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The *Dictyostelium* MAP Kinase DdERK2 Functions as a Cytosolic Protein in Complexes with Its Potential Substrates in Chemotactic Signal Transduction

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A polyclonal antibody against a MAP kinase (DdERK2) in *Dictyostelium* has been made and used to study DdERK2 activation and localization. The activation of DdERK2 by the chemoattractants cAMP and folate is rapid and transient. Its activity peaks between 15 and 60 seconds after cAMP stimulation and declines to basal levels after 5 minutes. In parallel with the DdERK2 activation is the appearance of a higher mobility band on Western blots. An antibody specific for activated MAP kinase shows that only the shifted band is tyrosine phosphorylated, suggesting that it is the active form. Both unstimulated and stimulated DdERK2 are soluble. In vitro phosphorylation with cell lysate supernatants or immunoprecipitates demonstrates the presence of several potential substrates, as identified by SDS-PAGE with mobility corresponding to molecular weights of 150, 25, and 19 kDa. Furthermore, immunoprecipitation studies suggest that these substrates are in a complex with DdERK2. These data suggest that DdERK2 works via cytoplasmic proteins to mediate signaling responses in *Dictyostelium*.

Extracellular signal regulated kinases (ERKs) are the output molecules for signal transduction cascades that are conserved from *S. cerevisiae* to mammals. The MAP kinase superfamily contains three subfamilies, ERKs, JNKs/SAPKs, and p38/ERKs (1,2). These molecules participate in a number of different cellular responses ranging from proliferation to differentiated cell responses. ERKs can be activated by a variety of receptors, including receptor tyrosine kinases and G protein coupled receptors, and have been shown to be involved in (among other responses) gene regulation, proliferative responses and chemotaxis (3–7).

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A useful system for analyzing the roles of ERKs/MAP kinases in chemotaxis, cell signaling, and gene expression is the cellular slime mold *Dictyostelium discoidum* (8–11). *Dictyostelium* cells grow on bacteria or axenic medium, and in this state, are chemotactic towards folic acid, probably as a food seeking mechanism. Upon removal of the food source, the amoebae undergo a developmental program in which sensitivity to folate is reduced, and chemotactic responses to extracellular cAMP are induced. Binding of extracellular cAMP to cell surface receptors also stimulates activation of adenylyl cyclase and secretion of the synthesized cAMP, which together with the chemotactic responses results in movement of the cells into aggregates. In the aggregates, cells sort into prespore and prestalk cells and form a primitive multicellular organism, termed the pseudoplasmodium or slug. The pseudoplasmodium in turn transforms into a fruiting body composed of spore cells in a sorus sitting on a stalk formed by stalk cells.

The ERK/MAP kinase, DdERK2, performs critical functions during this developmental process as demonstrated by mutants containing insertions in the *Dderk2* gene (12,13). Growth phase *Dderk2*$^-$ cells do not show growth defects in axenic medium, but are reduced in chemotactic responses to folate (14). Upon removal of the growth medium, the mutant cells undergo initial differentiation and expression of aggregation stage proteins such as the cAMP receptor and adenylyl cyclase, but are unable to activate adenylyl cyclase and show defects in cAMP chemotaxis (14). Finally, expression of late stage proteins is reduced or lacking in *Dderk2*$^-$ cells (15). Expression of DdERK2 in the mutant cells restores all these functions (12,15). Thus this system provides a convenient genetic approach to the analysis of the role of MAP kinases/ERKs in chemotaxis, activation of adenylyl cyclase, and gene expression without being required for cell division. It complements other systems which utilize pharmacological agents such as PD98059 (16). PD98059 inhibits the upstream activa-

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tor of ERKs, MEK1, but affects activation of both ERK1 and ERK2 (and potentially other members of the ERK family), and thus does not allow one to examine the specific role of a single member of the ERK family in signaling.

Given the importance of DdERK2 in signaling phenomena in *Dictyostelium*, our approach has been to develop tools to aid in the characterization of DdERK2 signaling and the identification of potential downstream mediators of DdERK2. This paper reports on the use of an anti-DdERK2 antibody to examine DdERK2 activation by the chemoattractants cAMP and folate, and the initial characterization of potential downstream substrates.

