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Activation of the Mitogen-activated Protein Kinase ERK2 by the Chemoattractant Folic Acid in *Dictyostelium**

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The *Dictyostelium* MAP kinase ERK2 is activated by extracellular cAMP in aggregation-competent cells and is required for receptor activation of adenylyl cyclase (Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996) *J. Biol. Chem.* 271, 3351–3354; Segall, J., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R., and Loomis, W. (1995) *J. Cell Biol.* 128, 405–413). This cAMP-dependent activation of ERK2 is mediated by the serpentine, G protein-coupled cAMP receptors. However, ERK2 activation by cAMP is at least partially heterotrimeric G protein-independent, with a level of activation in cells lacking the sole Gβ subunit or the G protein-coupled cAMP receptors-coupled Gα2 subunit that is ~50% that of wild-type cells (Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996) *J. Biol. Chem.* 271, 3351–3354; Segall, J., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R., and Loomis, W. (1995) *J. Cell Biol.* 128, 405–413). Folic acid, a chemoattractant in the vegetative cells that enables amoebae to find bacteria in the wild, also triggers the activation of adenylyl cyclase, which is impaired in the vegetative cells lacking the Gα protein subunit Gα4 (Hadwiger, J., Lee, S., and Firtel, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 10566–10570). In this study, we show that folic acid activates ERK2 in developmentally regulated manner and is required for ERK2 stimulation of adenylyl cyclase activity. Maximum levels of folate-stimulated ERK2 activity occur in cells from very early in development, prior to aggregation, and again at the tipped aggregate stages, corresponding to the stages in which folate receptors and the coupled Gα subunit Gα4 are maximally expressed. During the activation by folic acid, ERK2 is phosphorylated on tyrosine residue(s) and contemporaneously shows a mobility shift on SDS-PAGE. Interestingly, this activation is not elicited in the absence of Gβ subunits, in contrast to the response to cAMP. This response also requires the Gα4 subunit known to be required for other folic acid-mediated responses (Hadwiger, J., Lee, S., and Firtel, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 10566–10570). Furthermore, we show that the activation of ERK2 by cAMP is independent of the Gα4 subunit, while the activation of ERK2 by folate is independent of Gα2. Taken together, these data indicate that there are at least two pathways of ERK2 activation, heterotrimeric G protein-dependent and -independent pathways.

MAP[1] kinase cascades are used by eukaryotes to couple extracellular signals to diverse intracellular regulatory pathways. The diversity of these pathways is great and includes growth factor responses in mammalian cells, cell-type differentiation in *Drosophila* and *Caenorhabditis elegans*, mating and growth responses in yeast, and stress-mediated responses in yeast and mammalian cells. These cascades are mediated by various classes of G protein- and tyrosine kinase-coupled receptors (5–13). In *Dictyostelium*, three presumably independent MAP kinase cascades play essential regulatory roles during both growth and multicellular development. The MAP kinase kinase (MEK) DdMEK1 is required for chemotaxis toward cAMP during aggregation; *ddmek1* null cells form very small aggregates but continue to differentiate into normally proportioned, but very small, fruiting bodies (14). DdMEK1 is specifically required at the time of cAMP stimulation for the activation of guanylyl cyclase and the production of cGMP, the second messenger for chemotaxis in these cells. Two MAP kinases, ERK1 and ERK2, have been identified and their functions characterized. ERK1 is required for vegetative growth and is thought to play roles during multicellular development (15), while ERK2 is required for the activation of adenylyl cyclase by the chemoattractant cAMP during aggregation, prespore-specific gene expression, and morphogenesis (2, 16). *erk2* null cells are aggregation-deficient due to the inability to activate adenylyl cyclase and relay the cAMP signal but show normal activation of guanylyl cyclase, which couples to chemotaxis (17). *erk2* null cells expressing a temperature-sensitive ERK2 show abnormal morphogenesis during the multicellular stages, as well as the inability to induce prespore gene expression when shifted to a nonpermissive temperature. ERK2 activity is induce ~40-fold in aggregation-stage cells in response to extracellular cAMP. As expected from their aggregation-stage phenotypes, ERK2 activation is independent of DdMEK1 function. ERK2 is activated by cAMP through the G protein-coupled cAMP receptors cAR1 and cAR3, but the activation occurs to a level ~50% that of wild-type cells in cells in which the gene encoding the only Gβ subunit or the gene encoding the Gα subunit known to couple to cAR1 and cAR3 (Gα2) is disrupted, indicating that cAMP receptor-mediated ERK2 activation is at least partially G protein-independent (1, 2). This pathway is positively regulated by cAMP-dependent PKA and negatively

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[1] The abbreviations used are: MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; cAR, cAMP receptor; MEK, MAP kinase kinase; MBP, myelin basic protein; MES, 4-morpholineethanesulfonic acid.

regulated by Ras pathways (18), although another group has conflicting data on some of these points (Ref. 3; see below). ERK2 is also regulated by components of the adenylyl cyclase pathway such as CRAC (18).

