Association of the Cyclic AMP Chemotaxis Receptor with the Detergent-insoluble Cytoskeleton of Dictyostelium Discoideum

NANCY J. GALVIN, DIANNE STOCKHAUSEN, BETH L. MEYERS-HUTCHINS, and WILLIAM A. FRAZIER
Departments of Biological Chemistry and Neurobiology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63110. Dr. Meyers-Hutchins present address is Department of Surgery, Stanford University School of Medicine, Stanford, California 94305.

ABSTRACT   Treatment of 6-h differentiated Dictyostelium discoideum cells with the nonionic detergent Triton X-100 dissolves away membranes and soluble components, as judged by marker enzyme distributions, leaving intact a cytoskeletal residue that contains ~10% of the cell protein and 50% of the actin. Nitrobenzooxadiazo-phallacidin staining for F-actin and electron microscopy of detergent-extracted whole-mounts indicate that the cytoskeletons retain the size and shape of intact cells and contain F-actin in cortical meshworks. The cytoskeletons contain little if any remaining membrane material by morphological criteria, and the plasma membrane enzymes cyclic nucleotide phosphodiesterase and alkaline phosphatase are absent from the insoluble residue, which retains only 15% of the membrane concanavalin A-binding glycoproteins. This detergent-insoluble residue retains a specific [^{3}H]cAMP-binding site with the nucleotide specificity, rapid kinetics and approximate affinity of the cAMP receptor on intact cells. Upon detergent extraction of cells, the number of cAMP-binding sites increases 20–70%. The binding site is attached to the insoluble residue whether or not the cAMP receptor is occupied at the time of detergent addition. The pH dependence for recovery of the insoluble cAMP-binding site is much sharper than that on intact cells or membranes with an optimum at pH 6.1. Conditions of pH and ionic composition that lead to disruption of the cytoskeleton upon detergent treatment also result in the loss of cAMP binding. During differentiation, the detergent-insoluble cAMP binding increases in parallel with cell surface cAMP receptors and chemotaxis to cAMP.

Both human leukocytes (45, 57, 72, 74) and cellular slime molds (16, 21, 22), particularly the species *Dictyostelium discoideum*, have been studied extensively as paradigms for eucaryotic chemotaxis. Recently, a great deal of progress has been made in the study of chemotaxis receptors on leukocytes and slime molds. Receptors for the leukocyte chemoattractant *N*-formylmethionylleucylphenylalanine have been identified and characterized on intact cells (1, 47, 48, 64), plasma membranes (46) and in detergent solution (44). Receptors for cyclic AMP (cAMP) have been extensively studied on intact, aggregation-competent *D. discoideum* cells (14, 20, 24, 25, 29, 30, 42, 69) and their plasma membranes (29). Using a new hydrophobic immobilization assay (41), we detected a detergent-solublized cAMP-binding site with the properties

expected for the chemotaxis receptor (41) and succeeded in purifying this receptor (B. L. Meyers and W. A. Frazier, manuscripts in preparation). In spite of the progress in the study of chemotactic receptors themselves, the means by which receptor occupancy is transduced to yield directional informatioon is not understood (16, 21, 22, 74).

In both leukocytes and slime mold, a variety of postreceptor responses to chemoattractants have been described, but little evidence is available that directly shows a causal link between these responses to stimulation of receptors and the generation of directed cell motility. The situation is complicated by the fact that many of the responses to chemoattractants are not directly related to directional cell movement but to the cytotoxic functions of leukocytes (45, 57, 74) or to the signal relay

function of the adenylate cyclase in *D. discoideum* (16, 21, 22, 66). However, in the slime mold, responses of the cells related solely to chemotaxis can be identified by comparing the intracellular events in vegetative cells in response to folate, the chemoattractant of vegetative amebae (43, 51, 52, 71), with those occurring in differentiated cells in response to cAMP (6, 21, 22). It is based on reasoning of this sort that a rapid, transient accumulation of intracellular cyclic guanosine monophosphate has been proposed to be a link in the chemotactic transduction system in slime mold (22, 39, 71). Further comparative studies of the intracellular responses to chemoattractants are possible now that acrasins for other slime mold species have been identified (61, 68).

The components involved in the motility of eucaryotic cells are likely to be associated with the cytoskeleton (9, 12, 13, 19, 49, 58, 63). This structure is often operationally defined as the insoluble residue left after the extraction of cells with nonionic detergents such as Triton X-100 (TX-100)[1] (5, 9, 27, 35, 37, 49). Depending on the composition of the extraction medium, the three main filamentous systems of cells and nuclei are the primary components of cytoskeletons (5, 8, 27, 49). Cell surface events such as capping of surface IgG on lymphocytes (7, 59) or concanavalin A (Con A) receptors on *D. discoideum* (11) can lead to attachment of cell surface proteins via transmembrane linkages to the cytoskeleton. Recently, receptors for nerve growth factor (56, 67) and fibrinogen (50) have been found to associate with cytoskeletal elements of PC-12 cells and platelets, respectively. We reasoned that since the chemotaxis receptors regulate cellular motility, and since the directional and force-generating components of the response are likely to be cytoskeletal elements, the receptors may be linked to the cytoskeleton either directly or indirectly.

To determine whether the cAMP receptor can associate with the cytoskeleton of *D. discoideum*, we took advantage of the unique specificity and kinetics of the receptor to identify it with direct binding studies. A detergent-insoluble residue is obtained from *D. discoideum* cells (23, 63) that is of sufficient density that it can be centrifuged through a layer of silicone/ mineral oil to separate bound and free cAMP rapidly enough to measure binding to the chemotactic receptor (21, 22) as is done with intact cells. The residue contains a cAMP-binding site with many of the properties of the cell surface cAMP chemotactic receptor that clearly distinguish it from all other known cAMP-binding proteins. We characterize the detergent-insoluble residue by both biochemical and morphological criteria and show that it is a true cytoskeleton which retains the size and shape of intact cells but which is depleted of soluble and membrane material. This cytoskeletal preparation should prove valuable in the study of transduction events by which the receptor is linked to regulation of cellular motility.

MATERIALS AND METHODS

Materials: *D. discoideum* axenic strain A-3 and wild-type NC-4 cells were grown on HL-5 and *Escherichia coli* B/r, respectively, as described (2). Differentiation was initiated by washing the cells once (A-3) or six times (NC-4) with ice-cold 17 mM Pi (sodium/potassium phosphate) pH 6.2, and gyrating the washed cells in suspension at 10^7 cells/ml at room temperature for the

[1] *Abbreviations used in this paper:* cAMP, cyclic AMP; EM-720, emulphogene BC-720; Con A, concanavalin A; NBD, nitrobenzooxadiazol; mPDE, membrane phosphodiesterase; Pi, sodium/potassium phosphate; TX-100, Triton X-100.

times indicated. Cells for cAMP-binding studies were routinely gyrated for 6 h. TX-100, nucleotides, morpholinoethane sulfonic acid, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Emulphogene BC-720 (EM-720) was obtained from GAF, and the generic counterpart, polyoxyethylene-10-tridecyl ether from Sigma Chemical Co. (it is also referred to as EM-720). Its properties are similar to those of TX-100, octyl phenoxy polyethoxyethanol, except that it does not absorb at 280 nm. Acrylamide/bis was obtained from Bio-Rad Laboratories (Richmond, CA) and [³H]cAMP (25–35 Ci/mmole) and carrier-free Na[¹²⁵I] from New England Nuclear (Boston, MA). Silicone 550 fluid was purchased from Accumetric Inc. (Elizabethtown, KY) and scintillation counting fluid 3a70 from Research Products Inc. (Mount Prospect, IL). Nitrobenzooxadiazol (NBD)-phallacidin, and reaction product of phallacidin that contains a free amino group and 4-chloro-7-nitrobenz-2-oxa-1,3 diazole, was obtained from Molecular Probes (Plano, TX), and electron-microscope-grade glutaraldehyde, osmium tetroxide, and Spurr's embedding resin from Polysciences Inc. (Warrington, PA).

cAMP-binding Studies: Binding of [³H]cAMP to intact *D. discoideum* cells and detergent-insoluble residues was performed by adding 200 µl of cells at 10^8 per milliliter to chilled tubes containing 25 µl of 500 nM [³H]cAMP in 100 mM dithiothreitol (24, 25) with or without 10% detergent (TX-100 or EM-720) and 25 µl of blank buffer or competing nucleotide. All solutions were prepared in buffer that was routinely 17 mM Pi, pH 6.1, containing 1 mM EGTA. In some experiments, other buffers were substituted as indicated. After the indicated times on ice (usually 60 s), duplicate 100-µl samples were removed and layered above a 150-µl layer of oil (silicone 550:mineral oil, 8/3, vol/vol) resting on a cushion of 50 µl of 12% perchloric acid in a 400-µl microfuge tube. Sampling was timed so that the microfuge was started at the indicated time to terminate the binding reaction. The lower acid cushion dissociates all bound radioactivity from cellular material thus facilitating its dispersal in scintillation fluid for counting. After centrifugation for 15 s, each tube was clamped in the middle of the oil layer and cut so that the tip fell into a miniscintillation vial. 5 ml of 3a70 fluid was added and tubes were capped and shaken on a reciprocal shaker at 300 strokes/min for at least 2 h before counting to ensure complete solubilizatin of radioactivity. Vials were counted at least twice on a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Non-specific binding was determined by including 0.1 mM cAMP as the competing nucleotide, and was routinely <5% of total binding for intact cells and ~25% of total binding for the detergent-insoluble residues. The level of total binding at 50 nM cAMP in either case was in the range of 20,000 to 30,000 cpm, and the variation of duplicates was less than ± 5%. To collect samples of the detergent-insoluble material for SDS PAGE, we replaced the 12% perchloric acid cushion with 50 µl of SDS PAGE sample buffer: 50 mM Tris-HCl, pH 6.8, containing 5% 2-mercaptoethanol, 1% SDS, and 8 M deionized urea. After cutting the tips off, the sample buffer was collected and boiled for 2 min before loading on SDS slab gels (32) (1 mm, 10% acrylamide-bis). Con A-binding glycoproteins were visualized with the [¹²⁵I]Con A overlay technique as described by Burridge and Jordan (10). Marker enzymes were assayed in whole cell detergent lysates, supernatants and pellets collected in 17 mM Pi buffer containing 5% glycerol used as the bottom cushion under the oil layer. The enzymes used were the membrane-associated 3':5' cyclic nucleotide phosphodiesterase (EC 3.1.4.17) and alkaline phosphatase (EC 3.1.3.1) for plasma membrane, hexosaminidase (EC 3.2.1.30) for lysosomes, NADH oxidoreductase (EC 1.6.1.1) for endoplasmic reticulum, and succinic dehydrogenase (EC 1.3.99.1) for mitochondria. These enzymes have been used in previous characterization of subcellular fractions from *Dictyostelium* and were assayed as described (62). Protein content was assayed with the method of Lowry et al. (36) or of Bradford (8).