MATERIALS AND METHODS

Preparation of GSTERK2 fusion protein. A GST-DdERK2 was expressed in *E.coli* strain XL1 Blue and purified by a modified protocol (17). IPTG-induced bacteria were collected, rinsed with 10 mM Tris HCl pH 7.6/1 mM DTT, then resuspended and lysed by sonication in lysis buffer containing 20 mM phosphate buffer pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 μg/ml Leupeptin, 20 μg/μl Pepstatin, 1 mM PMSF. GST-DdERK2 was purified with Glutathione agarose (Sigma) from cell lysate supernatant. GST was also expressed and purified by Glutathione agarose under the same condition. GST and GST-DdERK2 fusion protein were eluted from the beads by 10 mM free glutathione in 20 mM Tris HCl pH 8.0. GST-DdERK2 fusion protein was dialyzed against PBS to remove glutathione. The concentration of purified GST-DdERK2 was quantitated by SDS-PAGE gel using BSA (Sigma) as a standard protein. Purified GST-DdERK2 fusion protein was used to raise a rabbit polyclonal antiserum (HRP, Inc. Denver, PA). The antiserum against GST-DdERK2 was bleed 79-4 and is designated as the anti-DdERK2 antibody and used in ECL Western blotting protocols (Amersham Life Science) in this paper. The antibody was used to detect or immunoprecipitate DdERK2 in wild type (DdERK2$^+$) and mutant (*Dderk2*$^-$) cells. For estimation of DdERK2 protein levels in *Dictyostelium* cells, the anti-DdERK2 antibody was first preabsorbed with purified GST to eliminate crossreaction to GST, and then used to detect DdERK2 in DdERK2$^+$ cell extracts using known amounts of GST-DdERK2 protein on the same blot as a quantitation standard.

cAMP and Folate stimulation in suspension. Cells were grown axenically in HL-5 medium. In cAMP stimulation experiments, cells were washed twice with 1× phosphate buffer (17 mM Soerensen's phosphate buffer), resuspended at 10^7/ml in 1× phosphate buffer with 1 mM $MgCl_2$, 0.2 mM $CaCl_2$ (termed Ca/Mg PB) and shaken at 170 rpm. After 2 hours they were pulsed with 100 nM cAMP every 6-8 minutes for 4-6 hours. Starved cells were washed twice with cold Ca/Mg PB containing 2.5 mM caffeine, resuspended in the same buffer at 2×10^7/ml and incubated on a shaker at 20–22°C for 10 minutes. The cells were then stimulated with 1/10 volume of 100 μM cAMP in phosphate buffer. At different time points, the cells were removed and lysed as described below. For folate stimulation, the cells were starved for only one hour before stimulation. Stimulated cells were added to SDS sample buffer, and the extracts analyzed by Western blotting with the anti-DdERK2 antibody. The relative proportion of the detected DdERK2 present in the activated form was quantitated and plotted to determine the relative activation level.

In vitro MBP kinase assay. The starved and pulsed cells were stimulated with cAMP, removed at different time points and lysed by the same volume of 2 × lysis buffer (16 mM phosphate buffer, 40 mM KCl, 4 mM EGTA, 2 mM DTT, 0.2 mM Na_3VO_4, 80 mM Na P_2O_7, 2% Triton X-100, 1% Sodium deoxycholate, 0.2% SDS, 0.2% BSA, 40 μg/ml Leupeptine, 40 μg/ml Aprotinin, 2 mM PMSF) (18). Cell lysates were centrifuged in a microcentrifuge at maximum speed at 4°C for 10 minutes. For each immunoprecipitation mixture, cell lysate supernatant equivalent to 6.25×10^4 cells was mixed with 1 μl of anti-ERK2 antibody on 20 μl of 1:1 protein A Sepharose CL-4B slurry (Pharmacia Biotech) and incubated in the cold room for 2 hours. The immunocomplexes were washed three times with IP wash buffer (150 mM NaCl, 20 mM Tris HCl pH 8.0, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.1 mM EDTA, 0.1% Triton X-100), and then equilibrated with 30 mM Tris HCl pH 8.0, 20 mM $MgCl_2$ and 2 mM $MnCl_2$ (19). In each 30 μl mixture for a phosphorylation reaction, the ERK2 immunocomplex was mixed with 30 mM Tris HCl pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mg/ml MBP, 1 mg/ml BSA, 10 uCi ^{32}P-ATP and 67 μM ATP. After shaking at room temperature for 20 minutes, the reaction was stopped by 2 × SDS sample buffer. The reaction mixtures were separated on 10% SDS PAGE, followed by ^{32}P autoradiography or Western blotting.

