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Random Mutagenesis of the cAMP Chemoattractant Receptor, cAR1, of *Dictyostelium*

EVIDENCE FOR MULTIPLE STATES OF ACTIVATION*

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cAMP receptor 1 (cAR1) of *Dictyostelium* couples to the G protein G2 to mediate activation of adenylyl and guanylyl cyclases, chemotaxis, and cell aggregation. Other cAR1-dependent events, including receptor phosphorylation and influx of extracellular Ca^{2+}, do not require G proteins. To further characterize signal transduction through cAR1, we performed random mutagenesis of the third intracellular loop (24 amino acids), since the corresponding region of other seven helix receptors has been implicated in the coupling to G proteins. Mutant receptors were expressed in *car1*$^-$ cells and were characterized for G protein-dependent and -independent signal transduction. Our results demonstrate that cAR1 is remarkably tolerant to amino acid substitutions in the third intracellular loop. Of the 21 positions where amino acid substitutions were observed, one or more replacements were found that retained full biological function. However, certain alterations resulted in receptors with reduced ability to bind cAMP and/or transduce signals. There were specific signal transduction mutants that could undergo cAMP-dependent cAR1 phosphorylation but were impaired either in coupling to G proteins, in G protein-independent Ca^{2+} influx, or in both pathways. In addition, there were general activation mutants that failed to restore aggregation to *car1*$^-$ cells and displayed severe defects in all signal transduction events, including the most robust response, cAMP-dependent cAR1 phosphorylation. Certain of these mutant phenotypes were obtained in a complementary study, where the entire region of cAR1 from the third to the seventh transmembrane helices was randomly mutagenized. Considered together, these studies indicate that the activation cycle of cAR1 may involve a number of distinct receptor intermediates. A model of G protein-dependent and -independent signal transduction through cAR1 is discussed.

G protein-coupled receptors mediate diverse cellular functions in eukaryotic cells. Several hundred of these receptors, which possess four extracellular domains, three intracellular loops, an intracellular C-terminal domain, and seven transmembrane helices, have been identified. Agonist association with the receptor triggers the exchange of GTP for GDP on the α-subunit of the associated heterotrimeric G protein, inducing dissociation of the activated Gα-subunit from the G$\beta\gamma$-complex (for reviews, see Refs. 1 and 2). These both modulate the activity of a number of effectors including adenylyl cyclases (3), phospholipases (4, 5), MAP[1] kinases (6), and ion channels (7).

A number of receptor domains are required for the activation of G proteins. In a variety of receptors, the three cytoplasmic loops act together with the membrane proximal region of the C-terminal domain during this process (8–13). Of these, the third intracellular loop has been most thoroughly characterized. Mutational analysis, use of synthetic peptides, and chimeric receptor studies suggest that the ends of this loop adjacent to the fifth and sixth transmembrane helices play a role in the formation of specific G protein-receptor complexes and in subsequent events required for the activation of G proteins (14–20). In support of this, the central portion of the third intracellular loops of several receptors can be deleted, without adverse effects on the coupling to G proteins (16, 21).

Recent evidence, however, suggests that the third loop domain may not couple directly to G proteins through specific amino acid side chain interactions, but may act as a hinge, which facilitates the exposure of binding domains for G proteins and kinases once the receptor is activated (22). First, synthetic peptides corresponding to the second intracellular loop or the tail domain of the *N*-formyl peptide receptor, but not the third intracellular loop, inhibited the association of G proteins with the receptor (23). Second, mutations in the human muscarinic acetylcholine receptor, subtype M1 (Hm1) in an amino acid motif that is thought to interact with G proteins, BB*XX*B or BB*X*B (where B is a basic amino acid, *X* is a nonbasic amino acid) (24) had minimal effects on receptor coupling to G proteins (25). Third, two distinct point mutations proximal to the sixth helix of Hm1 severely inhibited function and a third point mutation gave rise to a constitutively active receptor but the triple mutant was considerably less impaired (22). Fourth, a number of constitutively active G protein-coupled receptors resulting from amino acid substitutions within the third intracellular loop adjacent to the sixth membrane helix have been identified (26–30). Together, these findings suggest that receptor conformational changes can occur within the same domain thought to interact with G proteins, making it difficult to interpret how previously identified amino acid substitutions and deletions in the third loop influence coupling of G proteins to receptors.

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[1] The abbreviations used are: MAP, mitogen-activated protein; cAR, cAMP receptor; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate; wtl, wild-type-like; PB, phosphate buffer.

In the social ameba *Dictyostelium*, chemotaxis and differentiation are regulated by a family of cell surface cAMP receptors (cARs), which have seven transmembrane helices and are analogous to mammalian G protein-coupled receptors such as rhodopsin (31–33). cAR1 is maximally expressed in aggregating cells and interacts with the G protein α-subunit Gα2 to activate adenylyl and guanylyl cyclases, phospholipase C, and changes in cytoskeletal components required for chemotaxis (for review, see Ref. 34). cAR2 and cAR3 can substitute for cAR1 for many of these events[2] (35). A remarkable feature of cARs is that they also activate a number of signaling events in cells lacking functional G proteins. These include a stimulated Ca^{2+} entry (36, 37), activation of a MAP kinase (38), and regulation of several gene expression events occurring during development (39, 40). G proteins are also not required for cAMP-dependent cAR1 desensitization (37), where cAR1 is phosphorylated on several serine residues present in its C-terminal domain (41) and undergoes a reduction in affinity for cAMP (42).