Dictyostelium cells also undergo chemotaxis to other extracellular signals, including folic acid, which is produced by bacteria and is thought to be used by *Dictyostelium* cells to locate food in the wild (19). The folate response is present in vegetatively growing cells but is maximal in cells that have been starved for 0.5–2 h (19, 20), decreases significantly during aggregation (19, 20), which is the time of maximal cAMP responsiveness (17), and is reacquired at the tipped aggregate stage, when cells are responsive to both folate and the pterin monapterine. In addition to stimulating the activation of guanylyl cyclase and chemotaxis, folate also elicits the activation of adenylyl cyclase in vegetative cells and cells during early development. Folate and monapterine elicit a similar response during the multicellular stages (4, 20, 21). These folate-mediated responses require the Gα subunit Gα4 (4). The folate and monapterine responsiveness at the tipped aggregate stage may be involved in morphogenetic movements and cell-type differentiation and coincides with a stage-specific requirement of Gα4 for these processes (4, 21, 22).

In this report, we investigate the possible regulation of ERK2 by folate-mediated pathways. We show that folic acid activates ERK2 in developmentally regulated manner, this activation is paralleled by a mobility shift of ERK2 on SDS-PAGE and phosphorylation on tyrosine residue(s), ERK2 activation requires heterotrimeric G proteins containing the Gα4 subunit, and ERK2 is activated by monapterine in cells at the tipped aggregate stage. As has been observed previously for cAMP (2), we show that ERK2 is required for folate stimulation of adenylyl cyclase. We propose that there are two pathways leading to ERK2 activation; folic acid activates ERK2 via a completely G protein-dependent pathway, while cAMP activates ERK2 by pathways that are at least partially G protein-independent.

EXPERIMENTAL PROCEDURES

Strains—Wild-type developing *Dictyostelium discoideum* KAx-3 and JH10 (a thymidine auxotroph) were used as wild-type strains. Strains carrying disruptions in the genes encoding ERK2 (1, 2, 16), cAMP receptors cAR1 and cAR3 (23), the Gβ subunit (24), and the Gα4 subunit (22, 25) have been described previously. Wild-type and *erk2* null cells expressing a His-tagged ERK2 were created by C. Gaskins and L. Aubry (Department of Biology, University of California, San Diego, La Jolla, CA). A genomic *ERK2* clone into the *Bgl*II and *Xho*I sites of the Dep-j extrachromosomal vector, a derivative of pATANB42 and carrying an Act15 promoter, was engineered by polymerase chain reaction to add an in-frame His_6 tag at the carboxyl terminus of ERK2. After confirming the sequence of the open reading frame, the resulting vector was transformed into *erk2* null and wild-type cells and stable transformants were isolated.

Cell Culture—All strains used in this study were cultured in HL5 supplemented with or without G418 at 20 μg/ml or thymidine at 100 μg/ml. Cells were harvested at 2–4 $\times$ 10^6 cells/ml, washed twice with 12 mM sodium/potassium phosphate buffer (pH 6.1), and then resuspended in phosphate buffer at 5 $\times$ 10^7 cells/ml. After 30 min of starvation, cells were stimulated with folic acid at 50 μM. At appropriate intervals after the stimulation, 100 μl of cell suspension was withdrawn to 25 μl of 5 $\times$ sample buffer for SDS-PAGE and then boiled for 3 min. To see ERK2 activity during development, 0.5 ml of cell suspension was placed onto a Millipore filter (3 cm diameter, 0.45 μm pore size) supported by 1% phosphate-buffered agar and allowed to develop. Every 4 h of incubation, cells were harvested from two filters and resuspended in phosphate buffer at 5 $\times$ 10^7 cells/ml. For the preparation of cell suspension after 12 h of incubation, formed multicellular structures were dissociated by being passed through a needle in MES-EDTA as described previously (26) and washed twice in phosphate buffer. These suspensions were used for stimulation with 50 μM folic acid or 50 μM monapterine.

Purification of His_6-tagged ERK2 Protein by Ni^{2+} Beads—Log-phase, vegetative cells were washed and starved for 30 min before being stimulated as described in the preceding section. At specific intervals after the stimulation, 500 μl of the cell suspension was added to 500 μl of 2 $\times$ ice-cold lysis buffer (20 mM HEPES, pH 7.5, 30 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10 mM EGTA, 1% Triton X-100, and 300 mM NaCl). Lysates were clarified by centrifuging for 10 min at 15,000 rpm in a Tomy microcentrifuge at 4 °C. Fifty μl of 50% nickel-agarose beads in phosphate-buffered saline were added to the resulting supernatants. After 1 h of incubation with gentle mixing at 4 °C, Ni^{2+}-agarose beads were collected by centrifuging for 1 min at 10,000 rpm in the Tomy microcentrifuge and washed serially: four times with 200 μl of lysis buffer, once with 20 mM imidazole (pH 7.0) in lysis buffer, and then twice with 50 mM imidazole in lysis buffer with shaking for 5 min, all at 4 °C. Finally, proteins were eluted three times with 100 μl of 250 mM imidazole in lysis buffer. These eluates were boiled for 3 min after the addition of 25 μl of 5 $\times$ sample buffer for SDS-PAGE.

