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 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 MAP receptors, but is independent of the coupled Gβ2 subunit and the only known Gβi 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 Gβ2 and the only known Gβi subunit, and the second of which requires ERK2. Our results suggest that ERK2 may be activated by a novel receptor-mediated pathway.

MAP1 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 107 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 cyclase, and adenyllyl cyclases, triggering alterations in the cytoskeleton, and inducing gene expression (11–15). Recent results indicate that the Gβγ subunits released by the ligand activate adenyl cyclase, whereas the coupled Gα2 subunit is thought to regulate a variety of other effectors (11, 16, 17). The aggregation-stage adenyl cyclase in Dictyostelium has a similar structure to that of mammalian adenyl cyclases (18); however, the aggregation-stage adenyl cyclase has a more complex activation pathway compared with known activation pathways for mammalian adenyl cyclases (19). In addition to the Gβγ subunit, adenyl 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 adenyl 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 adenyl 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 Gβi subunit. Our data indicate that receptor activation of adenyl 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 adenyl cyclase is maximal. Vegetative Dictyostelium cells were washed and suspended in non-nutrient buffer (Na2HPO4 (pH 6.2) or MES-PDF) (23) at 5 × 106 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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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 quantify 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 band is labeled with an open arrowhead.

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 adenyl 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 ("Materials and Methods"), a band of 42 kDa was observed that was absent in erk2 null cells (Fig. 2A). A higher level of ERK2 was observed in the ERK2 overexpressor strain (Fig. 2A). This antibody did not interact with the other known Dictyostelium MAP kinase, ERK1 (Fig. 2B), 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...
is required for mediating aggregation-stage responses to cAMP (31, 32). These receptors are coupled to the G protein containing the Ga2 subunit, the only one of the eight known Ga subunits found to couple to cAMP receptors and mediate cAMP receptor-controlled pathways (11, 12, 14, 33–37). Cells lacking either Ga2, 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, ga2 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 ga2 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 ga2 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 Ga2, the known Ga subunit that couples to cAR1 and cAR3. Further, the activation of ERK2 occurred independently of the only known Dictyostelium Gβ subunit, that like Ga2, is required for the in vivo activation of adenylyl cyclase. The slower kinetics and the reduced level of activation of ERK2 in gβ and ga2 null cells compared with wild-type cells nevertheless suggest that a G protein-mediated pathway is important in obtaining a completely wild-type response.

Fig. 3. cAMP stimulation of ERK2 activity in mutant strains. Assays were done as described above. The gβ null and ga2 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.

erk2 null cells express adenyl cyclase aggregation-stage adenyl 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 adenyl cyclase, suggesting that activated ERK2 may be required continuously for the activation of adenyl 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 adenyl cyclase.

Two different receptor-mediated pathways are involved in cAMP activation of adenyl cyclase; one requires the Ga2 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 Ga2 or the known Gβ that are also required for adenyl 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 Ca2+ 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
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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