Journal of Cellular Biochemistry 18:181-196 (1982)
Cellular Recognition 587-602

Chemotactic Receptors of Dictyostelium discoideum

William A. Frazier, S.G. Nandini-Kishore, and Beth L. Meyers

Departments of Biological Chemistry and Neurobiology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63110

For over 30 years, the cellular slime molds, and in particular the species Dictyostelium discoideum, have been viewed as a eukaryotic model system for the study of a variety of regulatory and developmental processes [1]. In particular, the transition from the free-living amoeboid stage to the multicellular slug stage has been the focus of a great deal of attention by those investigators studying the regulation of development [1-3]. The first morphological evidence that differentiation of a starved population of cells is proceeding on schedule is the appearance of ripples or streams in a lawn of cells resting on a moist surface [1-3]. This prelude to the readily identified tight aggregate stage indicates the functioning of the cells' endogenous chemosensory system. In 1969, Bonner and coworkers [4] identified the endogenously produced chemoattractant or acrasin of these cells as cyclic AMP (cAMP). While cAMP is widely encountered as an intracellular regulatory agent or second messenger, the Dictyostelia provide one of the few well documented examples of cAMP as an extracellular messenger molecule.

The Polysphondylia such as P violaceum and P pallidum appear to employ a nonnucleotide molecule as their acrasin during starvation induced chemotaxis. This compound has been studied by Bonner and coworkers [5] and has been tentatively identified as a peptide with a blocked amino terminus, although further characterization is required for unambiguous identification. Bonner's group has also been responsible for the identification of folic acid and pterin compounds as chemoattractants of vegetative Dictyostelium amoebae [6-8]. This identification of a nonacrasin chemoattractant raises the possibility of studying the transition from a vegetative to a developmental mode of chemotaxis.

DEVELOPMENTAL CHEMOTAXIS IN D discoideum

The phenomenology of the cAMP chemotactic system in D discoideum has been well established [9,10]. It is convenient for descriptive purposes to imagine

Received August 3, 1981; revised and accepted October 12, 1981.

0730-2312/82/1802-0181$04.50

the chain of events which is initiated by the pulsatile release of cAMP [11] from cells at the center of an aggregation territory. The radially propagated [12] wave of cAMP [13] impinges on immediately outlying cells and binds to their cell surface receptors for the nucleotide [14–18]. These cAMP receptors are developmentally regulated and increase in number, reaching a maximum at about 6 h of differentiation [15,16,18]. The receptor binding event generates several transmembrane or intracellular responses within a few seconds of receptor stimulation. The most immediate response so far identified is the rapid accumulation and decline of intracellular cGMP [19,20]. This response is found in all species of slime mold, even those which use other chemoattractants [21,22] and thus is thought to be a general and causal event in signal transduction. At about the same time (10 sec) as the intracellular cGMP spike, the cell can be seen to begin extending a region of membrane toward the stimulus [23]. About 30 sec later, the cell's adenyl cyclase is activated [24–27] and intracellular cAMP accumulates. This increase is also transient as the cells quickly secrete the cAMP, thus generating the signal relay pulse [13,28]. It has been suggested that the adenyl cyclase of these cells is contained on or within membrane vesicles which sequester the newly synthesized cAMP and then fuse with the plasma membrane to provide rapid and efficient secretion of the cAMP as a pulse [29]. The function of this stimulus-secretion loop is of course to relay the cAMP signal to outlying cells in the aggregation territory thus allowing cells to communicate over distances up to 1 cm, 1,000 times the cell's diameter. Tomchik and Devreotes [30] have used a competitive, filter blotting technique to directly visualize waves of cAMP propagating from aggregation centers. Their data proves that the postulated waves of cAMP do in fact exist, and the fluographic images allow quantitative data on cAMP concentrations to be derived [30]. At the chemotactic stage of development, the cells have also synthesized a phosphodiesterase (PDE) which is specific for 3′-5′ nucleotides and serves the function of rapidly hydrolyzing extracellular cAMP [31,32]. Thus the enzyme maintains a high signal to noise ratio and prevents the system from becoming swamped in extracellular cAMP. It may well be that the PDE serves a much more subtle role in regulating the levels of cAMP impinging on the cell surface, thus aiding in the timing of the propagation of the response in order to maintain directional cell movement toward the aggregation center while allowing propagation of the wave of simulus [30] in the opposite direction.

In fact, this functional bidirectionality of the chemotactic system is somewhat enigmatic. From extensive time lapse cinematographic studies of aggregating cells, Robertson and coworkers [33] and others [12] have been forced to postulate a refractory period during which the cells which have just responded to the chemotactic signal remain unresponsive for a period of about 1 to 5 min duration. This seems necessary to account for the fact that cells which have moved toward a center do not move in an outward direction when the immediately outlying cells secrete cAMP in response to the propagated stimulus [33]. However,it has been impossible to detect such a refractory period in cell movement using direct stimulation of cells with a micropipette loaded with cAMP [23]. Cells seem able to respond to a new stimulus from the opposite direction after as little as 5 to 10 sec [23]. Further, Devreotes and coworkers [34] have not detected a refractory period in the ability of cells to secrete cAMP in response to a cAMP stimulus, so that relay also does not appear to have a refractory period when a population of cells is

tested that are not actively engaged in chemotaxis. However, using micropipette stimulation of aggregating cells, Robertson and Drage [35] found an apparent refractory period for relay of 6 to 9 min in cells near the beginning of differentiation and a shorter period of less than 3 min in cells differentiated longer. It thus seems possible or even likely that a population of cells entrained by an aggregation center may well develop properties in terms of the regulation of the chemotactic or relay response which are not evidenced by isolated single cells or even by cell populations which are not normally communicating. It seems reasonable to speculate that the cells may "learn" a polarity or a response period after repeated in situ stimulation and that this response has not yet been duplicated in suspensions of cells or in chemotactically responding single cells.

