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An unusual protein kinase phosphorylates the chemotactic receptor of *Dictyostelium discoideum*

(cAMP/plasma membrane/phosphorylation/desensitization)

KATHLEEN MEIER AND CLAUDETTE KLEIN

Edward A. Doisey Department of Biochemistry, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104

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ABSTRACT **We report the cAMP-dependent phosphorylation of the chemotactic receptor of *Dictyostelium discoideum* in partially purified plasma membranes. The protein kinase responsible for receptor phosphorylation is associated with this fraction and preferentially phosphorylates the ligand-occupied form of the receptor. 8-Azido[^{32}P]cAMP labeling of the cell surface has shown that the cAMP receptor exists in two forms. A 45-kDa protein is predominant on unstimulated cells. cAMP stimulation results in an increased receptor phosphorylation such that the receptor migrates on NaDodSO$_4$/PAGE as a 47-kDa protein. Phosphorylation of the chemotactic receptor is not detected in membrane preparations unless cAMP is added to the incubation mixture. Only under those conditions is the phosphorylated 47-kDa form observed. The requirement for cAMP reflects the fact that the kinase involved preferentially uses the ligand-occupied receptor as a substrate. *In vitro* phosphorylation of the receptor does not involve tyrosine residues. The enzyme does not appear to be a cAMP- or cGMP-dependent protein kinase nor is it sensitive to guanine nucleotides, Ca^{2+}/calmodulin, Ca^{2+}/phospholipid, or EGTA. Similarities with the β-adrenergic receptor protein kinase are discussed.**

How cells perceive and respond to diffusible substances is a general problem in biology. Cell-surface receptors mediate the effects of compounds that are impermeable to the plasma membrane. Activation of such receptors leads to the physiological response, whereas desensitization results in reduced responsiveness. The molecular events involved in excitation and desensitization appear to involve physical modulation of the receptor, either in terms of its conformation, covalent modification, or subcellular distribution (1).

In *Dictyostelium discoideum*, chemotactic signaling involves the activation of a cell-surface receptor by extracellular cAMP. Photoaffinity labeling of cell-surface proteins has demonstrated two forms of the chemotactic receptor: a 45-kDa form that predominates on unstimulated cells and a 47-kDa form that predominates on cells that have been stimulated with cAMP (2). This increase in the apparent molecular mass of the receptor, seen by NaDodSO$_4$/PAGE, results from the increased phosphorylation of the 45-kDa protein in response to cAMP stimulation (3–5). The time course and cAMP concentration dependence of receptor phosphorylation/dephosphorylation correlate well with the phenomenon of cell desensitization (4–6). To further characterize the relevant kinase(s) responsible for the phosphorylation of the chemotactic receptor and to eventually understand its role in signal transduction, we have developed a cell-free system in which the chemotactic receptor is phosphorylated in a cAMP-dependent manner. The enzyme responsible is an unusual protein kinase that preferentially phosphorylates the ligand-occupied receptor.

MATERIALS AND METHODS

Strains and Culture Conditions. Ax-2 amoebae (7) were grown in HL-5 medium (8). Cell differentiation to aggregation competence was induced by starving amoebae in 20 mM phosphate at pH 6.5 (9). Cell differentiation was monitored morphologically (10) and by changes in surface cAMP binding activity.

Membrane Phosphorylation. The plasma membrane-enriched fraction was prepared (from vegetative or starved cells) as described (2, 11). To obtain plasma membranes from "down-regulated" cells, aggregation-competent amoebae were incubated with 100 μM cAMP for 30 min and then washed extensively prior to cell lysis (12). Membranes were phosphorylated in a 100-μl reaction mixture containing 50 mM Tris (pH 8), 10 mM $MgCl_2$, and 25 μM [γ-^{32}P]ATP (40 μCi/mmol; 1 Ci = 37 GBq). Unless indicated otherwise, cAMP was present at a final concentration of 100 μM. At the indicated times, aliquots were removed, boiled for 2 min in NaDodSO$_4$/PAGE sample buffer, and analyzed on 10% NaDodSO$_4$/polyacrylamide gels according to Laemmli (13). Gels were stained (Coomassie blue), destained, and exposed to Kodak XR-5 film at −70°C with a DuPont Lightning Plus intensifying screen. Autoradiograms were scanned on a Bio-Rad video densitometer model 620. To determine the temperature at which the cAMP receptor kinase is inactivated, we incubated plasma membranes at different temperatures for 2 min. Samples were brought to room temperature and the phosphorylation reaction was carried out in the absence or presence of cAMP.

