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Seven Helix Chemoattractant Receptors Transiently Stimulate Mitogen-activated Protein Kinase in *Dictyostelium*

ROLE OF HETEROTRIMERIC G PROTEINS*

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Mitogen-activated protein (MAP) kinases are involved in controlling a cell's responses to a variety of stimuli and can be activated by both protein tyrosine kinase and G protein-coupled receptors. It was shown previously that *Dictyostelium* MAP kinase ERK2 is required for normal activation of adenylyl cyclase and *erk2* null cells are aggregation-deficient. In this manuscript, we show that the *Dictyostelium* MAP kinase ERK2 is rapidly and transiently activated in response to the chemoattractant cAMP. This response requires cAMP receptors, but is independent of the coupled Gα2 subunit and the only known Gβ subunit. These data indicate that ligand-mediated receptor activation of adenylyl cyclase requires two receptor-dependent pathways, one of which requires heterotrimeric G proteins, including Gα2 and the only known Gβ subunit, and the second of which requires ERK2. Our results suggest that ERK2 may be activated by a novel receptor-mediated pathway.

MAP[1] kinases (MAPKs) or ERKs control a wide array of cellular responses in eukaryotes and are stimulated by environmental stress and through receptor tyrosine kinases and G protein-linked/serpentine receptors (1–5). In the yeast *Saccharomyces cerevisiae* alone, five distinct MAP kinase activation pathways have been defined that control mating, pseudohyphal growth, cell integrity, response to osmotic shock, and sporulation (6). Little is known about the diversity of mechanisms that can lead to the activation of MAP kinase cascades. In *S. cerevisiae* and mammalian cells, some MAP kinase cascades can be controlled, at least in part, through Gβγ subunits (7–9), while the pathway that activates the MAP kinase Hog1 in response to osmotic shock is regulated through a transmembrane histidine kinase (10).

In *Dictyostelium*, the multicellular organism is formed via the chemotactic aggregation of up to 10^5 cells. The chemoattractant is extracellular cAMP that binds to the serpentine receptors cAR1 or cAR3, which are coupled to the heterotrimeric G protein containing the Gα2 subunit, activating guanylyl and adenylyl cyclases, triggering alterations in the cytoskeleton, and inducing gene expression (11–15). Recent results indicate that the Gβγ subunits released by the ligand activate adenylyl cyclase, whereas the coupled Gα2 subunit is thought to regulate a variety of other effectors (11, 16, 17). The aggregation-stage adenylyl cyclase in *Dictyostelium* has a similar structure to that of mammalian adenylyl cyclases (18); however, the aggregation-stage adenylyl cyclase has a more complex activation pathway compared with known activation pathways for mammalian adenylyl cyclases (19). In addition to the Gβγ subunit, adenylyl cyclase activation in *Dictyostelium* also requires a pleckstrin homology domain containing protein designated CRAC (20, 21), and, surprisingly, a MAP kinase ERK2. *erk2* null cells do not aggregate due to their deficiency in the ability to activate adenylyl cyclase and relay the cAMP signal (22).

In this communication, we show that the MAP kinase ERK2 is rapidly and transiently activated in response to cAMP. The kinetics of this response are similar to those of the activation of adenylyl cyclase. The response requires the cAMP receptor necessary for mediating other cAMP-mediated responses, but is independent of the coupled Gα2 and the only known Gβ subunit. Our data indicate that receptor activation of adenylyl cyclase requires two receptor-dependent pathways, one requiring Gβγ and involving ERK2.

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. Vegetative *Dictyostelium* cells were washed and suspended in non-nutrient buffer (Na/KPO_4 (pH 6.2) or MES-PDF) (23) at 5×10^6 cells/ml and pulsed with 30 nM cAMP for 4 h to induce cAMP receptors, Gα2, and other components of the aggregation response. Cells were washed, concentrated 2-fold, shaken for 10 min, and stimulated with 20 μM cAMP. Samples were taken at the time points indicated, immediately lysed in 5 × SDS sample buffer, and size-fractionated on SDS-polyacrylamide gel electrophoresis gels containing MBP as described previously (24–26). Proteins in the gels were denatured in guanidine hydrochloride and renatured in Tween and kinase activity was assayed *in situ* as described previously (24–26).