Microscopy: For NBD-phallacidin staining of F-actin in the detergent-insoluble residues (3, 4), cells were centrifuged (500 g, 5 min) from suspension at a density of 5×10^6 cells/ml in 17 mM Pi onto poly-L-lysine-coated coverslips. The buffer was changed to 17 mM Pi containing the indicated concentrations of EGTA and MgCl₂ (58) for 2 min and then to either 1% EM-720 or TX-100 in 17 mM Pi with EGTA and MgCl₂ for 1 min. Without further rinsing, the cytoskeletons were fixed immediately in 2% paraformaldehyde in the extraction buffer for 20 min at room temperature. After rinsing with 17 mM Pi, each coverslip was covered with 200 µl of NBD-phallacidin (165 nM) in 17 mM Pi and incubated overnight in the dark at 4°C. Coverslips were then washed in 17 mM Pi and mounted on glass slides. The edges were sealed with nail polish to prevent evaporation. Slides were viewed and photographed through an Aus-Jena Fluoval 2 microscope equipped with epifluorescence optics suitable for fluorescein fluorescence (the NBD-phallacidin emission and excitation maxima are similar to those of fluorescein).

For electron microscopic examination of whole-mounted cytoskeletons, cells were allowed to attach to Formvar-coated copper grids and detergent extracted as described above, except that the buffer contained either 10 mM EGTA, 2 mM MgCl₂, or 1 mM EGTA, 0.2 mM MgCl₂. After extraction, the cytoskeletons were fixed for 1 min in 1% glutaraldehyde in 17 mM Pi with the same EGTA

and MgCl₂ concentrations as in the extraction step for 30 min and rinsed three times in 17 mM Pi. Cytoskeletons were postfixed in 0.5% osmium tetroxide in 17 mM Pi with 1 mM EGTA and 0.2 mM MgCl₂, dehydrated, and critical-point-dried. Grids were then lightly coated with carbon for further stabilization. To obtain thin sections of cytoskeletons, washed cells were detergent-extracted for 1 min in suspension at 4°C under the conditions used above for whole-mounts. Concentrated glutaraldehyde was then added to bring the final concentration to 1%. After fixation for 30 min, the cytoskeletons were washed by centrifugation (500 g) in 17 mM Pi, postfixed in 1% osmium tetroxide in 17 mM Pi for 30 min, dehydrated through a series of graded ethanol solutions, and embedded in Spurr's resin. Thin sections were stained with 0.5% aqueous uranyl acetate and Reynolds' lead citrate. Both whole-mounts and thin sections of cytoskeletons were examined by transmission electron microscopy in a Philips EM 201.

RESULTS

Earlier work of Spudich and co-workers (23, 63) and Condeelis (11) suggested that, like many eucaryotic cells, *D. discoideum* cells could be treated with nonionic detergent to yield a particulate preparation which is enriched in actin. Condeelis (11, 12) found that this actin-enriched matrix could not be observed at a pH above 7 and Spudich's laboratory (23, 63) reported that the isolation of the cortical actin matrix was inhibited by Ca^{++}. We thus began by determining whether specific cAMP binding could be detected with the centrifugation assay after detergent treatment of 6-h differentiated cells in 17 mM Pi buffer that has a pH of 6.1 and no added Ca^{++}. 1% EM-720 or TX-100 was included in the cell incubation mixture for 1 min before centrifugation. This time of detergent treatment was sufficient for optical clearing of the cell suspension to occur, and was determined to produce maximal dissolution of cellular membrane by the criteria of electron microscopic examination and solubilization of membrane proteins. As seen in Table I, these conditions of detergent treatment result in slightly increased cAMP binding in the material recovered in the pellet beneath the oil layer, and either detergent is equally effective. While increasing the pH with Tris buffer or substituting MES buffer at pH 6.1 actually increases the specific binding of cAMP to intact cells, detergent treatment under these conditions leads to low recovery of binding in MES buffer and virtually no detectable binding in Tris, pH 8.1. SDS PAGE of the material recovered in the

pellet under these conditions indicates no detectable differences in the detergent-insoluble proteins in Pi or MES buffers at pH 6.1, while Tris, pH 8.1 leads to the loss of many proteins, suggesting a general dissolution of the residue (not shown). Table I shows that EGTA significantly increases the level of cAMP binding to the insoluble residue. Mg^{++} blocks this further enhancement, perhaps by acting as a Ca^{++} analog, while Mn^{++} has no effect beyond that of EGTA alone. As will be seen below, this increase of binding is due to an increase in apparent site number rather than an enhancement of affinity. To determine the total number of available cAMP-binding sites both inside and outside the cells, we used 0.1% saponin to permeabilize cellular membranes without dissolving them. After saponin treatment, the cAMP binding was the same as that found in the presence of TX-100 or EM-720, indicating that no net loss of cAMP-binding sites occurs upon solubilization of membranes with the nonionic detergents. We found that a saponin concentration of 0.01% or greater allows ferritin (60) to enter *D. discoideum* cells without dissolving cellular membranes (N. J. Galvin, unpublished observations).

A more detailed study of the dependence of cAMP-binding recovery on pH was performed (Fig. 1) using 17 mM Pi buffers in the presence of EGTA. A sharp optimum at pH 6.1 is seen with ~50% of the binding lost at pH 7.0 and 75% at pH 8.0. The less than total inhibition of binding at pH 8 in this experiment compared to Table I is probably due to the chelation of Ca^{++}. The binding of cAMP to the receptor on intact cells has a very different pH dependence, indicating that either the stability of the residue or the attachment of the cAMP-binding moiety to it is sensitive to increasing pH. Furthermore, EGTA has no effect on the binding of cAMP to intact cells at any pH (not shown).

Specificity of cAMP Binding to Detergent-insoluble Material

Using the optimized conditions of 17 mM Pi with 1 mM EGTA and 1% TX-100, we investigated the effect of nucleotides on cAMP binding (Table II). At a concentration of 1 μM, only 3':5' cAMP was able to substantially inhibit [³H]-cAMP binding to the detergent-insoluble binding site. Thus this binding site is neither the "nonspecific" nor the cGMP specific phosphodiesterase (21, 22), both of which are inhibited by cGMP. The intracellular regulatory subunit of the cAMP-dependent protein kinase of *Dictyostelium* does not have as high a selectivity for cAMP and would be significantly inhibited by 1 μM cGMP and even more so by dibutyryl cAMP (34). The insoluble binding site is not the soluble cAMP and adenosine-binding protein (17), since 5'-AMP

TABLE I

Effect of Detergents and Divalent Cations on [³H]cAMP Binding

	Additions to assay	[³H]cAMP binding % Control Level
Control, cells	17 mM Pi, pH 6.1	100
	20 mM MES, pH 6.1	120
	20 mM Tris, pH 8.1	140
1% EM-720	17 mM Pi, pH 6.1	120
	20 mM MES, pH 6.1	15
	20 mM Tris, pH 8.1	1
1% TX-100	17 mM Pi, pH 6.1	120
1% TX-100	17 mM Pi, pH 6.1 + 1 mM EGTA	171
	17 mM Pi, pH 6.1 + 1 mM EGTA + 1 mM Mg^{++}	122
	17 mM Pi, pH 6.1 + 1 mM EGTA + 1 mM Mn^{++}	168

Assays were performed as in Materials and Methods with an incubation time of 1 min with or without detergent present as indicated. This particular experiment was performed twice with essentially identical results. Several of the trends seen here such as the increase in binding upon detergent addition and with EGTA present have been observed in all experiments.
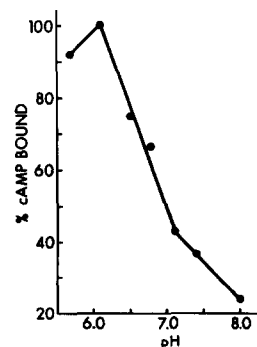

FIGURE 1 pH dependence of cAMP binding to the detergent-insoluble residue of 6-h differentiated cells in 1% TX-100, 17 mM Pi with 1 mM EGTA. The cAMP binding assays were incubated for 1 min and terminated by centrifugation through oil as in Materials and Methods.