An interesting and useful property of the cAMP chemotactic system of D discoideum is the fact that chemotactically competent cells both secrete and respond to cAMP. Thus when cells are placed in stirred suspension they become synchronized. This phenomenon was first observed by Gerisch and Hess [36] who found that an oxygenated cell suspension in a cuvette monitored in a spectrophotometer produced periodic changes in light scattering or turbidity. This oscillating light scattering signal could be modulated by exogenously applied cAMP [36] suggesting that it was in some way linked to the chemotactic system. These oscillations in turbidity apparently result from synchronous changes in cell shape which occur as the cells are stimulated by endogenous cAMP pulses and mimic the elongation of cells which is seen when they are stimulated with cAMP on a surface [12,23]. The oscillations in light scattering thus provide a convenient reference time scale for examining other components of the system. Thus, it has been found that the frequency of the oscillations in light scattering are first detectable in early differentiation at a frequency of one peak every 10 to 12 min. Later, the period drops to 4 to 6 min and at some critical point, the frequency drops further to about 2 min and remains constant thereafter [37]. It is also seen that intracellular cGMP levels [19] oscillate with the same frequency as the turbidity, as do intracellular and extracellular cAMP levels [19] and adenyl cyclase activity [27]. In addition, recordings of extracellular pH are also seen to oscillate [38] with the same frequency, and thus extrusion of hydrogen ions has been invoked as a part of the intracellular response mechanism. Intracellular cGMP levels are transiently elevated by stimulation of responsive cells with both cAMP and folate [39], leading to the suggestion that cGMP is a common second messenger in the transduction of chemotactic signals [21,38]. Recently, changes in protein and lipid methylation [40] have been implicated as potential intracellular regulatory events in the chemotactic response [41].

VEGETATIVE CHEMOTAXIS OF D discoideum

It is now appreciated that vegetative or undifferentiated D discoideum cells are chemotactically responsive to folate, pterin, and related compounds [6–8]. Since bacteria liberate folate-like substances [6], it is felt that this folate chemotactic system is a food finding mechanism. However, axenic strains of D discoideum accumulate or synthesize large amounts of folate-like compounds, and this material is secreted during differentiation [7]. It is not known whether this secretion is pulsatile or if folate plays any role during the early differentiation of the cells.

Recently it has been shown that folate pulses can accelerate the appearance of the PDE, and aggregation competence [42]. When cells have developed adenyl cyclase activity and retain a folate response, stimulation with folate can cause an artificial relay or secretion of cAMP [43]. As noted above, stimulation of vegetative cells with folate causes the transient rise of intracellular cGMP [39,43]. There is a degradative enzyme associated with the folate chemotactic system, a folate (or pterin) deaminase (8) that has a similar cellular distribution [44,45] and other properties to those of the PDE [Frazier et al, unpublished]. As expected, a receptor for folate and its analogs is present on the surface of vegetative cells [46,47]. However, until very recently, its characterization has been hindered by the fact that no useful inhibitors of the folate deaminase have been found [46,47] (see below). The mechanistic similarities and the potential structural and functional relationships between the folate and cAMP chemotactic systems, have led us to undertake a study of the folate receptor as described below. The main focus of our laboratory is on the properties of the receptors involved in these chemotactic responses. The remainder of this article will summarize work primarily from our laboratory on the cAMP and folate receptor systems.

THE CYCLIC AMP RECEPTOR

Cyclic AMP Binding Properties of Intact Cells

The binding of cyclic AMP to its receptor and the dissociation of the bound nucleotide are both extremely rapid processes [17,18,48]. It is this feature of the cyclic AMP chemotactic receptor system that makes it difficult to study with conventional assay techniques. The binding reaction occurs on the time scale noted for the association of small molecules with enzymes and it is impossible with any available assay to detect intermediate levels of receptor-bound cAMP after the start of a binding experiment, even if time points are taken as early as 1 to 2 sec. The dissociation rate, while also very rapid, can be estimated and has a half-time of dissociation of 2 to 5 sec. depending on assay methods [9,10,18,48]. We initially investigated the time course of [^{3}H]-cAMP binding to stirred suspensions of chemotactically competent cells using a rapid vacuum filtration assay in which cells are deposited on polycarbonate filters and washed in less than 2 sec. Like other laboratories, we use dithiothreitol at 10 mM to inhibit the PDE [17,18]. If samples are taken at intervals of 30 sec or less over a total time period of 10 to 20 min, it is seen that the amount of cAMP bound to cells does not reach a steady state or plateau level, but appears to oscillate with time [17,18]. Experiments in which unlabeled cAMP is included in the assay to assess specificity of binding indicate that the peaks or maxima of binding are specific and that the level of binding defined by the minima represents nonspecific binding to cells and filters. We suggested that the oscillations in binding may represent rapid changes in the affinity of a population of cell surface receptors rather than the internalization and reappearance of receptors [18]. A mechanism whereby the receptors remain on the cell surface but are transiently covered or masked can not, of course, be distinguished from rapid changes in affinity. That the binding affinity or capacity of the cell surface receptor for cAMP might oscillate, was not a terribly surprising idea, since as noted above, nearly every other component of this chemotactic system had been found to show oscillatory behavior in appropriate assays. With

the rapid filtration assay, data can be analyzed in terms of amplitude of maxima (peak height) and periodicity of maxima, or data from a time course experiment can be treated in a pseudo steady state manner such that total specific binding is averaged over the entire time course. In any experiment to determine the level of binding, time courses of at least 10 min are always performed to insure that a representative sampling of data (20 to 40 points) is taken.

The cAMP binding component of intact cells has the properties expected for the cAMP chemotactic receptor. When measured as amplitude of maxima or as time-averaged (pseudo steady-state) specific binding, this binding component increases in parallel with the acquisition of chemotactic competence as a function of differentiation for 6 to 7 h [18] and decreases with further differentiation to the tight aggregate stage [16]. As noted in the introduction, oscillations in light scattering, which represent functioning of the chemotactic system in a suspension of cells, decrease in period during development to a minimum period of 2 min [37]. In some experiments, we have been able to detect oscillations in cAMP binding with a shorter period of 3 to 4 min in 3 to 5 h differentiated cells, but with times of differentiation longer than 5 h, we find a period of 2 min between the maxima of binding [18,49]. Nucleotides that are not chemoattractants do not compete for the maxima in cAMP binding, while those that are less potent attractants such as cUMP and cXMP partially reduce the cAMP binding maxima [18].

