Localization of Functional Domains of the cAMP Chemotactic Receptor of Dictyostelium discoideum

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The topography and functional domains of the cAMP chemotactic receptor of Dictyostelium discoideum were investigated by protease sensitivity to chymotrypsin. Proteolytic digestion of intact cells produced a 23-kDa fragment of the receptor that retained the photoaffinity label used to identify the receptor. Additionally, this fragment contained the sites phosphorylated by CAR-kinase, the enzyme that phosphorylates the lipid-occupied form of the receptor. The fragment was also found to be phosphorylated in response to cAMP stimulation of cells. Proteolytic digestion of either intact cells or membrane preparations did not appreciably alter the binding properties of the receptor, indicating that the domains which determine the cAMP binding pocket are likely to be transmembrane regions of the protein. Additionally, the sensitivity of down-regulated receptors to chymotrypsin digestion suggests that the initial loss of cAMP binding activity upon incubation of cells with high concentrations of ligand does not require receptor internalization.

The aggregation of Dictyostelium discoideum amoebae is directed by external cAMP. Cyclic AMP is produced and secreted with a periodicity of 5–6 min by aggregation centers. Aggregation-competent cells within the vicinity of a center respond to these pulses by orienting their migration toward the emitting source and by relaying the chemotactic signal. The latter involves the activation of adenylyl cyclase for approximately 1–2 min and the secretion of the newly synthesized cAMP into the medium. Signal relay allows for the outward propagation of the chemotactic signal. Unidirectional movement of the cell population can be accounted for by a short refractory period during which time cells that had been stimulated with cAMP are no longer responsive (1, 2). The events involved in the transduction of an external pulse of cAMP into a physiological response have not been totally elucidated as cells develop aggregation competence and then transition into slugs (4–6). The binding specificity of the receptor for varied cAMP analogues correlates well with the ability of these compounds to effect chemotaxis (7). The protein is approximately 40–45 kDa in molecular mass, as determined by photoaffinity labeling (6, 9), but migrates on SDS-PAGE\(^1\) as a higher molecular weight protein when cells are stimulated with cAMP (8, 9). This protein, of approximately 43–47 kDa, represents a more highly phosphorylated form of the cAMP receptor (10–12). As in other signaling or hormonal systems, this increased phosphorylation is correlated with the desensitization of the system (11, 12). CAMP receptors also share other features with surface receptors in other systems, one of which is the ability to undergo down-regulation in response to chronic cAMP stimulation (13). Removal of the stimulus allows for the reappearance of binding activity on the cell surface. Both the loss and reappearance of this activity can occur in the absence of protein synthesis and thus may indicate reversible changes in the receptor on the cell surface or a recycling of internalized receptors (13). A cDNA for the cAMP receptor has been isolated and sequenced. The deduced amino acid sequence suggests that the binding pocket is likely to be transmembrane regions of the protein. Additionally, this fragment contained the sites phosphorylated by CAR-kinase, the enzyme that phosphorylates the lipid-occupied form of the receptor. The fragment was also found to be phosphorylated in response to cAMP stimulation of cells. Proteolytic digestion of either intact cells or membrane preparations did not appreciably alter the binding properties of the receptor, indicating that the domains which determine the cAMP binding pocket are likely to be transmembrane regions of the protein. Additionally, the sensitivity of down-regulated receptors to chymotrypsin digestion suggests that the initial loss of cAMP binding activity upon incubation of cells with high concentrations of ligand does not require receptor internalization.

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\(^1\)The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MES, 2-(N-morpholinoethanesulfonic acid; 8-N\(_3\)-cAMP, azido-cAMP.
or absence of 1 mg/ml of the indicated protease. Cells were maintained as spinner suspensions at 21 °C during the treatment period, which was generally for 1 h. In vitro phosphorylation of the cAMP receptor by CAR-kinase was achieved by incubating partially purified plasma membranes with [γ-32P]ATP and cAMP as described previously (22). To assess the susceptibility of the phosphorylated residues to chymotrypsin, membranes were subsequently incubated with 1 mg/ml protease for 30 min. The incubations were terminated by the addition of cold 10 mM Tris, pH 7.4, and repeated washes by centrifugation. Final washes were performed in the presence of a mixture of nine protease inhibitors (22) which were also present in the sample buffer used to solubilize preparations for SDS-PAGE. To also ensure that proteolysis was not occurring during the solubilization of the samples, we added sample buffer to either untreated cells or membranes, followed by the addition of the protease. Under those conditions, no receptor degradation was observed when either the photoaffinity label or phosphate label introduced by phosphorylation was monitored. When cAMP binding to plasma membranes was to be examined, membranes were incubated for 30 min in 10 mM Tris, pH 7.4, in the absence or presence of 1 mg/ml chymotrypsin. Membranes were then diluted into 20 mM phosphate buffer, pH 6.4, 10 mM dithiothreitol (6) containing the indicated concentration of [3H]cAMP. Membranes were incubated for 15 s and the reaction stopped by centrifugation at 15,000 x g. The supernatants were aspirated and the pellets solubilized in SDS and counted. Background radioactivity, which occurred when binding assays were performed in the presence of 10−4 M nonradioactive cAMP, was less than 2% of the total binding.