Other Assays. cAMP binding to the cell surface was measured as described (12). Binding to plasma membranes was monitored by vacuum filtration onto 0.2-μm polycarbonate filters as described (11). 8-Azido[^{32}P]cAMP (8-N_3[^{32}P]cAMP) labeling of intact cells was performed as described (2, 4). To obtain the higher molecular mass form of the receptor, aggregation-competent cells were stimulated with 100 μM cAMP for 20 min and washed extensively before photoaffinity labeling. Receptor phosphorylation by cAMP-stimulated and unstimulated cells was monitored as described (3, 4). Protein determinations were performed according to Lowry *et al.* (14). Immunoprecipitations were performed by using our anti-cAMP receptor antiserum as described (15). To differentiate between phosphorylation on serine and threonine residues versus tyrosine residues, gels were incubated in 1 M NaOH at 55°C for 1 hr as described (16).

Materials. [^{3}H]cAMP and [γ-^{32}P]ATP were purchased from New England Nuclear. 8-N_3[^{32}P]cAMP was purchased

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Abbreviations: 8-N_3cAMP, 8-azido-cAMP; CAR-kinase, protein kinase that phosphorylates the chemotactic receptor of *D. discoideum*.

from ICN. Polycarbonate filters were from Nuclepore. All other chemicals were from Sigma.

RESULTS

Cell-surface cAMP binding activity is developmentally regulated. Little or no activity is detected on vegetative cells, whereas maximal levels are present when cells express aggregation competence (2, 12, 17). The proteins phosphorylated in membranes prepared from cells at different stages of development were examined. Although a number of proteins were phosphorylated in vegetative cell membranes, we did not observe the phosphorylation of a protein that comigrated on $NaDodSO_4$/PAGE with either the 45- or 47-kDa forms of the cAMP receptor (Fig. 1*A*, lane 1). No phosphorylated 47-kDa protein was observed even when the reaction mixture contained cAMP (lane 2). No radiolabeled bands were immunoprecipitated from vegetative membranes by using our anti-cAMP receptor antiserum (5, 15). As with vegetative cell membranes, no receptor phosphorylation was observed when membranes from aggregation-competent cells were incubated in the absence of cAMP (lane 3). However, when cAMP was present in the reaction mixture, an additional phosphorylated band that migrated as a 47-kDa protein was observed (lane 4). No other band (within the entire gel) appeared in response to cAMP addition. The 47-kDa phosphorylated band could be specifically immunoprecipitated with our anti-cAMP receptor antiserum (Fig. 1*B*). The 47-kDa band comigrated with the higher molecular mass form of the chemotactic receptor identified either by 8-N_3[^{32}P]cAMP labeling of cell-surface proteins or by *in vivo* labeling of cells with [^{32}P]phosphate followed by cAMP stimulation.