In thinking about how the receptor affinity might be regulated in an oscillatory way, we were struck by the analogy to some kinase/phosphatase regulatory systems in which rapid phosphorylation and dephosphorylation of enzymes can alter their catalytic properties in a cyclic manner. In addition, the role of cAMP as a causal agent in these systems was intriguing. We thus performed a standard time course binding assay with [^{3}H]-cAMP in the presence of gamma [^{32}P]-ATP added externally to the intact cells. Not only does the incorporation of [^{32}P] radioactivity oscillate, but its maxima are precisely out of phase with the maxima of cAMP binding [17]. This result suggested a kinase/phosphatase regulation of the receptor. However, no proteins become covalently labeled with [^{32}P] with either an oscillatory or steady-state time course until after substantial degradation of ATP has occurred (20 to 30 min) [49], and the [^{32}P]is taken up by cells [50]. Instead, the [^{32}P] is transferred to a membrane-bound small molecule, which can be dissociated from the membrane with heat, acid, base or SDS treatment [49]. Identification of this phosphate acceptor molecule is currently being pursued. Transfer of radioactivity to membranes only occurs if the label is in the gamma phosphate of ATP, and [^{3}H]-ATP binds to cells with steady-state kinetics at levels 10-fold lower than the maxima of phosphate transfer [49]. These data suggest that the reaction is enzymatically mediated. Parish and Weibel [51] have found a cell-surface ATPase activity in D discoideum cells, but they did not investigate the possibility that the cleaved phosphate moiety was transferred to a membrane-associated acceptor. They also confirmed an earlier observation by L.J. Wallace (unpublished data) that the cells actively maintain an extracellular ATP concentration in the range of 0.1 to 1 uM.

More recently, we have attempted to develop assay methods other than the filtration assay with the time resolution necessary to detect oscillations in [^{3}H]-cAMP binding to intact cells. It should be noted that none of the methods employed by other workers who have studied this receptor have been sufficiently rap-

id to detect the oscillations we reported using the filtration assay [14–16,52]. We have modified a binding assay used by Stahl and co-workers [53] which employs microfuge centrifugation of a cell suspension layered above an oil cushion. The density of the oil is adjusted by mixing silicone and mineral oil such that cells centrifuge through the water/oil interface very quickly after the centrifuge is started. Duplicate samples can be taken as early as 5 sec of incubation and the cells are spun out of the aqueous layer within 2 sec after starting the centrifuge. With this assay, the on rate of [^{3}H]-cAMP binding is also found to be immeasurably fast, and the half time of dissociation is about 5 sec. Time course experiments in which duplicate samples are taken at 20- or 30-sec intervals reveal a constant, nonoscillatory level of cAMP binding. This plateau corresponds to the amount of cAMP bound at the maxima observed with the filtration assay (see above). The primary difference between the two assays is that the filtration assay incorporates a wash step, while the centrifugation assay does not. Hence the centrifugation assay may not detect differences in binding affinity, particularly if the lower affinity binding state is brought about by a reduction in on rate, rather than an increase in the off rate. We have attempted to resolve this issue by incorporating a third layer of aqueous wash buffer in the centrifugation assay. Due to volume restrictions, the resulting wash is very rapid. With this modification we also do not observe oscillations in binding time course data. It should be noted that the two assays are somewhat different in precision, the centrifugation assay yielding very good duplicates while the filtration assay is less precise. Thus, it seems possible that even slight variations in the filtration rate from one filter to the next during the washing step may allow dissociation of the bound ligand to occur in some time points and not others. Arguing aginst this artifactual and trivial interpretation of this data is the fact that double-label experiments with cAMP and ATP indicated inverse oscillatory association of the two labels with cells [17]. If minima in cAMP binding simply reflect low recovery due to slow filtration, the ATP radioactivity should yield parallel rather than inverse binding levels. In this context, the centrifugation assay also reveals steady-state binding of radioactivity from gamma-[^{32}P]-ATP, perhaps indicating that some aspect of this assay prevents the detection of oscillations in the binding of both cAMP and ATP.

Thus, we have as yet been unable to confirm with another method the validity of the oscillatory behavior of the cAMP receptor. However, we point out that all conclusions regarding the levels or numbers of cAMP receptors, their affinity, nucleotide specificity, developmental time course, sensitivity to treatments, and other properties are independent of assay method. Since we have always treated data from filtration assay in terms of both time averaged, pseudo-steady-state and averaged maximum amplitudes, it is only the details of the kinetic behavior of the receptor which in our hands appear to be assay dependent.

Photoaffinity Labeling Studies

The rapid association and dissociation kinetics of the chemotactic cAMP receptor made it extremely desirable to devise a means of attaching a stable, covalent radioactive label to the receptor to aid in further characterization and isolation. Such an approach was suggested by the success of the use by Haley and coworkers of 8-azido-cAMP as a photoaffinity labeling reagent for intracellular cAMP binding proteins such as the regulatory subunit of the cAMP-dependent

protein kinase from a variety of tissues [54-57]. The azido compound was particularly attractive since from cAMP analog studies [58] it was appreciated that substitution of the cAMP structure at any position reduced its affinity or efficacy at the receptor. It was hoped that the relatively small azido substituent at the C-8 position would minimally compromise receptor affinity for the label.

Initial studies with the nonradioactive 8-azido-cAMP established that the compound is a chemoattractant of D discoideum cells in the appropriate stage of differentiation [59] and that the slime mold PDE readily hydrolyzes the compound to 8-azido 5′-AMP [59,60]. Unfortunately, the potency of the affinity label as a chemoattractant is about 100-fold lower than that of cAMP [59]. In practical terms this means that concentrations of affinity label in excess of 10^{-7} M are required to give labeling of a receptor candidate to the extent that a band on an SDS gel can be visualized after autoradiography [59]. At these concentrations, nonspecific or nonaffinity labeling becomes a serious problem, and interpretation of the results is difficult. In addition, the PDE activity is problematic since the only effective inhibitor of the enzyme is dithiothreitol at concentrations above 10 mM, which effectively reduces the azido function of the labeling reagent to an amino group [61].