TABLE II

Effect of Cyclic Nucleotides on [³H]cAMP Binding to the Detergent Residue

Nucleotide	Concentration	Binding (% control)
None	—	100
3'-5' cAMP	1 mM	0
3'-5' cAMP	1 μM	23
3'-5' cGMP	1 μM	100
3'-5' cXMP	1 μM	101
3'-5' cIMP	1 μM	99
3'-5' cZMP	1 μM	86
Dibutyryl cAMP	1 μM	116
2'-3' cAMP	1 μM	92

Nucleotides were present with [³H]cAMP at the time cells were added to the detergent containing incubation mixture for assay as described in Materials and Methods. This experiment was performed twice. Averages shown varied <5% between the two experiments. cXMP is 3':5' cyclic xanthine monophosphate, and cZMP refers to the same derivative of the purine biosynthetic precursor 5-aminoimidazole-4-carboxamide.

does not inhibit cAMP binding even at 1 mM. Other non-cyclic nucleotides which have no effect on the level of cAMP binding at 1 mM concentration are GMP, ADP, GDP, and ATP. Cyclic ZMP (Z represents the purine biosynthetic precursor 5-aminoimidazole-4-carboxamide) has about 100-fold lower affity than cAMP for the cAMP receptor in intact cells (W. Frazier, unpublished observations), and 1 μM cZMP exhibits the same level of competition expected for 10 nM cAMP. The high specificity of this detergent-insoluble binding site for cAMP is characteristic of the cell surface chemotaxis receptor (21–25, 29) and not of any other nucleotide-binding protein present in these cells (17, 21, 22, 34).

Kinetics and Affinity of the Detergent-insoluble Binding Site

The time course of binding of [³H]cAMP to the detergent-insoluble material is shown in Fig. 2. Nonspecific binding (Fig. 2, open circles) in the presence of excess cAMP has not been subtracted from the total binding (Fig. 2, filled circles). Note that maximal binding has occurred as early as the first time point taken at 10 s of incubation and remains relatively constant through 60 s. The fact that nonspecific binding is also maximal at 10-s indicates that the detergent has effectively extracted the cells at this time since the increase in nonspecific binding over intact cells is apparently due to exposure of sites, perhaps in the nucleus, which bind cAMP nonspecifically. After 1 min, 0.1 mM cAMP was added to prevent rebinding of dissociated radiolabeled cAMP, and samples were taken to determine the rate of dissociation. By 10 s, >80% of the specifically bound cAMP has dissociated from receptors and, by 30 s, binding reaches the same level as determined for nonspecific binding in the association experiment (Fig. 2, dashed line). Thus, the rates of both association and dissociation are quite rapid and compare with those of the cell surface receptor that functions in cAMP-mediated chemotaxis (21–25, 29, 41, 42). To determine whether the association of the cAMP-binding site with the insoluble material was pre-existent at the time of detergent treatment or whether the presence of cAMP induced its attachment to the residue, a 2-min association time-course experiment was performed by first adding the TX-100, and at some later time adding the [³H]cAMP for the last 30 s of incubation. Thus, cells were extracted for various times in the absence of cAMP

before binding was measured. This protocol yielded data identical to those obtained when cAMP was present at the time of detergent extraction.

The binding of cAMP to intact cells and to the insoluble material obtained after detergent treatment was determined over the concentration range from 5 to 1,000 nM. These data are presented in the form of a Scatchard plot in Fig. 3A and in the form recently recommended by Klotz (31), bound vs. log free, in Fig. 3B. Fig. 3B indicates that data exist for a wide enough concentration range to justify determination of dissociation constants, i.e., that saturation has been approached (31). In this type of presentation, a sigmoid curve such as that found for the detergent-treated case (Fig. 3B, open circles) signifies a class of simple, independent binding sites, while the flattened curve (Fig. 3B, closed circles) found for the intact cells indicates complex behavior such as cooperative interactions. The dissociation constant (half-maximal level) for the detergent-treated cells is ~100 nM while that for the intact cells is near 50 nM. In the form of the more commonly used Scatchard plot (Fig. 3A), these data indicate a slightly curvilinear plot for intact cells with an approximate dissociation constant derived from the slope of 30 nM while the detergent-treated cells again show the increase in site number and a lower dissociation constant of 120 nM. In some experiments, more curvature is seen in Scatchard plots, including an indication of apparent positive cooperativity at very low cAMP concentrations (14). Thus, while some in-
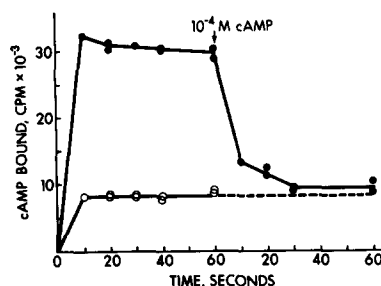

FIGURE 2 Time course of binding and dissociation of cAMP. 6-h differentiated cells were incubated in assay mixtures for the indicated times with 1% TX-100 and 1 mM EGTA, and the insoluble residues centrifuged through oil. Filled circles indicate the binding in the absence of unlabeled cAMP (total binding). After 60 s, the incubation mixture was made 0.1 mM in cAMP (arrow), and further time points were taken to determine the rate of dissociation. Note that the binding is reversible to the same level defined as nonspecific binding (open circles) determined in the presence of 0.1 mM cAMP present from the start of the experiment.
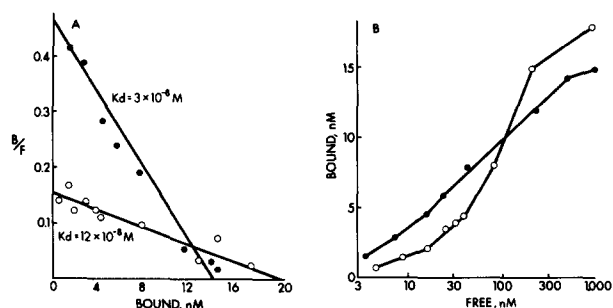

FIGURE 3 Concentration dependence of cAMP binding to 6-h differentiated cells (filled circles) and their TX-100 insoluble residues (open circles) presented as a Scatchard plot (A) and in the form recommended by Klotz (31) (B). K_d, dissociation constant.

crease in site number and reduction of affinity occurs upon detergent treatment of cells, the cAMP-binding site detected on the residue does not have an affinity for cAMP drastically different from that of the cell surface receptor. It is interesting that detergent extraction seems to result in conversion of all sites to a more homogeneous class, suggesting that the extraction of membrane proteins or factors that can modulate receptor affinity.

Developmental Regulation of Detergent-insoluble cAMP Binding

The cell surface cAMP chemotactic receptor is present in low numbers on vegetative cells and increases to maximum levels by 5 to 7 h of starvation in 17 mM Pi buffer (21-25). Cells were differentiated in suspension over this time period, and the cAMP-binding activity of cells before and after detergent treatment was compared. Fig. 4 shows the expected time course of appearance of cAMP binding to intact cells (closed circles). Binding to detergent-treated cells (Fig. 4, open circles) increases over the same time course in precise parallel fashion with the cell-surface binding sites. At all times, binding is increased upon detergent extraction as seen in other experiments (vida supra), and at the maximum level, attained at 5 h in this experiment, binding is ~50% higher than to intact cells.

Biochemical Characterization of Detergent-treated Cells

The protein content of the detergent insoluble residues obtained by TX-100 treatment of cells in phosphate buffer with and without EGTA and Mg^{++} was examined by SDS PAGE of the particulate and soluble fractions obtained after centrifugation under the conditions of Giffard et al. (23). As seen in Fig. 5, the amount of actin in the insoluble residue is enhanced by EGTA at either 1 or 5 mM and the presence or absence of Mg^{++} has no effect. The gel patterns seen in Fig. 5 for the residues obtained in the presence of EGTA compare quite closely to the pattern obtained by Giffard et al. in the presence of 2.5 mM EGTA (see Fig. 3 in reference 23). The actin bands were quantified by gel scanning and were found to contain ~50% (range of 44% to 57%) of the total actin. This compares with the 50% recovery of actin in the P1 (first) particulate fraction found by Giffard et al. (23).

To evaluate the extent of lysis of cellular membranes by the detergent under our conditions of assay (17 mM Pi, pH 6.1, with 1 mM EGTA), we assayed the activities of a battery of enzymes with known subcellular distributions in Dictyostelium (62) in the initial detergent lysates and in the supernatant and pellet fractions obtained after centrifugation through oil. These pellets were collected in lysis buffer containing 5% (vol/vol) glycerol so that enzyme assays could be performed on all samples under identical conditions. The
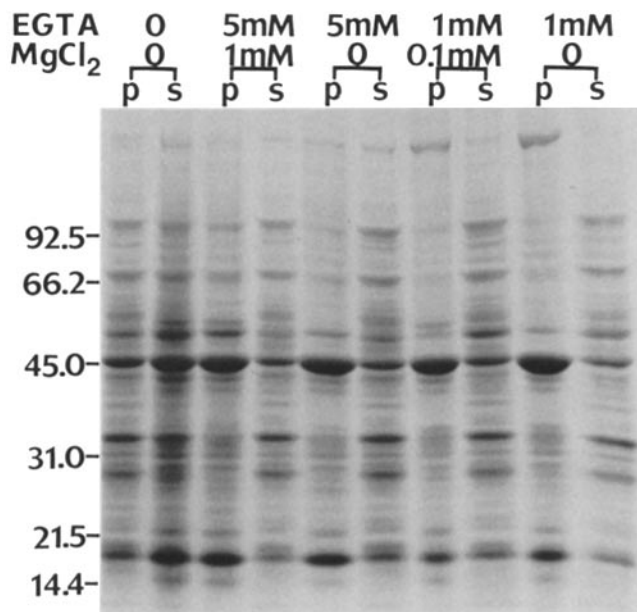
FIGURE 5 SDS gel electrophoresis of detergent-insoluble residues and the soluble material obtained from cells treated with 1% TX-100 for 1 min at 0°C under different buffer conditions. Insoluble material was separated from the soluble components by centrifugation at 400 g for 10 min. The components in the lysis buffer are indicated above the lanes. S and P are the supernatant and pellet of each condition.

results of these enzyme assays and the distribution of total protein are shown in Table III. Both plasma membrane markers, membrane phosphodiesterase (mPDE) and alkaline phosphatase, were more than 90% solubilized under these conditions and left in the supernatant fraction. Both the soluble but intralysosomal enzyme hexosaminidase and the mitochondrial matrix enzyme succinic dehydrogenase were released to the extent of ~80%, indicating virtually complete lysis of these intracellular membranes. NADH oxidoreductase, an endoplasmic reticulum enzyme, is also largely released into the soluble fraction. These data indicate that the membranous structures of the cell are solubilized to a large extent and that the plasma membrane is completely removed by our detergent extraction procedure used in the cAMP-binding assays. The detergent-insoluble residue contains only about 10% of the cellular protein (Table III), further suggesting complete release of soluble components.

