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Specific Photoaffinity Labeling of the cAMP Surface Receptor in *Dictyostelium discoideum**

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The recent observation that ammonium sulfate stabilizes cell-surface [^{3}H]cyclic AMP binding in *Dictyostelium discoideum* (Van Haastert, P., and Kien, E. (1983) *J. Biol. Chem.* 258, 9636–9642) led us to attempt to identify the surface cAMP receptor by photoaffinity labeling with 8-azido-[^{32}P]cAMP using this stabilization technique. 8-azido-[^{32}P]cAMP specifically labeled a polypeptide which migrates as a closely spaced doublet (M_r = 40,000 to 43,000) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Greater than 60% of the labeled polypeptide was found associated with membranes. This protein was distinguished from the cytosolic regulatory subunit of the cAMP-dependent protein kinase (M_r = 41,000) by differences in developmental regulation, specificity, and subcellular localization. No kinase regulatory subunit was detected in membranes by western blot analysis. Our preliminary observations show that labeling of this doublet correlates closely with cAMP-binding activity, suggesting that it is the surface receptor which mediates chemotaxis and cAMP signaling.

Acting as an extracellular transmitter, cAMP plays a pivotal role in directing the aggregation of developing *Dictyostelium discoideum* amoebae. The nucleotide is released in a pulsatile manner from aggregation centers and is detected by surface receptors of proximal cells (2). These cells respond by moving toward the source of the cAMP and also relay the chemical signal by releasing additional cAMP (reviewed in Ref. 3). Recent studies indicate that these and other responses are probably mediated by the same cAMP receptor which has stringent pharmacological specificity requirements (4, 5).[1] Receptor binding activity and physiological responsiveness increase dramatically in early development and then decline as aggregation progresses (6, 7). While others have reported photolabeling of the cAMP receptor (8–11), clear identification of the receptor has been hindered by the rapid dissociation rate of cAMP-receptor complexes (12), low affinity of available photoactive cAMP analogs, degradation of analogs by cell-surface phosphodiesterase, and the presence of cytoplasmic cAMP-binding proteins.

Recent reports indicate that binding of cAMP to intact cells, which normally has a dissociation rate of a few seconds, can be greatly stabilized by ammonium sulfate (1). We reasoned that this stabilization procedure would facilitate the removal of unbound ligand and also increase the chances of coupling a photoactive cAMP analog so that specific labeling would be observed. In the presence of ammonium sulfate, 8-N_3-[^{32}P]cAMP very specifically photoaffinity labels a major membrane polypeptide that migrates as a doublet (M_r = 40,000 to 43,000) on SDS-PAGE.[2] Labeling of this band is inhibited by excess unlabeled cAMP. It is distinguished from the regulatory subunit of the cAMP-dependent protein kinase (M_r = 41,000) (13) by numerous criteria. Our preliminary observations indicate that labeling of this band correlates quantitatively with receptor binding activity.[3]

MATERIALS AND METHODS

Ax-3 Growth and Development

Ax-3 cells were grown in HL-5 medium (14) and harvested at a density of less than 5×10^6/ml. Cells were washed in DB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.2), resuspended at 2×10^7 cells/ml and shaken at 100 rpm at 22 °C (15).

8-N_3-[^{32}P]cAMP Photoaffinity Labeling of Surface Receptor

Developed cells were centrifuged 700 × *g* for 4 min, resuspended in an equal volume of development buffer (DB), recentrifuged, resuspended in an equal volume of DB (minus Mg^{2+} and Ca^{2+}) plus 10 mM DTT, recentrifuged, resuspended in the latter buffer at 2×10^8 cells/ml, and used for binding or labeling. All steps were carried out at 0 °C.