Materials—8-N3-32PcAMP and [3H]cAMP were purchased from ICN and Amersham Corp., respectively. [32P]Orthophosphate and [γ-32P]ATP were obtained from Du Pont-New England Nuclear. Proteases were purchased from Boehringer Mannheim. Protease inhibitors, protein molecular weight standards, anti-actin antibody, and other chemicals were purchased from Sigma.

RESULTS

Chymotrypsin Hydrolyzes the Cell Surface cAMP Receptor—In our studies of chemotactic signaling in D. discoideum, we observed that cells starved in the presence of 1 mg/ml chymotrypsin developed aggregation competence and could migrate chemotactically toward central collection points.2 In contrast, aggregate formation did not occur when cells were treated with similar concentrations of substilbin, bromelain, or Pronase. However, these latter proteases were found to effectively hydrolyze gp80, a surface molecule involved in cell-cell cohesion during aggregation (23), while chymotrypsin did not.4 Thus, the lack of aggregation when cells were incubated with these latter proteases could be explained by the hydrolysis of surface components, other than the cAMP receptor, necessary for aggregation. The ability of cells to migrate chemotactically and form aggregates in the presence of chymotrypsin could reflect either a lack of appropriate cleavage sites for the protease on the receptor or their inaccessibility to the enzyme. A recent model for the chemotactic receptor, based upon a cDNA-derived amino acid sequence, suggests that the protein possesses seven transmembrane regions with external domains containing residues recognized by chymotrypsin (14). Thus, an alternative explanation that chymotrypsin can hydrolyze the receptor but that the proteolytic fragments generated can function appropriately in signal transduction should be considered. To address these alternatives, we performed several experiments to examine the susceptibility of the cAMP receptor to protease digestion and/or if occupation of the receptor alters its susceptibility.

Aggregation-competent cells were photolabeled with 8-N3-[32P]cAMP and then incubated for 1 h in the presence or absence of chymotrypsin. At the end of the incubation, samples were analyzed by SDS-PAGE and autoradiography. As seen in Fig. 1A, photolabeling of untreated cells identified p45, a protein of approximately 45 kDa that we have shown previously to be the cAMP receptor (8). Incubation of those cells in buffer without added protease did not result in a loss of radiolabel from p45 nor the appearance of a new radiolabeled band (lane 2). In contrast, incubation of cells in the presence of chymotrypsin resulted in almost the complete loss of radiolabeled p45, indicating that the receptor had been hydrolyzed by chymotrypsin (lane 3). Concurrent with the loss of radiolabeled p45, we observed the appearance of a protein of approximately 23 kDa which bore the 8-N3-[32P]cAMP radiolabel. Staining of the gel showed that the protein pattern of protease-treated and control cells was not detectably altered, as would be expected by the restricted action of protease to cell surface exposed proteins with the appropriate cleavage sites. In this particular experiment, only one or two other proteins of relatively high molecular weight were radiolabeled by the azido-cAMP. (Variation in the degree of nonspecific radiolabeling occurred with different batches of the radiolabel.) The extent to which these proteins were labeled was not significantly changed in the course of cell incubation with chymotrypsin indicating that the 23-kDa band generated was a degradation product of the cAMP receptor. This was substantiated in experiments in which cells were photolabeled in the presence of micromolar amounts of cAMP in order to specifically prevent the photolabeling of the receptor (8). Under such conditions, chymotrypsin digestion did not produce any lower molecular weight bands, in particular, a 22-kDa band (lane 4).

The converse experiment in which cells were first incubated with chymotrypsin and then photolabeled was also performed. As seen in Fig. 1B, chymotrypsin treatment resulted in a diminished incorporation of the photolabel into the intact receptor, p45, and produced a lower molecular protein of approximately 23 kDa that could be photolabeled specifically by azido-cAMP (lane 9). Proteins of chymotrypsin-treated cells that had been photolabeled in the presence of excess cAMP are shown in lane 1. No photolabeling of the 23-kDa band was observed.