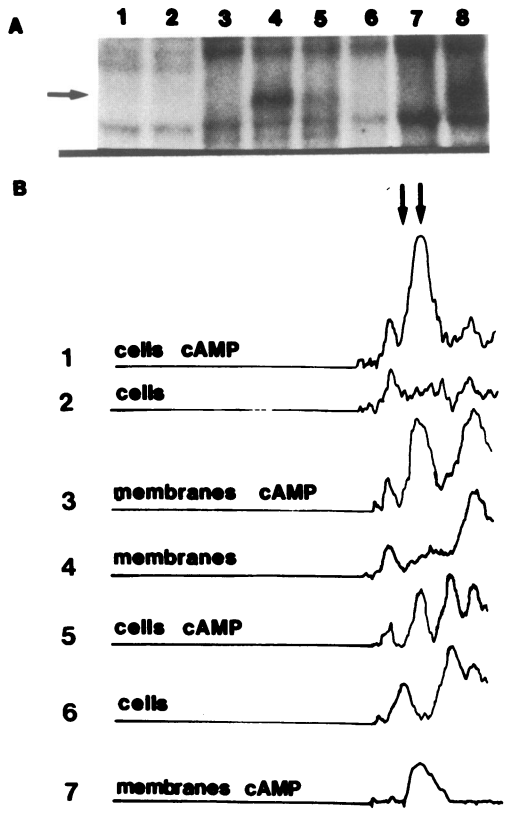

FIG. 1. cAMP-dependent phosphorylation in plasma membranes. (*A*) Membranes were prepared from vegetative cells (lanes 1 and 2), aggregation-competent cells (lanes 3 and 4), aggregation-competent cells that had been stimulated with either 100 μM cAMP for 20 min (lanes 5 and 6) or 1 μM cAMP for 15 sec (lanes 7 and 8). Membranes were phosphorylated in the absence (lanes 1, 3, 6, and 7) or presence (2, 4, 5, and 8) of cAMP. The arrow indicates the position of the 47-kDa protein. (*B*) Densitometer tracings of proteins phosphorylated by intact cells that had (tracing 1) or had not (tracing 2) been stimulated with 100 μM cAMP (also see ref. 3). Tracings 5 and 6 identify the two corresponding forms of the cAMP receptor as determined by photoaffinity labeling (see also ref. 4). Tracings 3 and 4 show the proteins phosphorylated by plasma membranes from aggregation-competent cells incubated with or without cAMP, respectively. Tracing 7 represents the immunoprecipitate obtained from the experiment depicted in tracing 3. The scans include the region between 38 kDa (left) and 58 kDa (right). The arrows indicate the positions of the 45-kDa (left) and 47-kDa (right) forms of the receptor.

The ability to catalyze the cAMP-dependent receptor phosphorylation was first observed in plasma membranes prepared from cells that had been starved for 3–4 hr. This coincides with the time cAMP-induced receptor phosphorylation is first observed in intact cells (3). The appearance of the phosphorylated 47-kDa band was very rapid—within 10 sec of membrane addition to the reaction cocktail containing cAMP. A similar rapid phosphorylation of the cAMP receptor occurs when intact cells are stimulated with cAMP (3). Extended periods of incubation of membranes in the absence of cAMP did not result in the presence of a 47-kDa phosphorylated band. We did not detect an obvious protein phosphatase activity under our experimental conditions. When membranes were incubated for 2 min with the reaction mixture containing cAMP and then 10 mM nonradioactive ATP was added, no loss of radioactivity from the 47-kDa band was observed (data not shown). Thus the changes in radiolabeling that occurred upon cAMP addition did not reflect changes in the rate of protein dephosphorylation but, rather, reflected an activation of a protein kinase.

The possibility that cAMP stimulation of intact cells could affect the level of receptor kinase activity present in the membrane preparations was examined. To do so, we stimulated cells with 1 μM cAMP for 15–30 sec or with 100 μM cAMP for 20 min. The latter treatment results in a down-regulation of receptors such that cells express 20–30% of their original binding activity (12). The receptor persists in the 47-kDa form (3, 4). In contrast, no receptor down-regulation is induced when cells are stimulated with 1 μM cAMP under our conditions (12). The 47-kDa form of the receptor is present only transiently (for $\approx$1 min). Rapid dephosphorylation of the receptor results in the regeneration of the 45-kDa form (4). As with intact cells, membranes prepared from cells that had been stimulated with 1 μM cAMP just before cell lysis showed no loss in receptor binding activity. Their ability to catalyze the cAMP-dependent phosphorylation of the receptor is shown in lanes 7 and 8 of Fig. 1*A*. Phosphorylation produced a 47-kDa band when cAMP was present in the reaction mixture. The amount of receptor phosphorylation, as determined by densitometer scanning of the autoradiograms, generally appeared equivalent to that of membranes from unstimulated cells that had been phosphorylated in the presence of cAMP. When membranes from down-regulated cells were incubated in the reaction cocktail in the absence of cAMP, phosphorylation leading to the presence of the 47-kDa band did not occur (lane 6). When cAMP was present, a much reduced level of the 47-kDa band was observed (lane 5).