In-gel Assay and Immunoblot Analyses—In-gel assays were performed as described previously using myelin basic protein (MBP) as the substrate (1). Cell lysates were analyzed by Western blot as described previously (1, 16).

Folate Binding Sites and Adenylyl Cyclase Activity—Folate binding sites were determined as described previously (4, 27, 28). Cyclic AMP mass assays were performed as published previously (29). Cells were starved for 0.5 h in phosphate buffer, washed, resuspended, and then stimulated with 50 μM folic acid, and accumulated cAMP was measured using the Cayman cAMP enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

RESULTS

Evidence for ERK2 Activation by Folic Acid—Folic acid elicits the activation of adenylyl cyclase through a G protein-coupled pathway containing the Gα subunit Gα4 with kinetics that are similar to the cAMP/cAR1/Gα2-mediated response during aggregation (4, 20, 21). As shown previously, cAMP stimulation of adenylyl cyclase requires ERK2 (2). To determine whether folate also stimulates ERK2 activity, we analyzed ERK2 activity in whole cell lysates and purified ERK2 protein after stimulation with folic acid by an in-gel assay (1) (see "Experimental Procedures"). As shown in Fig. 1*A*, addition of folate to very early developing cells (cells starved for 0.5 h) stimulates the activity of a kinase that has the mobility of ERK2 (Ref. 1; see below) and is absent from *erk2* null cells, strongly suggesting that the activity of endogenous ERK2 is stimulated by the chemoattractant folate. The kinetics and level of activation are similar to those we observed previously for ERK2 activation in response to cAMP in aggregation-stage cells (1).

To biochemically determine if this activity is ERK2, we expressed His_6-tagged ERK2 (His_6-ERK2) in *erk2* null cells under the control of the Act15 promoter, which is expressed throughout growth and development (30). Expression of His_6-ERK2 in *erk2* null cells complemented the *erk2* null aggregation-deficient phenotype (data not shown), as does the expression of unmodified ERK2 (1, 2), indicating that His_6-ERK2 can functionally replace the endogenous ERK2 *in vivo*. His_6-ERK2 was purified at each time point after folate stimulation by affinity chromatography using Ni^{2+}-agarose beads. As shown in Fig. 1*B*, folate-stimulated His_6-ERK2 activity in whole cell extracts with similar kinetics to those of the endogenous ERK2 (Fig. 1*A*). The in-gel activity of the Ni^{2+}-purified His_6-ERK2 samples (panel labeled *Ni-bead fr.*) shows that the kinase activity is either His_6-ERK2 or a kinase that is tightly bound to His_6-ERK2 and has a similar mobility in SDS-PAGE gels. When the supernatants of the Ni^{2+} resin-treated *erk2* null cell/His_6-ERK2 extracts were assayed by in-gel assay, only a low level of kinase activity remained at the mobility of the His_6-ERK2, indicating that the Ni^{2+} resin effectively purified the expressed His_6-ERK2 protein from the extracts. In control experiments using *erk2* null or wild-type cells in which cell extracts from cells not expressing His_6-ERK2 were treated with Ni^{2+} affinity resin, no kinase activity is detected at the position of the His_6-ERK2 (Fig. 1*C*; data not shown). Collectively, these re-

