EppA, a Putative Substrate of DdERK2, Regulates Cyclic AMP Relay and Chemotaxis in Dictyostelium discoideum

Songyang Chen and Jeffrey E. Segall*

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received 23 December 2005/Accepted 26 April 2006

The mitogen-activated protein kinase DdERK2 is critical for cyclic AMP (cAMP) relay and chemotaxis to cAMP and folate, but the details downstream of DdERK2 are unclear. To search for targets of DdERK2 in Dictyostelium discoideum, 32P,32P-labeled protein samples from wild-type and Dderk2−/− cells were resolved by 2-dimensional electrophoresis. Mass spectrometry was used to identify a novel 45-kDa protein, named EppA (ERK2-dependent phosphoprotein A), as a substrate of DdERK2 in Dictyostelium. Mutation of potential DdERK2 phosphorylation sites demonstrated that phosphorylation on serine 250 of EppA is DdERK2 dependent. Changing serine 250 to alanine delayed development of Dictyostelium and reduced Dictyostelium chemotaxis to cAMP. Although overexpression of EppA had no significant effect on the development or chemotaxis of Dictyostelium, disruption of the eppA gene led to delayed development and reduced chemotactic responses to both cAMP and folate. Both eppA gene disruption and overexpression of EppA carrying the serine 250-to-alanine mutation led to inhibition of intracellular cAMP accumulation in response to chemoattractant cAMP, a pivotal process in Dictyostelium chemotaxis and development. Our studies indicate that EppA regulates extracellular cAMP-induced signal relay and chemotaxis of Dictyostelium.

Directed cell movement, or chemotaxis, is vital to numerous biological processes and is displayed by many eukaryotic cells, including endothelial cells, neurons, and cells of the immune system. Chemotaxis is strikingly exhibited in the life cycle of the amoeba Dictyostelium discoideum (33). Upon starvation, cells move toward cyclic AMP (cAMP) signals and as many as 108 cells aggregate in ~8 h. Responses known to be activated by binding of extracellular cAMP to the plasma membrane cAMP receptor (cAR1) include activation of adenyl cyclase A (ACA) and guanylyl cyclase, production of phosphatidylinositol phosphates, synthesis of intracellular cAMP and relay of the extracellular cAMP signals, chemotaxis, and expression of development stage genes.

Mitogen-activated protein (MAP) kinase cascades are conserved signaling pathways in eukaryotes for the transfer of extracellular signals to a variety of intracellular regulatory pathways. These sequentially activated kinase cascades are induced by diverse G protein- and tyrosine kinase-coupled receptors. In Dictyostelium cells, three components of MAP kinase cascades have been identified. The MAP kinase kinase (MEK) DdMEK1 is required for chemotaxis toward cAMP during aggregation (13). Knockout of DdMEK1 impaired the formation of normal-sized aggregates. However, these small aggregates can still differentiate to form normally proportioned, small fruiting bodies. DdMEK1 is required for activation of guanylyl cyclase and the synthesis of cGMP, a second messenger regulating chemotaxis, in response to extracellular cAMP. There are two MAP kinases, DdERK1 and DdERK2, in Dictyostelium cells. Their activity peaks between 15 s and 1 min after stimulation (26, 32). In general, ERKs are proline directed in that they target substrates that contain a proline in the P2+1 site (Ser/Thr-Pro) (23). ERKs are uniformly distributed in the cytoplasm in quiescent cells, but a significant population of ERKs accumulates in the nucleus upon stimulation. Activated ERKs phosphorylate numerous substrates in all cellular compartments, including various nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, and STAT3), membrane proteins (CD120a, Syk, and calnexin), cytoskeletal proteins (neurofilaments and paxillin), and several protein kinases (RSK, MKS, and MNK) (20).

We and others have previously found that DdERK2 contributes to Dictyostelium chemotaxis (14, 15, 26, 34). Dderk2−/− cells show defects in folate and cAMP chemotaxis and fail to accumulate intracellular cAMP. Identification of a substrate(s) of DdERK2 will enhance our understanding of how DdERK2 regulates chemotaxis. In this study, we identified a new substrate of DdERK2, EppA. DdERK2 regulates phosphorylation of EppA at serine 250, and DdERK2-dependent phosphorylation of EppA is important in regulating directionality, velocity, and persistence during chemotaxis to cAMP. EppA is also required for accumulation of intracellular cAMP.

MATERIALS AND METHODS

Maintenance of strains, starvation, radiolabeling of Dictyostelium, and cAMP stimulation. The Dictyostelium discoideum axenic strains HS176 (wild type) and HS174 (DdERK2−) (26) were maintained in HL5 medium (30) in suspension at 22°C with aeration and supplied with 10 μg/ml G418 (Gibco). PCR reagents and restriction enzymes were from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively. The radioisotope 32P,32P used for in vivo labeling was from ICN (Irvine, CA). Starvation buffer consisted of 20 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH (pH 6.6), 2 mM MgCl2, and 0.2 mM CaCl2. A total of 5 x 107 starved cells were resuspended in 1 ml buffer, starved 2 h, and pulsed with 300 nM cAMP every 6 min for 4 h. A total of 4 x 107 starved cells were resuspended in 2 ml buffer, mixed with 50 μCi 32P,32P, and shaken for 25 min at 22 to 24°C. Then the cells were stimulated with 10 μM cAMP and folate (Sigma, St. Louis, MO). The stimulated cells were lysed by an equal volume of phenol, and protein pellets were washed with ether.