To verify the plasma membrane localization of the kinase and 47-kDa protein, we examined the ability of other subcellular fractions to catalyze the phosphorylation of the 47-kDa band in a cAMP-dependent manner. Specifically, we examined the cytosol and the fractions enriched in endoplasmic reticulum membranes or nuclei (18). We did not observe the phosphorylation of either a 45- or 47-kDa band in any of those fractions, even when the phosphorylation reactions were performed in the presence of cAMP. This result, however, could reflect the fact that the substrate for the kinase, the cAMP receptor, is not present in fractions other than the plasma membrane. To address further the question

of the localization of the protein kinase, we determined if the level of phosphorylation of the 47-kDa band in the plasma membrane could be enhanced by the addition of varied subcellular fractions to the reaction mixture. In some experiments, plasma membranes were first heated to 50°C for 2 min to inactivate the protein kinase activity present in that fraction and therefore increase the possibility of observing an effect of added fractions on receptor phosphorylation. In neither case did we observe an effect of other subcellular components on the phosphorylation of the 47-kDa band (data not shown).

Various nucleotides were examined for their ability to catalyze the phosphorylation resulting in a radiolabeled 47-kDa band. As seen in Table 1, cAMP concentrations as low as 1 μM could lead to the presence of the 47-kDa band; 0.1 μM was also effective, but not consistently. When 1 mM dithiothreitol was added to the reaction mixture to inhibit the phosphodiesterase activity present in the membranes, lower concentrations of cAMP were effective. The 47-kDa phosphorylated band was also induced by cGMP but only when present at 100 μM. At that concentration, cGMP can elicit a chemotactic response from cells and does so by binding to the cell-surface cAMP receptor (12, 19). 5'-AMP, adenosine, and folic acid—another chemoattractant for these cells (20)—were ineffective in eliciting receptor phosphorylation. The cAMP concentration dependence and specificity for receptor phosphorylation in our membrane preparations are those observed with intact cells (3).

The role of cAMP in the phosphorylation reaction was examined. It may be that cAMP is necessary to activate a cAMP-dependent protein kinase (21–23). Alternatively, cAMP binding to the chemotactic receptor may be necessary for the receptor to become a substrate for a protein kinase. These two alternatives were differentiated by examining the effects of dibutyryl cAMP or 2-deoxy cAMP on membrane phosphorylation. Dibutyryl cAMP can bind to the cAMP chemotactic receptor but does so with a reduced affinity when compared to the regulatory subunit of the cAMP-dependent protein kinase present in *D. discoideum* (19, 24). In contrast, 2-deoxy cAMP preferentially binds to the cAMP chemotactic receptor and does so with an affinity similar to that of cAMP (19). The results shown in Table 2 indicate that the addition of 100–1 μM 2-deoxy cAMP resulted in the presence of the 47-kDa band. In contrast, dibutyryl cAMP was effective only at 100 μM, a concentration at which receptor binding would occur (19, 24). Lower concentrations were ineffective.

Increasing evidence suggests that guanine nucleotides play an important role in regulating chemotactic signal reception and relay (11, 25–27). We have examined the possibility that guanine nucleotides effect receptor phosphorylation. Neither GTP, p[NH]ppG, nor GDP alone was found to elicit receptor phosphorylation. Nor did any of these compounds antagonize, or increase, the ability of cAMP to do so (data not shown).

Table 1. Specificity of receptor phosphorylation

Addition	Presence of 47-kDa band
None (control)	−
cAMP (100–1 μM)	+
Dithiothreitol	−
+ cAMP (0.1–0.01 μM)	+
cGMP (100 μM)	+
Dithiothreitol + cGMP (10–0.01 μM)	−
5'-AMP	−
Adenosine	−
Folic acid	−

The plasma membrane-enriched fraction was incubated with the phosphorylation cocktail in the presence of the indicated additions. Dithiothreitol, 5'-AMP, and adenosine were present at 1 mM; folic acid was present at 100 μM. + or − indicate the respective presence or absence of a phosphorylated 47-kDa band as determined by $NaDodSO_4$/PAGE and autoradiography. Experiments were performed four times. When the 47-kDa band was detected, no consistent differences in its degree of radiolabeling were observed.