Several batches of chymotrypsin were used to verify that the 23-kDa band was not generated as a result of a contaminating activity in the preparations. Additionally, similar experiments were performed using tosylphenylalanyl chloro-

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4 K. Meier and C. Klein, unpublished results.
methyl ketone-treated trypsin to observe if this protease could also digest the cAMP receptor of intact cells and the size of the radiolabeled product that could be generated. Using up to 200 μg/ml trypsin, we did not observe a loss of radioactivity in p45 nor the appearance of another radiolabeled protein. Technical difficulties prevented us from using higher concentrations of the protease. It proved difficult to wash cells free of added trypsin, and the residual activity associated with cells that had been incubated with higher concentrations resulted in extensive proteolysis of proteins upon addition of sample buffer. This was true even when additional trypsin inhibitor was added to the sample buffer.

Cell Surface cAMP Binding Activity Is Insensitive to Proteolysis.—The above experiments indicated that the CAMP receptor can be hydrolyzed when cells are incubated with chymotrypsin. To examine if this hydrolysis can influence the binding properties of the receptor, cells were incubated in the presence of 1 mg/ml chymotrypsin and then assayed for receptor activity. As seen in Table I, such treatment did not result in a significant decrease in cAMP binding activity. Assays were performed using two concentrations of radiolabeled ligand, 2 × 10⁻⁹ and 5 × 10⁻⁸ M, to measure preferential binding to the high and low affinity sites, respectively (4). The results were the same in both cases. Additional proteases were also examined. Pronase treatment generally resulted in a 30–35% reduction of cell surface cAMP binding activity while other proteases, by themselves or in a mixture, were ineffective. It would appear that CAMP binding activity is not appreciably altered when externally exposed portions of the receptor are attacked by proteases.

To assess if cytosolically exposed moieties influence receptor activity, experiments were performed using membrane preparations. Binding of cAMP to partially purified plasma membranes displayed a relative insensitivity to protease treatment. Only a 20–30% loss of binding activity occurred when membrane preparations were treated with chymotrypsin or trypsin. Pronase digestion resulted in a greater loss of binding activity. Although chymotrypsin had a limited effect on cAMP binding activity in membrane preparations, it effectively hydrolyzed other domains of the receptor, in particular the domain(s) phosphorylated by CAR-kinase (21), the enzyme that phosphorylates the ligand-occupied form of the receptor. This was demonstrated by phosphorylating the cAMP receptor in membrane preparations prior to incubation of the membranes with chymotrypsin. As seen in Fig. 2, the cAMP receptor, as detected by its phosphorylation in the presence of cAMP (lane 2), was no longer radiolabeled when membranes were subsequently incubated with chymotrypsin (lane 4). We did not observe the concurrent appearance of lower molecular weight phosphorylated bands, indicating that the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells Membranes</th>
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<tbody>
<tr>
<td>CT*</td>
<td>100 100</td>
</tr>
<tr>
<td>T*</td>
<td>99 75</td>
</tr>
<tr>
<td>CT/subtilisin</td>
<td>99 ND*</td>
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<tr>
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</tr>
<tr>
<td>Pronase</td>
<td>70 45</td>
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* CT, chymotrypsin.
* T, trypsin.
* ND, not determined.

Fig. 2. Hydrolysis of phosphorylated residues by chymotrypsin. Plasma membranes of aggregation-competent cells were incubated with [γ-³²P]ATP in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mg/ml chymotrypsin for 30 min (lanes 3 and 4), and the phosphorylated proteins were examined by SDS-PAGE and autoradiography. The data are representative of five experiments. The position of the ligand-induced phosphorylated receptor is indicated by the arrow.

**Table 2** Properties of protease-cleaved receptor

<table>
<thead>
<tr>
<th>Cells Pretreatment</th>
<th>Additions in assay</th>
<th>Binding</th>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10⁻⁹ M CAMP</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>1 M (NH₄)SO₄</td>
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<td>15</td>
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<tr>
<td>10⁻⁹ M CAMP</td>
<td>5 mM CaCl₂</td>
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<tr>
<td>10⁻⁹ M CAMP</td>
<td>1 M (NH₄)SO₄</td>
<td>53</td>
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<tr>
<td>CT*</td>
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<td>15</td>
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<tr>
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<td>220</td>
</tr>
<tr>
<td>10⁻⁹ M CAMP</td>
<td>1 M (NH₄)SO₄</td>
<td>48</td>
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</tbody>
</table>

* No addition or pretreatment.
* CT, chymotrypsin.

protein moieties generated under such conditions were smaller than could be resolved on our gel system, i.e. approximately 8 kDa in molecular mass. Staining of the gel revealed that extensive hydrolysis of proteins in general occurred when membranes were treated with chymotrypsin.