* Corresponding author. Mailing address: Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-4237. Fax: (718) 430-8996. E-mail: segall@acomm.yu.edu.
2-D electrophoresis. For isoelectric focusing (IEF), 18-cm precast ITP gels (pH 4 to 7) and an IGPophor IEF unit (Amersham Pharmacia Biotechnology, Sunnyvale, CA) were used as described elsewhere (8). Three hundred micrograms of protein was focused for 98,000 Vh in a sample buffer containing 6 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% Phosphate (pH 3 to 10), 0.5% Phosphate (pH 4 to 7), and 0.4% dithiothreitol. As soon as IEF was finished, the strips were either frozen at −70°C or equilibrated with buffer (0.375 M Tris-Cl [pH 8.8], 6 M urea, 1% sodium dodecyl sulfate [SDS], and 30% glycerol) containing 1% dithiothreitol or 4% iodoacetamide for 10 min each. Then the second dimension was carried out on 10% SDS-acrylamide gels. After electrophoresis, radioactive 2-dimensional gels were stained with colloidal Coomassie brilliant blue (Sigma; 0.5% in 50% methanol–10% acetic acid), wrapped in plastic film, exposed for 1 to 3 days to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA), and scanned with a Storm 4500 imager (Molecular Dynamics) at a resolution of 100 pixels/cm². From this 2D gel, 12 bands of interest were excised and eluted with 85% methanol. The concentrated bands were subjected to MS analysis of protein.

Expression constructs of myc-tagged EppA protein and point mutations of serine residues in myc-EppA. All DNA manipulations were carried out according to standard molecular biology techniques (21). All restriction enzymes were from Promega. The D. discoideum genome and cDNA database were searched using amino acid sequences acquired from LC-MS/MS, and as a result, a genomic sequence database.

All PCR amplifications were carried out using the high-fidelity PCR system. The PCR product thus amplified was purified using a QIAquick PCR purification kit (Qiagen). The insert was then released from the appropriate restriction enzyme sites (capitalized and italicized), respectively. The purified DNA fragment was cloned into the XbaI site of the P. pastoris P. pastoris expression vector pPICZα-A (Invitrogen), and sequenced.

To generate point mutations of serine residues in EppA, the pDNeo II-EppA tagging EppA KO plasmid vector. The SpeI site in primer 3 was used to determine the orientation of the insertion. From this vector DNA, the entire knockout cassette carrying the bacterial resistance cartridge in the middle was PCR amplified using primers 1 and 4. The amplified PCR DNA was purified and used for electroporation of HS176 cells as described previously (2). Transformants were selected in the presence of 10 μg/ml G418. Individual clones were isolated, amplified, and subjected to further analyses. Genomic DNA was extracted from each clone was analyzed by genomic PCR by using primers 1 and 4. Serum disruption in Dictyostelium. The disruption construct was assembled in plasmid pBsrBglII. A 440-bp DNA sequence was PCR amplified using primer 1 (5’TCTAGAcacgctccctagctagc-3) and primer 2 (5’ACTAGTCTACGAG-3) from the N terminus of the eppA gene. The (capitalized and italicized), respectively. The purified DNA fragment was cloned as an XbaI restriction fragment into the XbaI site of pBsrBglII to generate the pBsrBglII-EppA vector. Similarly, another DNA fragment of 626 bp was PCR amplified using primer 3 (5’AACTGATTACTGAGTCGTTGCTGATA-3) and primers 4 (5’-AAGCTTACTGTCGTTGCTGATA-3) from the HindIII site to the C terminus of the eppA gene. The purified fragment was cloned into HindIII and cloned into the HindIII site of the pBsrBglII-EppA vector to create the pBsrBglII-EppA-KO plasmid vector. The Plasmid was cut from each clone was analyzed by genomic PCR by using primers 1 and 4.

To generate point mutations in the myc-EppA vector was used as a template. The PCR amplification was carried out using the pBluescript vector. The PCR product was then inserted into the pBluescript KS + plasmid vector (Stratagene, La Jolla, CA) by following the manufacturer’s instructions. The pBluescript vector was used to transform E. coli DH5α (Invitrogen). The plasmid was then isolated from the E. coli transformants. Plasmids carrying the correct point mutation were confirmed by sequencing. Generation of stable cell lines. The expression of myc-tagged EppA proteins was confirmed as described previously (19) with the following modifications. HS176 and HS174 cells growing in suspension were used to transform the expression of myc-tagged EppA proteins by performing Western blot analyses with an anti-myc antibody (Upstate, Charlottesville, VA).
Folate chemotaxis assays. Axenically grown cells were centrifuged and cells from the pellet dotted on an agar dish containing 1% Bacto agar (Difco, Sparks, MD) in 17 mM phosphate buffer, 1 mM MgCl₂, and 1 mM CaCl₂. They were placed 3 to 4 mm from the edge of a well containing 1 mM folate. The positions of the cells were recorded by photography at the beginning and after 7 to 9 h. The distance of the moving front was measured and used to calculate an average speed of spread. Then the average speeds were converted to the percentage of the speed of wild-type cells. Results are means ± standard errors of the means from three experiments.

Reverse transcription-PCR (RT-PCR) analysis of epp1 expression. At different rearing times during the development at 22°C, total RNA was isolated from HS176 cells by using TRIzol reagent, followed by synthesis of cDNA with random hexamers as primers. Using synthesized cDNA as a template, PCR amplifications were performed with epp1 gene-specific primers epp1A(361-382) (5'-G GTAATGCTAGACCACTGTG-3') and epp1B(889-865) (5'-CACGATCTG GAGTACCTCTGTAACC-3') to amplify a 528-bp fragment. As a control, the constitutively expressed gene IG7 (18) was amplified with primer pairs IG7-S (5'-TTACATTATTTAGACCCGAAACCG-3') and IG7-A (5'-TTCCC TTAGAGCTATGGACCCGAAACCAAGCG-3') on the same template sample.

RESULTS

Separation of cAMP-responsive phosphoproteins by 2-D electrophoresis. To identify substrates of DdERK2, we compared the protein phosphorylation patterns of wild-type and Dderk2− cells after stimulation with the chemotacticant, cAMP. Cells were starved, labeled with ³²P, and stimulated with cAMP for 1 min, and then lysed. Solubilized proteins were separated by 2-D electrophoresis, and phosphorylation was quantitated by a PhosphorImager. In Coomassie brilliant blue-stained gels, there are several hundred protein spots resolved by the 2-D gel. The overall pattern of proteins is similar between wild-type and Dderk2− cells (Fig. 1A).