Affinity Purification of Anti-ERK2 Antibody—Rabbits were immunized with a GST-ERK2 fusion protein expressed in *Escherichia coli*. Rabbit anti-GST/ERK2 serum was affinity-purified by the method of Harlow and Lane (27). The whole serum was diluted 10-fold with PBS and then passed over a GST column (see below) to remove anti-GST antibodies. The flow-through was passed three additional times over the GST column, which had been regenerated by washing with 0.2 M glycine (pH 2.2) followed by PBS. The final flow-through was applied to

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[1] MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MES, 4-morpholineethanesulfonic acid; MBA, myelin basic protein; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); cAR, cAMP receptors.

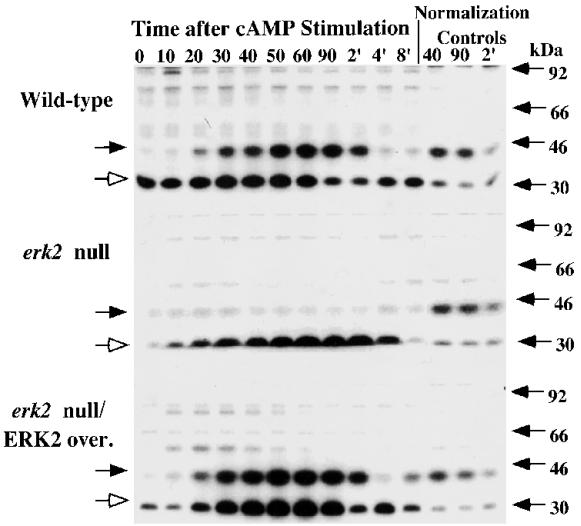

FIG. 1. **cAMP stimulated ERK2 activation.** In-gel assay of cAMP stimulation of ERK2 activity. The activation of ERK2 was measured in aggregation-competent cells (cells pulsed with nM cAMP for 4 h, see "Materials and Methods"). The lanes on the *right* are "normalization" controls containing extracts of a time course that allows an internal standardization of the results between gels and between experiments. An aliquot is also taken to quantitate total protein for normalization of activity between different strains and experiments. The same amount of total extract protein is loaded in each lane. In general, the same amount of protein is loaded for each sample between the different experiments. The autoradiographs are quantitated by densitometry and relative activity is normalized using the internal controls and protein levels. The band corresponding to ERK2 is marked with a *solid arrowhead*. This band is missing in *erk2* null cells. The 30-kDa kinase is labeled with an *open arrowhead*.

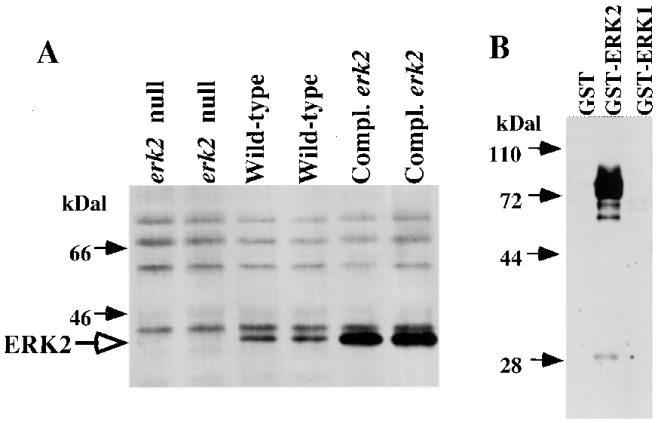

FIG. 2. **Western blot analysis with immunopurified anti-ERK2 antibodies.** *A*, Western blot of *erk2* null, wild-type, and ERK2 overexpressing cells. *B*, Western blot of glutathione-Sepharose-purified GST, GST-ERK2 fusion protein, and GST-ERK1 fusion proteins expressed in *E. coli*. See "Materials and Methods."

a GST-ERK2 column in PBS. The column was washed with PBS and then with PBS containing 1 M KCl, and bound antibody was eluted with 10 bed volumes of 0.1 M glycine (pH 2.2). The eluate was collected in a tube containing 1 bed volume of 1 M Tris (pH 8.0). GST and GST-ERK2 columns were prepared according to the method of Koff *et al*. (28).