Standard 8-N_3-[^{32}P]cAMP Photolabeling Assay—300 μl of cells plus 300 μl of 8-N_3-[^{32}P]cAMP (usually 300 nM, ICN Cat. No. 37003) in the same buffer were mixed in 15-ml cortex tubes. After 30 s, 12 ml of 98% saturated AS was added, samples were mixed and, after 10 min, samples were centrifuged at 7000 rpm for 6 min (Sorval SS-34). Pellets were resuspended in 1 ml of AS, vortexed, 12 ml AS added, and samples were recentrifuged. Pellets were resuspended in 600 μl of AS and 550 μl were spotted on a 100-mm plastic Petri dish. After irradiation (Minerallight UVG-54) from 4.5 cm for 15 min (3 and 81 min gave the same result), 500 μl were transferred to 5 ml of receptor buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM DTT containing per 10 ml: 10 mg of benzamidine, 10 mg of phenanthroline, 2.5 mg of benzyloxycarbonyl-phenylalanine, 1 mg of phenylmethylsulfonyl fluoride, 20 μg of chymostatin, 20 μg of pepstatin, 10 μg of leupeptin, 50 μg of antipain, 100 μg of aprotin, 1 mg of 1-chloro-3-tosylamido-7-amino-2-heptanone, 1 mg of *p*-tosyl-L-arginine methyl ester, 1 mg of L-1-tosylamido-2-phenylethyl chloromethyl ketone). After centrifugation at 16,000 rpm for 30 min (Sorvall SS-34) samples were resuspended in 600 μl of sample buffer (1.5% SDS, 2.5% DTT, 10% glycerol, 31.25 mM Tris, pH 6.8). After 30 to 60 min at 22 °C, 20 to 100 μl were analyzed by SDS-PAGE (16). Gels were stained with Coomassie Blue, destained, dried on Whatman 3MM paper, and autoradiographed on Kodak AR x-ray film at −80 °C with intensifying screens for 15 to 48 h.

Miniassay—100 μl of cells were mixed with 100 μl of 8-N_3-[^{32}P]cAMP (300 nM) in the same buffer in a glass tube (12 × 75 mm). After 30 s, 3 ml of AS was added and the tube was vortexed. After 5 min tubes were centrifuged 4000 rpm for 12 min (Sorvall HS4). Pellets were resuspended in 1 ml of AS and vortexed, and 3 ml of AS was added. Tubes were recentrifuged and pellets were resuspended in 200 μl of AS. 180 μl were irradiated for 10 min and 160 μl were

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‡ American Heart Association Established Investigator.

[1] A. Theibert, M. Palmisano, B. Jastorff, and P. N. Devreotes, manuscript in preparation.

[2] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AS, ammonium sulfate; DTT, dithiothreitol.

[3] Klein, P., Theibert, A., Fontana, D., and Devreotes, P. N. (1984) *J. Biol. Chem.* **259**, in press.

transferred to 1.3 ml of receptor buffer in a 1.5-ml microfuge tube. After centrifugation at 19,000 rpm for 30 min (Sorvall SS-34), pellets were resuspended in 200 μl of sample buffer then analyzed as for standard assay. For whole cell assays, receptor buffer was omitted, cells were microfuged for 10 min, and pellets were resuspended in 200 μl of sample buffer containing the same protease inhibitors as receptor buffer.

Western Blot Analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose as described in Ref. 17. Samples were overlayed with a 1/100 dilution of serum directed against the regulatory subunit of protein kinase of *D. discoideum* (a generous gift from Dr. B. Leichtling, Department of Molecular and Cellular Biology, National Jewish Hospital and Research Center, Denver, CO) and stained with ^{125}I-Protein A.

RESULTS AND DISCUSSION

Developed, intact cells were mixed at 0 °C with 8-N_3-[^{32}P]cAMP and DTT (to inhibit phosphodiesterase), and ammonium sulfate was added to stabilize specifically bound ligand. Cells shrank upon addition of the high salt but did not lyse. Cells were centrifuged and washed to remove unbound 8-N_3-[^{32}P]cAMP, irradiated, and lysed by hypo-osmotic shock. Membranes were collected by centrifugation and analyzed by SDS-PAGE. The results are illustrated in Fig. 1. A polypeptide that migrates as a doublet (M_r = 40,000 to 43,000) was labeled with this technique. The accompanying gel scan shows that this doublet represents about 90% of the labeled material in the membranes. Over 80% of the radioactivity was consistently found associated with this doublet although the relative intensity of the upper and lower bands of the doublet varied in independent experiments. Labeling of the doublet was completely eliminated by including 100 μM cAMP in the binding step. Comparison of the autoradiograph with the Coomassie Blue staining pattern shows that the radioactivity is not associated with a major membrane protein.