A total of 35 phosphorylation spots could be consistently identified (Fig. 1B; Table 1) in the autoradiograms. The majority showed increases in both wild-type (31 out of 35) and Dderk2− (22 out of 35) cells after cAMP stimulation. Three spots in wild-type cells and 12 spots in Dderk2− cells showed no change in phosphorylation after cAMP stimulation. Both wild-type and Dderk2− cells showed one spot with reduced phosphorylation after stimulation. Five spots showed statistically significant (P < 0.05) DdERK2-dependent phosphorylation increases (spots 3, 4, 10, 18, and 31).

We then aligned the Coomassie brilliant blue-stained gels with the autoradiographs to identify the corresponding phosphoproteins. Spots 4, 10, 18, and 31 could not be identified—the corresponding protein spot in the Coomassie brilliant blue gel could not be found due to a small amount of protein or was masked by overlapping highly abundant proteins—and thus, these potential substrates have not been further pursued. However, spot 3 had a clear protein in the corresponding Coomassie brilliant blue gel (Fig. 1C) with an estimated molecular size of 45 kDa and will be referred to as EppA (ERK2-dependent phosphoprotein A). In wild-type cells, the phosphorylation intensity of EppA increased 2.95-fold in response to cAMP, while in Dderk2− cells, the intensity increased 1.2-fold (Fig. 1C; Table 1). The difference is statistically significant (P < 0.01). We therefore focused on this protein as a candidate DdERK2 substrate or downstream effector.

Identification of EppA. To identify the potential DdERK2 substrate EppA, the protein was excised from the Coomassie brilliant blue-stained gels and digested with trypsin, and the peptides extracted from the digestion were used for MS/MS sequencing and MALDI-TOF analysis. Upon a search of the Dictostelium genomic database with the MS/MS sequencing results (Table 2), sequence matches with a single 45-kDa open reading frame in expressed sequence tag and genomic database (7, 17) were identified with high probability. The MALDI-MS spectrum covered 34.6% of the open reading frame, confirming the MS/MS sequencing identification (Fig. 2). The epp1A gene (DDB0218473) is located on chromosome 4 with one intron. We have not yet found a homologue of EppA in other species. There are four potential ERK phosphorylation sites (Ser/Pro) in the protein (Fig. 2B) and possible evidence for a phosphopeptide containing serine 250 (shown in Fig. 2C).

To confirm that EppA is phosphorylated in vivo, a 10-amino-acid myc tag was added to the N terminus of the open reading frame (Fig. 3A) and the tagged protein was expressed in both wild-type and Dderk2− cell lines. myc-tagged protein was immunoprecipitated from the cells, and phosphorylation of the protein increased 3.9-fold in wild-type cells after cAMP stimulation, with no change in phosphorylation in Dderk2− cells (Fig. 3B). This confirmed that EppA phosphorylation was cAMP stimulated and DdERK2 dependent. Analysis of the kinetics of EppA phosphorylation showed that EppA phosphorylation reached a maximum after the maximum of DdERK2 activation, consistent with EppA being a substrate of DdERK2 (Fig. 3C).

To further characterize the phosphorylation sites of EppA, the four potential DdERK2 recognition serine sites in EppA were mutated to alanine individually and reintroduced into wild-type cells. Three point mutants (S64A, S126A, and S325A) showed increased phosphorylation after cAMP stimulation, comparable to that of wild-type EppA protein (Fig. 3D). However, mutation of Ser250 to alanine abolished DdERK2-dependent phosphorylation of EppA in response to cAMP stimulation. In addition, MALDI-TOF analysis showed a pair of peaks consistent with unphosphorylated and phosphorylated forms of a peptide containing Ser250 (Fig. 2C). These data indicate that Ser250 is the major DdERK2-dependent phosphorylation site in EppA.

Expression of epp1A was also assessed. RT-PCR of a 528-bp fragment of the epp1A cDNA from total RNA indicated that on the mRNA level there is little alteration during development.
To assess the potential role of EppA in chemotaxis and morphogenesis in this developmental process, we have previously reported that DdERK2 is required for the formation of multicellular fruiting bodies and dormant cells entering a multicellular developmental program leading to slow growth.

Expression of EppA in wild-type cells had no significant effect, overexpression of the Ser250Ala mutation in wild-type cells resulted in a defect similar to that produced by disrupting EppA, consistent with phosphorylation of Ser250 being important for EppA function.

eppA disruption also had an effect on cell growth. Wild-type cells divided every 13 h, an interval similar to the 12-h doubling time of the Ax-2 cell line, widely used in Dictyostelium studies (Fig. 4C). However, eppA-disrupted cells grew significantly more slowly, with a doubling time of 21 h. Reintroduction of myc-EppA into eppA− cells restored the normal growth rate, with a doubling time of 13.8 h. Mutation of serine 250 or serine 325 also had severe effects on cell growth and increased doubling times to 21 and 27 h, respectively.

**EppA is involved in regulation of Dictyostelium chemotaxis.** To determine the function of EppA in Dictyostelium chemotaxis, we analyzed responses of wild-type, EppA-overexpressing, and eppA− cells to an exogenous cAMP gradient. Disruption of the eppA gene led to significant decreases in both velocity and chemotaxis (Table 3). These cells also made turns more frequently than wild-type cells (reduced persistence). Introduction of the myc-tagged eppA gene into the EppA-disrupted cells restored fruiting body formation by 24 h. Although overexpression of the myc-tagged full-length EppA in wild-type cells had little effect, overexpression of the Ser250 Ala mutation in wild-type cells resulted in a defect similar to that produced by disrupting EppA, consistent with phosphorylation of Ser250 being important for EppA function.