Table 2. Requirement for ligand binding for receptor phosphorylation

Addition	Presence of 47-kDa band
None (control)	−
2-Deoxy cAMP, μM	
100	+
10	+
1	±
Dibutyryl cAMP, μM	
100	+
10	−
1	−

The presence or absence of the phosphorylated 47-kDa band is indicated by + or −, respectively; ± indicates that phosphorylation was observed but inconsistently. Experiments were performed three times. When the 47-kDa band was present, its degree of phosphorylation was similar at all concentrations of the analogues.

A number of hormone plasma membrane receptors possess intrinsic kinase activities that result in receptor autophosphorylation upon binding of hormone. In such instances, the phosphorylation occurs primarily on tyrosine residues of the receptor (1). Phosphorylation of the chemotactic receptor by intact cells does not involve tyrosine residues (3, 5). We examined whether the cAMP-induced phosphorylation we have reported here reflects the activity of a tyrosine kinase. To do so, membranes were phosphorylated in the presence or absence of cAMP and duplicate samples were analyzed by $NaDodSO_4$/PAGE. One-half of the gel was stained, destained, and analyzed by autoradiography, whereas the other half was incubated in 1.0 M NaOH at 55°C for 1 hr prior to autoradiography. This treatment eliminates phosphate present on serine and threonine residues but not on tyrosine residues (16). Most of the radiolabel was removed by this treatment, in particular all that had been present in the 47-kDa band (data not shown).

In addition to the cAMP- and cGMP-dependent protein kinases, Ca^{2+}/phospholipid- and Ca^{2+}/calmodulin-dependent protein kinases also phosphorylate proteins on serine and threonine residues. Ca^{2+}-requiring protein kinases are also important mediators in hormone signal transduction (1). To examine if the protein kinase evidenced here bears similarity to those enzymes, the experiments reported in Table 3 were performed. Membranes were incubated with 100 μM Ca^{2+} in the absence or presence of diacylglycerol,

Table 3. Effect of various protein kinase modulators on phosphorylation resulting in a 47-kDa band

Addition	Phosphorylation of the 47-kDa band,* %
Ca^{2+}/calmodulin	0
+ cAMP	100
Ca^{2+}/PtdSer/diacylglycerol	0
+ cAMP	100
EGTA	0
+ cAMP	100

Membranes were incubated in the reaction mixture containing the indicated compounds: 100 μM Ca^{2+}, 5 μg of calmodulin, 10 μg of phosphatidylserine (PtdSer), 2 μg of diacylglycerol, 100 μM EGTA, and 100 μM cAMP. The degree of phosphorylation was monitored by scanning densitometry and normalized to that induced by cAMP alone.
*% of cAMP-induced level.

phosphatidylserine, or calmodulin. These components did not induce a phosphorylation leading to a 47-kDa band, nor did the presence of any of those compounds effect the ability of cAMP to do so.

DISCUSSION

We have described the characteristics of the protein kinase that phosphorylates the chemotactic receptor of *D. discoideum* in a cAMP-dependent manner. The enzyme activity, to be referred to as CAR-kinase, is observed in partially purified plasma membranes. The ability to detect CAR-kinase in that fraction is developmentally regulated and parallels the ability of intact cells to phosphorylate the receptor in a cAMP-dependent manner. We observed that radiolabeling of the receptor in membranes prepared from cells that had been incubated with 100 μM cAMP for 20 min is reduced when compared to membranes prepared from untreated cells. Amoebae respond to such stimulation by decreasing their receptor binding activity and maintaining the receptor in its more highly phosphorylated state (3, 4, 12). We have demonstrated that the loss in binding activity does not involve receptor internalization and that the receptor protein remains on the cell surface (5). Thus, although membranes from down-regulated cells contain 20% of the binding activity present in control cell membranes, the substrate for the kinase in such membrane preparations is still present to the same degree as in control membranes. Although we cannot exclude the possibility that CAR-kinase may dissociate from the membrane of down-regulated cells, it is more likely that the decreased level of the radiolabeled 47-kDa band reflects the fact that a majority of the receptor had already been phosphorylated by intact cells in response to cAMP stimulation. This schema would then argue that the amino acid residues of the receptor that are phosphorylated in isolated membranes are identical to those phosphorylated by intact cells. In both cases, those are serine/threonine residues as opposed to tyrosine residues.