Two minor labeled bands (M_r = 220,000 and 70,000) were noted but together comprised only about 3% of the radioactivity. The former is competed poorly by 100 μM cAMP. The latter may be the cAMP-binding protein that Meyers-Hutchins and Frazier (11) have partially purified from membranes of *D. discoideum*. The diffuse band (M_r = 32,000 to 36,000), comprising about 8% of the radioactivity, is most likely derived from proteolysis of the larger doublet (M_r = 40,000 to 43,000) since it is the major species present if protease inhibitors are omitted. It also appears as a doublet, and it is labeled to exactly the same extent as the larger doublet under a variety of experimental conditions (see below).

The photolabeling of a single major species in intact *D. discoideum* cells is unprecedented. While others have reported specific photolabeling of cells with 8-N_3-[^{32}P]cAMP (8–10), their results were overshadowed by the large degree of nonspecific labeling. Using ammonium sulfate to stabilize ligand binding, the problem of nonspecific binding has been minimized. As shown in Table I, about 5–20% of the 8-N_3-[^{32}P]cAMP is bound nonspecifically. Both specifically and nonspecifically bound radioactivity attaches covalently to macromolecules that are excluded from Sephadex G-50 (Table I). While the specific, covalently bound radioactivity migrates as a single major band, the nonspecific material is covalently bound to proteins that migrate throughout the gel lane (see Fig. 2, *lanes 1* and *2*). Nearly all of the nonspecifically labeled proteins are lost when membranes are prepared (Table I and Fig. 2, *lanes 3* and *4*). More than 80% of the radioactivity bound to membranes and excluded from the G-50 column is recovered when the (M_r = 40,000 to 43,000) region is excised from the gel. While not yet optimized, 2–5% of the specifically bound 8-N_3-[^{32}P]cAMP becomes covalently attached upon irradiation (Table I).