To determine whether many membrane proteins remained associated with the detergent-insoluble residue or whether the association of the cAMP receptor is relatively specific, we examined the distribution of Con A-binding glycoproteins after separating the detergent lysates into pellets and supernatants by centrifugation through the oil layer as done in the cAMP-binding assay. The fractions were subjected to SDS PAGE and the gels were "stained" for Con A-binding glycoproteins with the [^{125}I]Con A overlay method of Burridge (10). Fig. 6 (center panel) shows these results for lanes containing the detergent-insoluble pellet fraction, the detergent-soluble fraction, and the whole cells lysed with detergent but not centrifuged. Most of the Con A-binding glycoproteins present in the cell lysate are found in the soluble fraction after centrifugation. It should be noted that all of these glycoproteins are associated with cell membranes and are not soluble before detergent treatment (not shown). After autoradiogra-
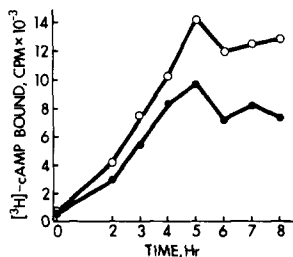
FIGURE 4 Developmental time-course of cAMP binding to cells (filled circles) and their TX-100-insoluble residues (open circles). Cells were differentiated in suspension in 17 mM Pi, pH 6.2, for the indicated times and assayed in the presence and absence of detergent as detailed in Materials and Methods.

TABLE III

Distribution of Marker Enzymes after Detergent Treatment of Cells

	Percent Activity		
Enzyme	Pellet	Supernatant	Recovery
Membrane 3':5' cyclic phos-phodiesterase	8	93	101
Alkaline phosphatase	<1	99.5	100
Beta-D-N-Acetyl-Hexosamini-dase	15	85	100
Succinate dehydrogenase	20	78	98
NADH oxidoreductase	36	76	112
Protein	11	85	96

Cells were lysed by the addition of 1% TX-100 to 17 mM Pi, pH 6.1, with 1 mM EGTA and centrifuged over an oil layer to separate the supernatant from the pellet that was recovered below the oil in 17 mM Pi buffer with 5% glycerol. Fractions were prepared immediately before assay. At least two preparations were made from vegetative and 6-h differentiated cells, and assays were performed in duplicate. All data were pooled for this table since results were identical for vegetative and differentiated cells. All assays were performed as described by Siu et al (62).

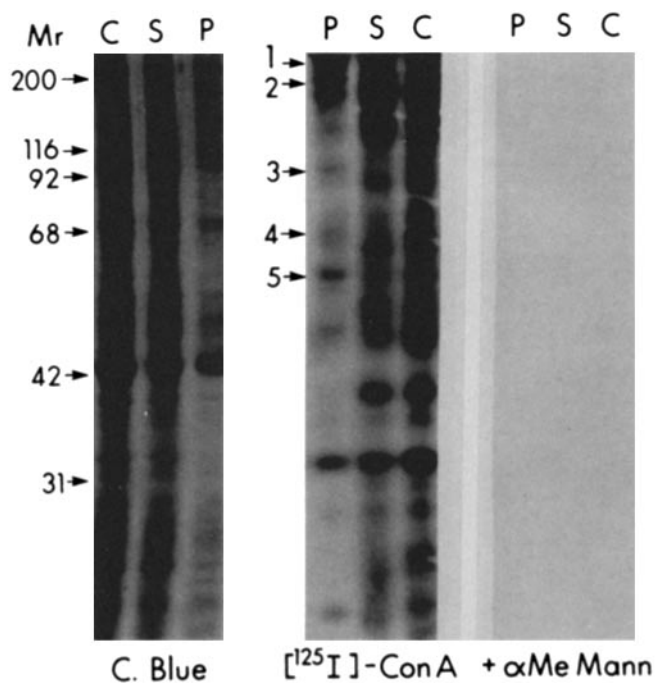C. Blue [^{125}I]-Con A + αMe Mann

Figure 6 Distribution of Con A-binding glycoproteins after TX-100 lysis of cells. In each case the sample run on the gel was whole cells (C); the supernatant after centrifugation of the detergent-treated sample (S); the pellet recovered below the silicone/mineral oil layer (P). The middle panel shows the autoradiograph of the same gel after incubation with [^{125}I]Con A, washing and drying. The *right* panel is a duplicate gel that was treated identically, except that incubation with [^{125}I]Con A and washing were performed in the presence of 0.1 M alpha methyl-D-mannoside to determine nonspecific labeling. Positions of protein molecular weight standards are shown at the extreme left, and the arrows numbered 1 through 5 in the center panel indicate Con A-binding glycoproteins found in the pellet which do not appear to be present in the supernatant fraction.

phy, the gels were cut into lanes and the radioactivity in each was quantified. Only 15 to 17% (two determinations) of the radioactive Con A is found associated with the detergent-insoluble pellet fraction. More importantly, it appears that only a few specific Con A-binding glycoproteins are found associated with this residue. Glycoproteins that appear to be recovered largely in the pellet and not in the soluble fraction are marked with the arrows numbered 1 through 5 in Fig. 6. These correspond to molecular weights of > 200,000, 180,000, 95,000, 65,000 and 55,000, respectively, and, with the possible exception of the band at M_r 95,000, do not correspond to major Coomassie Blue-stained bands (left panel, Fig. 6). The cAMP receptor is a Con A-binding glycoprotein of M_r 70,000 (B. Meyers-Hutchins and W. Frazier, manuscripts submitted for publication), but it is undoubtedly a minor component of the pellet fraction shown in Fig. 6. The right panel (Fig. 6) shows that all "staining" by the [^{125}I]Con A is eliminated by alpha-methyl-D-mannoside, the specific sugar inhibitor. Thus, of the many Con A-binding glycoproteins associated with *D. discoideum* membranes, only a few are associated with the detergent-insoluble residue. This suggests that association of membrane proteins with the potential cytoskeleton prepared by our methods is specific and does not occur through adventitious trapping or nonspecific adherence.

Morphological Characterization of Detergent-treated Cells

To further characterize the putative cytoskeletons and to determine whether detergent treatment under the conditions adopted for the cAMP-binding assays resulted in a cytoskeletal structure that was representative of the morphology of the cells, we examined the detergent-extracted cells by phase-contrast and fluorescence microscopy after staining with NBD-phallacidin to visulaize F-actin-containing structures (3, 4) and by electron microscopy of critical-point-dried whole-mounts and of thin sections.

Upon detergent treatment, the phase-bright boundary of the cells rapidly disappeared, but otherwise little change in appearance ensued (Fig. 7, compare *A* and *B* with *C* and *D*). Staining of both fixed cells and cytoskeletons with NBD-phallacidin reveals similar patterns and intensities of staining. In particular, staining of both cells and cytoskeletons was most intense in peripheral regions near the boundaries of cells (18). Fig. 7*A* shows intense staining of what appear to be lamellipodia of cells, and such staining is also seen in cytoskeletons in Fig. 7, *C* and *D*. Thus the detergent-extracted cells retain the size, shape, and F-actin distribution of intact cells as expected on the basis of observations from previous studies of these and other cells (4, 9, 18, 19, 27, 35, 49, 58, 63). NBD-phallacidin staining was competed by the unlabeled toxin phalloidin to the level of the faint, uniform autofluorescence always seen with these cells.