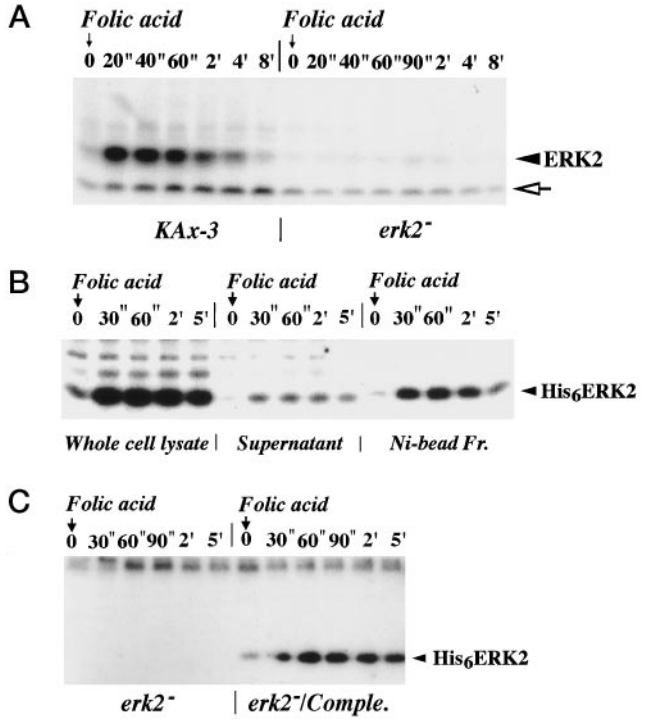

FIG. 1. **ERK2 activation by folic acid.** *A*, folate activation of ERK2 in wild-type (KAx-3) and *erk2* null cells. Cells starved for 0.5 h were stimulated with 50 μM folate as described under "Experimental Procedures." Kinase activity was measured using the in-gel assay. Briefly, in the "in-gel" assay, the *in vitro* ERK2 substrate MBP is embedded in the SDS-PAGE gel (1). After electrophoresis of the samples, the proteins in the gel are denatured and renatured and then *in situ* kinase activity is assayed with the addition of [γ-^{32}P]ATP and detected by autoradiography (see "Experimental Procedures"). *Closed arrowhead* marks the position of the mobility of ERK2. The *open arrowhead* marks the position of an ~30-kDa kinase (p30) that can phosphorylate MBP and has been described previously (1). *B*, *erk2* null cells constitutively expressing His_6-ERK2 were assayed for ERK2 activity using an in-gel assay after stimulation with folic acid. The *left-hand panel* shows ERK2 activity in whole cell lysates. Lysates were also fractionated using Ni^{2+} affinity resin into "supernatant," containing materials that did not bind to the resin, and the bound and eluted fraction (labeled *Ni-bead fr.*). *C*, comparison of ERK2 activities of Ni^{2+}-bead purified samples from *erk2* null cells and *erk2* null cells constitutively expressing His_6-ERK2. See "Experimental Procedures" and Maeda *et al.* (1) for details. There is no endogenous ERK2 band in *B* and *C* since the strain used for the complementation is the ERK2 knockout strain (*erk2* null; Refs. 1 and 16).

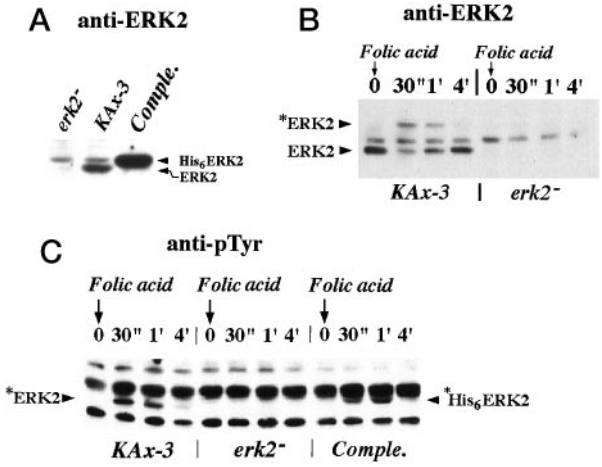

FIG. 2. **Mobility shift and phosphorylation of ERK2 on tyrosine during activation by folic acid.** *A*, characterization of endogenous ERK2 and His_6-tagged ERK2 by immunoblot analysis with anti-ERK2 antibody. Whole cell lysates were prepared from *erk2* null cells, wild-type cells (strain KAx-3), and *erk2* null cells expressing His_6-tagged ERK2 (*Comple.*), size-fractionated by SDS-PAGE, blotted, and probed with anti-ERK2 antibody (1, 16). His_6-ERK2 shows a slightly slower mobility than endogenous ERK2. A band of mobility similar to the His_6-ERK2 is present in all strains. This band is not a form of ERK2 or due the a disruption of *ERK2*, as it is present in cells in which the ERK2 gene is disrupted within the open reading frame (*erk2* null cells) as well as the parental strain (1, 16). *B*, whole cell lysates prepared from KAx-3 and *erk2* null cells at 0 s, 30 s, 1 min, and 4 min after stimulation with folic acid were analyzed by immunoblot with anti-ERK2 antibody. The shifted ERK2 is labeled **ERK2*. *C*, whole cell lysates prepared from KAx-3 cells, *erk2* null cells, and *erk2* null cells expressing His_6-ERK2 (*Comple.*) were size-fractionated by SDS-PAGE, blotted, and probed with anti-phosphotyrosine antibody. Each lane contains 5×10^5 cells. The shifted ERK2 His_6-ERK2 are labeled **ERK2* and **His$_2$ERK2*, respectively. The anti-ERK2 antibody has been described previously (1, 16).

sults demonstrate that the ~40-kDa protein is ERK2 and its activity is stimulated by folic acid with kinetics similar to what we have observed previously in response to cAMP in aggregation-competent cells.

The whole cell extracts show additional bands of kinase activity, including a major non-ERK2 band at 30 kDa, which we have observed previously in in-gel assays of cAMP-stimulated extracts (1). This band is also present in *erk2* null cell extracts and those from other strains, including some in which ERK2 is not present or not activated (see Figs. 1*A*, 3, and 5; Refs. 1 and 14). Under the cell culture conditions used, the basal activity of the exogenous His_6-ERK2 in unstimulated cells was very low. The relative activity of the purified His_6-ERK2 protein from unstimulated cells is even lower than that observed in whole cell extracts, suggesting that some of the activity previously found at the same mobility as ERK2 in unstimulated cells may be due to background kinases and not ERK2 (1, 2, 18). Consistent with this, whole cell extracts of *erk2* null cells show a low level of background in-gel kinase activity against MBP at the mobility of ERK2 (Fig. 1*A*).