Wallace and Frazier [59] used the 8-azido-[^{32}P]-cAMP reagent at 0°C in the presence of cGMP to obtain labeling of a protein with an Mr of 40,000 on SDS-gel electrophoresis. Although many other proteins were labeled, only the Mr 40,000 protein was specifically labeled. Neither cGMP nor a variety of nonchemotactic nucleotides inhibited labeling, while cAMP prevented labeling at low concentrations. In the absence of cGMP, little or no labeling of the Mr 40,000 protein was seen, and a protein of Mr 48,000 was labeled. The PDE is now known to have an Mr of 48,000 [32]. The Mr 40,000 protein was developmentally regulated with the appropriate time course, including its disappearance from the membrane after chemotaxis. Labeling of the protein could not be detected at reagent concentrations below 10^{-7} M. Specific labeling of the Mr 40,000 protein could not be detected in plasma membrane preparations due to the tremendous increase in nonspecific labeling of contaminants and cytoplasmic face proteins [59,60]. The PDE of plasma membranes converted the label to the 5′-AMP form which was found to efficiently label membrane associated actin at a nucleotide site that may be distinct from the ATP/ADP site [60]. Soluble actin was only labeled if the extract was allowed to undergo endogenous proteolysis [60]. More recently, Juliani and Klein [62] have reported the use of the same reagent to label a protein of Mr 45,000 in a PDE-deficient mutant. Upon treatment of the cells with high concentrations of cAMP which causes a decrease in the number of cAMP binding sites, the amount of label associated with the Mr 45,000 protein is reduced and a protein of Mr 47,000 is labeled [62]. These data are very difficult to interpret due to the overwhelming amount of nonspecific labeling on the autoradiograms presented [62]. The disparity of 5,000 in the molecular weights of the putative receptors identified in our studies and those of Klein and Juliani [62] is as yet difficult to explain. However, further photoaffinity labeling studies with the detergent solubilized and partially purified receptor (see below) offer a possible explanation. Difficulties with nonspecific labeling have not made this approach as rewarding as initially hoped as a means of further receptor characterization. Thus, studies were continued using [^{3}H]-cAMP binding as a receptor assay for isolated plasma membranes and detergent solubilized preparations.

Cyclic AMP Binding Properties of Isolated Plasma Membranes

To further investigate the properties and mechanism of the cAMP receptor, we sought to prepare plasma membranes that retained specific cAMP binding. By controlling proteolysis with alkaline buffers such as Tris-HCl, pH 8.1, and with phenylmethanesulfonyl fluoride (PMSF), we have prepared plasma membranes with both a discontinuous sucrose gradient method (18) and an aqueous two phase polymer method (63) which exhibit specific cAMP binding. Binding is assayed with the rapid filtration method using 0.2 μm pore diameter filters. We have recently found an oil density that allows the centrifugation assay (see above) to be used with plasma membranes, but the assay is neither as rapid nor precise as it is with intact cells. The receptor in isolated membranes displays many of the same properties as that on intact cells, including apparent oscillatory binding kinetics in the filtration assay.

The nucleotide specificity of the receptor in isolated plasma membranes is the same as that found for the receptor on intact cells [18] with the possible exception that the selectivity for cAMP versus cGMP appears to be decreased slightly. The cAMP binding activity of membranes is not affected by varying levels of Ca^{++} or Mg^{++} or the presence of EDTA or EGTA. Specific binding is abolished by chymotrypsin or pronase but not by trypsin treatment, a result also found with intact cells [49]. Binding studies conducted as a function of pH indicate a broad optimum from pH 6 to 7.5 [49]. Specific bindng is abolished by heating the membranes at 80°C for 10 min [18]. In the course of experiments aimed at stabilization of the receptor in plasma membrane preparations, we found that sulfonyl fluorides such as phenylmethyl sulfonyl fluoride (PMSF) and DANSYL fluoride inhibit binding to the membrane bound receptor, but in a reversible manner, suggesting that the mode of inhibition does not involve covalent reaction with the receptor. Inhibition is concentration-dependent, being half-maximal at 10 μM. At present, other protease inhibitors are being tested for potential covalent derivatization of the receptor. Interestingly, it has been reported that TPCK and the chymotrypsin subtrates phenylalanine and tyrosine benzyl ester tosylate will all irreversibly inhibit binding to the NFM peptide receptor of human neutrophils and their membranes [64]. PMSF and TLCK have no direct effect on the neutrophil NFM-peptide receptor [64].

Solubilization of the cAMP Receptor

Treatment of plasma membranes from chemotactically competent cells with nonionic detergents leads to the loss of specific cAMP binding activity [18]. To determine if this represented release of receptor from the membrane in a soluble state, it was necessary to develop an assay for detergent solubilized receptor. Because of the very fast off rates of the receptor, all available assays for detergent solubilized receptors were too slow in separation of bound and free ligand to allow detection of binding. We initially employed an assay in which the soluble detergent extract was bound to DEAE-filters [49]. Although specific binding to a component of the detergent extract could be detected with this method, background or nonspecific binding was extremely high due to the direct interaction of the negatively charged cAMP with the positively charged DEAE-filters. In fact, we found that a fraction of the specific binding was due to high affinity sites for cAMP on the filters themselves. We have recently developed an assay [63]

for solubilized receptors based on the hydrophobic binding of membrane proteins to agarose beads derivatized with alkyl chains or other hydrophobic groups [65-67]. A detergent extract of membranes is prepared with 0.5% Emulphogene BC-720 (polyoxyethylene 10 tridecyl ether, E-720). This extract is then diluted with a suspension of hydrophobically derivatized agarose beads and [^{3}H]-cAMP which contains no detergent, thus reducing the detergent concentration to a value near the CMC. Under these conditions a variety of membrane proteins insert among the hydrophobic groups and become noncovalently bound to the matrix. Subsequent elution of the beads with detergent has been used to partially purify membrane proteins [67]. To our knowledge, this is the first use of this method to provide an immobilized membrane protein preparation for use as a rapid and convenient assay system for a detergent solubilized receptor. To determine nonspecific binding, an excess of unlabeled cAMP is included. After incubation, the bound and free ligand can be rapidly separated by filtration on large pore size (2 to 10 μM) polycarbonate filters.

We first examined the influence of the chain length of the alkyl groups on the agarose on the level of specific and nonspecific cAMP binding observed when an E-720 extract of 6 h differentiated membranes was immobilized. At alkyl chain lengths of 2 to 6 carbons, nonspecific binding is high, but drops precipitously as the alkyl chain length on the agarose is increased from 6 to 10 carbons. A ten-carbon chain gave the optimal ratio of specific to nonspecific binding. In addition to the better ratio of specific to nonspecific binding, further advantages of this assay over the DEAE filter method include good precision among replicates, lack of sensitivity to changes in ionic strength and no "specific" cAMP binding in the absence of immobilized membrane proteins. Since the hydrophobic binding of detergent solubilized membrane proteins to alkyl agarose is a rather general phenomennon, this approach to the assay of solubilized receptors should be widely applicable to a variety of systems.