To visualize the cytoskeletons at higher magnification under conditions allowing their extraction while attached to a surface, we centrifuged cells onto Formvar-coated copper grids and allowed to spread. The cells were then treated with detergent for 1 min, and glutaraldehyde was then added to a final concentration of 1% without disturbing the attached cells. This method was used to screen various extraction conditions for their effects on the morphology of the cytoskeleton. It was found that extraction with either 0.5% or 1% TX-100 in 17 mM Pi for 1 min in the presence of either 1 or 10 mM EGTA and with 0.2 or 2 mM MgCl$_2$ gave optimal preservation of filamentous cytoskeletal structure. Fig. 8 shows an example of a cytoskeleton prepared in 1% TX-100 and 1 mM EGTA which has been critical-point-dried to
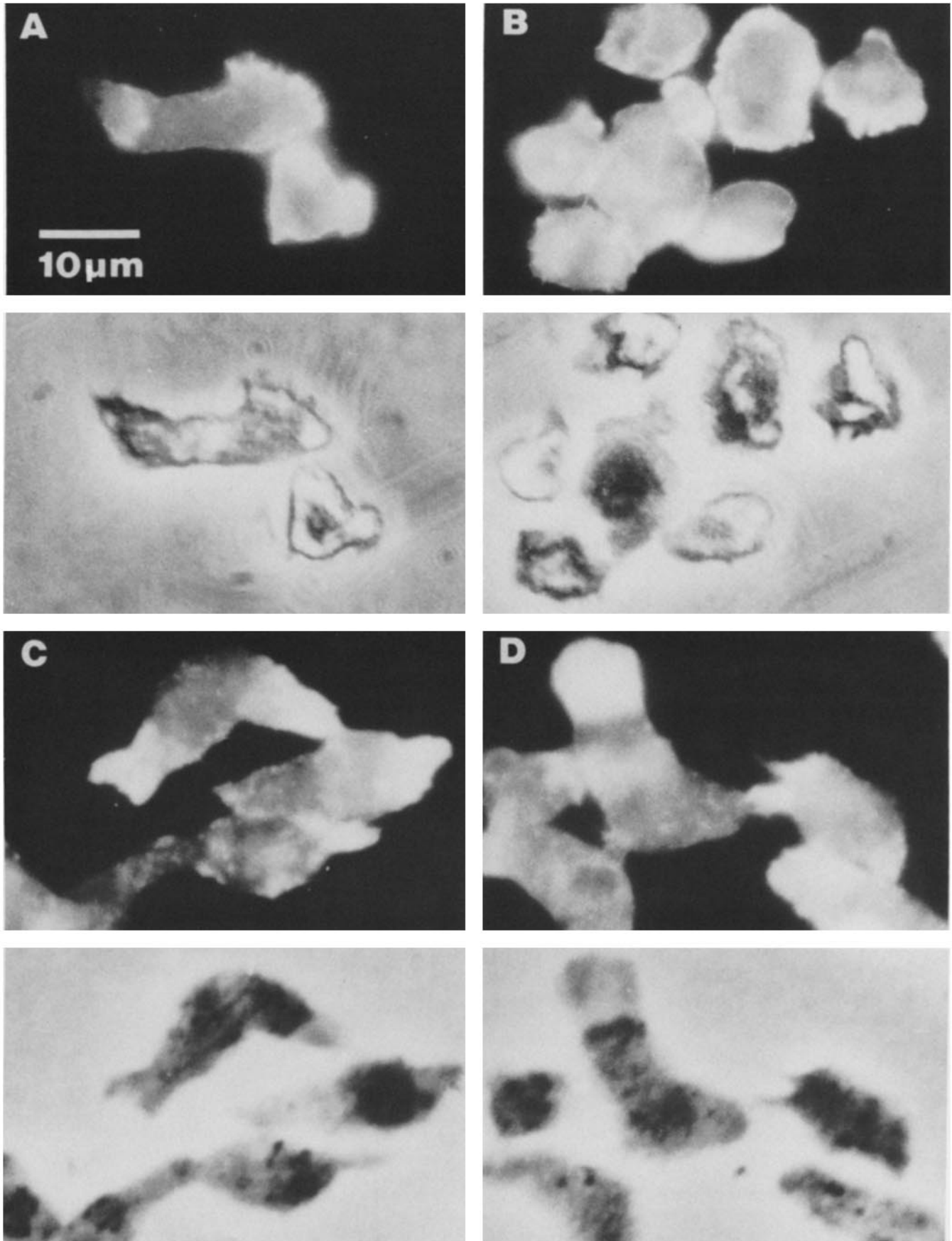
FIGURE 7   NBD-phallacidin staining of 6-h differentiated intact and TX-100-extracted cells. Cells were fixed and stained with or without detergent treatment as in Materials and Methods. (A and B) Corresponding phase-contrast and fluorescence micrographs of cells fixed with no detergent treatment, showing the distribution of F-actin by NBD-phallacidin staining. Note the phase-bright appearance of the cells. (C and D) Phase-contrast and fluorescence micrographs of cells extracted with 1% TX-100 in 17 mM Pi, pH 6.1 with 1 mM EGTA. Here the cytoskeletons are not phase-bright, but the distribution of F-actin staining is very similar to that seen in intact cells.
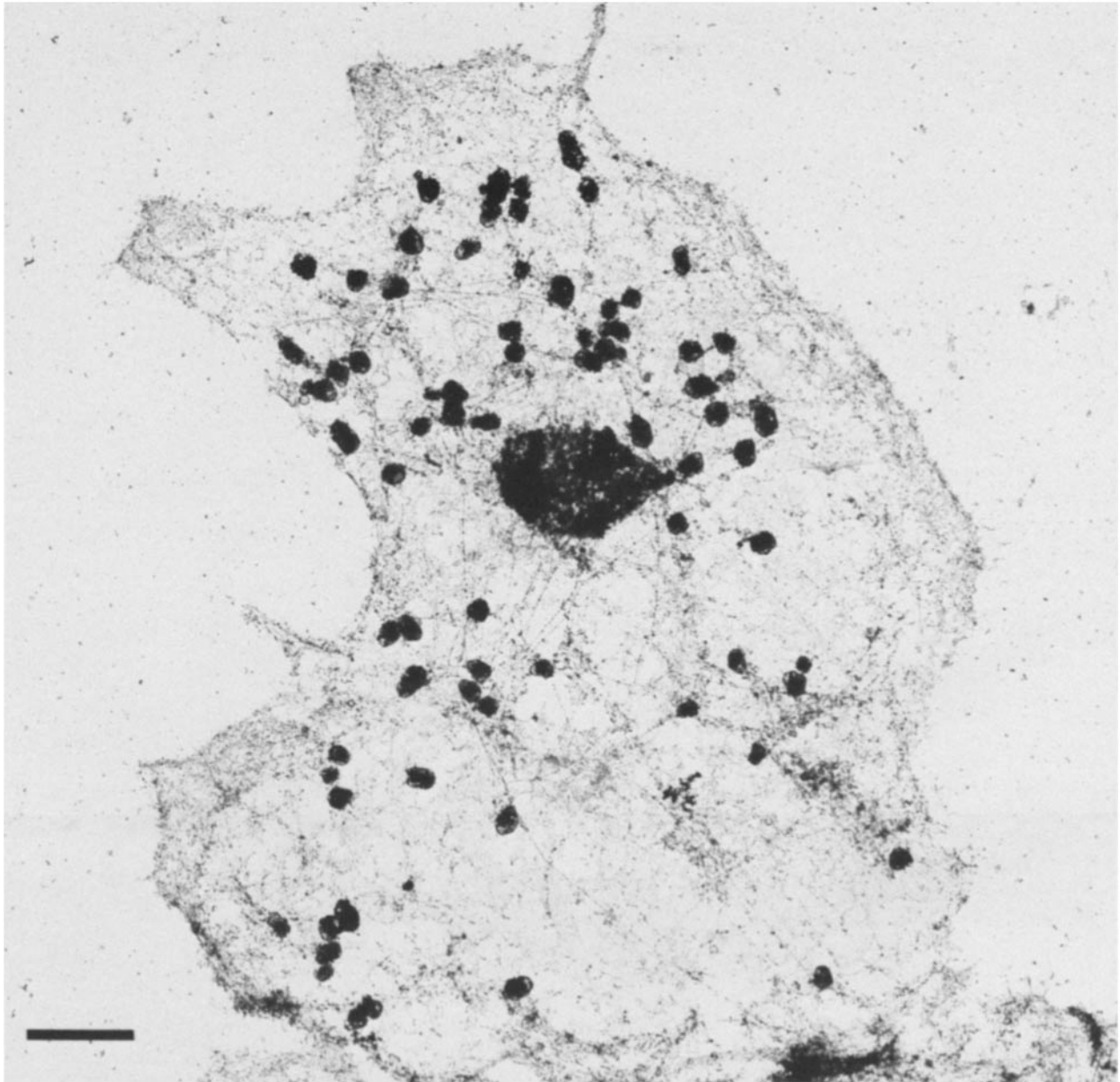
FIGURE 8 Critical-point-dried cytoskeleton prepared in 1% TX-100 in 17 mM Pi, pH 6.1 with 1 mM EGTA and 0.2 mM MgCl₂. Bar, 1 μm. × 16,000.

prevent collapse of its three-dimensional structure. The complex filamentous structure occupies all regions of the cell, but appears most dense in the periphery. It is presumably in this region that cell surface receptors associate with cytoskeletal elements.

Ben-Ze'ev et al. (5) found that HeLa cells and fibroblasts when extracted with TX-100 appeared to have a continuous surface lamina surrounding the cells that they attributed to membrane proteins. Clearly, inclusion of the cAMP receptor in such a nonspecific accretion of protein would not have the same significance as attachment to a cytoskeleton which has been freed of most membranous components, as suggested by the loss of membrane enzymes and most Con A-binding glycoproteins (above). Giffard et al. (23) found that the cortical actin matrix prepared under their conditions appeared to be attached to fragments of undissolved plasma membrane (see their Fig. 2a or Fig. 5 in reference 63). To determine