Cell fractionation and Western blots. To prepare particulate fractions, the cells were added to a syringe and lysed by pushing through two layers of nucleopore membrane (5 um polycarbonate membranes, Poretics Corp, (20)) into a microtube on ice containing (final concentrations) 1 × phosphate buffer, 2.5 mM EGTA, 1 μM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.5 μM PMSF. Cell lysates were centrifuged in a microcentrifuge at maximum speed at 4°C for 10 minutes. Supernatant samples were mixed with 2 × SDS sample buffer. The pellets were resuspended in SDS sample buffer. Protein samples were separated on 10% SDS-PAGE. To prepare Triton X-100 insoluble fractions, at different time points after cAMP stimulation, the cells in 1× phosphate buffer were lysed by 2× Triton X-100 lysis buffer (1× phosphate buffer, 2.5 mM EGTA, 5 mM MgCl2, 0.5 mM ATP, 1% Triton X-100, and protease inhibitors). Cell lysates were centrifuged in a microcentrifuge at maximum speed at 4 °C for 10 minutes. Triton X-100 insoluble pellets were resuspended in SDS sample buffer. DdERK2 protein in the supernatant and pellet samples was detected by ECL Western blotting protocols (Amersham Life Science) using anti-DdERK2 antibody or anti-Phospho MAPK antibody (New England BioLabs).

In vitro ^{32}P incorporation. The cell lysate supernatant from nucleopore membrane lysis was used for in vitro ^{32}P incorporation experiments. For reactions with cell lysate supernatant, each mixture contained supernatant equivalent to 2×10^5 cells, 30 mM Tris HCl pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 0.17 uCi ^{32}P-ATP, 11.2 μM ATP. For each phosphorylation reaction with DdERK2 immunocomplexes, DdERK2 was immunoprecipitated by 16ul of anti-DdERK2 antibody on 40 μl of 1:1 protein A beads from nucleopore cell lysate supernatants equivalent to 10^6 cells in the cold room, washed three times with IP wash buffer, and equilibrated with 30 mM Tris HCl pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$. ^{32}P incorporation with immunocomplex was carried out under the same condition as for cell lysate supernatant (see above). Reaction samples were separated by SDS-PAGE, followed by Coomassie Blue staining and ^{32}P autoradiography.

RESULTS

Activation of DdERK2 correlates with increased mobility in SDS-PAGE. A GST-DdERK2 fusion protein was expressed in bacteria, purified and utilized to generate a polyclonal antiserum to DdERK2 as described in Materials and Methods. This anti-DdERK2 antibody was then utilized for Western blotting and immunoprecipitation. In Western blots of whole cell extracts, this antibody identifies a major band at about 42kD in unstimulated DdERK2$^+$ cells with variably crossreacting bands of 50 kD or higher (Figure 1A). *Dderk2*$^-$ cell extracts showed little of the 42 kD band and a weakly