**Peptide**

*Peptides from EppA sequenced by tandem MS/MS*

| Peptide | m/z | Sequence |
|---------|-----|----------|
| 1       | 2916.8 | $^{60}$KVASPDTEDDFPALGOEK |
| 2       | 2949.3 | $^{51}$TYGSQYSSSSSSSPQE |
| 3       | 2852.7 | $^{79}$KVASPDTEDDFPALGOEK |
| 4       | 2752.8 | $^{23}$KVASPDTEDDFPALGOEK |
| 5       | 712.1  | $^{36}$NPSEDYTSNAFK |
| 6       | 596.6 | $^{79}$NFCNLNLVOTK |
| 7       | 5908.3 | $^{79}$OQSSSSQOPPSSGER |
| 8       | 6228.0 | $^{28}$FNSGVSPFNFK |
| 9       | 7113.3 | $^{59}$TKFCNLNLVOTK |

---

**TABLE 1. Comparison of protein phosphorylation changes**

| Spot no. | Mol mass (kDa) | pH | Relative phosphorylation change (1 min/0 min)# | P |
|----------|----------------|----|-----------------------------------------------|---|
| 1        | 50             | 6.3| 1.63 ± 0.45                                   | 0.44 |
| 2        | 44             | 6.5| 2.72 ± 0.73                                   | 0.29 |
| 3        | 45             | 5.9| 2.95 ± 0.29                                   | 0.22 |
| 4        | 46             | 5.7| 2.00 ± 0.39                                   | 0.22 |
| 5        | 42             | 5.9| 1.59 ± 0.40                                   | 0.32 |
| 6        | 40             | 4.8| 3.52 ± 1.43                                   | 0.07 |
| 7        | 40             | 4.2| 0.98 ± 0.37                                   | 0.28 |
| 8        | 39             | 4.2| 1.94 ± 0.74                                   | 0.43 |
| 9        | 38             | 4.7| 2.52 ± 0.55                                   | 0.10 |
| 10       | 38             | 4.7| 3.94 ± 0.71                                   | 0.01 |
| 11       | 37             | 4.4| 3.61 ± 2.20                                   | 0.14 |
| 12       | 36             | 4.6| 1.49 ± 0.43                                   | 0.20 |
| 13       | 38             | 5.9| 0.98 ± 0.34                                   | 0.34 |
| 14       | 34             | 4.7| 3.82 ± 1.12                                   | 0.06 |
| 15       | 34             | 4.5| 3.45 ± 1.56                                   | 0.14 |
| 16       | 30             | 5.7| 1.78 ± 0.41                                   | 0.44 |
| 17       | 27             | 5.3| 0.78 ± 0.16                                   | 0.13 |
| 18       | 29             | 5.0| 2.62 ± 0.37                                   | 0.04 |
| 19       | 27             | 6.6| 0.68 ± 0.40                                   | 0.34 |
| 20       | 26             | 4.8| 1.89 ± 0.45                                   | 0.08 |
| 21       | 25             | 6.5| 2.07 ± 0.76                                   | 0.15 |
| 22       | 24             | 5.1| 2.18 ± 0.75                                   | 0.09 |
| 23       | 24             | 4.5| 2.85 ± 1.31                                   | 0.48 |
| 24       | 22             | 6.7| 1.46 ± 0.22                                   | 0.20 |
| 25       | 22             | 6.4| 2.19 ± 0.23                                   | 0.35 |
| 26       | 20             | 5.7| 2.06 ± 1.21                                   | 0.45 |
| 27       | 22             | 5.3| 1.73 ± 0.66                                   | 0.27 |
| 28       | 22             | 5.2| 2.14 ± 0.98                                   | 0.32 |
| 29       | 20             | 4.8| 3.20 ± 1.19                                   | 0.08 |
| 30       | 20             | 4.7| 4.75 ± 1.94                                   | 0.45 |
| 31       | 18             | 5.3| 2.79 ± 0.45                                   | 0.03 |
| 32       | 14             | 6.0| 2.43 ± 0.61                                   | 0.22 |
| 33       | 17             | 4.9| 1.49 ± 0.50                                   | 0.38 |
| 34       | 17             | 4.8| 1.86 ± 0.69                                   | 0.13 |
| 35       | 17             | 4.4| 2.65 ± 1.00                                   | 0.09 |

*# Incorporation into spots of 2-D gels was quantified by PhosphorImager and normalized to the protein amount of actin in gel. Phosphorylation changes of proteins are expressed as the fold change in 32P incorporation after cAMP stimulation versus before stimulation. Values are means ± standard errors from three experiments. Only proteins that were phosphorylated in three independent experiments were quantified.

---

**FIG. 2. Identification of EppA by mass spectrometry.** (A) MALDI-TOF spectrum of EppA protein. The spot corresponding to phosphorylated EppA was excised and digested with trypsin. The tryptic peptides were extracted and subjected to MALDI-TOF analysis. Peaks that match theoretical tryptic digested peptide masses from EppA are marked by asterisks. (B) Amino acid sequence of EppA. The amino acid sequence was derived from full-length cDNA sequences (clone SVD141 and AFK 496) provided by the Japanese Dictyostelium cDNA project. Four potential Erk2 phosphorylation sites (Ser/Pro) are labeled with asterisks. Amino acids sequenced by LC-MS/MS are boldfaced, and sequences covered by MALDI-TOF are underlined. (C) Mass spectrum of potential phosphopeptide. A portion of panel 2A was enlarged to show a peak (1,244.45 Da) (left arrow) potentially representing the peptide containing serine 250 (amino acids 245 to 255) and the peak (1,323.42 Da) (right arrow) potentially representing the phosphorylated peptide.

**TABLE 2. Peptides from EppA sequenced by tandem MS/MS**
A.

B.