**Proteolytically Cleaved Receptors Show Characteristic Behavior**—It would appear that chymotrypsin digestion of the external or cytosolically exposed domains of the receptor do not dramatically alter its binding capacity. Thus, we examined other features of the cAMP receptor. Receptor binding activity is enhanced by Ca²⁺ (24) and by ammonium sulfate treatment (25). As seen in Table II, the effects of these treatments were the same whether or not the receptor had been digested with chymotrypsin. The effectiveness of different concentrations of ligand to induce receptor down-regulation (13) was also unaltered by chymotrypsin treatment. Shown in this table are the results obtained when cells were incubated with 10⁻³ or 10⁻⁴ M cAMP for 15 min prior to determination of their residual binding activity.

As mentioned earlier, receptor occupation with cAMP results in enhanced receptor phosphorylation (10–12). The ability of proteolytically cleaved receptors to undergo increased phosphorylation in response to cAMP stimulation was examined. This was performed by incubating cells with chymotrypsin for 1 h after which time [³²P]orthophosphate was added for 15 min. One-half of the population was treated with
CAMP to induce receptor phosphorylation. Plasma membranes were then prepared according to Klein et al. (19) and analyzed by SDS-PAGE and autoradiography. Fig. 3 shows that, as expected, CAMP treatment of control cells resulted in the appearance of the phosphorylated form of the cAMP receptor (lane 1). No labeling occurred when cells were incubated without CAMP (lane 2). In chymotrypsin-treated cells, CAMP stimulation induced the phosphate labeling of a band of approximately 23 kDa (lane 3). Some labeling of the intact receptor also occurred, but this level was reduced when compared with untreated cells. The presence of some radiolabel in the intact receptor protein would suggest that, in this experiment, hydrolysis of the receptor by chymotrypsin was not complete. Neither the 23-kDa band nor that corresponding to the intact receptor was observed in chymotrypsin-treated cells that were not stimulated with cAMP (lane 4). The converse experiment in which proteins were phosphorylated and the cells then treated with chymotrypsin was attempted but yielded inconclusive results due to the generation of a number of low molecular weight phosphorylated bands under these conditions.

**Down-regulated Receptors Are Sensitive to Protease**—The hydrolysis of the cAMP receptor by chymotrypsin was exploited to investigate the mechanism of down-regulation in these cells. We have previously demonstrated that 80-90% of the receptors can be down-regulated in response to stimulation with 10^{-7} M cAMP, as determined by the loss of cAMP binding activity (13). This loss of active receptor is associated with a conversion of the receptor to a form from which CAMP slowly dissociates (26). This form of the receptor could be generated by modifying the ligand-occupied receptor present on the cell surface or by internalizing the receptor-ligand complex. To assess if receptor internalization is involved in the mechanism of down-regulation, we examined the accessibility of down-regulated receptors to chymotrypsin. Cells were labeled with 8-Nrz^{[32P]}cAMP and then incubated with nonradioactive azido-cAMP to induce receptor down-regulation (Fig. 4). In the experiments shown, azido-cAMP, as opposed to cAMP, was used to induce receptor down-regulation to avoid the possibility that receptors occupied with the analogue behave differently from those occupied with cAMP. Other experiments, however, were performed using cAMP, and the results obtained were identical to those reported here. After a 15-min incubation of the cells with 10^{-4} M azido-

cAMP, which resulted in an 80% loss of receptor binding activity, chymotrypsin was added to one-half of the population, and the incubation was continued for an additional hour. In lanes 3 and 4, additional 8-Nrz-cAMP (or CAMP) was added again immediately prior to dividing cells (lane 3) and chymotrypsin addition (lane 4). Cells were then analyzed by SDS-PAGE and autoradiography to determine if the pattern of azido-cAMP labeling had been altered. Under the conditions of these experiments, the photoaffinity-labeled receptor remained susceptible to hydrolysis by chymotrypsin. Coincident with the loss of radiolabel from the band corresponding to the undigested protein was the appearance of another radiolabeled band of approximately 23 kDa (lanes 2 and 4). Lanes 5 and 6 of this figure show the pattern obtained when cells that had been photolabeled in the presence of excess cAMP to prevent radiolabeling of the receptor were down-regulated and then incubated with chymotrypsin, respectively. No lower molecular weight band was generated upon protease treatment.