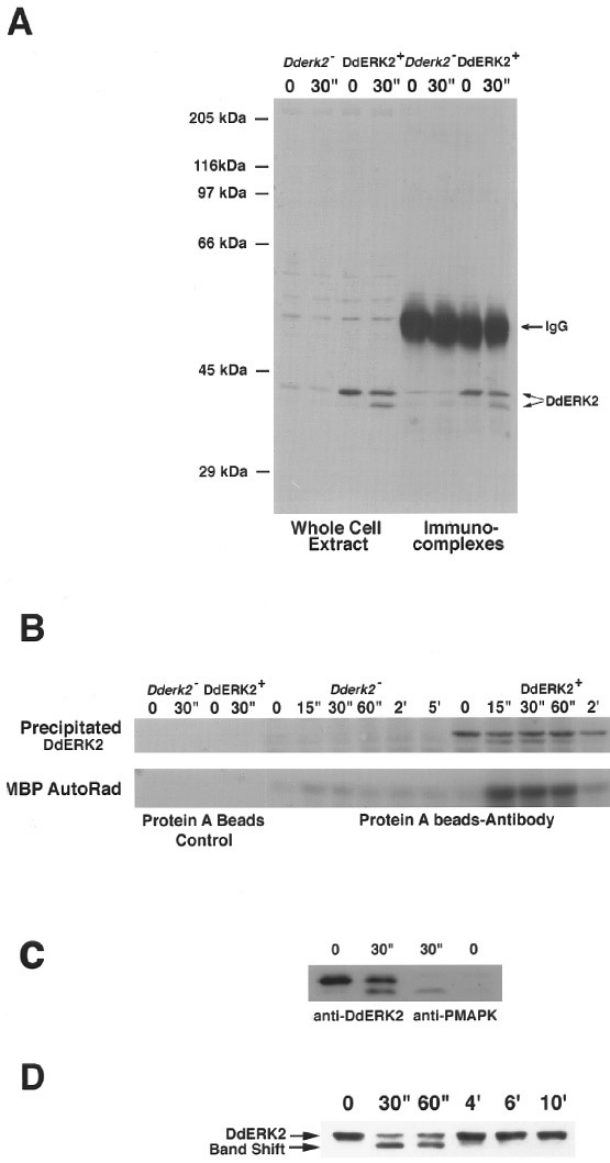

FIG. 1. The change in mobility of DdERK2 in SDS-PAGE reflects activation. (A) Samples were prepared from cells before and 30 seconds after stimulation with cAMP. DdERK2 was detected by the polyclonal antibody 79-4. For the whole cell extracts, cells were directly placed in SDS sample buffer. For immunocomplexes, cells were lysed by nucleopore membrane and immunoprecipitated as described in the Materials and Methods. (B) The immunocomplexes were used to phosphorylate MBP as described in Materials and Methods, followed by separation by SDS-PAGE. The upper part of the gel was used for Western blotting for detection of DdERK2 and the lower part was used for MBP autoradiography. (C) Cell extract samples were prepared from wild type cells before and 30 sec after cAMP stimulation. The protein samples were separated on SDS-PAGE in two sets on a single gel and blotted onto a single sheet of nitrocellulose. The nitrocellulose was then divided in two parts, each part probed by the anti-ERK2 antibody or the anti-phospho-MAPK antibody, and then realigned to allow comparison of the positions of the bands that were detected. (D) Wild-type cells were stimulated with cAMP and lysed by SDS sample buffer at 0, 30″, 60″, 4′ and 6′. DdERK2 and shifted DdERK2 were detected on Western blot.

crossreacting band of slightly higher molecular weight. Immunoprecipitation using the anti-DdERK2 antibody followed by Western blotting with the same antibody again revealed a major band at 42 kD in unstimulated DdERK2$^+$ cells that was much reduced or missing in *Dderk2*$^-$ ells. In stimulated DdERK2$^+$ cell extracts, a second band of higher mobility appears concomitant with a reduction in the level of the 42 kD band, suggesting a shift in the mobility of a portion of the DdERK2 molecules (see below).

To estimate the concentration of DdERK2 in *Dictyostelium* cells, the anti-DdERK2 antibody was preabsorbed with purified GST protein to remove GST reacting antibodies, and then used to detect DdERK2 in DdERK2$^+$ cell extracts using purified bacterially expressed GST-DdERK2 as a quantitation standard. The concentration of DdERK2 in growth phase *Dictyostelium* cells is estimated to be about 0.7 μM, corresponding to 650,000 molecules per cell. This amount is typical of ERKs, with concentrations in other systems ranging from .1-3 μM (21).

To examine the significance of the change in mobility of a portion of the DdERK2 molecules upon stimulation with cAMP, the MBP kinase activity of immunoprecipitated DdERK2 was correlated with the mobility shift observed by Western blotting with the anti-DdERK2 antibody (Figure 1B). Immunoprecipitated DdERK2 from DdERK2$^+$ cells showed increased MBP kinase activity between 15 and 60 seconds that correlated well with the presence of the higher mobility band detected by the anti-DdERK2 antibody. Immunoprecipitates from *Dderk2*$^-$ cells or immunoprecipitates using blank protein A beads showed 6% or less of the DdERK2$^+$ MBP kinase activity.

The kinetic data in Figure 1B showed that the higher mobility band detected by the anti-DdERK2 antibody was correlated with activation of DdERK2. To provide confirmation that the higher mobility band was an activated form of DdERK2, cell extracts were probed with an antibody that detects the activated state of ERKs by recognizing the phosphotyrosine within the context of the TEY(P) motif of ERKs (phospho-MAPK antibody) (22) (Figure 1C). Whole cell extracts blotted with the anti-PMAPK antibody showed the appearance of a band of similar mobility upon stimulation with cAMP. Alignment of Western blots using the anti-DdERK2 antibody and the phospho-MAPK antibody demonstrated that the higher mobility band detected by the anti-DdERK2 antibody had the same mobility as the activated DdERK band detected by the phospho-MAPK antibody. In addition, the mobility shift is reversible, with the shifted band disappearing and the unstimulated DdERK2 band returning back to its initial intensity at later time points (Figure 1D), ruling out the likelihood of involvement of proteolysis. We conclude that the higher mobility band corresponds to the activated form of DdERK2.