1 MSNVTKFNIN DLVQTKQSSN LPSPRSSYS EDDENQGQYS GGGYNNRGRP TYGSQYSSSS
61 SSALPQKKEV SAPTEDDFFPA LGQEKKEPRQ ROOSQSSSQP QPPSSGERVD PFQGRAPPTDG
121 QRGEDSPSER DSEEBREBRN DDDQPRQQ NDRDDQTDEG GGGPNRGYNYR GGYGNNRGG
181 DREGYNGRDD YGGYRGRGYGG GGDREGGGYNR GGYGNRGGGD RYNRRDRNDR
241 KDDRFNSGVS PPNFKNNNMR DRGDDDRYNN NNRNSSDGDRDG GYRGTDQREG GDYNNRRND
301 GGNRGGGGG NRDDGDRNBRN PGGSRPQNYD NNRRNPDREE RRPPFSKVEDS VDDWRNPSED
361 VSTNAPKQR KPAWNNEEQ RSYNNNNNYQ RRDNNRQHDQ QN

C.
significantly reduced the directionality of cells in the cAMP gradient, with moderate inhibition of velocity and persistence (Table 3). These results suggest that EppA is important in regulating Dictyostelium chemotaxis and that ERK2-mediated phosphorylation of serine 250 is required for appropriate chemotaxis.

To evaluate the developmental stage of the cells during the cAMP chemotaxis assay, the expression of cAR1, an early developmental marker, was examined. eppA/H11002 cells showed cAR1 regulation similar to that of wild-type cells (Fig. 4D). Expression of cAR1 was first seen at 4 h after the onset of starvation and continued to increase, peaking at 6 h, indicating that disruption of eppA did not change gene expression in early developmental stages. Thus, the reduced chemotactic response

### TABLE 3. Quantitation of cAMP chemotaxis

| Strain (n) | Ave cosθ | Ave velocity (μm/min) | Persistence |
|-----------|-----------|-----------------------|-------------|
| wt/pBsr (853) | 0.49 ± 0.01 | 6.31 ± 0.39 | 0.64 ± 0.03 |
| eppA− (424) | 0.29 ± 0.04** | 4.46 ± 0.35* | 0.55 ± 0.01* |
| eppA−/myc-EppA (941) | 0.56 ± 0.02 | 6.67 ± 0.26 | 0.69 ± 0.03 |
| wt (638) | 0.58 ± 0.04 | 6.41 ± 0.71 | 0.73 ± 0.05 |
| wt/vector (602) | 0.62 ± 0.02 | 6.17 ± 0.75 | 0.75 ± 0.03 |
| wt/myc-EppA (925) | 0.58 ± 0.01 | 6.29 ± 0.16 | 0.75 ± 0.01 |
| wt/myc-EppA(S250A) (506) | 0.36 ± 0.06* | 5.75 ± 0.48 | 0.61 ± 0.02 |

* Cell chemotaxis was measured on 8-h-starved cells in a Zigmond chamber with a 2 μM cAMP gradient.

** wt, wild type; n, total number of cells analyzed in three experiments.

* Data are means ± standard errors from three experiments. *, P < 0.05; **, P < 0.01.
FIG. 4. Disruption of the eppA gene and evaluation of development and growth of Dictyostelium cells. (A) Expression of the eppA gene during development. Wild-type cells (HS176) growing in suspension culture were collected by centrifugation, followed by a wash with phosphate buffer. The cell pellet was resuspended in the buffer, and $1 \times 10^7$ cells (in 1 ml buffer) were placed on top of Ca/Mg-phosphate agar plates. RNA was isolated at the indicated times. RT-PCR was performed with eppA-specific primers after generation of first-strand cDNA by reverse transcriptase. PCR mixtures were subjected to electrophoresis on a 1% agarose gel. As mentioned in Materials and Methods, IG7 is a control constitutively expressed gene. com is an eppA disrupted by targeting construct. (B) EppA is required for normal Dictyostelium development. A total of $10^6$ cells in 100 μl of phosphate buffer were placed on phosphate agar plates, and cells were allowed to develop at 22°C in a moist chamber. Pictures were taken at the indicated times. Bar, 1 mm. wt, wild-type parental cells; eppA, cells with the eppA gene disrupted by targeting construct; wt/myc-EppA, wild-type cells overexpressing myc-EppA; wt/myc-EppA(S250A), wild-type cells overexpressing myc-EppA carrying the S250A point mutation; eppA/myc-EppA, eppA knockout cells expressing myc-EppA. (C) Effect of EppA on Dictyostelium cell growth. Cells were grown in suspension, and the doubling time was calculated from cell density measurements made during the exponential-growth phase. Results are means ± standard errors of the means from three experiments. (D) Expression of cAR1 in early development of Dictyostelium. Cells were starved in PB and pulsed with 100 nM cAMP for varying times after 2 h of starvation. Aliquots of cells were taken at different time points, lysed by 2× SDS sample buffer, and Western blotted (WB) with an anti-cAR1 antibody. (E) cAMP-dependent activation of DdERK2. Cells were starved and pulsed for 7 h, treated with caffeine for 10 min, stimulated with 10 μM cAMP, lysed by an equal volume of 2× SDS sample buffer at 0 and 30 s after stimulation, and Western blotted with an anti-DdERK2 antibody. After stimulation, a higher-mobility band indicates the activated DdERK2 protein under these gel electrophoresis conditions.
to cAMP of eppA− cells indicates a role for EppA in chemotaxis. Expression and activation of DdERK2 were also tested. Wild-type and eppA− cells expressed similar amounts of DdERK2, and DdERK2 was phosphorylated 30 s after cAMP stimulation (Fig. 4E) in all strains, indicating that upstream signaling and activation of ERK2 were not affected by the loss of EppA function. To determine whether EppA is generally required for chemotaxis or selectively affects cAMP responses, chemotaxis to folic acid was studied using the agar well assay. Disruption of EppA function. To determine whether EppA is generally required for chemotaxis or selectively affects cAMP responses, chemotaxis to folic acid was studied using the agar well assay. eppA− cells responded to folate, but not as efficiently as wild-type cells, suggesting that EppA is required for both cAMP and folate chemotaxis (Fig. 5).