To explore the functional role of the third intracellular loop of cAR1 in G protein-dependent and -independent signal transduction, we extensively mutagenized the entire loop region. In another study, the entire region from transmembrane III through transmembrane VII was randomly mutagenized (77). Importantly, *Dictyostelium* provides a useful system to screen for random mutations in the receptor; *car1*$^-$ cells have been constructed and these fail to aggregate, a phenotype that is reversed when the cells are transformed with an extrachromosomal vector containing the gene encoding cAR1 (43). The presence of both G protein-dependent and -independent signal transduction pathways mediated by cAR1 provides a unique opportunity to determine whether mutants defective in coupling to G proteins are, in fact, activation mutants defective in both signal transduction pathways. In this study, 22 individual mutants were characterized for their ability to carry out G protein-dependent and -independent signal transduction. Thirteen mutants previously characterized for G protein-independent signal transduction (44) were also characterized for their ability to couple to G proteins. Analysis of both sets of mutants has led to the identification of affinity mutants, general activation mutants, and selective signal transduction mutants that decouple G protein-dependent signaling events, G protein-independent Ca^{2+} influx, and cAR1 phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—96-well polyvinylidene difluoride-bottomed filtration plates (0.65 μm) were from Millipore, silicon oil was from Wacker Silicones Corporation, MI, GTPγS was from Boehringer Mannheim, and Renaissance Western blot chemiluminescence reagent was from Dupont NEN. Other materials used were of analytical grade and purchased from the suppliers indicated in Milne and Devreotes (36).

Cell Strains, Culture Conditions, and Development—In this study, the *car1*$^-$ G418-sensitive strain JB4 (44) was transformed with plasmids containing wild-type or mutant versions of cAR. Transformants were grown in HL5 (45) supplemented with 20 μg of Geneticin/ml of HL5. JB4 was grown in HL5. Cells were maintained in Petri dishes. For biochemical experiments to screen many transformants ([^{32}P]cAMP binding, Ca^{2+} influx, receptor phosphorylation, and development), cells were grown in shaking suspension in 5-ml cultures to a density of ~5 $\times$ 10^6 cells/ml in sterile 50-ml Corning tubes. For all other experiments, cells were grown in Erlenmeyer flasks. To initiate development, cells were washed in developmental buffer, resuspended to 1 $\times$ 10^7 cells/ml, and plated on non-nutrient agar as described previously (46).

cAR1 Library Construction—Two partially degenerate oligonucleotides were synthesized. The first, corresponding to nucleotides 649–681 of the cAR1 gene, was comprised of the sequence TTCTCTTTa′tta′tca′cta′aca′cca′tta′tga′atAACAACAT (region B). The second, corresponding to the nucleotides 673–713 (region C) had the sequence AATTTGAAt′tgg′tat′gtt′a′aa′tgt′ttc′tct′ttATTATCAC. Capital letters represent a homogeneous position. Positions without a prime contain 93% of the indicated nucleotide and 2.3% of the other nucleotides. Positions with a prime are as follows: a′ = 92% A, 8% G; t′ = 92% T, 4% A, 4% G; g′ = 92% G, 8% C; c′ = 92% C, 4% A, 4% G. The cAR1 cDNA, subcloned into bacteriophage M13, was randomly mutagenized using these degenerate oligonucleotides as described elsewhere (44). A *Bam*HI-*Bst*XI fragment from the replicative form DNA was subcloned as described into pMC34, an extrachromosomal *Dictyostelium* expression vector carrying a neomycin resistance gene, to generate a library of mutant cAR1 plasmids. In this construct, cAR1 DNA is flanked by the actin-15 promoter, which is active during growth and aggregation, and the 2H3 terminator. Two mutant cAR1 sequences identified from sequencing of phage clones, IIIa-1 and I-11, were individually subcloned into pMC34.

Transformation of car1$^-$ *Cells, Plasmid Rescue, and Sequencing*—JB4 cells were transformed with the degenerate cAR1 libraries by electroporation as described elsewhere (47). After 12–16 h in HL5, cells were resuspended in HL5 containing 20 μg of Geneticin/ml and divided into 96-well plates. Viable cells were streak-plated on SM agar in association with *Klebsiella aerogenes* to obtain isolated clones, which were reselected into 24-well plates containing selective media. For plasmid rescue, total DNA was recovered from 4 $\times$ 10^7 cells as described previously (48) and used to transform MC1061 bacteria. Plasmids were isolated and sequenced using standard techniques. Examination of the sequences of mutant cARs (B and C region clones, Table I) indicates that the mutagenesis procedure introduced, by unknown means, mutations throughout the entire third intracellular loop, rather than in the expected central one-third or the C-terminal one-third of the third cytoplasmic loop. In contrast, mutagenesis of the N-terminal one-third of the third intracellular loop yielded mutants present in the anticipated region (A region clones) (44). While we have not ruled out the possibility of mutations elsewhere in every B and C region clone, full-length sequencing of mutants wild-type-like (wtl)-4, IIIa-1, I-14, and IV-8 did not reveal the presence of additional mutations. No unexpected mutations were seen in partial sequencing of mutants wtl-9 (nucleotides 350–800), wtl-12 (nucleotides 350–890), I-8 (nucleotides 333–530 and 780–900), and IV-6 (nucleotides 333–901).