Mobility Shift of ERK2 and Phosphorylation on Tyrosine Residue(s) by Folic Acid Stimulation—MAP kinases are known to be activated by phosphorylation on conserved tyrosine and threonine residues by an upstream MEK (6) and can often be followed by a mobility shift of the MAP kinase on SDS-PAGE (31). To examine these properties for ERK2, we analyzed whole cell lysates prepared after stimulation with folic acid by Western blot analysis with affinity purified anti-ERK2 antibody (1). Western blot analysis using the ERK2 antiserum demonstrates that His_6-ERK2 has a slightly slower mobility than that of the endogenous ERK2, presumably due to the His_6 tag (Fig. 2*A*). To detect a possible ERK2 mobility shift after folate stimulation, we used longer gels for SDS-PAGE than those used for the in-gel assays. As shown in Fig. 2*B*, ERK2 transiently exhibits a reduced mobility after stimulation that is coincident with the increase in ERK2 activity. The shifted band is detectable in the 30-s and 1-min time points but not in unstimulated cells or cells 4 min after stimulation, when the level of endogenous ERK2 activity has already decreased (see above; Fig. 1*A*). No band at the position of the unshifted (labeled *ERK2*) or shifted ERK2 (labeled **ERK2*) is observed in *erk2* null cells. In addition, we observed a tyrosine-phosphorylated band at the mobility of *ERK2 from wild-type (KAx-3 cells) at the same time points as we observed the mobility-shifted ERK2 in Fig. 2*B*. No tyrosine-phosphorylated band of this mobility was observed in *erk2* null cells; however, *erk2* null cells complemented with His_6-ERK2 did show a new tyrosine-phosphorylated band at the 30-s and 1-min time points with the expected mobility (slightly slower than the endogenous tyrosine-phosphorylated ERK2 in wild-type cells, consistent with a slower mobility of the His_6-tagged protein).

The Heterotrimeric G Protein Containing the Gα4 Subunit Is Required for Folic Acid-induced ERK2 Activation—Previously,

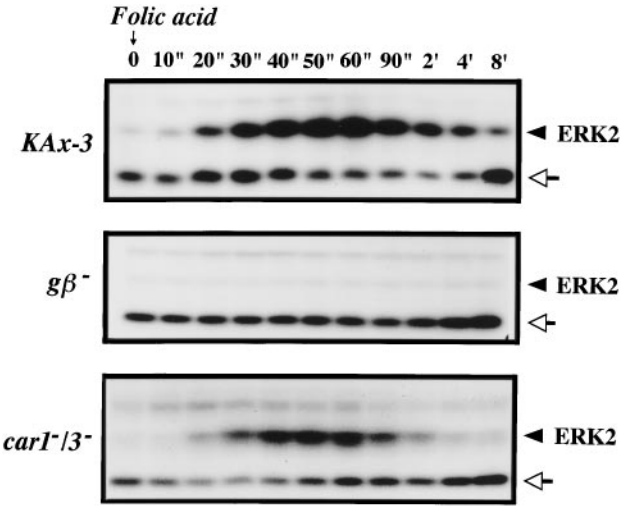

FIG. 3. **Kinetics of ERK2 activation by folic acid in wild-type (KAx-3), *gβ* null, and car1/3 double knockout strains.** Whole cell lysates were prepared from wild-type cells (KAx-3), *gβ* null cells (51), and *car1/3* double knockout cells (23) at the intervals indicated after stimulation with 50 μM folic acid and analyzed by in-gel assay containing MBP as a substrate for ERK2. The position of ERK2 (*solid arrowhead*) and p30 (*open arrowhead*) are indicated. Each lane contains 5×10^5 cells.