Regulation of cAMP accumulation by EppA. Previous studies have demonstrated that DdERK2 activity is required for cAMP accumulation and signal relay (14, 26). Therefore, we tested the role of EppA in intracellular synthesis of cAMP. In contrast to wild-type cells, eppA− cells displayed only a weak and short increase in cAMP production after stimulation with the cAR1 agonist 2′-deoxy-cAMP (Fig. 6). Expression of myc-EppA in disruptants restored stimulation of cAMP. Expression of the S250A EppA protein in wild-type cells reduced cAMP accumulation to the level of EppA disruptants.

**DISCUSSION**

In this study we used 2-dimensional electrophoresis to look for proteins that were phosphorylated by DdERK2 in response to the chemoattractant cAMP. We detected five proteins that showed DdERK2-dependent phosphorylation. By LC-MS/MS and MALDI-TOF, we were able to identify one of them and have named it EppA. The EppA gene is located on chromosome 4, and there is one intron in the genomic sequence. We have found that phosphorylation of EppA on serine 250 is dependent on DdERK2 activity. In eppA− cells, extracellular cAMP-stimulated accumulation of intracellular cAMP was inhibited. Disruption of the eppA gene also led to a deficiency in chemotaxis to cAMP and folate. EppA is required for proper development of Dictyostelium.

For aggregation-stage Dictyostelium cells, binding of cAMP to the plasma membrane receptor cAR1 triggers a series of downstream events involving DdERK2. DdERK2 is activated by stimulation of cells with cAMP, and its kinase activity peaks between 30 s and 1 min after cAMP binding to the receptor. DdERK2 is important for accumulation of intracellular cAMP. Two minutes after binding of cAMP to the receptor cAR1, DdERK2 activity begins to drop; it returns to basal levels after 5 min. Before the identification of EppA, the only ERK2 substrate in Dictyostelium that had been proposed was RegA, a phosphodiesterase that degrades cAMP specifically (27, 28, 31). It has been proposed that upon stimulation of cells with cAMP, inhibition of RegA activity results in increased intracellular cAMP levels (16). Cells with a disruption of regA accumulated more cAMP than wild-type Dictyostelium cells in response to cAMP stimulation. regA disruptants suppress the defect in aggregation and cAMP production of Dderk2− mutants, and control of RegA activity may be due to DdERK2-dependent phosphorylation (16). There are four potential DdERK2 recognition sites in RegA, including threonine 676. Expression of RegA protein carrying a mutation of threonine 676 blocks the stimulated production of intracellular cAMP (16). Although threonine 676 is important for the regulation of RegA, there is no direct evidence of phosphorylation of RegA at threonine 676.

We describe here the identification and characterization of EppA as a novel substrate of DdERK2, which regulates chemotaxis and development of Dictyostelium cells. cAMP-induced and DdERK2-dependent phosphorylation of EppA was first found by comparing 2-D gels of wild-type and Dderk2− samples. MS/MS analysis of the protein excised from 2-D gels provided sequences of nine peptides, which covered 29.6% of the EppA coding sequence. Ten peaks in the MALDI-TOF spectrum of tryptic peptides from EppA matched theoretical sequences. Immunoprecipitation of myc-tagged EppA from wild-type cells displayed a threefold increase in EppA phosphorylation after cAMP stimulation, while Dderk2− cells showed only a
Slight increase, confirming that EppA is phosphorylated by a DdERK2-dependent pathway. Disruption of the eppA gene decreased the growth rate and caused delayed development of *Dictyostelium* cells, and the disruptants formed smaller fruiting bodies. The disruptants showed significantly reduced cAMP-stimulated cAMP production, which could delay aggregation kinetics. In addition, in the Zigmond chamber assay, eppA− cells showed a significant decrease in directionality in a cAMP gradient. Cell velocity and persistence also dropped significantly. The combination of reduced cAMP production and reduced chemotactic response is consistent with the delayed aggregation that is observed. DdERK2 was properly activated by cAMP stimulation in eppA− cells, indicating that the disruptants were able to develop to the aggregation-competent state and that the major function of EppA is downstream of DdERK2. In addition to chemotaxis to cAMP, EppA disruptants were defective in folate chemotaxis, implying that EppA is required for both cAMP and folate chemotaxis, within the qualitative resolution of the folate chemotaxis assay that was used.

EppA has four potential DdERK2 phosphorylation sites. Mutation of serine 250 to alanine impaired DdERK2-dependent phosphorylation of EppA in response to cAMP stimulation, indicating that serine 250 is required for DdERK2-dependent phosphorylation of EppA. We did not detect DdERK2 coimmunoprecipitated with myc-EppA, implying that DdERK2 interactions with EppA must be relatively brief. Alternatively, it is possible that phosphorylation of EppA at serine 250 is performed by another kinase that is in turn activated by DdERK2. While expression of wild-type EppA in wild-type cells had no effect on any of the phenotypes we have measured, expression of the S250A mutant of EppA in wild-type cells (S250A/EppA) caused a delay in development, consistent with phosphorylation of EppA being important for signaling during development. When we looked at chemotaxis and signal relay, EppA/S250A cells showed decreased directionality, velocity, and persistence in cAMP gradients and lower intracellular cAMP accumulation. These data suggest that the S250A mutant may be a dominant-negative form of EppA, raising the possibility that EppA forms a complex with downstream components that is altered by phosphorylation of EppA.