Nomenclature of Previously Identified Mutant cAR1s—In order to maintain a consistent mutant nomenclature between this study and that described in a companion study (77) and to allow that the names convey the general properties of the mutants, the following mutant cARs identified previously (44) were renamed as follows (old name = new name): A2 = wtl-1; A22 = wtl-2; A62 = IIIa-2; A5 = I-3; A16 = I-9; A60 = I-10; A42 = IV-1; A53 = IV-2; A3 = IV-3; A55 = IV-4; A81= IV-5.

cAMP Binding Assays—For [^{32}P]cAMP binding, transformants (4 $\times$ 10^5) washed once in phosphate buffer (PB; KH_2PO_4/Na_2HPO_4, pH 6.1) were resuspended in PB and loaded into wells of a 96-well filtration plate. Surface [^{32}P]cAMP binding sites were assayed as described (44) in the presence of 3 M ammonium sulfate, which stabilizes the binding of cAMP to cAR1 (49).

For Scatchard analysis, cells were starved in shaking suspension for 6 h in the presence of 100 nM cAMP pulses, harvested by centrifugation, and resuspended in 50 ml of ice-cold PB. After shaking (22 °C, 250 rpm, 30 min) cells were harvested, washed once in ice-cold PB, and resuspended to 1 $\times$ 10^8 cells/ml in PB. After shaking 10 min on ice, [^{3}H]cAMP binding was performed in triplicate in the presence of either 1 $\times$ 10^{-9} M or 1 $\times$ 10^{-8} M [^{3}H]cAMP and various concentrations of nonradioactive cAMP (0–10^{-6} M) (49). Scatchard plots were generated and analyzed using the computer program LIGAND (50).

GTPγS Inhibition of cAMP Binding—2 $\times$ 10^8 cells were starved in shaking suspension for 6 h in the presence of 100 nM cAMP pulses to induce the expression of the G protein G2, harvested by centrifugation, and resuspended in 50 ml of ice-cold PB. After shaking (22 °C, 250 rpm, 30 min) cells were harvested, washed once in ice-cold PB, and resuspended in 5 ml of the same buffer. After shaking 10 min on ice, cells were lysed through 3-μm Nucleopore filters, and crude membranes were recovered by centrifugation (10,000 rpm, 5 min, 4 °C, SS34 rotor). After resuspending the membranes to 4 $\times$ 10^7 cell equivalents/ml, binding of 2 nM cAMP was measured in triplicate in the presence or absence of 100 μM GTPγS (5 min, on ice) by spinning the membranes through silicon oil (80% light AR20: 20% heavy, P-AR-200) as described previously (51).

Filter Plate $^{45}Ca^{2+}$ Assay—Growth stage cells were washed once in H buffer (20 mM Hepes/KOH, 5 mM KCl, pH 7.0), resuspended to 2 $\times$ 10^7 cells/ml in H buffer, and shaken (15 min, 300 rpm, 22 °C). Aliquots (50 μl) were pipetted in duplicate into wells of a 96-well filtration plates, which were prewashed with 400 μl of H buffer. Using a multichannel pipetter, 100 μl of $^{45}Ca^{2+}$ uptake mix (H buffer, 10 μM $CaCl_2$, 500 μM

[2] J. Y. Kim and P. N. Devreotes, manuscript in preparation.

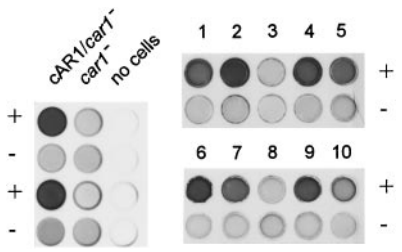

FIG. 1. **Filtration assay for cAMP-induced Ca^{2+} entry.** Growth stage cAR1/*car1*$^-$ cells, *car1*$^-$ cells containing a control vector, or 10 different *car1*$^-$ cell lines expressing different mutant cAR1s were loaded into columns of a 96-well filtration plate and assayed for $^{45}Ca^{2+}$ uptake in the absence (−) or presence (+) of 100 μM cAMP, as described under "Experimental Procedures."