The residual binding activity on down-regulated cells was equally sensitive to chymotrypsin. Cells were incubated with azido-cAMP (or cAMP) to down-regulate 80% of their binding activity. Subsequently, cells were washed and photolabeled with 8-Nrz^{[32P]}cAMP to photolabel the residual active binding sites. Photolabeled cells were then incubated with chymotrypsin and, in some cases, with additional ligand to ensure that cells remained in the down-regulated state. Chymotrypsin treatment resulted in the degradation of the receptor and the production of the 23-kDa product (data not shown).

**DISCUSSION**

Proteolytic susceptibility has been successfully used in a number of systems to determine the topography of specific membrane proteins and the orientation of specific sites on those proteins. We have applied this approach to the cell surface cAMP chemotactic receptor of *D. discoideum*. Since these amoebae aggregate normally when incubated with high concentrations of chymotrypsin, we have primarily focused our attention on the effects of chymotrypsin on the receptor. However, extensive proteolytic treatment of either intact cells or membrane preparations with a number of different proteases was shown not to affect the ability of the receptor to bind cAMP. We have verified that chymotrypsin does indeed cleave the receptor. Such treatment generates a 23-kDa band.
when only the externally exposed domains are exposed to the enzyme (i.e. when intact cells are treated), and this band is extensively hydrolyzed if the cytosolic exposed domains are also accessible (in the case of membrane treatment). A cDNA clone for the cAMP chemotactic receptor has been isolated and a derived amino acid sequence used to propose a model for its structure in the membrane (14). The model simulates the structure of the β-adrenergic receptor and rhodopsin and places seven hydrophobic domains as likely transmembrane regions. Scattered throughout the protein are numerous sites for cleavage by the proteases used in our studies. The proposed structure for the receptor could, however, readily explain the protease insensitivity of the cAMP binding activity if we place the domain(s) necessary for that activity within the transmembrane region(s). Such a location would be consistent with the finding that proteolytic cleavage of either the external or cytosolic exposed domains of the receptor did not appreciably affect its binding capacity. This hypothesis would also be in keeping with the analogy of the chemotactic receptor with rhodopsin and the β-adrenergic receptor where a number of experiments, including proteolytic sensitivity and mutagenesis, have indicated that the binding sites for their respective ligands are defined by the transmembrane regions of the protein (27, 28).

The chemotactic cAMP receptor, once cleaved by chymotrypsin, appeared to be unhampered with respect to a number of its properties, in addition to cAMP binding. The receptor activity was still enhanced in response to Ca2+ or ammonium sulfate, and it could be down-regulated in a manner indistinguishable from the uncleaved receptor. Given the extent of receptor hydrolysis, we would expect that any changes in the biochemical properties of the hydrolyzed receptor, should they exist, would be readily detected and distinguished from those of the nonhydrolyzed protein. Since amoebae are able to develop aggregation competence and migrate chemotactically toward one another when starved in the presence of chymotrypsin, the receptor also appeared capable of effective signal transduction upon binding of cAMP. In this latter case, however, it should be noted that chymotrypsin treatment of the receptor was not always quantitative, and any small amount of remaining undigested receptor could be sufficient for normal development.

As mentioned previously, digestion of the receptor by chymotrypsin was demonstrated by the generation of a 23-kDa band bearing the photoaffinity label used to identify the receptor. That fragment could be photoaffinity-labeled either before or after chymotrypsin treatment of cells and, in either case, could undergo phosphorylation in response to cell stimulation with cAMP. We do not wish to imply, however, that the information for displaying these properties and those mentioned in the above paragraph resides uniquely in that 23-kDa fragment. Since the cytosolically exposed loops of the protein remain intact when cells are incubated with chymotrypsin, it is likely that varied domains of the receptor are still interactive and retain normal function.

The approach used in this paper can provide some information concerning the role of the cytosolic regions of the receptor in signal transduction. As mentioned earlier, extensive digestion of plasma membrane preparations with chymotrypsin did not dramatically alter their cAMP binding capacity. This was true despite the fact that the moiety(s) phosphorylated by CAR-kinase was removed. Although it is possible that more subtle features of receptor binding were affected by the removal of the sites for CAR-kinase activity, the results are consistent with the proposed role of receptor phosphorylation in its uncoupling to an intermediate in the signal transduction pathway, e.g. a G protein (29–31), as opposed to altering directly the binding properties of the receptor.