The precise mechanism(s) by which EppA regulates cAMP synthesis and chemotaxis is still unclear. With respect to cAMP synthesis, EppA may act directly on RegA or in parallel. Although RegA has a potential phosphorylation site (threonine 676) and mutation of the threonine impairs DdERK2-dependent inhibition of RegA (16), there is no direct evidence that RegA is phosphorylated at that site. Thus, it is formally possible that ERK2 phosphorylates EppA and EppA regulates inhibition of RegA in cAMP-stimulated cells. Alternatively, EppA may act in parallel with RegA to control cyclase activity. The regulatory mechanism of ACA activity is complex (22). It is known that CRAC (cytosolic regulator of adenyl cyclase) (5, 6, 25), RasC (12), Rips3 (10), RasGEF (9), and Pia (4) are required for activation of ACA. How these proteins interact with each other and ACA is unclear. For the adaptation of ACA, Go9 was found to have an inhibitory effect on ACA (3). Since Go9-null cells still showed periodic signaling, there must be other mechanisms to negatively regulate ACA. With respect to the reduction in chemotaxis, the defects in EppA disruptants may be due to the alteration in intracellular cAMP. Proper accumulation and degradation of cAMP is important for protein kinase A function and inhibition of lateral pseudopod formation in chemotaxis (29). Mutants lacking ACA have reduced chemotactic responses in cAMP gradients, and thus, the reduced cAMP production in eppA− cells could result in a phenotype similar to that of acac− mutants.

The sequence of EppA does not show strong homologies to other proteins, as is also true for CRAC, another regulator of adenyl cyclase (11). The presence of strings of arginines and a repeated motif involving glycine can be seen in DNA and RNA binding proteins, but sequence searches have not identified a standard DNA or RNA binding domain. Thus, it is possible that EppA is also involved in regulation of transcription or translation. The glycine-rich domain of TAFIh68 has been shown to interact with the DNA binding domain in cis or in trans (1), and thus, EppA’s glycine-rich domain may also play a role in intramolecular or intermolecular interactions. The myc-tagged EppA construct, which fully restores chemotaxis and development in the EppA disruptants, is mainly cytoplasmic and not localized to the nucleus. Thus, we currently favor models focusing on a cytoplasmic function for EppA.

In summary, we have identified a novel protein, EppA, whose phosphorylation is regulated by DdERK2 and which is necessary for chemotaxis to cAMP and intracellular production of cAMP. We have identified the DdERK2-dependent phosphorylation site on EppA and shown that mutating this site to alanine generates a dominant-negative form, demonstrating that phosphorylation is important in the regulation of EppA. Identification of the interacting partners of EppA will aid in determining the mechanism of action of EppA in the regulation of cAMP production and chemotaxis.

Acknowledgments

We thank P. DeVreotes for providing the anti-cAR1 antibody, R. Gomer for providing the pBsr3AβGalIII plasmid, and H. Urushihara and the Japanese *Dictyostelium* cDNA project for providing the full-length cDNA of EppA. We also thank R. Angeletti and E. Neives for help on MALDI-TOF analysis and J. Chubb for help on establishing knockout strains. We appreciate the suggestion of EppA as the name for pS5 from a reviewer.

This work was supported by MCB9728324 and CA100324.

References

1. Alex, D., and K. A. Lee. 2005. RGG-boxes of the EWS oncoprotein repress a range of transcriptional activation domains. Nucleic Acids Res. 33:1323–1331.
2. Betapudi, V., K. Shoebbotham, and T. T. Egelhofer. 2004. Generation of double gene disruptions in *Dictyostelium discoideum* using a single antibiotic marker selection. BioTechniques 36:106–112.
3. Brzostowski, J. A., C. Johnson, and A. R. Kimmel. 2002. Goα-mediated inhibition of developmental signal response. Curr. Biol. 12:1199–1208.
4. Chen, M. Y., Y. Long, and P. N. Devreotes. 1997. A novel cytosolic regulator, Pianissimo, is required for chemoeffectant receptor and G protein-mediated activation of the 12 transmembrane domain adenyl cyclase in *Dictyostelium*. Genes Dev. 11:3218–3231.
5. Comer, F. I., C. K. Lippincott, J. J. Masbad, and C. A. Parent. 2005. The PI3K-mediated activation of CRAC independently regulates adenylyl cyclase activation and chemotaxis. Curr. Biol. 15:134–139.
6. Dormann, D., G. Weijer, C. A. Parent, P. N. Devreotes, and C. J. Weijer. 2002. Visualizing PI3 kinase-mediated cell-cell signaling during *Dictyostelium* development. Curr. Biol. 12:1178–1188.
7. Eichinger, L., J. A. Pachecat, G. Glocker, M. A. Rajandream, R. Suggang, M. Berriman, J. Song, R. Olsen, K. Szalfrinski, Q. Xue, T. Tunggal, S. Kummerfeld, M. Maderia, B. A. Konfortov, F. Riviero, A. T. Bankrier, R. Lehnmann, N. Hamlin, R. Davies, P. Gaudet, P. Fey, K. Pilger, G. Chen, D.
Saunders, E. Sodergren, P. Davis, A. Kerhornou, X. Nie, N. Hall, C. Anjard, L. Hempfling, N. Bason, P. Farbrother, B. Desany, E. Just, T. Morio, R. Rost, C. Churcher, J. Cooper, S. Haydock, N. van Driessche, A. Cronin, I. Goodhead, D. Muzny, T. Mourié, A. Pain, M. Lu, D. Harper, R. Lindsay, H. Hansen, K. James, M. Quiles, M. Madan Babu, T. Saito, C. Buchrieser, A. Wardrop, M. Felder, M. Thangavelu, D. Johnson, A. Knights, H. Lousegèd, K. Munagall, K. Oliver, C. Price, M. A. Quail, H. Urukshihara, J. Hernandez, E. Rabinowitsch, D. Steffen, M. Sanders, J. Ma, Y. Kohara, S. Sharp, M. Simmonds, S. Spiegel, A. Tivey, S. Sugano, B. White, D. Walker, J. Woodward, T. Winckler, Y. Tanaka, G. Shaulsky, M. Schleicher, G. Weinstock, A. Rose, K. Morita, R. A. Firtel, M., L. Aubry, R. Insall, C. Gaskins, P. N. Devreotes, and R. A. Firtel. 1997. Activation of the mitogen-activated protein kinase ERK2 by the chemoattractant folic acid in Dictyostelium discoideum. J. Biol. Chem. 272: 304: 4332.