$CoCl_2$, ~5 μCi of $^{45}CaCl_2$) was added to each of one set of wells. To measure cAMP-stimulated Ca^{2+} uptake, the same mix containing 150 μM cAMP was added to the duplicate wells. The reaction was terminated by the addition of 100 μl of 775 mM nonradioactive $CaCl_2$ at 40 s, a time at which cAMP-stimulated Ca^{2+} entry into suspensions of cells is complete. Samples were filtered using a filtration manifold, and washed three times with 200 μl of ice-cold H buffer containing 10 mM $CaCl_2$. Filters were air-dried and exposed to autoradiographic film for 24 h. For quantitation, filters were punched out, vortexed in 100 μl of 1% SDS, and assessed for radioactivity using scintillation counting. Fig. 1 illustrates this rapid filtration assay. Nonstimulated *car1*$^-$ cells accumulated low levels of $^{45}Ca^{2+}$, and this level did not increase detectably in the presence of cAMP. In contrast, *car1*$^-$ cells expressing wild-type cAR1 accumulated significantly more $^{45}Ca^{2+}$ in the presence of 100 μM cAMP than did untreated controls. Eight randomly selected clones expressing different mutant cARs also showed cAMP-stimulated $^{45}Ca^{2+}$ entry, while two clones showed no stimulated Ca^{2+} entry. Wells not receiving cells did not bind appreciable amounts of $^{45}Ca^{2+}$. Quantitation of the wild-type cAR1-induced Ca^{2+} response indicated that it was ~4-fold higher than Ca^{2+} entry into unstimulated cells (Table I). For certain experiments, cAMP-dependent $^{45}Ca^{2+}$ influx into suspensions of cells was assessed as described elsewhere (36).

cAR1 Phosphorylation and Immunoblot Analysis—Growth stage amebae (1×10^6) were washed once in PB and resuspended in PB containing 5 mM caffeine, to restore cAR1 to the 40-kDa form, and 10 mM dithiothreitol, to inhibit phosphodiesterase activity. cAMP-induced cAR1 phosphorylation was measured as described previously (52). Cells were solubilized in Laemmli buffer, electrophoretically separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with cAR1 antiserum as described elsewhere (53), except that an enhanced chemiluminescence kit was used for detection. Autoradiographs of immunoblots were digitized and analyzed as described previously (44).

RESULTS

Screen for Loss-of-function Mutations in cAR1—We performed mutagenesis of wild-type cAR1 using two degenerate oligonucleotides designed to introduce 1–4 mutations into two regions of the third intracellular loop designated B (Ile^{190}–Asn^{197}) and C (Lys^{198}–Gln^{205}). The mutant cAR1 libraries were subcloned into an extrachromosomal expression vector (pMC34), and transformed into *car1*$^-$ cells. The cells were divided into 96-well plates, and after selection, growth-positive wells were individually streak-plated on bacterial lawns to obtain clones. After 7 days, 18 aggregation-negative clones as well as 53 aggregation-positive clones (one per plate) were reselected into 24-well plates containing selective media.

car1$^-$ cells do not show significant levels of cAMP binding sites, but expression of exogenous cAR1 in these cells increases the number of binding sites at least 20-fold (43). The selected clones were assessed for cell surface [^{32}P]cAMP binding sites in the presence of ammonium sulfate using a filtration assay. Fifty-seven clones showed high levels of cAMP binding, 10 clones showed low levels of cAMP binding, and 4 clones did not bind cAMP (data not shown). Clones exhibiting cAMP binding sites and two with no detectable cAMP binding sites were selected for further characterization.

TABLE I

G protein-dependent and -independent signal transduction in car1$^-$ *cells expressing wild-type or mutant cAR1s*

The amino acid sequence of the third intracellular loop of wild-type (WT) cAR1 is shown. For the mutants, deviations from the wild-type sequence are shown; blank spaces indicate identity to the wild-type sequence. The oligonucleotide used to construct each mutant (A, B, or C) is indicated in the right-hand column. cAR1 phosphorylation induced by 50 nM (left hand shift column) or 10 μM cAMP (right-hand shift column) was measured as described under "Experimental Procedures." White circles; complete shifting response; gray triangles, impaired response; black circles, no shifting. cAMP-induced Ca^{2+} accumulation, GTPγS inhibition of [^{3}H]cAMP binding to membranes and development assays were performed as described under "Experimental Procedures." Each experiment was performed at least twice. Ca^{2+} entry was followed for 40 s in the presence or absence of cAMP. Values shown are the ratio of Ca^{2+} uptake into cAMP-treated cells/Ca^{2+} uptake into nonstimulated cells. White circles, ratio ≥2; gray triangles, ratio 1.2–1.9; black circles, ratio ≤1.1. For GTPγS inhibition of cAMP binding, values represent the amount of [^{3}H]cAMP binding sites in treated membranes expressed as a percentage of the levels in untreated controls. White circles, ≤70% remaining binding; gray triangles, 70–95% remaining binding; black circles, 96–100% remaining binding. For development, white circles, aggregation-positive; black circles, aggregation-negative. nd, not determined.

