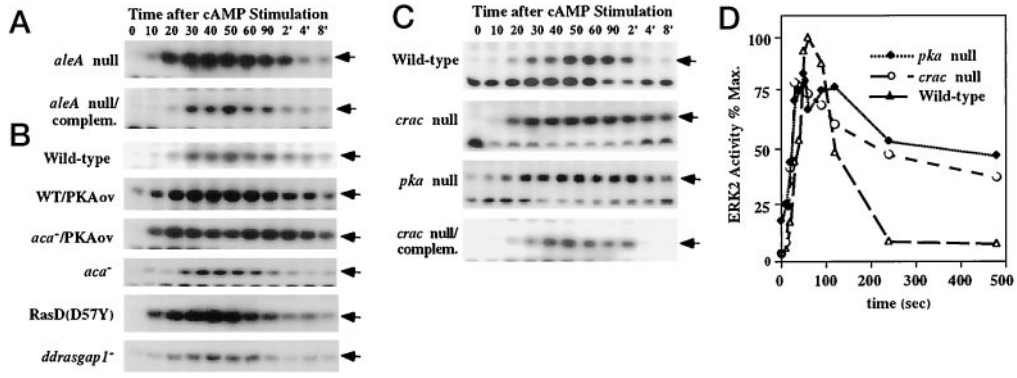

FIG. 2. **Effect of Ras, PKA, and components of the adenylyl cyclase activation pathway on cAMP-stimulated ERK2 activation.** *A*, complementation of *aleA* null cells with an *AleA* expression vector. *B*, assay of wild-type, wild-type cells overexpressing the PKA catalytic subunit (WT/PKAov), null mutants of the aggregation-stage adenylyl cyclase *ACA* (*aca*$^-$ cells), aca$^-$ cells overexpressing PKA (*aca*$^-$/PKAov), RasD(D57Y) cells, and DdRasGAP1 null cells (12). For the RasD(D57Y) transformants only, the gene was expressed from the *Act15* promoter on an extrachromosomal vector (see text) because obtaining transformants with Ras(D57Y) mutant of *RasD* on an integrating vector has been problematic.[2] *C*, assay of wild-type, *crac* null, complemented *crac* null, and *pka* null cells. The wild-type strain samples are those previously reported (1). *D*, quantitation of ERK2 activity.

ever, expression of ACA in *pka* null cells, while restoring chemoattractant activation of adenylyl cyclase, does not restore the ability of the cells to aggregate (19). To examine whether aspects of the activation/adaptation pathway may require PKA, cAMP stimulation of ERK2 activity was examined in *pka* null cells. Both the kinetics and level of stimulation were similar to those found in *crac* null cells (Fig. 2, *C* and *D*). The maximum level of activation was consistently slightly lower than that of wild-type cells, and the adaptation of the activity was suppressed with the activity being high for more than 8 min. In addition, the basal level of ERK2 activity in unstimulated *pka* null cells was higher than that in wild-type cells (Fig. 2*C*). In *pka* null:ACA cells, the kinetics of activation and adaptation of ERK2 are similar to those in *pka* null cells (data not shown), indicating that ACA, which is not expressed in *pka* null cells (19), was not the limiting factor in these cells for maximal ERK2 activation. The reduced level of ERK2 activation observed in *aca* null cells (Fig. 2*B*), is possibly due to the absence of endogenous cAMP that would be expected to result in a reduction in the level of active endogenous PKA. Because the lack of PKA activity altered ERK2 activation and adaptation, we examined the effect of overexpressing PKA in wild-type cells from the *Act15* promoter, which results in a high level of constitutive PKA activity (21). As shown in Fig. 2*B*, these cells exhibit a more rapid increase, a higher maximum level of activation, and a more extended period of activation compared with wild-type cells. This stimulation is independent of ACA, as this pattern is observed in *aca* null cells expressing PKA (Fig. 2*C*).

Constitutive PKA Bypasses the Requirement of ERK2 for Aggregation—When plated for development, *erk2* null cells are aggregation-deficient and are unable, even in response to exogenous cAMP, to proceed through development (8, 9). However, the *erk2* null cells overexpressing the PKA catalytic subunit aggregate and produce fruiting bodies with wild-type morphology (data not shown), indicating that these cells are able to bypass the requirement of ERK2 for aggregation (see "Discussion").

DISCUSSION

The pathways required for the activation of adenylyl cyclase in *Dictyostelium* are significantly more complex than the textbook paradigm. The direct activation *in vivo* in response to cAMP stimulation or *in vitro* in the presence of GTPγS requires the G$\beta\gamma$ subunit and CRAC (13, 14, 22). *In vivo* stimulation also requires ERK2 and the putative Ras GEF (8, 10). The kinetics of ERK2 activation/adaptation are similar to those of adenylyl cyclase, suggesting a model in which ERK2 activity is required at the time of adenylyl cyclase stimulation (1). In contrast to the situation in metazoans where MAP kinase pathways can be stimulated by Ras (23), our results suggest that Ras is a negative regulator of chemoattractant receptor-mediated ERK2 activation. ERK2 activation is delayed and the level significantly reduced in cells overexpressing RasD(Q61L), whereas activation is enhanced in *aimless* null cells and cells overexpressing RasD(S17N) or RasD(D57Y). Moreover, reduced ERK2 activation is also observed in a Ras GAP null strain, and the elevated ERK2 activation in *aimless* null cells is suppressed by RasD(Q61L). At present, we cannot reconcile the effects of Ras pathway mutants on ERK2 activation with those on adenylyl cyclase activation. The effects of the RasD(S17N) are more severe than those in *aimless* null cells, yet *aimless* null cells have a broader range of phenotypes than the RasD(S17N) overexpression cells: RasD(S17N)-expressing cells aggregate, whereas *aimless* cells do not and overexpression of RasD(Q61L) does not complement the aggregation defect of *aimless* cells. We expect, therefore, that one or more Ras proteins must positively regulate other pathways required for aggregation and that differences in Ras protein function account for different effects on chemotaxis and adenylyl cyclase activation, both required for aggregation. Indeed, cells overexpressing an activated RasG do not aggregate or activate adenylyl cyclase (24).