whether our conditions resulted in the retention of a surface lamina after extraction, we prepared cytoskeletons as described above, and fixed, embedded, and thin sectioned them as done by Ben-Ze'ev et al. (5). Fig. 9 shows an example typical of the appearance of cytoskeletons prepared with our conditions. No surface lamina is found to enclose the cellular remnants, and membranes appear to be totally dissolved in agreement with the membrane markers (Table III) and the distribution of Con A-binding glycoproteins (Fig. 6). Very rarely, small patches of cell surface material that appear as short segments with the approximate thickness of membrane are seen in the sections. These structures were apparently seen with some frequency by Giffard et al. (23) and Spudich and Spudich (63). In both these studies the glutaraldehyde fixative was added at the same time as the detergent, perhaps leading to the fixation of membrane patches that would otherwise be soluble.
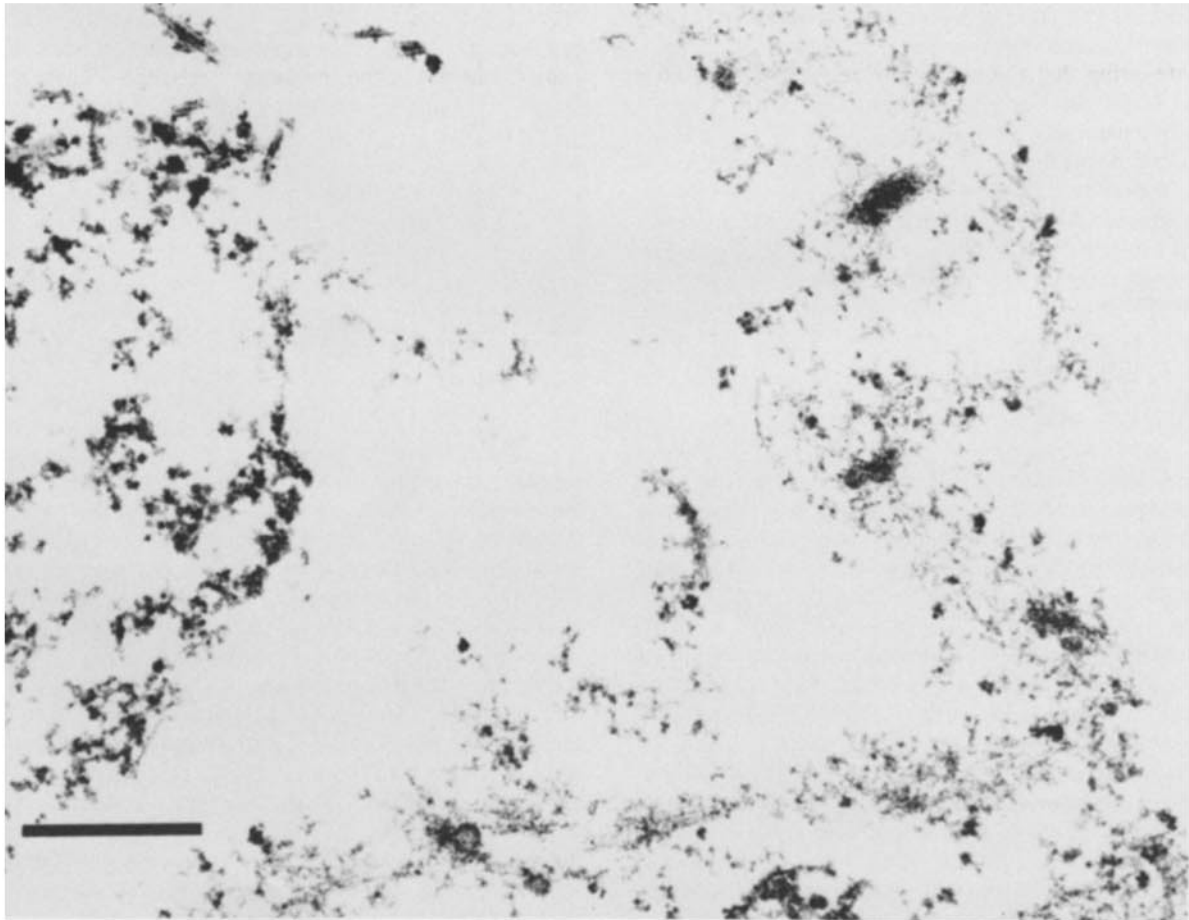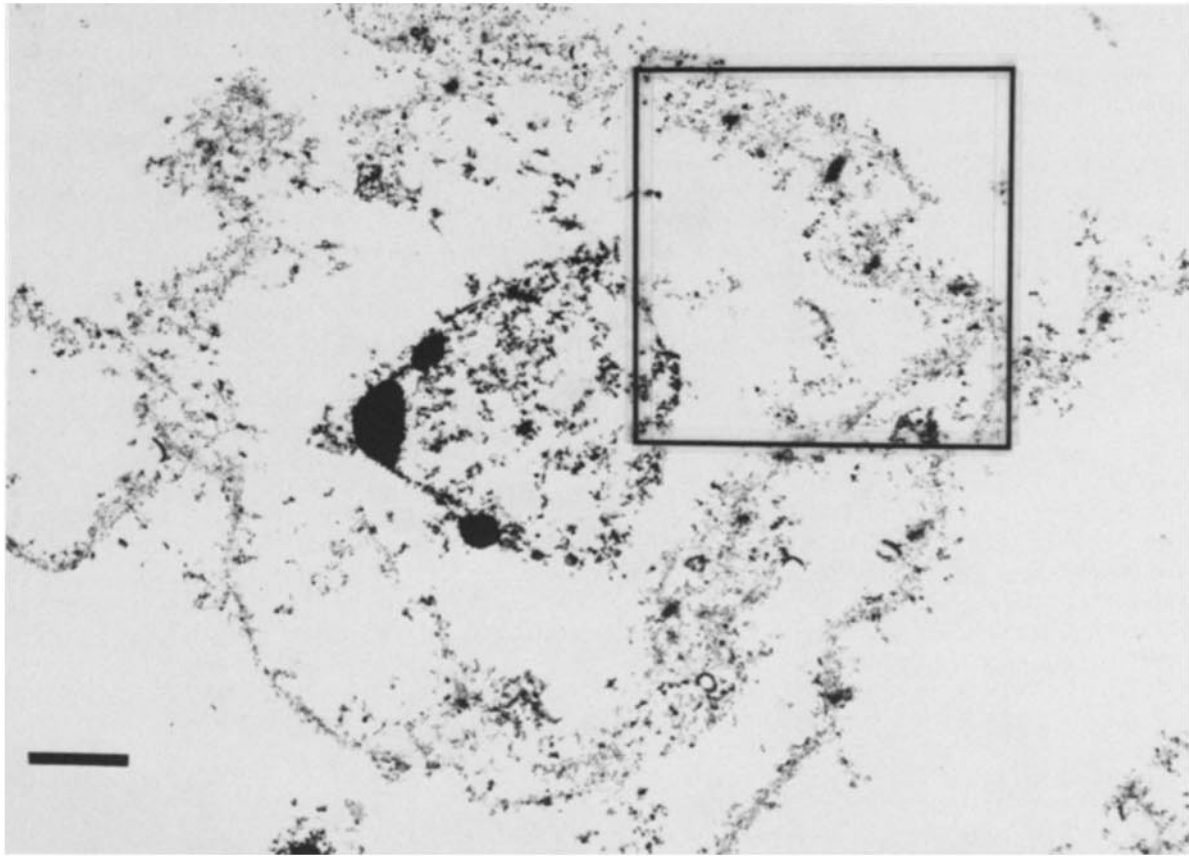
FIGURE 9   Thin section of a cytoskeleton prepared as in Fig. 8 at two magnifications. Bar, 1 µm. × 13,000. × 23,500.

DISCUSSION

The data presented here indicate that extraction of *D. discoideum* cells with nonionic detergents produces a cytoskeleton that retains specific cAMP-binding sites with properties that identify them as the cAMP chemotaxis receptors present on the surface of intact cells. The criteria for this identification include their developmental regulation and affinity and, more stringently, their high specificity for cAMP and the characteristic kinetics of cAMP binding and release (14, 21–25, 29, 41, 42). While *Dictyostelium* cells contain a developmentally regulated cAMP-binding protein that is the regulatory subunit of protein kinase (34) and a cGMP-binding protein (16, 22), neither of these has the high selectivity for cAMP vs. cGMP or the rapid rates of association and dissociation found for the cAMP chemotaxis receptor (34, 42). The mPDE has been assayed and is solubilized under conditions that result in retention of the cAMP receptor (Table III). In addition, the assays are performed in 10 mM dithiothreitol that completely inhibits the enzyme (25). A recently identified cAMP- and adenosine-binding protein (17) has much slower kinetics than the chemotaxis receptor and is inhibited by 5'-AMP which the cytoskeletal cAMP-binding protein is not. The fact that a receptor which communicates information to cytoskeletal components is found physically associated with this structure is highly suggestive of a functionally significant interaction. Further investigation will be required to establish a functional correlate of receptor-cytoskeleton association reported here. In this regard, McRobbie and Newell (40) have found that chemotactic stimulation of *D. discoideum* cells with folate or cAMP leads to a rapid (5 s) increase in the amount of actin that can be recovered with the cytoskeleton.

It is interesting that the number of receptors found after detergent extraction to produce cytoskeletons is actually higher than the number accessible for cAMP binding on intact cells. Depending on the extraction conditions, the increase is between 20 and 70%. That these receptors that are recovered in the cytoskeletal pellet contain as a subset the receptors initially present on the cell surface and are not a totally distinct pool of cryptic receptors is indicated by the results of permeabilization of cells with saponin. This compound permeabilizes membranes without dissolving them (60). The levels of cAMP binding in saponin-permeabilized and TX-100 extracted cells are about the same. If cell surface receptors were being lost in the TX-100 extraction, the binding of saponin-treated cells would be higher by the amount due to the cell surface receptors. Furthermore, cell fractionation studies carried out by us (29, 41) and others (16, 21, 22) have failed to reveal particulate cAMP-binding proteins other than the cAMP receptor, making it unlikely that a large class of cryptic cAMP-binding proteins with properties nearly identical to those of the chemotactic receptor has been discovered in this study. Thus, the additional receptors would appear to be somewhere inside the cell, yet still attached in some way to the cytoskeleton. This is an attractive idea since it has been suggested that receptors can become internalized upon binding cAMP (30), and receptors for N-formylated peptides on neutrophils appear to be rapidly internalized upon the binding of ligand (47, 48, 64). Alternatively, the association of receptors with the cytoskeleton as soon as they appear in development (Fig. 4) suggests that, from the time of synthesis, new receptors immediately attach to the cytoskeleton.