	T S R Y T Y V V I H N G V S D N K E K H L T Y Q F K	shift low	shift high	Ca^{2+}	Dev	GTP	
WT		○	○	4.5 ○	○	36 ○	A
wtl-1	D	○	○	4.4 ○	○	39 ○	A
wtl-2	F L	○	○	2.9 ○	○	57 ○	C
wtl-3	A Y	○	○	6.1 ○	○	36 ○	C
wtl-4	D	○	○	5.3 ○	○	30 ○	C
wtl-5	N	○	○	2.3 ○	○	58 ○	C
wtl-6	A L	○	○	3.9 ○	○	30 ○	C
wtl-7	L D N	○	○	2.5 ○	○	69 ○	C
wtl-8	D F I	○	○	2.4 ○	○	55 ○	C
wtl-9	I	○	○	2.4 ○	○	63 ○	C
wtl-10	Y S	○	○	5.3 ○	○	17 ○	C
wtl-11	I	○	○	3.2 ○	○	nd	C
m11.12	G G	○	○	2.8 ○	○	33 ○	C
IIIa-1	G	△	○	3.2 ○	○	25 ○	A
IIIa-2	C A A	△	○	2.1 ○	○	48 ○	A
I-1	I	○	○	2.2 ○	○	75 △	C
I-2	T N	●	○	2.2 ○	○	70 △	C
I-3	S H	○	○	1.1 ●	○	54 ○	A
I-4	D R V	○	○	1.4 △	○	64 ○	C
I-5	I D I	△	○	1.4 △	○	54 ○	C
I-6	N A K N	●	○	1.2 △	○	49 ○	C
I-7	N	○	○	1.4 △	○	83 △	A
I-8	D S	○	○	1.6 △	○	75 △	C
I-9	H S	△	○	1.3 △	○	92 △	A
I-10	A D	△	○	1.2 △	○	80 △	A
I-11	N	△	○	1.9 △	○	74 △	A
I-12	F N G	△	○	1.4 △	○	89 △	C
I-13	Y S R N	△	○	1.5 △	○	75 △	C
I-14	N	●	○	1.3 △	○	75 △	C
I-15	Q N	●	○	1.4 △	○	nd	C
IV-1	D	△	△	1.0 ●	●	91 △	A
IV-2	S T R	●	△	1.3 △	●	95 △	A
IV-3	L N S	●	△	1.4 △	●	90 △	A
IV-4	A P F	●	△	1.0 ●	●	nd	A
IV-5	N N H D	●	△	1.0 ●	●	86 △	A
IV-6	S R	△	△	1.3 △	●	86 △	B
IV-7	F I N D	●	△	1.0 ●	●	92 △	C
IV-8	P S	●	●	1.0 ●	●	100 ●	B

Plasmids from 60 individual clones were rescued and sequenced through the third intracellular loop. Of these, 22 mutant receptors (B and C region clones) are shown in Table I. Of the rest, 5 receptors were partially characterized and are not shown, 14 receptors contained the wild-type cAR1 sequence, 12 receptors had a single $Lys^{207} \rightarrow Asn$ which was observed in clone Ia-9, 2 receptors had frameshift mutations, and the other 5 possessed sequences identical to certain of the mutant receptors presented in Table I. Further characterization of the clones (discussed below) indicated the presence of at least four classes of mutant receptors: those with properties indistinguishable from wild-type (wtl), cAMP binding affinity mutants with normal signal transduction (class III), general activation mutants that are defective in all responses (class IV), and signal transduction mutants that are defective in specific G protein-dependent responses and/or G protein-independent Ca^{2+} influx (class I). Each of the mutant cell lines was reconstructed fol-

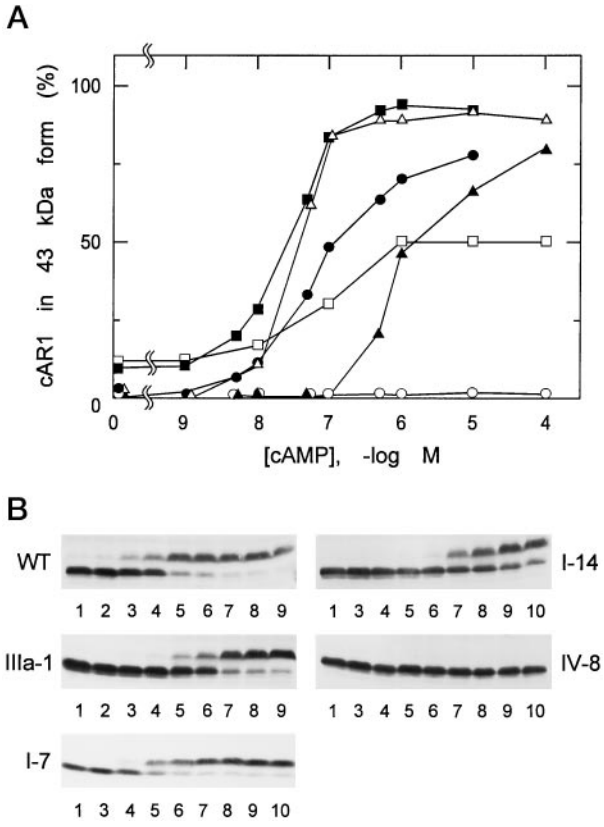

FIG. 2. **Effect of cAMP concentration on phosphorylation of mutant cARs.** Growth stage *car1*$^-$ cells overexpressing wild-type cAR1 (■), mutant IIIa-1 (●), mutant I-7 (△), mutant I-14 (▲), mutant IV-1 (□), or mutant IV-8 (○) were stimulated with the indicated dose of cAMP for 15 min, solubilized, electrophoretically separated, and immunoblotted with cAR1 antiserum as described under "Experimental Procedures." *A*, percentage of the phosphorylated receptor (43 kDa) was calculated by image analysis of three experiments, except for I-7, which represents data from a single experiment. Data for mutant IV-1 was taken from Caterina *et al.* (44). *B*, representative autoradiographs used to derive the values plotted in *A*. *Lanes 1–10* represent the following concentrations of cAMP: 0, 1 nM, 5 nM 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 10 μM, and 100 μM.