Using this hydrophobic immobilization technique in conjunction with filtration, we have proceeded to characterize the cAMP binding site detected in detergent extracts of plasma membranes from chemotactically competent cells. The choice of 0.5% E-720 is critical since lower concentrations do not solubilize the activity and higher concentrations lead to rapid proteolysis of the solubilized binding site (63). The rates of association and dissociation are very rapid, on the order of a few seconds. Many association time course experiments have been carried out for 10 to 20 min to determine if the solubilized cAMP binding site displays oscillatory behavior. While some variations in binding levels with time were noted in some experiments, these lack regular periodicity and most experiments appear steady state in character. Thus, it seems that the detergent solubilized cAMP binding site detected in these experiments does not bind cAMP in an oscillatory manner. Preliminary nucleotide specificity studies indicate that, like the cell surface receptor, this binding site has a marked preference for cAMP rather than cGMP and noncyclic nucleotides do not compete for binding [63]. The apparent affinity of the solubilized binding site is very similar to that found for the cAMP receptor on intact cells and plasma membranes [15-18,48], the dissociation constant being about 10 nM.

While the mPDE is solubilized from membranes under these conditions and also becomes bound to the decyl-agarose beads (H. Blair and W.A. Frazier, un-

published), the binding site we detect is distinct from mPDE by four criteria: [1] dithiothreitol is an effective inhibitor of detergent solubilized mPDE [49] and is included in the assay, [2] the affinity for cAMP is greater than that of the mPDE [32], [3] the mPDE and the cAMP binding site can by physically separated by ion exchange chromatography on DEAE cellulose, and [4] sulfonyl fluorides inhibit cAMP binding to plasma membranes and to the detergent solubilized binding site, but do not inhibit the mPDE.

The inhibition of cAMP binding by PMSF and DANSYL fluoride is a novel and potentially useful property of the cAMP receptor. Thus far, we have not found conditions that lead to the irreversible inactivation of the receptor, but the reversible inhibition is a rather uncommon property that serves to help identify the detergent solubilized binding site as the receptor characterized on plasma membranes. Inhibition of both the membrane-bound and detergent-solubilized cAMP binding protein is concentration dependent with half-maximal inhibition at about 10 uM PMSF or DANSYL fluoride. Attempts are being made to use more reactive reagents to covalently label the site of sulfonyl fluoride interaction.

Partial purification of the detergent solubilized cAMP binding site has been achieved using DEAE cellulose chromatography. The binding site detected in detergent extracts with the decyl-agarose assay binds very tightly to DEAE cellulose and thus is cleanly separated from the bulk of the proteins in the detergent extract, including the PDE. Silver staining of SDS gels indicates that only a few proteins are found in the active fraction. We have also performed photoaffinity labeling experiments [59,60] with the detergent extract of plasma membranes and on the partially purified fractions from the DEAE column. Several proteins in the extract are labeled by 8-azido-cAMP, three of them specifically. These have molecular weights of 40,000, 45,000, and 70,000. The Mr 70,000 protein appears to be the protein responsible for the great majority of the cAMP binding activity in the detergent extract. Thus, it may be that both the Mr 40,000 protein identified by Wallace and Frazier [59] and the Mr 45,000 protein identified by Juliani and Klein [62] are relatively stable proteolytic fragments of the Mr 70,000 protein. Experiments to test this idea are now in progress. The three radioactive, affinity labeled proteins will be convenient reagents with which to screen for monoclonal antibodies for use in further receptor characterization and purification.

THE FOLATE CHEMOTACTIC SYSTEM

Folate Deaminase

Vegetative cells chemotax to folic acid and several related compounds such as pterin. Like the cAMP chemotactic system, the components of this folate chemotactic system include a degradative enzyme, in this case a folate deaminase [8,44,45]. We have undertaken studies of this enzyme with the aim of controlling its activity so that radioligand binding assays of the chemotactic receptor can be performed in the absence of ligand degradation. Thin layer [8] and column [44] chromatographic methods have been employed to separate radiolabeled folate and the product of the enzyme 2,4-diamino folate. We have developed simple and reliable continuous spectrophotometric and fluorometric assays for the folate deaminase, and have characterized the enzyme [W.A. Frazier et al, submitted]. The axenic strain A3 has ten times the level of enzyme activity found in the wild type NC-4. Enzyme activity does not change dramatically during growth, but when

cells are differentiated in suspension, a soluble, extracellular form of the enzyme is released. The cell associated enzyme is nearly all found on the external face of the plasma membrane. The membrane enzyme is quantitatively solubilized with nonionic detergent. Both the soluble, extracellular enzyme and the detergent solubilized enzyme have identical Km values of 1.4×10^{-5} M folate. Like the extracellular PDE, the soluble deaminase is heterogeneous in molecular size and in isoelectric point with a variety of acidic species.

During this study, a variety of potential enzyme inhibitors such as substrate analogs, reducing agents and chemical modification reagents were found to be ineffective or too harsh for inclusion in ligand binding assays. We did find however, that the folate analogs methotrexate (MTX) and aminopterin, both 2,4-diaminopterin compounds, inhibited the deamination of folate. Both UV-visible spectral analysis and HPLC analysis indicated that these compounds were not acted upon by the deaminase preparation or by any other enzymes present in cell lysates or in the extracellular medium. These data suggested that radiolabeled MTX might be a useful ligand for binding studies of the folate receptor [68].

Chemotactic Activity of Methotrexate (MTX)

Using a semiquantitative chemotaxis assay developed in our laboratory [59], we tested both MTX and aminopterin for their ability to attract cells differentiated for increasing times from the vegetative state through the time when they have acquired a chemotactic response to cAMP. This assay detects both oriented cell movement (chemotaxis) and the stimulation of random cell motility rate (chemokinesis) [68]. Cells at each time of development were tested against gradients of known concentrations of MTX and of cAMP [68]. Vegetative (0 h) cells respond directionally to MTX and the response is of the same magnitude as their response to folate. These cells do not respond at all to cAMP. After 3 h of development, the response to MTX is slightly less well oriented and there is a slight random or chemokinetic response to cAMP. By 7 h the cAMP response is large and well oriented, while the response to MTX, though still extensive in terms of cell number, has become radial rather than oriented. At 9 h the magnitude of the radial MTX response is diminished, and the cAMP response remains directional. Varnum and Soll [69] have found that if cells in the state corresponding to 7 h are refed to erase [70] the differentiated state, they rapidly interchange their responses to cAMP and folate (MTX) such that the cAMP response becomes radial or chemokinetic and the folate response becomes directional or chemotactic.