RasD is only one of five known *Dictyostelium* Ras genes (25–27), some of which, including RasD, are essentially identical to mammalian Ras proteins within the highly conserved N-terminal domain, whereas others show nonconserved amino acid substitutions in this region, including the effector domain. Our results on the effect of Ras on ERK2 activation are in direct conflict with those of Knetsch *et al.* (11), who show that cells expressing the dominant active RasD(G12T) have a high basal level of ERK2 and that activity decreases with cAMP stimulation but also only show a <5-fold cAMP-stimulated ERK2 activation. We cannot account for this discrepancy but emphasize that our results are internally consistent with the analysis being performed on multiple mutants affecting Ras function. It is possible that the Knetsch group examined a different kinase; we have confirmed that we are examining ERK2 using a purified, *in vivo* activated His-tagged ERK2.[3]

[3] M. Maeda, L. Aubry, and R. A. Firtel, manuscript in preparation.

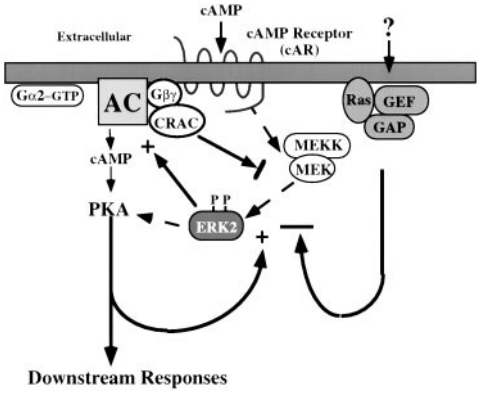

FIG. 3. **Model for regulation of cAMP stimulation of ERK2.** See "Discussion" for details. CRAC and the heterotrimeric G protein containing Gα2 are required *in vivo* for cAMP stimulation of adenylyl cyclase (*AC*). ERK2 is presumed to be activated through a classic MAP kinase cascade as is indicated by the MAP kinase kinase (*MEK*) and the MEK kinase (*MEKK*) in the pathway. ERK2 is required for cAMP-stimulated activation of adenylyl cyclase. CRAC is required for the proper adaptation of ERK2, and PKA activity is required for maximal stimulation and normal adaptation. Overexpression of the PKA catalytic subunit results in a higher cAMP-stimulated ERK2 activity, suggesting that PKA is a positive regulator of ERK2 activation. Ras is a negative regulator of ERK2 activation. The data presented in this manuscript suggest that the Aimless Ras GEF regulates Ras activity during aggregation. What activates Ras GEF is unknown. ERK2 may mediate downstream responses by modulating PKA function, as constitutive PKA suppresses the *erk2* null mutation.

The activation and adaptation pathways also involve CRAC. However, this requirement is not dependent on the ability to synthesize cAMP or activate cAMP-dependent protein kinase. When CRAC translocates to the plasma membrane after cAMP stimulation it may interact with upstream components of the ERK2 MAP kinase pathway to down-regulate ERK2 activation. Our results also present evidence for a role of PKA in regulating ERK2 activity. *pka* null cells have a higher basal activity and a lower level, but more extended time, of activation. Overexpression of PKA yields a more rapid, extended, and higher level of activation, suggesting that PKA may be essential for maintaining low basal levels and maximally stimulating ERK2 activation. The more extended activation in *pka* null cells may be associated with other roles of PKA, which is known to be required for multiple aspects of aggregation and later multicellular development (19, 28).

Lastly, we showed that cells overexpressing the PKA catalytic subunit, which has been shown to lead to constitutive PKA activity (21), suppresses the *erk2* null phenotype. This suggests that ERK2 is upstream from PKA and functions in part to control the activation of PKA, which is required for aggregation. In wild-type cells, cAMP produced through the activation of adenylyl cyclase would activate PKA. We expect that a component of this pathway may be a direct substrate for ERK2. ERK2 might function to directly regulate the PKA catalytic or regulatory subunit to mediate the interaction of these subunits or another protein that may control the level of cAMP in cells or other aspects of the pathway. As constitutive PKA activity suppresses the *erk2* null phenotype, we expect that such a protein would lie downstream from ERK2 and upstream of PKA. A model depicting the possible inter-relationships between different components in the ERK2 pathway is shown in Fig. 3. Further analyses should continue to elucidate the mechanisms controlling chemoattractant receptor-mediated signaling during aggregation.

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