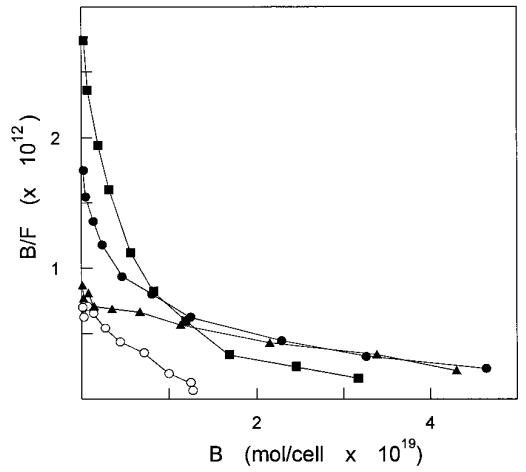

FIG. 3. **Scatchard analysis of cAMP binding to cAR1 mutants.** Phosphate buffer [^{3}H]cAMP binding assays were performed on 6-h starved cells expressing wild-type cAR1 (■), mutant IIIa-1 (●), mutant I-14 (▲), and mutant IV-8 (○). A representative example of a Scatchard plot of each cAR1 is shown. Scatchard data were fitted using the computer program LIGAND; the K_d and B_{max} values are indicated in Table II.

lowing initial biochemical characterization by reintroducing isolated plasmid DNA into *car1*$^-$ cells to recapitulate the phenotype and to eliminate the possibility of multiple plasmids in a single clone.

Characterization of Representative Mutants from Classes I, III, and IV for G Protein-dependent and -independent Responses and cAMP Binding Affinity—We assessed the cAMP-dependent cAR1 phosphorylation of wild-type cAR1 and several representative mutants (IIIa-1, I-7, I-14, and IV-8) by monitoring the parallel change in the apparent molecular mass of the receptor from 40 to 43 kDa on polyacrylamide gels. The reduction of electrophoretic mobility arises from the phosphorylation of Ser^{303} and Ser^{304} present in the C-terminal domain of cAR1 (41). The EC_{50} of this response can be determined if the reaction is carried out to steady-state at increasing doses of cAMP. Fig. 2*A* illustrates the profile of each mutant; Fig. 2*B* shows data representative of that used to derive this plot. Wild-type cAR1 began to respond at low nanomolar concentrations of cAMP and responded maximally between 10 and 100 nM. The EC_{50} of the response was 23 nM. A similar profile was obtained for I-7. Higher concentrations of cAMP were required to induce receptor phosphorylation in mutant IIIa-1 (EC_{50} = 78 nM) and in I-14 (EC_{50} = 428 nM). IV-8 was the most severely impaired, showing no detectable response even in the presence of 100 μM cAMP.

To determine whether the high EC_{50} values of IIIa-1, I-14, and IV-8 were due to a reduced ability to bind cAMP, Scatchard analysis was performed under physiological conditions (Fig. 3 and Table II). Wild-type cAR1 exhibited a minor cAMP binding site with a K_d of 9 nM and and a major site with a K_d of 450 nM, values similar to earlier estimates (54). Mutant IIIa-1, showed a 2-fold reduction in affinity with K_d values of 22 and 1000 nM. Mutant I-14 displayed an even lower affinity with K_d values of 96 and 1600 nM. Despite lacking any detectable phosphorylation response even at 100 μM cAMP, IV-8 bound cAMP comparatively well, with a single affinity site of 220 nM. All of these receptors displayed high levels of high affinity cAMP binding sites in the presence of ammonium sulfate, which stabilizes the binding of cAMP to cAR1 (data not shown).

A rapid filtration assay was used to assess cAMP-dependent Ca^{2+} entry into *car1*$^-$ cells expressing wild-type cAR1, IIIa-1, I-7, I-14, or IV-8 (Table I). The relative ability of IIIa-1, I-14, and IV-8 to trigger the Ca^{2+} response was the same as their relative ability to elicit agonist-induced cAR1 phosphorylation. For example, IIIa-1 was as effective as wild-type cAR1 in promoting cAMP-induced uptake of extracellular Ca^{2+}, I-14 responded weakly, and IV-8 did not respond. Similar results were obtained when cAMP-mediated Ca^{2+} entry was measured by the standard centrifugation assay (36), rather than by the rapid 96-well filtration assay (data not shown). However, despite having the same EC_{50} as wild-type cAR1 for agonist-induced phosphorylation, I-7 showed an impaired cAMP-dependent Ca^{2+} response. The amount of cAMP-dependent Ca^{2+} entry into I-7 and I-14 was standardized to the levels of cAR1 binding sites measured in the presence of saturating concentrations of [^{3}H]cAMP and ammonium sulfate as described in Milne and Devreotes (36). Wild-type cells accumulated 11 ± 2.3 Ca^{2+} ions/receptor, I-7 accumulated 2.1 ± 0.7 Ca^{2+} ions/receptor, and I-14 accumulated 0.6 ± 0.3 Ca^{2+}/receptor (±S.E., n = 3).