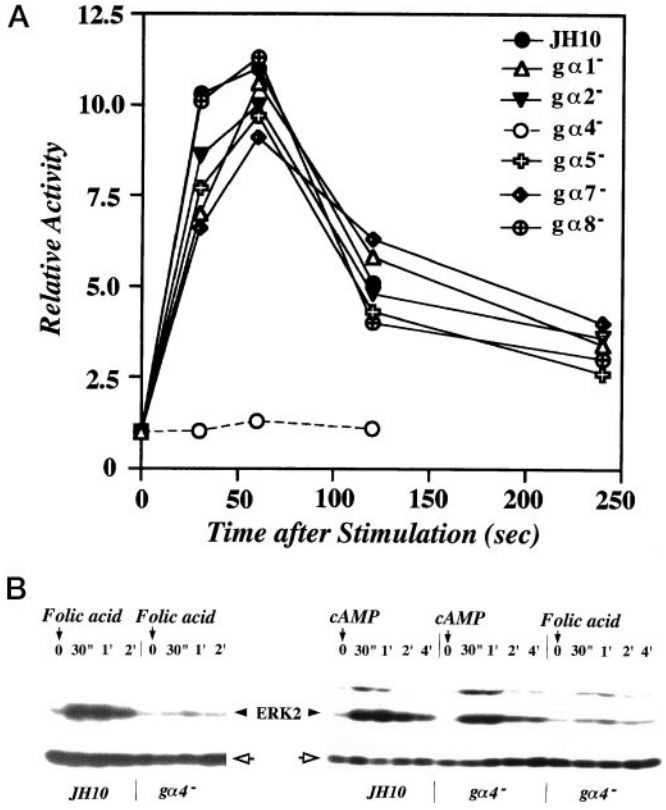

FIG. 4. **Requirement of the heterotrimeric G protein containing the Gα4 subunit in the activation of ERK2 by folic acid.** *A*, analysis of folate-mediated activation of ERK2 in *gα1* null, *gα2* null, *gα4* null, *gα5* null, *gα7* null, and *gα8* null strains. Activity was calculated by measuring the relative density of of the bands at different time points and comparing this to internal wild-type controls assayed and analyzed simultaneously (1). The activity is compared with an internal wild-type standard run on each gel as described previously (1). *B*, comparison of ERK2 activities in *gα4* null cells and the wild-type-developing, thymidine auxotroph parental strain (JH10). Whole cell lysates were prepared before (0 time point) and 30 s, 1 min, and 2 min after stimulation with 50 μM folic acid and 30 μM cAMP and analyzed by in-gel assay containing MBP as a substrate for ERK2.

we demonstrated that ERK2 is activated in aggregation-stage cells by cAMP through the cAMP G protein-coupled receptors, cAR1 and cAR3 (1). A level of ERK2 activation that was ~40–60% that observed in wild-type cells was found in strains in which the genes encoding either the cAR-coupled Gα subunit Gα2 or the only *Dictyostelium* Gβ subunit were deleted, suggesting that cAMP-stimulated ERK2 activation was at least partially G protein-independent (1). To examine whether heterotrimeric G proteins are required for ERK2 activation by folic acid, we assayed ERK2 activity in whole cell lysates of appropriate strains. As presented in Fig. 1*A*, wild-type cells (strain KAx-3) showed an increase in ERK2 activity that was detected by 20 s after the stimulation, reaching maximal levels at ~1 min (Fig. 3, *upper panel*). In contrast to our observations with cAMP-stimulated ERK2 activity in aggregation-competent cells, no folate-stimulated ERK2 activity was detected in *gβ* null cells (Fig. 3, *middle panel*). To determine if this was due to differences in the amount of folic acid binding, this was directly assayed (see "Experimental Procedures"). Wild-type cells starved for 1 h gave 60.8 ± 8.9 [^{3}H]folate binding sites/cell, while *gβ* null cells had 52.4 ± 7.4 sites/cell, indicating no significant differences between *gβ* null and wild-type cells that would account for the lack of ERK2 activation by folate. In addition, we compared the amount of ERK2 protein in the *gβ* null to that in wild-type cells by Western blot analysis. The blots showed no significant differences (data not shown). Moreover, parallel experiments performed simultaneously demonstrated cAMP-stimulated activation of ERK2 in *gβ* null cells (1). The results with the *gβ* null cells suggest that ERK2 activation by folic acid requires heterotrimeric G proteins.

In *Dictyostelium*, genes encoding eight distinct Gα subunits have been cloned, and six have been disrupted by homologous recombination (*Gα1*, *Gα2*, *Gα4*, *Gα5*, *Gα7*, and *Gα8*) (22, 25, 32–35). Folate-stimulated induction of ERK2 was assayed in the single gene disruptants of each of the above Gα subunit encoding genes. ERK2 activation was normal in all strains except that carrying a deletion in *Gα4*, the only Gα subunit known to be required for folate-mediated chemotaxis and activation of adenylyl cyclase and guanylyl cyclase (Ref. 4; Fig. 4*A*). Fig. 4*B* shows a more detailed analysis of the *gα4* null strain in comparison to its parental, wild-type-developing, thymidine auxotrophic strain JH10. These data also show the lack of folate-mediated ERK2 activation in *gα4* null cells. In contrast, cAMP stimulation of ERK2 activity is normal in *gα4* null cells (Fig. 4*B*). Although in conflict with previous observations by another group (3), the activation of ERK2 by cAMP in *gα4* null cells is expected, as cAMP activation of adenylyl cyclase is normal in *gα4* null cells (4) and ERK2 is required for cAMP activation in wild-type cells (2). Control experiments showed that the *gα4* null cells did not exhibit folated-mediated chemotaxis, as expected from previous analyses (4). As expected, the activation is independent of cAMP receptors, as exhibited by folate stimulation of ERK2 activity in *car1/car3* double knockout cells (Fig. 3, *bottom panel*), although the activity levels are slightly lower in these cells than in wild-type cells.