RESULTS AND DISCUSSION

Addition of the Chemoattractant cAMP Activates ERK2 Activity—We examined the activity of ERK2 in response to the chemoattractant cAMP using an in-gel assay in which a MAP kinase substrate, MBP, is embedded in the gel. The samples were size-fractionated using standard SDS-polyacrylamide gel electrophoresis techniques, renatured, and their ability to phosphorylate MBP assayed (24, 25). The addition of cAMP to aggregation-stage, wild-type cells resulted in a rapid activation of an ~42-kDa kinase (the predicted size of ERK2) (Fig. 1). Gels lacking MBP showed no phosphorylation at this mobility (data not shown; see Ref. 26). Stimulation of kinase activity was observed within 10 s and peaked at ~50 to 60 s. After 2 min, the activity was significantly reduced, and by 4 min the activity had returned to near-basal levels. These kinetics of the activation and subsequent adaptation of kinase activity were similar to those of cAMP activation of adenylyl cyclase and significantly slower than those of cAMP activation of guanylyl cyclase (12, 15, 29). In addition to ERK2, other phosphorylated bands were observed, the strongest of which is at ~30 kDa. In some genetic backgrounds (see below and data not shown), this kinase was differentially stimulated or repressed by cAMP stimulation.

To confirm that cAMP-stimulated MBP phosphorylating activity was ERK2, we examined the activation profile in *erk2* null cells and cells overexpressing ERK2. No stimulation of this activity was observed in *erk2* null cells, while expression of ERK2 in the *erk2* null background (complemented *erk2* null cells) resulted in the restoration of the activity (Fig. 1). In addition, these *erk2* null complemented cells showed normal aggregation and morphological differentiation that was indistinguishable from that of wild-type cells (Ref. 30; data not shown). The maximum level of kinase activity in the *erk2* null complemented cells was higher than that in wild-type cells, probably due to an overexpression of the kinase (see below). Overexpression of ERK2 in wild-type cells resulted in an ~4-fold higher level of stimulated kinase activity (data not shown). When the samples were analyzed by Western blot with a rabbit polyclonal anti-ERK2 antibody made against a GST-ERK2 fusion (see "Materials and Methods"), a band of 42 kDa was observed that was absent in *erk2* null cells (Fig. 2*A*). A higher level of ERK2 was observed in the ERK2 overexpressor strain (Fig. 2*A*). This antibody did not interact with the other known *Dictyostelium* MAP kinase, ERK1 (Fig. 2*B*), or with other proteins with the same mobility as ERK2, although it did interact with some other proteins (Fig. 2, *A* and *B*). In the complemented null cells overexpressing ERK2, the increased protein was greater than the increased maximal level of activity seen in this assay (Fig. 1). We assume that either not all of the kinase could be activated or the assay was not linear at high kinase levels.

A background activity at the same molecular weight as ERK2 was seen in *erk2* null cells and in unstimulated wild-type cells (Fig. 1). Notably, this unstimulated activity was not detectably increased by overexpression of ERK2, suggesting that the kinase activity at this mobility may not be ERK2 and that ERK2 basal activity was very low. There is at least one other MAP kinase, ERK1, in *Dictyostelium* and may be responsible for the background activity in these assays. ERK1 has a similar electrophoretic mobility to that of ERK2 on these gels, uses MBP as a substrate, and is active at the same time in development (26). The absence of stimulation of the band at this mobility in *erk2* null cells suggests ERK1 is not stimulated in response to cAMP, consistent with previous results (26). When the ERK2 stimulation in response to cAMP in wild-type cells was quantitated, we observed an ~40-fold increase in the level of kinase activity at this mobility. If the background band observed in *erk2* null cells were subtracted, the relative level of stimulation would be higher.

Signaling Pathway Regulating ERK2 Activation—To determine which components of the known cAMP-dependent signal transduction pathways may regulate the activation of ERK2, we examined the ability of cAMP to stimulate ERK2-MBP phosphorylating activity in various genetic backgrounds. Previous results showed that either cAMP receptor cAR1 or cAR3