The ability of the mutant receptors to mediate G protein-dependent events was examined in several assays. The ability of cells to aggregate when plated on starvation agar was used as an initial test. Cells lacking cAR1, Gα2, or Gβ cannot carry out chemotaxis or cell-cell signaling and remain as smooth monolayers (43, 55–57). Expression of IIIa-1, I-7, or I-14 in *car1*$^-$ cells restored aggregation and supported later development, although not as efficiently as wild-type cAR1. Expression of

TABLE II
Scatchard analysis of WT cAR1 and mutants IIIa-1, I-14, and IV-8

Scatchard plots were generated as described under "Experimental Procedures" and analyzed using the computer program LIGAND. Binding curves were fitted for models with one site and two sites, and affinities were determined. The model that statistically best fits the data is presented. For each receptor, the data represent the results of at least four experiments. R_1 and R_2 are the percentage of high and low affinity sites, respectively.

Receptor	Sites/cell	K_{d1}	K_{d2}	R_1	R_2
	$\times 10^{-3}$		nM		
WT	279 ± 48	9 ± 1	450 ± 50	10	90
IIIa-1	291 ± 43	22 ± 6	1000 ± 100	3.1	96.9
I-14	325 ± 15	96 ± 24	1600 ± 400	2.9	97.1
IV-8	129 ± 17		220 ± 30		>99

IV-8 did not restore aggregation (Fig. 4). Next, GTPγS inhibition of the binding of 2 nM [^{3}H]cAMP to membranes was measured. This response, used generally to assess G protein coupling to seven helix receptors (1), is absent in cells lacking Gα2 or Gβ (58, 59). The measurement was carried out on membrane preparations from cells that were starved in the presence of exogenous cAMP pulses to induce expression of G2. All of the cell lines expressed similar levels of cAR1 and the G2 α- and β-subunits (data not shown). 100 μM GTPγS effectively reduced the binding of 2 nM cAMP to wild-type cAR1 and IIIa-1, but mutants I-7 and I-14 were noticeably impaired, and IV-8 showed no detectable response (Fig. 5). Similar results were obtained when cAMP binding was measured in the presence of 10 nM [^{3}H]cAMP (data not shown), which provided greater sensitivity for the lower affinity mutants.

Characterization of Additional car1$^-$ Clones Expressing Mutant Receptors for G Protein-dependent and -independent Functions—Selected cAR1 mutants obtained from an earlier mutagenesis of the N-terminal one-third of the third intracellular loop (Thr^{182}–Val^{189}, A region clones) were also expressed in *car1$^-$* cells for analysis of G protein-dependent and -independent responses. In our previous work, most of these mutant cARs were analyzed in wild-type AX3 cells, which also contain endogenous cAR1 (44). The functional properties of each mutant cAR1 are illustrated in Table I. Immunoblot analysis of each of these mutant cell lines revealed that they expressed receptor protein at levels ~0.5–2-fold of levels seen in the wild-type cAR1/*car1$^-$* control (data not shown).

One striking result is apparent. Despite its small size, many mutations can be introduced throughout the third loop of cAR1 with no loss of function. For example, 11 mutants (wtl-1 through wtl-11) induced wild-type patterns of electrophoretic mobility shift with maximal responses occurring in the presence of 50 nM cAMP (Table I). Similar results were obtained for M11.12, a mutant cAR1 in which Ser^{183} and Ser^{195} present in the third intracellular loop have been replaced with Gly residues by site-directed mutagenesis.[3] Each of these mutants showed a cAMP-dependent Ca^{2+} uptake that was at least 2.3-fold higher than the amount of Ca^{2+} accumulated by nonstimulated cells. This degree of stimulation is comparable to that elicited by wild-type cAR1, which shows ~2-fold stimulation when cells are assayed in suspension for cAMP-dependent Ca^{2+} uptake (44). Each of the mutants coupled to G proteins since they effectively rescued the aggregation-deficient phenotype of *car1$^-$* cells. Moreover, binding of 2 nM [^{3}H]cAMP to membranes prepared from each of these cAR1-containing mutants (wtl-11 not examined) was reduced by at least 30% in the presence of 100 μM GTPγS.