Lim, C. J., G. B. Spiegelman, and G. Weeks. 2000. The genome of the social amoeba Dictyostelium discoideum. Nature 435: 43–57.

Hermann, T., M. Finkemeyer, W. Pfeffer, G. Wersch, R. Kramer, and A. Burkovski. 2000. Two-dimensional electrophoretic analysis of Corynebacterium glutamicum membrane fraction and surface proteins. Electrophoresis 21: 654–659.

Insall, R. H., J. Borleis, and P. N. Devreotes. 1996. The aimless RasGEF is required for processing of chemoattractant signals through G-protein-coupled receptors in Dictyostelium. Curr. Biol. 6: 719–729.

Lee, S., C. A. Parent, R. Insall, and R. A. Firtel. 1999. A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate signal relay in Dictyostelium. Mol. Biol. Cell 10: 2829–2845.

Lilly, P. J., and P. N. Devreotes. 1994. Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenyl cyclase in Dictyostelium. J. Biol. Chem. 269: 14123–14129.

Lim, C. J., G. B. Spiegelman, and G. Weeks. 2001. RasC is required for optimal activation of adenyl cyclase and Akt/PKB during aggregation. EMBO J. 20: 4490–4499.

Ma, H., M. Gamper, C. Parent, and R. A. Firtel. 1997. The Dictyostelium MAP kinase DdMEK1 regulates chemotaxis and is essential for chemotactant-mediated activation of guanylyl cyclase. EMBO J. 16: 4317–4332.

Maeda, M., L. Aubry, R. Insall, C. Gaskins, P. N. Devreotes, and R. A. Firtel. 1996. Seven helix chemotactant receptors transiently stimulate mitogen-activated protein kinase in Dictyostelium. Role of heterotrimeric G proteins. J. Biol. Chem. 271: 3351–3354.

Maeda, M., and R. A. Firtel. 1997. Activation of the mitogen-activated protein kinase ERK2 by the chemotactic folic acid in Dictyostelium. J. Biol. Chem. 272: 23690–23695.

Maeda, M., S. Lu, G. Shaulsky, M. Miyazaki, H. Kuwayama, Y. Tanaka, A. Kuspa, and W. F. Loomis. 2004. Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA, Science 304: 875–878.

Morio, T., H. Urukshihara, T. Saito, Y. Uegawa, H. Mizuno, M. Yoshida, R. Yoshino, B. N. Mitra, M. Pi, T. Saito, K. Takemoto, H. Yasukawa, J. Williams, M. Maeda, I. Takeuchi, H. Ochiai, and T. Yamada. 1998. The Dictyostelium development eDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. DNA Res. 5: 335–340.

Nagasaki, A., G. Itoh, S. Yumura, and T. Q. Uyeda. 2002. Novel myosin heavy chain kinase involved in disassembly of myosin II filaments and efficient cleavage in mitotic Dictyostelium cells. Mol. Biol. Cell 13: 3333–3432.

Pang, K. M., M. A. Lynes, and D. A. Nechaev. 1999. Variables controlling the expression level of exogenous genes in Dictyostelium. Plasmid 41: 187–197.

Roux, P. P., and J. Blenis. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. 68: 320–344.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Saran, S., M. E. Meina, E. Alvarez-Curto, K. E. Weening, D. E. Rozen, and P. Schaap. 2002. cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection. J. Muscle Res. Cell Motil. 23: 793–802.

Schaeffer, H. J., and M. J. Weber. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 19: 2435–2444.

Segall, J. E. 1992. Behavioral responses of streamer F mutants of Dictyostelium discoideum: effects of cyclic GMP on cell motility. J. Cell Sci. 101: 589–597.

Segall, J. E. 1999. Cell polarization: chemotaxis gets CRACKing. Curr. Biol. 9: R46–R48.

Segall, J. E., A. Kuspa, G. Shaulsky, M. Ecke, M. Maeda, C. Gaskins, R. A. Firtel, and W. F. Loomis. 1995. A MAP kinase necessary for receptor-mediated activation of adenyl cyclase in Dictyostelium. J. Cell Biol. 128: 405–413.

Shaulsky, G., R. Escalante, and W. F. Loomis. 1996. Developmental signal transduction pathways uncovered by genetic suppressors. Proc. Natl. Acad. Sci. USA 93: 15260–15265.

Schaulsky, G., D. Fuller, and W. F. Loomis. 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development 125: 691–699.

Stepanovic, V., D. Wessels, K. Daniels, W. F. Loomis, and D. R. Soll. 2005. Intracellular role of adenyl cyclase in regulation of lateral pseudopod formation during Dictyostelium chemotaxis. Eukaryot. Cell 4: 775–786.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of Dictyostelium under controlled experimental conditions. Methods Cell Biol. 28: 9–29.

Thomason, P. A., D. Traynor, G. Cavet, W. T. Chang, A. J. Harwood, and R. R. Kay. 1998. An intersection of the cAMP/PKA and two-component signal transduction systems in Dictyostelium. EMBO J. 17: 2838–2845.

van Es, S., and P. N. Devreotes. 1999. Molecular basis of localized responses during chemotaxis in amoebae and leukocytes. Cell. Mol. Life Sci. 55: 1341–1351.

Van Haastert, P. J., and P. N. Devreotes. 2004. Chemotaxis: signalling the way forward. Nat. Rev. Mol. Cell Biol. 5: 626–634.

Wang, Y., J. Liu, and J. E. Segall. 1998. MAP kinase function in amoeboid chemotaxis. J. Cell Sci. 111: 373–383.

Wang, Y., and J. E. Segall. 1998. The Dictyostelium MAP kinase DdERK2 functions as a cytosolic protein in complexes with its potential substrates in chemotactic signal transduction. Biochem. Biophys. Res. Commun. 244: 149–155.