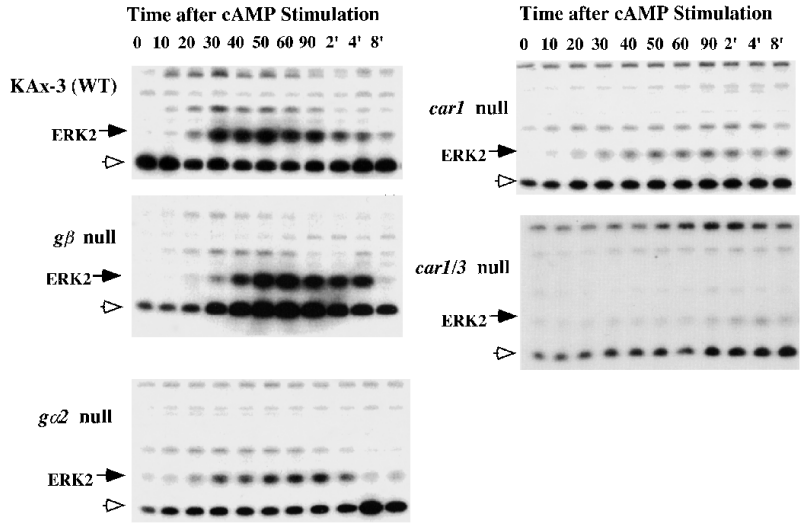

FIG. 3. **cAMP stimulation of ERK2 activity in mutant strains.** Assays were done as described above. The *gβ* null and *gα2* null cells carry a vector that constitutively expresses cAR1 from an actin (*Act15*) promoter, since these strains express a lower number of receptors than wild-type cells after cAMP stimulation.

is required for mediating aggregation-stage responses to cAMP (31, 32). These receptors are coupled to the G protein containing the Gα2 subunit, the only one of the eight known Gα subunits found to couple to cAMP receptors and mediate cAMP receptor-controlled pathways (11, 12, 14, 33–37). Cells lacking either Gα2, cAR1, cAR3, or the only known Gβ subunit through engineered gene disruptions, showed no cAMP stimulation of adenylyl cyclase or guanylyl cyclase *in vivo* (14, 15, 17, 31). The *gβ* null cells are well characterized (17). These cells lack known receptor/G protein/effector interactions, including: the absence of detectable cAMP receptor high affinity binding sites and the absence of GTPγS-stimulated adenylyl cyclase activity in cell lysates. These results suggest that cAMP receptors are no longer coupled to a heterotrimeric G protein in the *gβ* null cells and that there is only a single Gβ subunit in this organism (17). When ERK2 activation was examined in cells lacking cAR1 (*car1* null cells) (38), the major aggregation-stage receptor, the level of ERK2 activation was significantly reduced (~80%) compared with that of the wild-type cells (Fig. 3). This residual stimulation was absent in the *car1/car3* double knockout strain, indicating that cAMP-mediated ERK2 activation requires cAMP receptors (Fig. 3).

To examine the possible role of heterotrimeric G proteins in ERK2 activation, *gα2* and *gβ* null strains (14, 39) were first transformed with a plasmid that constitutively expresses cAR1 during growth and development. This measure ensured that these cells had appropriate levels of receptors. Surprisingly, in *gα2* and *gβ* null cells constitutively expressing cAR1, the ERK2 pathway was also stimulated. However, the onset of activation was delayed, and its peak was reduced compared to that in wild-type cells. When quantified, the maximal level of activation in the *gα2* and *gβ* null strains varied between ~40 and 80% of that observed in wild-type cells (six separate experiments) and between ~50 and 60% of that in wild-type cells constitutively expressing cAR1 (data not shown).

Our analysis has shown that the MAP kinase ERK2 is transiently activated in response to chemotactic signaling by cAMP and that this activation is dependent on cARs but is independent of Gα2, the known Gα subunit that couples to cAR1 and cAR3. Further, the activation of ERK2 occurred independently of the only known *Dictyostelium* Gβ subunit, that like Gα2, is required for the *in vivo* activation of adenylyl cyclase. The slower kinetics and the reduced level of activation of ERK2 in *gβ* and *gα2* null cells compared with wild-type cells nevertheless suggest that a G protein-mediated pathway is important in obtaining a completely wild-type response.

erk2 null cells express adenylyl cyclase aggregation-stage adenylyl cyclase and CRAC, another required component for this pathway (22), indicating that ERK2 is not required for the expression of these genes. The kinetics of activation and deactivation parallel those of adenylyl cyclase, suggesting that activated ERK2 may be required continuously for the activation of adenylyl cyclase. Other results have shown that receptor-mediated activation of at least some aggregation-stage genes in response to cAMP pulses is normal in *erk2* null cells (30). However, we cannot exclude that ERK2 is required for the expression of some unknown gene and that the kinetics of activation are fortuitously similar to those of adenylyl cyclase.