By varying the conditions of detergent extraction and pre-

treatment of the cells, we tried to deduce some information about the component or components of the cytoskeleton with which receptors associate. Cells are thoroughly chilled on ice before being extracted and assayed, and performing these steps at room temperature results in the recovery of little or no cAMP-binding activity with the cytoskeleton. Since *D. discoideum* microtubules are sensitive to depolymerization at low temperature (19, 70), it is unlikely that intact microtubules are the primary site of interaction of the receptor with the cytoskeleton. This conclusion is supported by the fact that pretreatment of cells for 1 h with high (0.1 mM) concentrations of colchicine (19) before detergent extraction has no effect on the retention of cAMP binding by the cytoskeletons. The enhanced recovery of activity in the presence of EGTA suggests that some Ca^{++}-dependent process results in receptor dissociation or dissolution of the cytoskeleton. Giffard et al. (23) found that EGTA was required for maximal recovery of insoluble actin in their procedure. Attempts to investigate the potential coupling of receptors to actin microfilaments have been equivocal. High concentrations of DNAse I (28, 38) causes some loss of actin from cytoskeletons but much remains, and this treatment has little effect on cAMP-binding activity (not shown). Painter and Ginsberg (50) found that some platelet actin was resistant to extraction from cytoskeletons with DNAse I and the retention of glycoproteins IIb and III was disproportionately high. Thus, when 90% of the actin was extracted, only ~50% of the IIb and III was removed (50), suggesting that DNAse I-resistant actin might be preferentially involved in anchorage of IIb and III. Recovery of cytoskeletal cAMP binding was also not affected by 1-h preincubation of cells with 10 μg/ml cytochalasin b before extraction (not shown), further suggesting that actin filament association may not be the anchorage mechanism. These negative experiments suggest that perhaps intermediate filaments (33) might be sites of receptor-cytoskeleton association. Unfortunately, virtually nothing is known about the properties and protein composition of slime mold intermediate filaments. Conditions that lead to dissolution of the cytoskeleton (Tris buffer, pH 8) without destroying the nucleus lead to loss of cAMP binding, indicating that the nucleus is probably not the site of cAMP receptor attachment. Recently, Dang et al. (15) have found methionyl-tRNA synthetase, an endoplasmic reticulum enzyme, to remain associated with the detergent-insoluble residue of PtK_2 cells. However, the enzyme does not co-localize with any of the filamentous structures, and they suggest that it is retained by a residue of the endoplasmic reticulum membrane (15). In the case of the cAMP receptor, the majority is in the plasma membrane, which is completely solubilized by both morphological and biochemical criteria. Thus, while some part of the intracellular receptor pool may be associated with the residue of internal membranes, it seems unlikely that the complement of cell surface receptors is trapped by poorly solubilized membrane material.

The pH dependence of the recovery of cAMP binding (Fig. 1) indicates a very acidic optimum of pH 6.1 for either retention of the receptor or the stability of the cytoskeletal component(s) with which it interacts. Using fluorescein diacetate, we found that *D. discoideum* cells maintain an intracellular pH of about 6.2 during early differentiation (G. A. Jamieson, W. A. Frazier and P. H. Schlesinger, manuscript in preparation). Thus the intracellular pH is close to the optimum for receptor-cytoskeleton association which, it should be noted, is somewhat more acidic than the optimum

found for cytoplasmic gelation (12) or actin cross-linking (13) by an M_r 95,000 protein from *Dictyostelium*.

There exists some precedent for the idea that receptors and their effector mechanisms may be associated with cytoskeletal elements. Sayhoun et al. (54, 55) have detected binding of a detergent-solubilized catalytic unit of adenylate cyclase to TX-100 extracted avian erythrocytes, and some evidence has been presented that favors the idea that the guanyl nucleotide-binding protein or G/F protein also interacts with the cytoskeleton (53). Direct data are not yet available on the association of the adrenergic receptor with the cytoskeleton, but photobleaching/recovery data indicate that in Chang liver cells the receptor is essentially immobile in the plane of the membrane (26), suggesting attachment to some submembranous, perhaps cytoskeletal, structure. As noted above, receptors for nerve growth factor (56, 67) and fibrinogen (50) can attach to the cytoskeleton, but it is not clear whether these receptors interact with effector mechanisms under these conditions.

Since the function of the cAMP receptor and other chemotactic receptors is to impose a polarity on the cell's contractile machinery or cytoskeleton (65, 73), it is intriguing that the cAMP receptor is found physically associated with this structure and that cAMP can cause an increase in cytoskeletal actin (40). The generality of chemotactic receptor-cytoskeletal interaction is suggested by our finding that the receptor for folate present on vegetative cells is also associated with the cytoskeleton (N. Galvin and W. Frazier, manuscript in preparation). Further study is required to determine the means by which these receptors associate with the detergent insoluble material. The protein components of this association and the factors that modulate it must be identified. Other proteins that have been postulated to be involved in chemotactic transduction events may also be bound to the cytoskeleton or may participate in the coupling of receptors to the cytoskeleton. These may include guanylate cyclase (22, 39, 71), calmodulin (22), and cGMP- (39) or guanyl nucleotide-binding proteins (34). Factors that modulate such receptor-cytoskeleton coupling may be those found to change as a result of chemotactic stimulation of cells such as intracellular cGMP and Ca^{++} levels, intracellular pH, phospholipid methylation and protein phosphorylation (21, 22, 39, 71). Since the cytoskeletal preparation is enriched for cAMP receptors and devoid of soluble proteins and nucleotide pools, it should provide a useful system with which to study receptor regulation and transduction.

REFERENCES

1. Aswanikumar, S., B. A. Corcoran, E. Schiffmann, A. R. Day, R. J. Freer, H. J. Showell, E. L. Becker, and C. B. Pert. 1977. Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochem. Biophys. Res. Commun.* 74:810–817.
2. Bartles, J. R., and W. A. Frazier. 1982. Discoidin I-membrane interactions. I. Discoidin I binds to two types of receptor on fixed *Dictyostelium discoideum* cells. *Biochim.*

Biophys. Acta. 687:121–128.
3. Barak, L. S., and R. R. Yocum. 1981. Nitrobenzoxadiazole phallacidin: synthesis of a fluorescent actin probe. *Anal. Biochem.* 110:31–38.
4. Barak, L. S., R. R. Yocum, and W. W. Webb. 1981. In vivo staining of cytoskeletal actin by autointernalization of nontoxic concentrations of nitrobenzoxadiazole-phallacidin. *J. Cell Biol.* 89:368–372.
5. Ben-Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. *Cell.* 17:859–865.
6. Bonner, J. T., D. S. Barkley, E. M. Hall, T. M. Konijn, J. W. Mason, G. O'Keefe III, and P. B. Wolfe. 1969. Acrasin, acrasinase and sensitivity to acrasin in *Dictyostelium discoideum. Dev. Biol.* 20:72–87.
7. Bourguignon, L. Y. W., and S. J. Singer. 1977. Transmembrane interactions and mechanism of capping of surface receptors by their specific ligands. *Proc. Natl. Acad. Sci. USA.* 74:5031–5035.
8. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye binding. *Anal. Biochem.* 72:248–254.
9. Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of duck embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119–130.
10. Burridge, K., and L. Jordan. 1979. Glycoproteins of *Dictyostelium discoideum*: Changes During Development. *Exptl. Cell. Res.* 124:31–38.
11. Condeelis, J. S. 1979. Isolation of con A caps during various stages of formation and their association with actin and myosin. *J. Cell Biol.* 80:751–758.
12. Condeelis, J. S., and D. L. Taylor. 1977. The contractile basis of ameboid movement. V. The control of gelation, solation, and contraction in extracts from *Dictyostelium discoideum. J. Cell Biol.* 74:901–927.
13. Condeelis, J., and M. Vahey. 1982. A calcium and pH-regulated protein from *Dictyostelium discoideum* that cross-links actin filaments. *J. Cell Biol.* 94:466–471.
14. Coukell, M. B. 1981. Apparent positive cooperativity at a surface cAMP receptor in *Dictyostelium. Differentiation.* 20:29–35.
15. Dang, C. V., D. C. H. Yang, and T. D. Pollard. 1983. Association of methionyl tRNA synthetase with detergent-insoluble components of the rough endoplasmic reticulum. *J. Cell Biol.* 96:1138–1147.
16. Devreotes, P. N. 1982. Chemotaxis. *In* The Development of *Dictyostelium discoideum*. W. F. Loomis, editor. Academic Press, Inc., New York. 117–168.
17. de Gunzburg, J., R. Hohman, D. Part, and M. Vernon 1983. Evidence that a cAMP binding protein from *Dictyostelium discoideum* carries S-adenosyl-L-homocysteine hydrolase activity. *Biochimie.* 65:33–41.
18. Eckert, B. S., and E. Lazarides. 1978. Localization of actin in *Dictyostelium* amebas by immunofluorescence. *J. Cell Biol.* 77:714–721.
19. Eckert, B. S., R. H. Warren, and R. W. Rubin. 1977. Structural and biochemical aspects of cell motility in amebas of *Dictyostelium discoideum. J. Cell Biol.* 72:339–350.
20. Finney, R., B. Varnum, and D. R. Soll. 1979. Erasure in *Dictyostelium*: a dedifferentiation involving the programmed loss of chemotactic functions. *Dev. Biol.* 73:290–303.
21. Frazier, W. A., S. G. Nandini-Kishore, and B. L. Meyers. 1982. Chemotactic receptors of *Dictyostelium discoideum. J. Cell. Biochem.* 18:181–196.
22. Gerisch, G. 1982. Chemotaxis in *Dictyostelium. Annu. Rev. Physiol.* 44:535–552.
23. Giffard, R. G., J. A. Spudich, and A. Spudich. 1983. Ca^{++}-sensitive isolation of a cortical actin matrix from *Dictyostelium* amoebae. *J. Muscle Res. Cell Motil.* 4:115–131.
24. Green, A. A., and P. C. Newell. 1975. Evidence for the existence of two types of cAMP binding sites in aggregating cells of *Dictyostelium discoideum. Cell.* 6:129–136.
25. Henderson, E. J. 1975. The cyclic adenosine 3':5'-monophosphate receptor of *Dictyostelium discoideum. J. Biol. Chem.* 250:4730–4736.
26. Henis, Y. I., M. Hekman, E. L. Elson, and E. J. M. Helmreich. 1982. Lateral motion of beta receptors in cultured liver cells. *Proc. Nat. Acad. Sci. USA.* 79:2907–2911.
27. Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86:212–234.
28. Hitchcock, S. E. 1976. Depolymerization of F-actin by deoxyribonuclease I. *Cell.* 7:531–542.
29. King, A. C., and W. A. Frazier. 1979. Properties of the oscillatory cAMP binding component of *Dictyostelium discoideum* cells and isolated plasma membranes. *J. Biol. Chem.* 254:7168–7176.
30. Klein, C. 1979. A Slowly Dissociating Form of the Cell Surface Cyclic Adenosine 3':5'-Monophosphate Receptor of *Dictyostelium discoideum J. Biol. Chem.* 254:12573–12578.
31. Klotz, I. M. 1982. Numbers of receptor sites from scatchard graphs: facts and fantasies. *Science (Wash. DC).* 217:1247–49.
32. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
33. Lazarides, E. 1982. Intermediate filaments: a chemically heterogeneous, developmentally regulated class of proteins. *Annu. Rev. Biochem.* 51:219–250.
34. Leichtling, B. H., I. H. Majerfeld, D. S. Coffman, and H. V. Rickenberg. 1982. Identification of the regulatory subunit of a cAMP dependent protein kinase in *Dictyostelium discoideum. Biochem. Biophys. Res. Commun.* 105:949–955.
35. Lenk, R., L. Ransom, Y. Kaufmann, and S. Penman. 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell.* 10:67–78.
36. Lowry, O. M., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
37. Luna, E. J., V. M. Fowler, J. Swanson, D. Branton, and D. L. Taylor. 1981. A membrane Cytoskeleton from *Dictyostelium discoideum.* I. Identification and partial characterization of an actin-binding activity. *J. Cell Biol.* 88:396–409.
38. Mannherz, H. G., J. Barrington-Leigh, R. Leberman, and H. Pfrang. 1975. A specific 1:1 G-actin:DNase I complex formed by the action of DNase I on F-actin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 60:34–38.
39. Mato, J. M., and T. M. Konijn. 1977. Chemotactic signaling and cGMP Accumulation in *Dictyostelium. In* Development and Differentiation in the Cellular Slime Molds. P. Cappuccinellie, and J. M. Ashworth, editors. Elsevier/North Holland, Amsterdam. 93–103.
40. McRobbie, S. J., and P. C. Newell. 1983. Changes in actin associated with the cytoskeleton following chemotactic stimulation of *Dictyostelium discoideum. Biochem. Biophys. Res. Commun.* 115:351–359.
41. Meyers, B. L., and W. A. Frazier. 1981. Solubilization and hydrophobic immunobilization assay of a cAMP binding protein from *Dictyostelium discoideum* plasma membranes. *Biochem. Biophys. Res. Commun.* 101:1011–1017.
42. Mullens, I. A., and P. C. Newell. 1978. cAMP binding to cell surface receptors of *Dictyostelium. Differentiation.* 10:171–176.
43. Nandini-Kishore, S. G., and W. A. Frazier. 1981. [3H]Methotrexate as a Ligand for the Folate Receptor of *Dictyostelium discoideum. Proc. Natl. Acad. Sci USA.* 78:7299–7303.
44. Neidel, J. 1981. Detergent solubilization of the formyl peptide chemotactic receptor. *J.*