Induced receptor phosphorylation in membranes incubated with cAMP did not reflect an activation of a cAMP-dependent protein kinase. Rather, it indicated that the preferential substrate of the enzyme is the ligand-occupied receptor. This was demonstrated by the findings that cAMP analogues only induce receptor phosphorylation at the concentrations at which they bind to the receptor. Also, 2-deoxy cAMP, which does not activate the cAMP-dependent protein kinase but which binds to the cAMP receptor with relatively high affinity, is a potent effector of receptor phosphorylation. The preference of the kinase for occupied receptor is consistent with results obtained in *in vivo* studies of receptor phosphorylation (5). For example, although numerous treatments can perturb the plasma membrane of intact cells and indirectly alter cAMP binding activity, receptor phosphorylation is only induced by ligand binding.

CAR-kinase does not have the properties of a cAMP- or a cGMP-dependent protein kinase, nor does it resemble protein kinases that are regulated by Ca^{2+}. Neither Ca^{2+} alone nor Ca^{2+} in conjunction with the various compounds shown to coregulate Ca^{2+}-dependent protein kinases was found to effect receptor phosphorylation *in vitro*. Nor did these agents effect the ability of cAMP to do so. CAR-kinase more closely resembles β-adrenergic receptor protein kinase (28). Both are unusual enzymes that preferentially phosphorylate serine/threonine residues of the ligand-occupied form of its respective receptor. In that regard, it is of interest to note that both enzymes function to modify receptors that are coupled to adenylate cyclase and do so in a manner consistent with a role in homologous desensitization. It has been suggested that the "coupling domains of all adenylate cyclase-coupled receptors might be structurally homologous" (29). The enzymes responsible for altering such coupling may also bear such homology. It is tempting to consider CAR-kinase an ancestor to the more complex systems that developed as cell specialization required the integration of varied external regulatory signals.

In the β-adrenergic receptor system, the association of β-adrenergic receptor protein kinase with the receptor membrane fraction may occur to different degrees depending upon whether hormone-stimulated or unstimulated cells are examined (1). Although we did not observe a CAR-kinase activity in cell fractions other than the plasma membranes, the data do not unequivocally rule out this possibility. However, if the level of plasma membrane-bound enzyme determines the degree of receptor phosphorylation, then the association of the enzyme with the membranes must occur very rapidly: cAMP-stimulated phosphorylation of the receptor occurs within 5 sec of stimulation of intact cells (3). The rapidity with which this occurs may preclude a regulation at the level of membrane association. In addition, cAMP stimulation of cells prior to the preparation of plasma membranes does not lead to increased receptor phosphorylation in response to cAMP. Although it seems probable that all of the CAR-kinase activity is present in the plasma membrane fraction of the cell, this will best be determined when a purified substrate is available to more accurately assay possible enzymic activity in other subcellular locales.

In summary, we have demonstrated a cAMP-dependent phosphorylation of the chemotactic receptor in *D. discoideum* in plasma membrane preparations. Characterization of the enzyme responsible for such phosphorylation indicates that it is an unusual protein kinase that preferentially phosphorylates the ligand–receptor complex. Studies may be designed to determine the physiological role of this phosphorylation in the transduction of the chemotactic signal and to modify the conditions for cAMP-dependent receptor phosphorylation to those that are more favorable for the stability and activity of the receptor, adenylate cyclase, and G protein present in that fraction (26, 30). Once this is accomplished, examination of the effects of receptor phosphorylation on the properties of the receptor and its ability to couple to the adenylate cyclase will be possible.

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