Activation of ERK2 by Monapterine and Developmentally Regulated ERK2 Activation by Folic Acid—The number and ligand specificity of folic acid binding sites, which are thought to correspond to folate receptors as the binding affinities are altered by GTP, change during *Dictyostelium* development (20, 27, 36–39). Cells show chemotaxis toward folic acid and maximal folate binding sites during vegetative growth and early stages of development (4, 20, 21). The number of sites decreases during aggregation and then increases at the tipped mound stage (4, 20, 21). This pattern also corresponds to the developmental pattern of expression of Gα4 (22, 25). Folate binding sites expressed on the cell surface during this later stage also

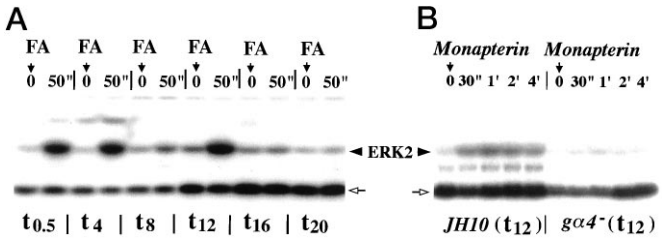

FIG. 5. **Developmentally regulated ERK2 activation by folate.** *A*, whole cell lysates were prepared from wild-type KAx-3 cells at various developmental stages before (0 time point) and 50 s after stimulation with 50 μM folic acid and analyzed by in-gel assay containing MBP as a substrate. Data presented in previous figures indicated that folate stimulation of ERK2 activity has a broad peak of maximal activity centered at ~50 s after stimulation. Cells were assayed at 0.5 and 4 h after starvation (preaggregation stages), 8 h (aggregation), 12 h (tipped aggregate stage), 16 h (slug), and 20 h (early culmination) of development. *B*, similarly, whole cell lysates were prepared from 12 h developed (tipped aggregate stage) JH10 and *gα4* null cells before (0 time point) and 30 s, 1 min, 2 min, and 4 min after stimulation with 50 μM monapterine and analyzed by the in-gel assay.

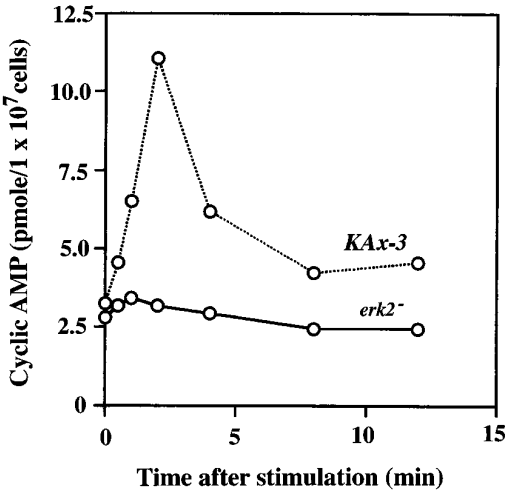

FIG. 6. **ERK2 is required for folate activation of adenylyl cyclase.** Stimulation of adenylyl cyclase activity by folate was assayed in wild-type (KAx-3) and erk2 null cells developed for 0.5 h by cAMP mass measurement (see "Experimental Procedures"). The experimental results are reproducible, and the curve shown is a representative experiment.

bind the pterin monapterine, suggesting a shift in the type of putative folic acid receptor on the cell surface (20, 21). Fig. 5*A* shows that the developmental kinetics of folate-stimulated ERK2 activity parallel the known kinetics of folate binding sites, folate responsiveness, and expression of Gα4 (4, 20, 22, 25). Folate stimulation of ERK2 is high in 0.5-h starved, 4-h starved, and 12-h starved cells, but low in 8-h aggregation-stage cells, or cells developed for 16 h or longer. Fig. 5*B* shows that monapterine activates ERK2 in 12-h starved cells. The results indicate that folate stimulates ERK2 activation during the multicellular and preaggregation stages.

ERK2 Is Required for Folate-mediated Stimulation of Adenylyl Cyclase—ERK2 is required for cAMP-mediated stimulation of adenylyl cyclase (2). To determine whether ERK2 is required for folate activation of adenylyl cyclase, wild-type and *erk2* null cells starved for 1 h were stimulated with folic acid and the level of cAMP was quantified at different times after stimulation. As indicated in Fig. 6, wild-type but not *erk2* null cells showed a significant increase in cAMP produced in response to folate stimulation.

DISCUSSION

Extracellular signal-regulated kinases, ERKs or MAP kinases, are mediators for the transmission of extracellular signals to their respective targets. In *Dictyostelium*, ERK2 plays essential roles in cell aggregation, prespore-specific gene expression, and morphogenesis during multicellular development (2, 16). Previously, it has been demonstrated that ERK2 is required for cAMP stimulation of adenylyl cyclase activity and *erk2* null cells are aggregation-deficient, as they are unable to relay the cAMP signal (1, 2). Analysis of an ERK2 temperature-sensitive mutation suggests that the requirement of ERK2 for prespore gene expression, and possibly morphogenesis, is distinct from its role in activating adenylyl cyclase (16). In this manuscript, we show that ERK2 is also activated by folic acid in cells at the interface between vegetative growth and starvation and at the time of tip formation, the times during development when folate receptors (binding sites) and Gα4 expression are maximal. We demonstrated ERK2 activation using in-gel assays of whole cell extracts made from stimulated and unstimulated cells as previously employed for cAMP-stimulation studies. In addition, for the first time, we directly assayed purified His_6-tagged ERK2. These later analyses present additional proof that we are directly measuring ERK2 activity. We show that ERK2 is activated by both folate, which binds to and activates the early (growth/early development) and late (tip stage) receptors, and monapterine, a ligand that binds to and activates late receptors (4, 21). We also show that folate stimulation of ERK2 activity is G protein-dependent and requires the Gβ and Gα4 subunits. Moreover, we demonstrate that ERK2 is required for folate stimulation of adenylyl cyclase activity, consistent with ERK2's known role in cAMP stimulation of adenylyl cyclase activity during aggregation.