Biol. Chem. 256:9295-9299.

45. Neidel, J. E., and P. Cuatrecasas. 1980. Formyl peptide chemotactic receptors of leukocytes and macrophages. *Curr. Top. Cell. Reg.* 17:137-170.

46. Neidel, J. E., J. Davis, and P. Cuatrecasas. 1980. Covalent affinity labeling of the formyl peptide chemotactic receptor. *J. Biol. Chem.* 255:7063-7066.

47. Neidel, J. E., I. Kahane, and P. Cuatrecasas. 1979. Receptor-mediated internalization of fluorescent chemotactic peptide by human neutrophils. *Science (Wash. DC).* 205:1412-1414.

48. Neidel, J., S. Wilkinson, and P. Cuatrecasas. 1979. Receptor-mediated uptake and degradation of [125-I]-chemotactic peptide by human neutrophils. *J. Biol. Chem.* 254:10700-10706.

49. Osborn, M., and K. Weber. 1977. The detergent resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. *Exp. Cell Res.* 106:339-349.

50. Painter, R. G., and M. Ginsberg, with B. Jaques. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:565-573.

51. Pan, P., E. M. Hall, and J. T. Bonner. 1972. Folic acid as the second chemotactic substance in the cellular slime moulds. *Nature New Biol.* 237:181-182.

52. Pan, P., E. M. Hall, and J. T. Bonner. 1975. Determination of the active portion of the folic acid molecule in cellular slime mold chemotaxis. *J. Bacteriol.* 122:185-191.

53. Sahyoun, N. E., H. Levine III, J. Davis, G. M. Hebdon, and P. Cuatrecasas. 1981. Molecular complexes involved in the regulation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA.* 78:6158-6162.

54. Sahyoun, N. E., H. LeVine III, G. M. Hebdon, R. Hemadah, and P. Cuatrecasas. 1981. Specific binding of solubilized adenylate cyclase to the erythrocyte cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 78:2359-2362.

55. Sahyoun, N. E., H. LeVine III, G. M. Hebdon, R. K. Khouri, and P. Cuatrecasas. 1981. Evidence for cytoskeletal associations of the adenylate cyclase system obtained by differential extraction of rat erythrocyte ghosts. *Biochem. Biophys. Res. Commun.* 101:1003-1010.

56. Schechter, A. L., and M. A. Bothwell. 1981. Nerve growth factor receptors on PC-12 cells: evidence for two receptor classes with differing cytoskeletal association. *Cell.* 24:867-874.

57. Schiffmann, E. 1982. Leukocyte chemotaxis. *Annu. Rev. Physiol.* 44:553-68.

58. Schliwa, M., and J. van Blerkom. 1981. Structural interactions of cytoskeletal components. *J. Cell Biol.* 90:222-235.

59. Schreiner, G. F., K. Fukiwara, T. D. Pollard, and E. R. Unanue. 1977. Redistribution of myosin accompanying capping of surface Ig. *J. Exp. Med.* 145:1393-1398.

60. Seeman, P. 1967. Transient holes in the erythrocyte membrane during hypotonic hemolysis and stable moles in the membrane after lysis by saponin and lysolecithin. *J. Cell Biol.* 32:55-70.

61. Shimomura, O., H. L. B. Suthers, and J. T. Bonner. 1982. Chemical identity of the acrasin of the cellular slime mold *Polysphondylium violaceum. Proc. Natl. Acad. Sci. USA.* 79:7376-7379.

62. Siu, C. H., R. A. Lerner, and W. F. Loomis, Jr. 1977. Rapid accumulation and disappearance of plasma membrane proteins during development of wild type and mutant strains of *Dictyostelium discoideum. J. Mol. Biol.* 116:469-488.

63. Spudich, J. A., and A. Spudich, 1982. Cell motility *In* Development of *Dictyostelium discoideum.* W. F. Loomis, editor. Academic Press, Inc., New York. 169-194.

64. Sullivan, S. J., and S. H. Zigmond. 1980. Chemotactic peptide receptor modulation in polymorphonuclear leukocytes. *J. Cell Biol.* 85:703-711.

65. Swanson, J. A., and D. L. Taylor. 1982. Local and spatially coordinated movements in *Dictyostelium discoideum* amoebae during chemotaxis. *Cell.* 28:225-232.

66. Tomchik, K. J., and P. N. Devreotes. 1981. Adenosine 3',5'-monophosphate waves in *Dictyostelium discoideum*: a demonstration by isotope dilution fluorography. *Science (Wash. DC).* 212:443-446.

67. Vale, R. D., and E. M. Shooter. 1982. Alteration of binding properties and cytoskeletal attachment of nerve growth factor receptors in PC-12 cells by wheat germ agglutinin. *J. Cell Biol.* 94:710-717.

68. van Haastert, P. J. M., R. J. W. de Witt, Y. Grijpma, and T. M. Konijn. 1982. Identification of a pterin as the acrasin of the cellular slime mold *Dictyostelium lacteum. Proc. Natl. Acad. Sci. USA.* 79:6270-6274.

69. Varnum, B., and D. R. Soll. 1981. Chemoresponsiveness to cAMP and folic acid during growth, development, and dedifferentiation in *Dictyostelium discoideum. Differentiation.* 18:151-160.

70. White, E., E. M. Tolbert, and E. R. Katz. 1983. The identification of tubulin in *Dictyostelium discoideum*: characterization of some unique properties. *J. Cell Biol.* In press.

71. Wurster, B., K. Schubiger, U. Wick, and G. Gerisch. 1977. Cyclic GMP in *Dictyostelium discoideum.* Oscillations and pulses in response to folic acid and cyclic AMP signals. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 76:141-144.

72. Zigmond, S. H. 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* 75:606-616.

73. Zigmond, S. H., H. I. Levitsky, and B. J. Kreel. 1981. Cell polarity: an examination of its behavioral expression and its consequences for polymorphonuclear leukocyte chemotaxis. *J. Cell Biol.* 89:585-592.

74. Zigmond, S. H. 1978 Chemotaxis by polymorphonuclear leukocytes. *J. Cell Biol.* 77:269-287.