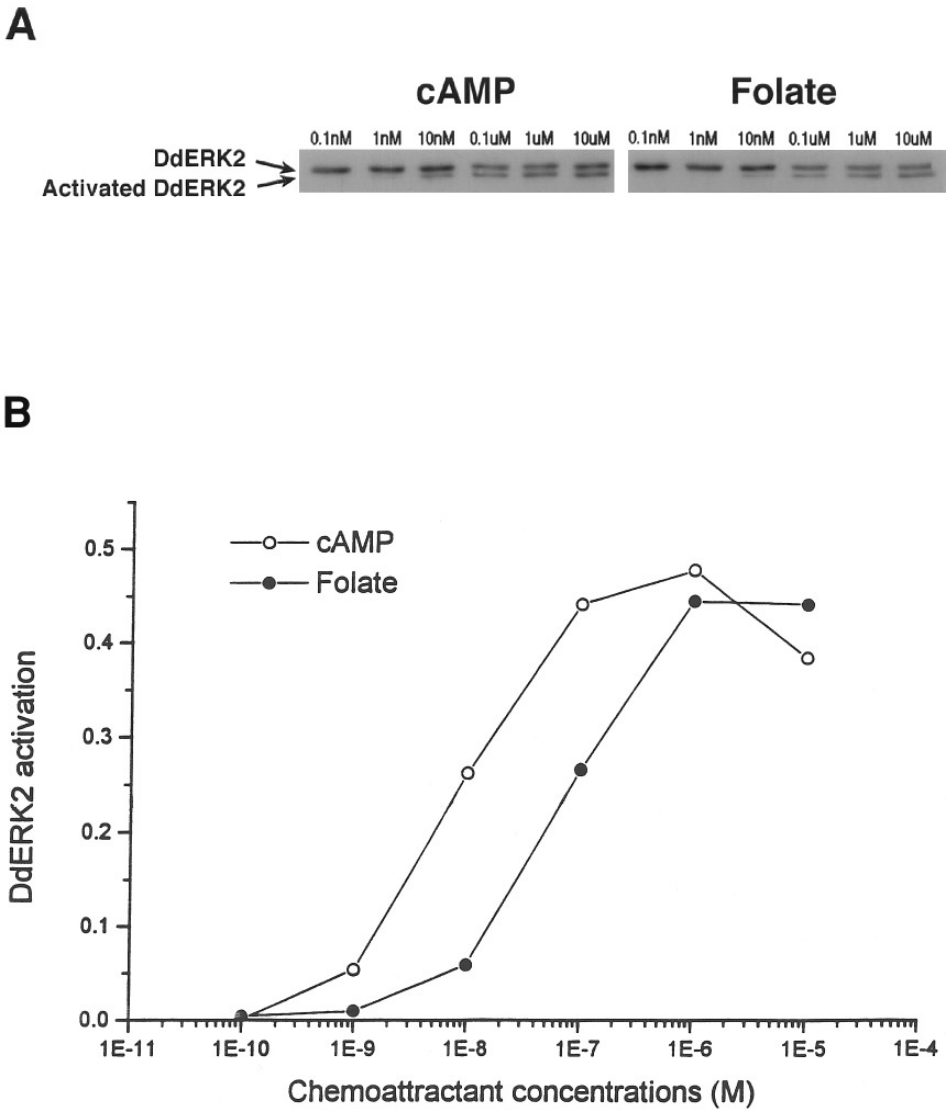

FIG. 2. Dose response curves for activation of DdERK2 by cAMP and folate. (A) DdERK2$^+$ cells (HS176) were stimulated with cAMP or folate at different concentrations for 30 seconds, added to SDS sample buffer, and blotted using the anti-DdERK2 antibody. (B) The relative proportion of the detected DdERK2 present in the activated form was quantitated and plotted to determine the relative activation level in response to varying concentrations of chemoattractants. The average standard errors of the mean at the maximal responses were 0.1.