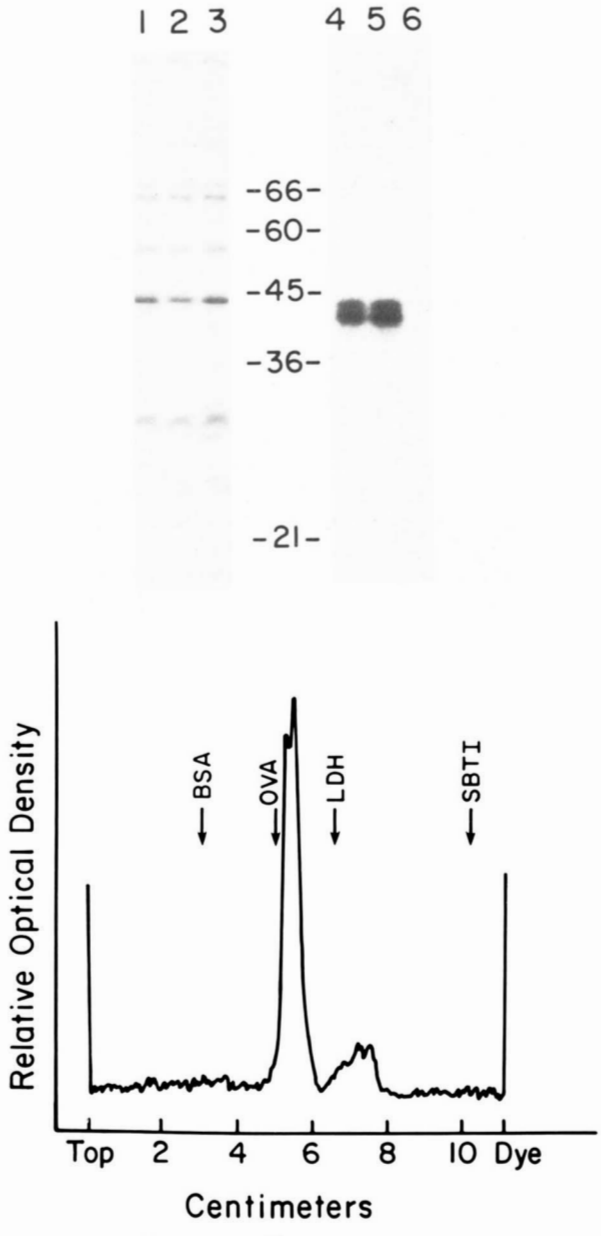

FIG. 1. **Specific photoaffinity labeling with 8-N_3-[^{32}P]cAMP.** Cells were developed for 5 h and photoaffinity labeled with 8-N_3-[^{32}P]cAMP according to the miniassay described under "Materials and Methods." The samples were then run on 10% SDS-PAGE and the Coomassie Blue stained gel (*lanes 1–3*) was autoradiographed (*lanes 4–6*). *Lanes 4* and *5* are duplicates showing specific photoaffinity labeling. *Lane 6* shows nonspecific labeling in the presence of 100 μM cAMP. An optical density scan of *lane 5* is shown in the *bottom* of Fig. 1. Molecular weight markers were bovine serum albumin (*BSA*) (M_r = 66,000), carbonic anhydrase (M_r = 60,000), ovalbumin (*OVA*) (M_r = 45,000), lactate dehydrogenase (*LDH*) (M_r = 36,000), and soybean trypsin inhibitory (*SBTI*) (M_r = 21,000). Autoradiographs were exposed for 18 h. The experiment was repeated 17 times with similar results.

We were initially concerned that the membrane fraction may contain the cytoplasmic regulatory subunit of the cAMP-dependent protein kinase (M_r = 41,000). This possibility was ruled out by the following experiments. First, receptor binding activity peaks at 5 h of development when less than 40% of the maximum amount of kinase regulatory subunit is present (kinase slowly increases during development and is maximal after 11 to 14 h (18)). Cells developed for 5 h showed strong labeling of the surface receptor doublet, while 9- to 11-h cells

TABLE I

Photolabeling of D. discoideum

Cells were prepared for photoaffinity labeling according to the standard assay described under "Materials and Methods." Prior to the irradiation step, aliquots were taken for measurements of total and nonspecifically bound 8-N_3-[^{32}P]cAMP and (no light) controls. Samples were divided, whole cells or membranes were prepared as described under "Materials and Methods," and final pellets were dissolved in sample buffer. 200-μl aliquots were chromatographed on 1-ml Sephadex G-50 columns equilibrated in sample buffer. The excluded peak, containing covalently bound 8-N_3-[^{32}P]cAMP and all of the cellular protein, was clearly separated from noncovalently bound 8-N_3-[^{32}P]cAMP. The combined data from several experiments is presented as average percentages with standard errors. Numbers in parentheses are cpm recorded in a typical experiment.

	Whole cells		Membranes	
	%	*cpm*	%	*cpm*
Bound radioactivity				
Total	100	(79,494)		
Nonspecific	6.68 ± 4.91	(5,701)		
G-50 excluded peak				
Total	3.85 ± 0.70	(3,028)	2.00 ± 0.42	(1,359)
Total (no light)	0.22 ± 0.02	(162)	0.02	(21)
Nonspecific	0.87 ± 0.12	(725)	0.03 ± 0.01	(35)

showed little or no labeling.[3] Second, 8-N_3-[^{32}P]cAMP photolabeling of the regulatory subunit of protein kinase in cytosolic extracts was completely eliminated by 1 μM *N*-6-monobutyryl-cAMP while cAMP surface receptor binding activity and the photoaffinity labeling of the doublet was decreased only 30% by a 50-fold higher concentration of *N*-6-monobutyryl-cAMP (50 μM). Third, the photolabeled polypeptide is a membrane protein while the protein kinase is a cytosolic protein (13). As shown in Fig. 2, membranes retained about 60 to 80% of the 8-N_3-[^{32}P]cAMP-labeled polypeptide that was present on whole cells. No protein kinase was detected in the same membranes by immunoblotting using an antibody to the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum*. Fourth, the regulatory subunit of the kinase, detected by 8-N_3-[^{32}P]cAMP photoaffinity labeling or immunoblotting, does not appear as a doublet band (Fig. 2) (13, 18).[4]

We have begun an extensive quantitative comparison of 8-N_3-[^{32}P]cAMP binding and photolabeling of the doublet band. Our evidence indicates that both have identical developmental regulation, peaking at 5 h and declining by 10 h. Both saturate at 1 μM 8-N_3-[^{32}P]cAMP and are competed by cAMP in the range 10 nM to 1 μM. In addition, both are competed by 2′-H-cAMP in the range 50 nM to 5 μM, 6-Cl-cAMP and 8-bromo-cAMP in the range 1 μM to 100 μM, and *N*-6-monobutyryl-cAMP in the range of 10 μM to 1 mM. 5′-AMP at 2 mM inhibits the binding and photolabeling by about 5%. Both are lost when cells are washed once in phosphate buffer prior to addition of ammonium sulfate. These results strongly suggest that the doublet band contains the surface cAMP-binding site of the receptor mediating chemotaxis and cAMP signaling.[3]