According to the model of Klein et al. (14), three of the four external domains of the cAMP receptor possess sites recognized by chymotrypsin: those between the transmembrane regions 2/3, 4/5, and 6/7. (Transmembrane regions are numbered from 1 to 7 starting with the N terminus.) Digestion at all possible sites would not generate a 23-kDa peptide. However, the 6/7 external domain, that most proximal to the C terminus of the protein, is unique in that it contains only one site for chymotrypsin action. That site is positioned at the start of the seventh transmembrane region and is likely to be inaccessible to the protease. Under such circumstances digestion of the 4/5 external domain would generate a fragment of 232 amino acids, a size very similar to that of the peptide that retains the photolabel after chymotrypsin treatment. Other polypeptides that could be generated would be significantly smaller, composed of 77 amino acids or less. This would place the residue(s) modified by the photolabel within the last three transmembrane regions. For the β-adrenergic receptor and rhodopsin, residues in the seventh hydrophobic domain have been shown to participate in ligand binding (32, 33). Continued experimentation in this system to determine the specific amino acid residue(s) modified by azido-cAMP and the sequence of neighboring residues should help identify the domains that define the binding pocket for cAMP.

It is also recognized that the consistency of the data obtained upon chymotrypsin digestion of the receptor with that predicted by the model of Klein et al. (14) lends credence to that model and suggests that experiments using an expanded repertoire of proteases or site-specific reagents can be employed to gain further insights into the topography of the receptor in the membrane and the localization of its functional domains. The results reported here show that the residues phosphorylated by CAR-kinase are also localized to the 23-kDa moiety that retains the photolabel. Cells that had been incubated with chymotrypsin to generate the 23-kDa moiety of the receptor phosphorylated that band when stimulated with cAMP. Although the amino acid modified by the photoaffinity label appears to reside in a transmembrane domain, it is likely that the phosphorylated residues are located on a cytosolic exposed moiety of this fragment since they are readily removed by chymotrypsin digestion of membranes. The model proposed for the cAMP receptor shows an abundance of Ser/Thr residues concentrated in the C-terminal cytosolic moiety. That C-terminal moiety would be a predicted component of the 23-kDa fragment generated upon chymotrypsin digestion of the receptor on intact cells.

When cells were photoaffinity-labeled with azido-cAMP and then incubated with ligand for 10 min to induce receptor down-regulation, the photoaffinity-labeled receptor remained susceptible to hydrolysis by chymotrypsin. In neither case was receptor sensitivity to chymotrypsin a reflection of cell leakiness or accessibility of intracellular proteins to the protease. Proteolytic treatment of membranes, where domains of the receptor other than those that are externally exposed are accessible, results in extensive receptor degradation such that the products could not be observed in our gel system. In particular no 23-kDa band was generated. In addition, staining of gels showed that the protein pattern was not detectably altered when intact cells were treated with chymotrypsin, in contrast to the extensive hydrolysis seen when internal proteins were accessible. For example, when membranes were digested with chymotrypsin, membrane-associated actin was completely hydrolyzed but remained unaltered when intact
cells were treated. This aspect was further verified by monitoring the stability of actin by Western blots. The finding that receptors remain accessible to chymotrypsin would argue that under the experimental conditions used receptor down-regulation does not reflect the internalization of the receptor. We would have expected the receptor to be resistant to proteolytic cleavage if that had occurred. A possible caveat is that the photolabeled receptor is unable to undergo down-regulation. Currently we do not believe this to be the case since the cell's responses to azido-CAMP stimulation (e.g., receptor phosphorylation and down-regulation) are identical to those produced by cAMP stimulation and are not altered by UV irradiation (8). It is more likely that ligand occupancy induces a change in receptor binding properties, either directly or by modifying the surface interaction or clustering of receptors as they exist on the cell surface. We have not investigated the effects of more prolonged cell incubation with cAMP on receptor sensitivity to chymotrypsin. It may be that additional events, including receptor internalization, occur subsequent to the actual loss of binding activity and as a result of more protracted cell stimulation. Continued application of the approaches discussed in this paper, and complemented by that of in vitro mutagenesis, should elucidate the answers to these and other questions concerning the functional domains of the receptor and their regulation.

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Y P Tao and C Klein


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