The appearance of the higher mobility form of DdERK2 was used as a simple assay to determine the dose response curves for DdERK2 activation by the chemoattractants cAMP and folate (Figure 2). Cells starved for 1 hour (for folate) or 6 hours (for cAMP) were stimulated with varying concentrations of chemoattractant, added to SDS sample buffer, and the extracts analyzed by Western blotting with the anti-DdERK2 antibody. The relative level of DdERK2 activation was determined by the relative amount of the higher mobility band as described in Materials and Methods. Half maximal activation occurred at around 10 nM cAMP and 100 nM folate respectively. Maximal activation was seen between .1 and 1 μM cAMP, and between 1 and 10 μM folate. This correlates with activation of adenylyl cyclase and chemotactic responses to cAMP, and chemotactic responses to folate (23–26). Consistent with previous studies on folate chemotaxis, after starvation for 6 hours, no DdERK2 activation was detected after folate stimulation (data not shown) (27).

An in vitro assay reveals potential DdERK2 substrates. To identify potential DdERK2 substrates, we first examined whether most of the DdERK2 present was soluble or attached to the cytoskeleton. Triton lysis or nucleopore membrane lysis followed by low speed centrifugation (8,000×g) left the majority of the DdERK2 in the supernatant (Figure 3). High speed centrifugation of Triton X-100 lysates showed no in-

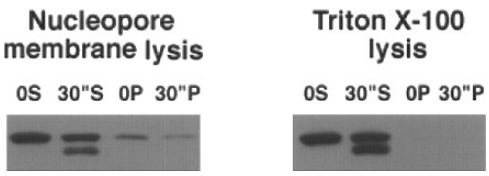

FIG. 3. DdERK2 is soluble. DdERK2$^+$ cells (HS176) were lysed before and 30 sec after cAMP stimulation by either filtration through nucleopore membranes or Triton X-100 as described in Materials and Methods. DdERK2 in the supernatant and pellet was detected on Western blots using the anti-DdERK2 antibody.

crease in the amount of DdERK2 pelleted, indicating that the majority of the DdERK2 present in cells was soluble under our lysis conditions. All the activated form of DdERK2 was soluble as well.

Given that DdERK2 is present in relatively high concentration in cells and is soluble, we tested whether addition of gamma labeled ATP to activated cell lysates would produce specific bands that were dependent on the presence of DdERK2. cAMP-stimulated DdERK2$^+$ and *Dderk2*$^-$ cells were lysed through Nucleopore filters, membranes and cytoskeleton removed by centrifugation, and labeled ATP was added to the lysate as described in Materials and Methods (Figure 4A). Comparing stimulated DdERK2$^+$ and *Dderk2*$^-$ extracts revealed labeling in bands of 150 kD, 40 kD, 25 kD and 19 kD that was dependent upon the presence of DdERK2.

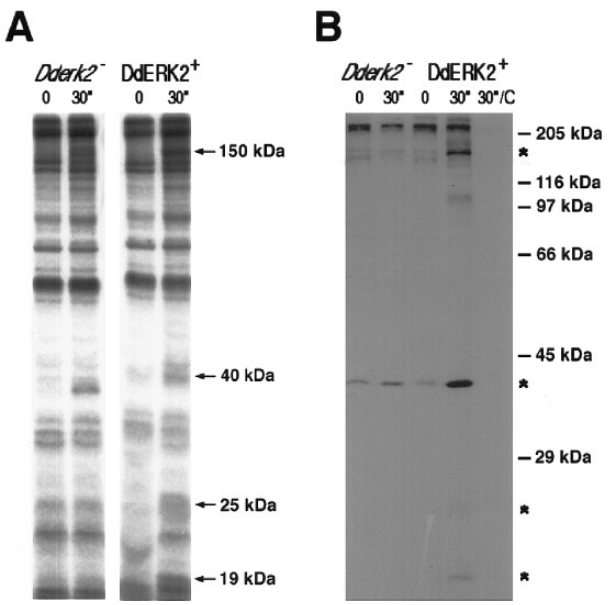

FIG. 4. Identification of potential DdERK2 substrates. *Dderk2*$^-$ (HS174) and DdERK2$^+$ cells (HS176) were lysed by filtration through nucleopore membranes 30″ after cAMP stimulation. In vitro phosphorylation reactions were carried out with the cell lysates (A) and immunocomplexes (B) as described in Materials and Methods, separated by SDS-PAGE and examined by autoradiography. 30″/C is a control in which preimmune Ab was used for immunoprecipitation from stimulated DdERK2$^+$ cell lysates.