Two different receptor-mediated pathways are involved in cAMP activation of adenylyl cyclase; one requires the Gα2 and Gβ subunits and the other requires ERK2 (11, 12, 22) (see model in Fig. 4). We now find that ERK2 is activated by cAMP and that this requires cAMP receptors, but is independent of either Gα2 or the known Gβ that are also required for adenylyl cyclase activation. While the possibility of another Gβ subunit can never be completely excluded, there is significant biochemical evidence that *Dictyostelium* has only one Gβ subunit as described above (17, 39). On the weight of this evidence, we entertain the intriguing possibility that ERK2 is activated by a pathway that may be independent of heterotrimeric G proteins. One model to accommodate our observations is that the receptor functions as a docking site for proteins that stimulate downstream pathways, one of which leads to the stimulation of the ERK2 activation cascade (Fig. 4). This model has precedent in the known docking of different effectors to activated receptor tyrosine kinases. The underlying mechanism for docking to serpentine receptors such as cAR1 could be similar to the binding mechanisms that allow heterotrimeric G proteins to interact with ligand-bound ("activated") receptors or receptor kinases to interact with and phosphorylate the ligand-bound but not free receptors (40, 41).

There is evidence for G protein-independent pathways mediated through serpentine receptors in mammalian cells. As described above, the binding and phosphorylation of rhodopsin and β-adrenergic receptors by their respective kinases in mammals requires ligand binding, but is independent of G proteins (40, 41). In *Dictyostelium*, cAMP stimulation of Ca^{2+} influx and cAMP-mediated activation of post-aggregative gene expression in the multicellular stages (which is activated through the transcription factor GBF and a high, continuous level of cAMP) are known to be independent of the identified Gβ subunit and have been proposed to be G protein-independent (42, 43). Both

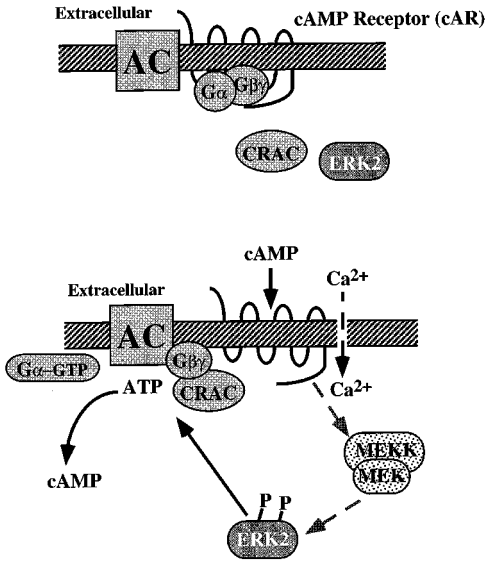

FIG. 4. **Model for cAMP stimulation of adenylyl cyclase and ERK2.** *Upper panel* shows proposed location of relevant components in an unstimulated cell. The *bottom panel* shows the G protein-dependent (G$\beta\gamma$) pathway, CRAC, and possible input of ERK2. It is expected that ERK2 is activated by a conserved upstream activating cascade containing a MEK and MEKK (shown as *speckled ovals*). The mechanism of activation of this putative MAP kinase cascade is not known but it is independent of the Gβ subunit required for interactions with CRAC and aggregation-stage adenylyl cyclase. One model proposes that the receptor acts as a docking site for the unknown required components, as tyrosine kinase receptors act as a docking site. In addition, the activation of calcium influx is also a receptor-dependent, but is independent of the Gβ subunit. Whether this functions with another receptor-mediated process or directly to promote the activation of the MAP kinase cascade is not known. A relevant example is the ligand-dependent but G protein-independent binding of both βARK and rhodopsin kinase to their respective receptors (see text).

G proteins and receptor kinases interact with receptors in response to ligands, and recent evidence indicates that STAT transcription factors are activated through the angiotensin II receptor (44), although it is not known whether a JAK kinase directly couples to this receptor. With the biochemical evidence that receptor kinase binding and phosphorylation is ligand-dependent but G protein-independent, it is possible that other heterotrimeric G protein-independent pathways will be identified as future genetic analysis permits further dissection of pathways controlled via serpentine receptors.

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