These data indicate that even though MTX (and aminopterin) are not degraded they can elicit a chemotactic response, thus ruling out the obligatory participation of signal degradation in the chemotactic response. Further, they suggest that MTX and folate probably interact with the same chemotactic receptor. This notion is strengthened by the observation that a constant level of MTX or folate in the agar assay plates prevents the chemotactic response to a gradient of either compound. The fact that both the cAMP response and the folate response can be chemotactic or chemokinetic at different times suggests that each receptor can exist in two states of coupling to the intracellular apparatus responsible for the generation and direction of motility. The rapid change of coupling state seen

by Varnum and Soll [69] may indicate that both receptors use the same transmembrane pathways for these processes, and that the cells' developmental state dictates which receptor will be coupled to directional motility (chemotaxis) and which to motility rate stimulation (chemokinesis). These considerations make the D discoideum chemotactic systems an easily manipulated and experimentally accessible model for studying the mechanism(s) by which chemotactic receptors are coupled to the transducer molecules which then communicate with the cells contractile apparatus.

Radioligand Binding Studies of the Folate Receptor

As expected from the data presented above, [^{3}H]-MTX has proven to be the ligand of choice for the study of the folate receptor. Not only is it stable to enzymatic attack during the course of incubation with cells, but it is also much more stable to light and oxygen than folate, and the tritiated compound yields much lower levels of nonspecific binding than tritiated folate [68]. The labeled MTX binds to cells rapidly, reaching a maximal level by 1 min. Binding is constant for up to 30 min showing no apparent oscillations and if an excess of unlabeled MTX or folate is added, more than 90% of the binding is rapidly (less than 15 sec) reversed, indicating that MTX is not internalized. While binding assays are routinely conducted at low temperature, cells also fail to internalize MTX or folate at room temperature. Binding studies have been conducted with both the filtration and centrifugation assays (see above) with identical results. In equilibrium binding experiments, NC-4 cells were found to have many more receptors than axenic A3 cells. Scatchard analysis of the binding data yield a dissociation constant in the range of 20 to 100 nM MTX. We have directly measured the local concentration of [^{3}H]-MTX in chemotaxis assay plates and find that cells respond to a gradient of MTX when the local concentration is as low as 5×10^{-9} M or as high as 10^{-6} M. If the cells detect a spatial gradient, they do so remarkably well, since at 10^{-7} M, they detect a difference in concentration across a cell diameter of only 0.5%. This is on the order of the figure of 1% difference in NFM-peptide concentration found for polymorphonuclear leukocytes by Zigmond [71].

Binding studies as a function of differentiation were performed in parallel with the motility experiment described above. These indicated that the number of receptors on NC-4 cells decreases from about 40,000 per cell at 0 h to about 12,000 per cell at 9 h. This lower number of receptors does not in itself account for the change from a tactic to a kinetic response to MTX, since many batches of cells were found to respond chemotactically even though they had as few as 5,000 receptors per cell.

Inhibition of [^{3}H]-MTX binding was used to test for receptor specificity. Both MTX and aminopterin were good competitors while folate competed for MTX binding but with 10- to 20-fold lower potency than MTX itself. This is probably due to the degradation of folate by the deaminase. Folate can totally block MTX binding, establishing that MTX is in fact binding to the folate receptor. On the other hand, pterin, a compound that we and others [7] have found to be a chemoattractant of these cells, does not inhibit MTX binding suggesting that it interacts with a distinct receptor. This idea is supported by the findings of Butz and Wurster [46] who found that pterin would not compete for [^{3}H]-folate binding and by pterin ana-

log studies [7]. No nucleotides, including cAMP and cGMP were found to compete for MTX binding [68].

At present, characterization of the folate receptor in plasma membranes and the detergent solubilized state is being carried out along with attempts to synthesize affinity reagents from [^{3}H]- and [^{125}I]-MTX for direct identification of the receptor protein as well as the deaminase polypeptide.

CORRELATIONS WITH LEUKOCYTE CHEMOTAXIS

As with other developmental functions, it is tempting to imagine that D discoideum is in many ways a model system for the chemotactic response of eukaryotic cells. As noted by Zigmond [72], the morphologic and motility responses to chemoattractants are quite similar in leukocytes and slime mold amoebae. As suggested above for cAMP chemotaxis in populations of aggregating slime mold cells, leukocytes may "learn" or acquire a polarity as a result of responding to a gradient of attractant [72]. In either cell type, little is known about the molecular mechanisms which are responsible for signal transduction. However, cGMP, Ca^{++}, and lipid methylation and degradation by phospholipase have been implicated in both systems [see 73 and the Developmental Chemotaxis section]. An obvious correlation is the fact that in both cell types the ultimate site of transduction is interaction with the actomyosin system of the cell. In a broad evolutionary sense, there are inherent similarities between the attraction of slime mold amoebae by bacterially secreted folate and the attraction of leukocytes by bacterial chemotactic factors, some of which may be more similar to folate compounds than the N-formylmethionyl (NFM) peptides.

Properties of the N-formylpeptide Receptor

Thus far, the only eukaryotic chemotactic system, other than the cellular slime molds, which has been studied in molecular terms is the chemotactic response of polymorphonuclear leukocytes (PMNs) and macrophages to N-formylmethionyl (NFM) peptides [73–75]. These cell types respond chemotactically to a great variety of other stimuli as well, such as oxidized lipids, 12-hydroxy unsaturated fatty acids, complement components such as C5a and denatured proteins [73, 75,76]. In addition to the response of oriented movement these cells respond to all of these stimuli with changes in respiration, cell adhesiveness, enzyme exocytosis, and motility rate [73,75,76], thus complicating the identification of cellular responses which are involved only in generating the directional motility response. A receptor has been identified on responsive cells which binds NFM peptides with high affinity and specificity [77,78]. The receptor with bound peptide is internalized by cells as reflected in a "down regulation" of receptor number [77,78]. The internalization process may also be an intimate part of the mechanism which generates the chemotactic response [73,79]. Neidel and co-workers [80] have recently developed a variety of affinity labeling methods for the NFM peptide receptor. With several reagents they obtain specific covalent incorporation of [^{125}I]-peptide into a protein which shows a broad band on SDS gels in the molecular weight range of 55,000 to 70,000.