Previous studies have shown that Gα4 has multiple functions during the life cycle, and it is required for all previously known folate-mediated responses (4). These responses include folate-mediated chemotaxis, which is thought to be part of a food-seeking response of starved cells that is present during growth, for the first few hours after food is depleted, and before the cells initiate aggregation (19). Gα4 also plays essential roles at the tip/finger stages of development (22), being required for proper morphogenesis and cell-type differentiation. The time of Gα4 function corresponds with the time of expression of Gα4 from its late promoter and the appearance of the late folate receptors (4, 20, 21). It has been proposed that Gα4 is required to respond to specific morphogens at this stage of development that function through the late folate receptors and that these pathways are essential for the developmental transitions after tip formation (4). It is not thought that folate *per se* is the ligand at the tipped mound stage, but it is a convenient agonist for at least some of the pathways studied in the laboratory. Analysis of the $ERK2^{ts}$ mutant indicated that ERK2 also plays an essential role at this stage of development (16). It is thus possible that the late ERK2 functions are mediated by both cAMP and the endogenous folate receptor ligand through cAMP and folate receptors, respectively. The possible role of ERK2 in mediating the activation of adenylyl cyclase by folate in these pathways is not known.

Our analysis has shown that folate activation of ERK2 exhibits kinetics of activation and adaptation similar to those we previously observed for cAMP stimulation of ERK2. However, in contrast to our findings that cAMP can stimulate ERK2 activity through the serpentine cAMP receptors to a level that is ~50% that of wild-type cells in *gβ org2* null cells and is thus at least partially G protein-independent, ERK2 activation by folate is fully G protein-dependent. Activation requires both the only known Gβ subunit and the Gα4 subunit, which is required for all other known folate-mediated responses (see above). Thus, our present analysis of the various available mutations suggests that ERK2 can be activated by both G

protein-dependent and -independent pathways. Activation by cAMP may proceed through these two pathways, both of which may be required for maximal activation during aggregation, while the folate pathway requires heterotrimeric G proteins. However, it is not known if this pathway is directly mediated by subunits of the heterotrimeric G protein containing Gα4 or if this G protein couples to the putative G protein-independent pathway used by the cAMP receptors. As the same *g*β null strain was used for both the folate and cAMP studies on ERK2 activation (1) and some of these studies were performed simultaneously, we conclude that the observed differences in the requirement for G proteins for cAMP and folate responses are not due to strain differences or other uncontrolled experimental effects. Furthermore, we show that single mutations in the genes encoding Gα1, Gα2, Gα5, Gα7, and Gα8 have no discernible effect on folate stimulation of ERK2 activity, suggesting that they are not required in this pathway. We cannot exclude redundant functions of these Gα subunits, although all previous data have suggested that the different Gα subunits have distinct functions (33–35, 40).

We find that cAMP stimulation of ERK2 activity is normal in *g*α*4* null cells. This represents a control for the lack of folate stimulation of ERK2 in this strain, as it indicates that *g*α*4* null cells are not deficient in a possible, essential component of the signaling pathway that is required for both folate and cAMP stimulation of ERK2. While these results are in conflict with a finding of another group that Gα4 is required cAMP-stimulation of ERK2 (3), our observations are consistent with the facts that ERK2 is required for cAMP stimulation of adenylyl cyclase (2) and that cAMP stimulation of adenylyl cyclase is normal in *g*α*4* null cells (4).

Folate-mediated chemotaxis in vegetative and very early starved cells is considered a food-seeking mechanism (see above). As with cAMP-mediated chemotaxis during aggregation, this pathway requires receptor/G protein stimulation of guanylyl cyclase activity, and chemotaxis is thought to proceed via the same pathway as during aggregation. However, folate can also stimulate adenylyl cyclase, which we have shown requires ERK2 activation. The physiological significance of folate-mediated ERK2 and adenylyl cyclase activity is probably not for the relay of cAMP but for the activation of cAMP-dependent protien kinase, which is required at multiple stages of the life cycle (41–49). Interestingly, a more primitive *Dictyostelium* species, *D. minutum*, exploits a folic acid-related compound as a chemoattractant in the aggregation stage, suggesting chemotaxis toward folic acid and other folate-mediated responses may be very ancient in this group of organisms (50). The requirement of ERK2 and Gα4 at the tipped mound stage may be due in part to a potential role of folate-related molecules in controlling both cell movement and cell-type differentiation, as is known to be the case for extracellular cAMP. Further study of the downstream pathways regulated by ERK2 should help elucidate additional components in and the role of these signal transduction cascades.

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