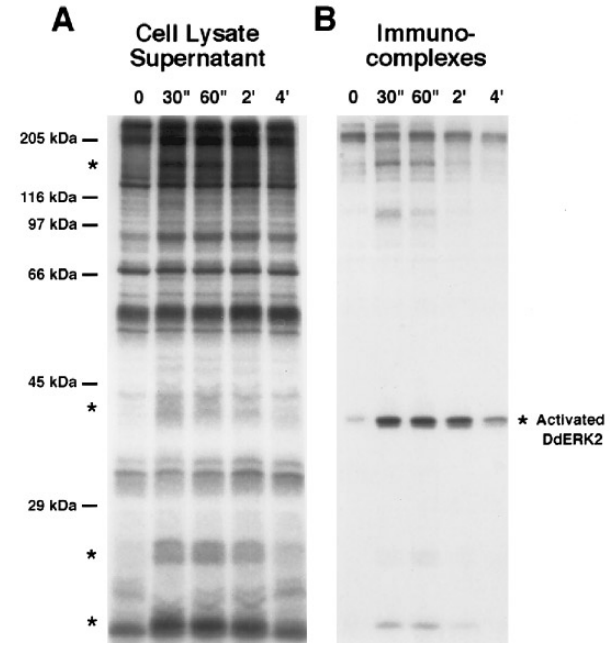

FIG. 5. DdERK2 substrates form a complex with DdERK2. Kinetics of ^{32}P incorporation in DdERK2$^+$ cell lysates (A) and DdERK2 immunocomplexes from lysates (B). Wild type cells were lysed by filtration through nucleopore membranes at 0, 30″, 60″, 2′, and 4′ after cAMP stimulation. In vitro phosphorylation reactions were carried out with the cell lysate supernatants directly or with DdERK2 immunoprecipitates from the lysates as described in Materials and Methods, followed by separation by SDS-PAGE and autoradiography.

To determine if a complex was formed between DdERK2 and these substrates, cells were stimulated, lysed and then DdERK2 immunoprecipitated from the cell lysates using the anti-DdERK2 antibody. The immunocomplexes were washed and then incubated with gamma labeled ATP, and incorporation of ^{32}P into protein was examined (Figure 4B). Incorporation of ^{32}P into the 150, 40, 25 and 19 kD bands was consistently seen in the immunoprecipitates, indicating the presence of these substrates in a complex with the immunoprecipitated DdERK2. The band in the 40 kDa region is likely to be the activated form of DdERK2, since it is located at the position of activated DdERK2 as seen on the Coomassie blue-stained gel. Immunoprecipitates from *Dderk2*$^-$ cells with anti-DdERK2 antibody or from DdERK2$^+$ cells with preimmune serum did not show incorporation of ^{32}P into these bands, indicating a specific requirement for DdERK2 in the immunoprecipitate for labeling of these bands.

To determine whether the phosphorylation of these bands followed the time course of activation of DdERK2, cell lysates were made at various times after stimulation with cAMP and incorporation of ^{32}P examined by SDS-PAGE and autoradiography (Figure 5A). Incorporation of ^{32}P into all 4 bands followed a time course similar to the activation of DdERK2, with maximal incorporation occurring 30 - 60 seconds after stimulation with cAMP.

These results indicate that DdERK2 or a kinase activated by DdERK2 is involved in phosphorylation of the 150, 40, 25 and 19 kD proteins. DdERK2 immunocomplexes from DdERK2$^+$ cell lysates also clearly showed ^{32}P incorporation into those 4 bands (Figure 5B). One possible downstream kinase activated by DdERK2 is protein kinase A. Stimulation of cells with cAMP results in synthesis of cAMP within the cells and the possible subsequent activation of protein kinase A (28). However, in *Dderk2*$^-$ cells, adenylyl cyclase is not activated, and thus protein kinase A would not be activated. To rule out the possibility that phosphorylation of these substrates requires activation of adenylyl cyclase or a cAMP dependent protein kinase, cell lysates of a *Crac*$^-$ mutant were utilized. Cells lacking the CRAC protein are unable to activate adenylyl cyclase in response to stimulation with cAMP, but still show activation of DdERK2. In extracts from *Crac*$^-$ cells, phosphorylation of the 150, 40, 25 and 19 kD bands was still observed (data not shown), supporting the likelihood of direct phosphorylation by DdERK2.