Comparison With Slime Mold Chemotactic Receptors

It should soon be possible to draw detailed comparisons between the slime mold chemotactic receptors for cAMP and folate and the NFM-peptide receptor of leukocytes as more information regarding receptor structure and function becomes available. At present it is of interest that the molecular weight of the NFM-peptide receptor and the cAMP binding site identified in detergent extracts of D discoideum plasma membranes are quite similar, both being about 70,000. Another potential hint of similarity is found in the inhibition of the cAMP receptor by PMSF and DANSYL fluoride compared to the inhibition of NFM-peptide binding and function by TPCK [64] and diisopropyl phosphorofluoridate [81]. Becker et al [81] found that both phosphorylating and nonphosphorylating analogs of the latter reagent would prevent NFM-peptide mediated secretion of lysozomal enzymes from neutrophils. This suggests that covalent modification of the receptor need not occur for the reagent to act as an inhibitor and that its mode of inhibition may be similar to that of PMSF in the case of the slime mold cAMP receptor (see above).

A further analogy between the leukocyte NFM peptide receptor and the slime mold cAMP receptor is found in the evidence presented by Klein's group for the "down regulation" and potential internalization of the cAMP receptor [82,83]. Due to the fact that substituents of any kind on the cAMP molecule lower its receptor affinity, it is not possible to do the direct localization experiments with fluorescent cAMP analogs such as those done by Neidel et al [79] with a fluorescent NFM-peptide derivative. We are attempting to use fluorescent, electron-dense multivalent cAMP and MTX conjugates of ferritin to localize and study the dynamics of the receptors for these compounds on slime mold cells. An interesting finding in preliminary studies with a multivalent conjugate of cAMP is that the conjugate appears to be hyperactive in eliciting a chemotactic response. The conjugate is more potent than the equimolar amount of cAMP derivative which it contains. This finding suggests that simultaneous occupation of adjacent cAMP receptors may be a more powerful stimulus than univalent cAMP. It may also indicate that receptor clustering, as seen by Neidel et al [73,79] is an important step in signal transduction, since the multivalent cAMP conjugate would be expected to promote receptor aggregation. As noted above, both leukocytes and slime mold cells are able to respond to a spatial gradient across the cell of only 0.5%-1% change in the absolute local concentration of attractant [71,75, and the Folate Chemotactic section]. If simultanous occupancy of more than one receptor binding site is a more sensitive function of the concentration gradient than simply the number of receptor sites occupied on opposite ends of the cell, this mechanism may provide a more sensitive way of "reading" the gradient. This mechanism does not necessarily invoke an apparent positive cooperativity in binding of the attractant, but simply requires that some post-binding event in signal transduction be synergistically amplified when two (or more) such events occur within a limited time window. Comparative studies of multivalent cAMP and MTX conjugates are currently underway to test the generality of conjugate hyperactivity. It would be of obvious interest to know if multivalent conjugates of NFM peptides display hyperactivity in leukocyte chemotaxis. If the MTX conjugates are hyperactive in causing chemotaxis of vegetative slime mold cells, this would suggest that both the cAMP and the folate system utilize similar transduction mechanisms, and would

strengthen our hypothesis that both receptors are linked in a similar way to the control of cellular motility.

It is hoped that through comparative studies of the structure and function of the cAMP and the folate receptor we will gain some clues with which to begin studies of the mechanism by which these two receptors communicate their information across the plasma membrane to the components which regulate the cell's contractile machinery. It is perhaps not too unreasonable to expect that the means by which cell surface receptors communicate with the interior machinery of the cell is a critically important process that has been highly conserved during the evolution of higher eukaryotes. With this in mind, we feel that continued study of the chemotactic systems of the cellular slime molds will soon begin to provide the mechanistic insights needed to approach questions of receptor-effector coupling in higher, more complex organisms.

REFERENCES

1. Bonner JT: "The Cellular Slime Molds," ed. 2. Princeton, New Jersey: Princeton University Press, 1967, p 205.
2. Loomis WF Jr: "Dictyostelium discoideum: A developmental System," New York: Academic Press, 1975, p 214.
3. Jacobson A, Lodish HF: Annu Rev Genet 9:145–185, 1975.
4. Bonner JT, Barkley DS, Hall EM, Konijn TM, Mason JW, O'Keefe G, Wolfe PB: Dev Biol 20: 72–87, 1969.
5. Wurster B, Pan P, Tyan G-G, Bonner JT: Proc Natl Acad Sci USA 73:795–799, 1976.
6. Pan P, Hall EM, Bonner JT: Nature New Biol 237:181–182, 1972.
7. Pan P, Hall EM, Bonner JT: J Bacteriol 122:185–191, 1975.
8. Pan P, Wurster B: J Bacteriol 136:955–959, 1978.
9. Newell PC: In Reissig JL (ed): "Microbial Interactions. Receptors and Recognition." London: Chapman and Hall, series B, vol 3, 1977, pp 3–49.
10. Darmon M, Brachet P: In Hazelbauer GL (ed): "Taxis and Behavior. Receptors and Recognition." London: Chapman and Hall, series B, vol 5, 1979, pp 101–139.
11. Gerisch G, Wick U: Biochem Biophys Res Commun 65:364–370, 1975.
12. Alcantara F, Monk M : J Gen Microbiol 85:321–334, 1974.
13. Shaffer BM: Nature 255:549–552, 1975.
14. Malchow D, Gerisch G: Proc Natl Acad Sci USA 71:2423–2427, 1974.
15. Green AA, Newell PC: Cell 6:129–136, 1975.
16. Henderson EJ: J Biol Chem 250:4730–4736, 1975.
17. King AC, Frazier WA: Biochem Biophys Res Commun 78:1093–1099, 1977.
18. King AC, Frazier WA: J Biol Chem 254:7168–7176, 1979.
19. Wurster B, Shubiger K, Wick U, Gerisch G: FEBS Lett 76:141–144, 1977.
20. Mato JM, Krens FA, Van Haastert PJ, Konijn TM: Proc Natl Acad Sci USA 76:2348–2351, 1977.
21. Mato JM, Konijn TM: In Cappuccinelli P, Ashworth JM (eds): "Development and Differentiation in the Cellular Slime Molds." Amsterdam: Elsevier/North Holland, 1977, pp 93–103.
22. Wurster B, Bozzaro S, Gerisch G: Cell Biol Int Rep 2:61–69, 1978.
23. Gerisch G, Hulser D, Malchow D, Wick U: Phil Trans R Soc Lond, Series B, 272:187–192, 1975.
24. Roos W, Gerisch G: FEBS Lett 68:170–172, 1976.
25. Klein C: FEBS Lett 68:125–128, 1976.
26. Roos W, Scheidegger C, Gerisch G: Nature 266:259–261, 1977.
27. Devreotes PN, Derstine PL, Steck TL: J Cell Biol 80:291–299, 1979.
28. Dinauer MC, MacKay SA, Devreotes PN: J Cell Biol 86:537–544, 1980.
29. Hintermann R, Parish RW: Exp Cell Res 123:429–434, 1979.
30. Tomchik KJ, Devreotes PN: Science 212:443–446, 1981.
31. Chang YY: Science 161:57–59, 1968.
32. Orlow SJ, Shapiro RI, Franke J, Kessin RH: J Biol Chem 256:7620–7627, 1981.