Das and Henderson (19) observed that a surface iodinated membrane protein (M_r = 40,000) increases early in development while most other proteins do not change. It is possible that this is the receptor we have identified. Juliani and Klein (10) and Wallace and Frazier (9) have reported 8-N_3-[^{32}P]cAMP photolabeling of bands (M_r = 40,000 and 45,000) in Ax-3, Ax-2, and a phosphodiesterase-deficient mutant of Ax-2 cells. In several attempts to label wild-type cells without prior ammonium sulfate stabilization of bound ligand, we were unable to get significant specific incorporation of radioactivity, although we did not expose these autoradiographs longer than one week. Therefore, it is difficult to compare our current results with these previous reports.

[4] A. Theilbert, P. Klein, and P. N. Devreotes, unpublished observations.

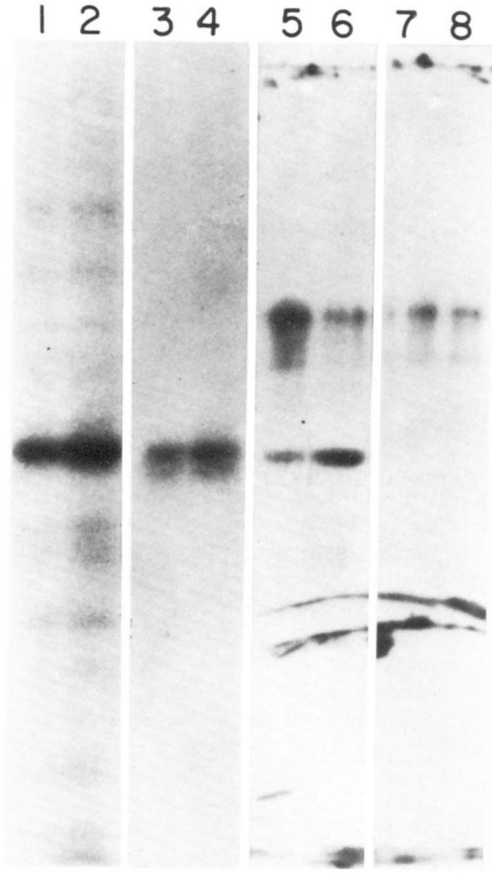

FIG. 2. **Membranes contain the majority of the surface receptor present in whole cells and the regulatory subunit of protein kinase is not detected in membranes.** Two identical aliquots of cells were carried through all the steps of the miniassay labeling procedure described under "Materials and Methods" except that 8-N_3-[^{32}P]cAMP was omitted from one of the samples. After irradiation, each sample was divided and either whole cells or membranes were prepared as described under "Materials and Methods." The autoradiographs of the samples that were labeled with 8-N_3-[^{32}P]cAMP are shown in *lanes 1* through *4*. *Lanes 1* (20 μl) and *2* (50 μl) are whole cells; *lanes 3* (20 μl) and *4* (50 μl) are membranes. The sample that was not exposed to 8-N_3-[^{32}P]cAMP was run in *lanes 5* through *8* and western blotting was carried out as described under "Materials and Methods" using antiserum directed against the regulatory subunit of *D. discoideum* protein kinase. *Lanes 5* (20 μl) and *6* (50 μl) are whole cells; *lanes 7* (20 μl) and *8* (50 μl) are membranes. The autoradiograph shown here was also exposed for 20 times longer and no band was seen in *lanes 7* and *8* (membranes). The same experiment was also done with a larger dilution series (from 1 to 50 μl of whole cells or membranes). When the autoradiographs were scanned, there was a concentration-dependent increase in optical density of the protein kinase band (M_r = 41,000) for the whole cell samples. These data indicated that the protein kinase present in 3 μl of whole cells would have been detectable. When the 50-μl lane of membranes was scanned, even after the prolonged exposure of the autoradiograph, no band was detected. Thus, while less than 6% of the kinase in cells is detectable, none was observed in membranes.

Our identification of the receptor should open the door to its biochemical characterization. Since essentially all the covalently bound radioactivity in membranes is specifically bound to the receptor (Table I), it should be possible to purify it simply by monitoring radioactivity during purification.

These studies have also opened an exciting line of investigation; we have preliminary evidence that the upper band of the doublet represents a modified form of the lower band. Physiological concentrations of cAMP elicit a time-dependent shift from the lower to upper bands.[3]

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