DISCUSSION

In this paper, we utilize an anti-DdERK2 antibody to examine DdERK2 activation in *Dderk2*$^-$ and DdERK2$^+$ cells. First, the antibody has been used to quantitate DdERK2 in DdERK2$^+$ cells and to demonstrate a significant decrease in DdERK2 expression in *Dderk2*$^-$ cells. Second, under our SDS-PAGE conditions, the activation state of DdERK2 can be ascertained as a change in mobility. This change in mobility has been used to determine dose response curves for stimulation with chemoattractants. Third, we have demonstrated the presence of several potential substrates for DdERK2 in cell lysates, and the presence of a complex with DdERK2 using immunoprecipitation.

Using the anti-DdERK2 antibody to quantitate DdERK2 in *Dictyostelium* cells, we find that growth phase cells contain about 650,000 molecules/cell, corresponding to a concentration of about 0.7 μM. This concentration is within the range of concentrations of MAP kinases found for other cell types. However, consistent with earlier data regarding mRNA expression (12), we find that DdERK2 expression increases during development by a factor of 3. Although in growth phase cells, DdERK2 is important for chemotaxis to folate (14), during development DdERK2 function is important for chemotaxis to cAMP, activation of adenylyl cyclase, and prespore gene expression (12,14,15). DdERK2 activation by folate and cAMP shows a similar dose dependence to these functions, supporting a role for DdERK2 in mediating them (23-26). The increase in expression during development may reflect a requirement for additional DdERK2 molecules in order to perform these additional functions adequately. Consistent with other work, this antibody demonstrates a significant loss of DdERK2 expression in *Dderk2*$^-$ cells (13).

In this manuscript, we describe the use of a band shift assay for the analysis of DdERK2 activation in *Dictyostelium*. Although mobility changes of mammalian ERKs upon activation have been noted previously, such changes have not been reported for DdERK2. Indeed, under other gel conditions using commercially available antibodies, a mobility shift was not detected (29). Under our SDS-PAGE conditions, we reproducibly observe an increase in mobility of DdERK2 upon activation. As the intensity of the higher mobility band increases, there is a corresponding reduction in intensity of the unstimulated DdERK2 band, indicating a shift in protein from the unstimulated to the stimulated state. The mobility shift correlates kinetically with increased MBP kinase activity in DdERK2 immunoprecipitates, and the anti-PhosphoMAPK antibody recognizes the higher mobility form of DdERK2 and not the lower mobility form. These data all consistently support our conclusion that the higher mobility band corresponds to the activated form of DdERK2. The reason that activation of DdERK2 results in an increase in mobility rather than a decrease, as is commonly seen for other MAP kinases is unclear. However, under different gel conditions activated MAP kinase has shown a higher mobility (30), and it is possible that this capability is accentuated for DdERK2. Since DdERK2 is only about 40% identical to other members of the ERK family, the sequence differences could lead to subtle changes in mobility upon activation which would differ from mammalian ERKs.

The relatively high concentration of DdERK2 in *Dictyostelium* cells, combined with its solubility, led us to examine whether major substrates could be identified in cell extracts. Several potential substrates have been identified by SDS-PAGE, with mobility corresponding to molecular weights of 150kD, 25 kD, and 19 kD. Phosphorylation of these substrates was dependent upon stimulation with cAMP, was strongly reduced in *Dderk2*$^-$ cells, and followed a time course similar to the time course of activated DdERK2. In addition, *Crac*$^-$ mutants which are unable to activate adenylyl cyclase showed the same pattern of phosphorylation as DdERK2$^+$ cells, indicating that phosphorylation of these substrates is not due to activation of adenylyl cyclase or protein kinase A. Immunoprecipitation studies demonstrated that these substrates are in a complex with DdERK2, consistent with studies in other systems indicating that ERKs can form complexes with substrates that are stable enough to be maintained through such purification procedures (31–34).

The characterization of the properties of DdERK2 presented in this manuscript provide the basis for future studies aimed at delineating the role played by DdERK2 in chemotaxis, activation of adenylyl cyclase, and gene expression. The combination of mutants lacking DdERK2 with the ability to directly identify potential substrates in cell lysates provides a powerful ap-

proach towards identifying the *in vivo* substrates for DdERK2 and their functions.

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