33. Cohen MH, Robertson A: J Theor Biol 31:101–118, 1971.
34. Dinauer MC, Steck TL, Devreotes PN: J Cell Biol 86:554–561, 1980.
35. Robertson A, Drage DJ: Biophys J 15:765–775, 1975.
36. Gerisch G, Hess B: Proc Natl Acad Sci USA 71:2118–2122, 1974.
37. Gross J, Kay R, Lax A, Peacey M, Town C, Trevan D: In Cappuccinelli P, Ashworth JM (eds): "Development and Differentiation in the Cellular Slime Molds." Amsterdam: Elsevier/North Holland, 1977, pp 135–147.
38. Gerisch G, Maeda Y, Malchow D, Roos W, Wick U, Wurster B: In Cappuccinelli P, Ashworth JM (eds): "Development and Differentiation in the Cellular Slime Molds," Amsterdam: Elsevier/North Holland, 1977, pp 105–124.
39. Mato JM, Van Haastert PJM, Krens FA, Rhijnsberger EH, Dobbe FCPM, Konijn TM: FEBS Lett 79:331–336, 1977.
40. Mato JM, Marin-Cao D: Proc Natl Acad Sci USA 76:6106–6109, 1979.
41. Alemany S, Gil MG, Mato JM: Proc Natl Acad Sci USA 77:6996–6999, 1980.
42. Wurster B, Schubiger K: J Cell Sci 27:105–114, 1977.
43. Wurster B, Schubiger K, Brachet P: Cell Differentiation 8:235–242, 1979.
44. Kakebeeke PIJ, DeWitt RJW, Konijn TM: J Bacteriol 143:307–312, 1980.
45. Bernstein RL, Rossier C, Van Driel R, Brunner M, Gerisch G: Cell Diff 10:79–86, 1981.
46. Wurster B, Butz U: Eur J Biochem 109:613–618, 1980.
47. Van Driel R: Eur J Biochem 115:391–395, 1981.
48. Mullens IA, Newell PC: Differentiation 10:171–176, 1978.
49. King AC: PhD thesis, Washington University, St. Louis, Mo, 1979.
50. Rahmsdorf HJ, Malchow D, Gerisch G: Cell Biol Int Rep 3:237–245, 1979.
51. Parish RW, Weibel M: FEBS Lett 118: 263–266, 1980.
52. Klein C, Brachet P, Darmon M: FEBS Lett 76:145–147, 1977.
53. Stahl P, Schlesinger P, Sigardson E, Rodman J, Lee YC: Cell 19:207–215, 1980.
54. Pomerantz AH, Rudolph SA, Haley BE, Greengard P: Biochemistry 14:3858–3862, 1975.
55. Walter U, Greengard P: J Cyclic Nucleotide Res 4:437–444, 1978.
56. Owens JR, Haley BE: J Supramol Struct 5:91–102, 1976.
57. Skare K, Black JL, Pancoe WL, Haley BE: Arch Biochem Biophys 180:409–415, 1977.
58. Mato JM, Jastorff B, Morr M, Konijn TM: Biochim Biophys Acta 544:309–314, 1978.
59. Wallace LJ, Frazier WA: Proc Natl Acad Sci USA 76:4250–4254, 1979.
60. Wallace LJ, Frazier WA: J Biol Chem 254:10109–10114, 1979.
61. Staros JV, Bayley H, Standring DN, Knowles JR: Biochem Biophys Res Commun 80:568–572, 1978.
62. Juliani MH, Klein C: J Biol Chem 256:613–619, 1981.
63. Meyers BL, Frazier WA: Biochem Biophys Res Commun 101:1011–1017, 1981.
64. Neidel J, Frothingham R, Cuatrecasas P: Biochem Biophys Res Commun 94:667–673, 1980.
65. Shaltiel S: Methods in Enzymol 34:126–140, 1974.
66. Hofstee BHJ: Biochem Biophys Res Commun 53:1137–1147, 1973.
67. Homcy CJ, Wrenn SM, Haber E: J Biol Chem 252:8957–8964, 1977.
68. Nandini-Kishore SG, Frazier WA: Proc Natl Acad Sci USA 78:7299–7303, 1981.
69. Varnum B, Soll DR: Differentiation 18:151–160, 1981.
70. Soll DR, Waddell DR: Dev Biol 47:292–302, 1975.
71. Zigmond SH: J Cell Biol 75:606–616, 1977.
72. Zigmond SH, Levitsky HI, Kreel BJ: J Cell Biol 89:585–592, 1981.
73. Neidel JE, Cuatrecasas P: Curr Top Cell Regul 17:137–170, 1980.
74. Schiffmann E, Corcoran BA, Wahl SM: Proc Natl Acad Sci USA 72:1059–1062, 1975.
75. Zigmond SH: J Cell Biol 77:269–287, 1978.
76. Wilkinson PC: In Hazelbauer GL (ed): "Taxis and Behavior. Receptors and Recognition." London: Chapman and Hall, series B, vol 5, 1979, pp 293–329.
77. Neidel J, Wilkinson S, Cuatrecasas P: J Biol Chem 254:10700–10706, 1979.
78. Sullivan SJ, Zigmond SH: J Cell Biol 85:703–711, 1980.
79. Neidel JE, Kahane I, Cuatrecasas P: Science 205:1412–1414, 1979.
80. Neidel JE, Davis J, Cuatrecasas P: J Biol Chem 255:7063–7066, 1980.
81. Becker EL, Koza EP, Sigman M: Immunology 35:373–380, 1978.
82. Klein C, Juliani MH: Cell 10:329–335, 1977.
83. Klein C: J Biol Chem 254:12573–12578, 1979.