Twenty of the remaining mutants showed a defective phosphorylation response at 50 nM cAMP but responded fully at 10 μM cAMP. Only 2 receptors, IIIa-1 and IIIa-2, had defects strictly related to cAMP binding. These latter receptors displayed a decreased sensitivity for cAMP in the mobility shift assay, but at saturating concentrations of cAMP behaved like wild-type cells in this response and in cAMP-dependent Ca^{2+} entry. They also effectively underwent GTPγS inhibition of cAMP binding. Mutant IIIa-1 had a single amino acid substitution Arg^{184} → Gly close to the N-terminal side of the loop. Mutant IIIa-2 altered the same amino acid residue Arg^{184} → Cys, although it had several additional alterations. The remaining receptors appeared to have defects in cAMP binding affinity, as assessed by the mobility shift assay; however, these mutants also had additional defects in signal transduction.

Examination of the class I mutants indicated that all of these receptors showed essentially wild-type levels of cAR1 phosphorylation at 10 μM cAMP, but displayed specific defects in signal transduction. Certain mutant receptors appeared to separate the pathways leading to G protein-dependent responses and G protein-independent Ca^{2+} entry. For example, I-1 and I-2 had wild-type levels of stimulated Ca^{2+} entry, but were impaired in GTPγS inhibition of cAMP binding. In contrast, I-3, I-4, I-5, and I-6 displayed the opposite pattern of coupling: they all showed good GTPγS inhibition of cAMP binding, but I-3 displayed no stimulated Ca^{2+} entry, and I-4, I-5, and I-6 were markedly defective. (These findings were confirmed in two independently constructed clones of I-3 and I-4.) The other class I mutants, however, were defective in both signaling pathways, showing less than 30% inhibition of cAMP binding in the presence of GTPγS and reduced levels of stimulated Ca^{2+} entry. Surprisingly, even the most defective of the class I mutants still restored development of *car1$^-$* cells.

Certain amino acid substitutions gave rise to mutant receptors with severely compromised function. These receptors, designated as class IV, appeared to be general activation mutants, since they were uniformly impaired in all G protein-dependent and -independent responses. These mutants typically showed less than 50% of the agonist-induced phosphorylation response at saturating concentrations of cAMP, displayed an absent or highly impaired ability to promote Ca^{2+} entry, displayed little GTPγS inhibition of [^{3}H]cAMP binding, and did not rescue the aggregation-minus phenotype of *car1$^-$* cells (Table I). Detailed data for a representative class IV mutant, IV-1, is shown in Figs. 2 and 5. It shows impaired cAR1 phosphorylation responses, even at saturating concentrations of cAMP (EC_{50} = 178 nM), binds cAMP with a K_d of 117 nM, and shows strongly impaired or absent G protein-dependent signaling (44). We previously found several other mutants in the N-terminal region of the third loop (IV-2, IV-3, and IV-5) that were impaired in their ability to promote cAMP-induced receptor phosphorylation, even at high concentrations of cAMP. Other mutants (IV-4, IV-6, and IV-7) showed similar cAR1 phosphorylation profiles. All of these mutants were strongly impaired or blocked in their ability to activate G protein-dependent events and G protein-independent Ca^{2+} influx (Table I). Several of these mutants (IV-1, IV-2 IV-3, IV-4, IV-5, and IV-6) introduced or deleted charged amino acid residues in the N-terminal side of

[3] J. A. Borleis and D. Hereld, unpublished results.

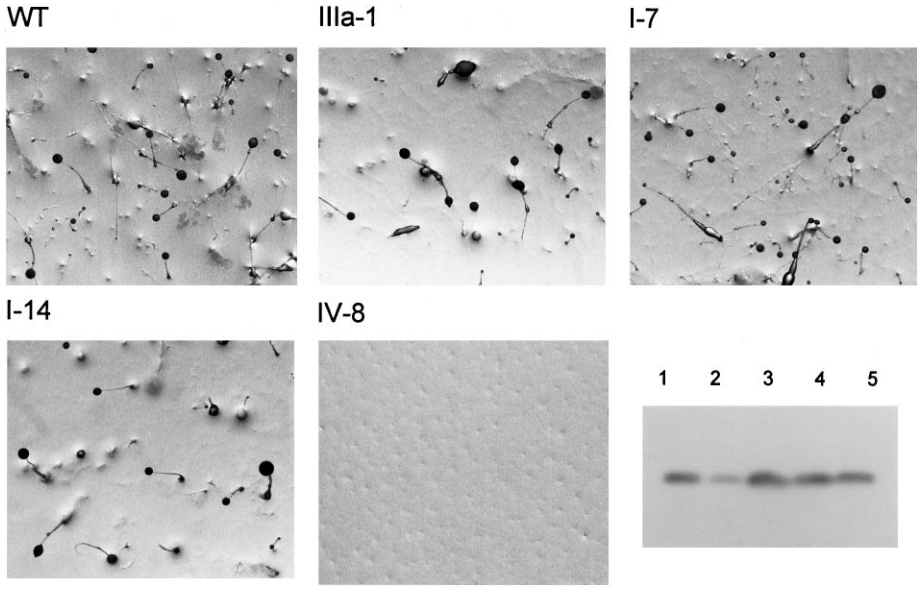

FIG. 4. **Rescue of the aggregation-minus phenotype of *car1*$^-$ cells by wild-type or mutant cAR1 receptors IIIa-1, I-7, I-14, or IV-8.** Growth stage *car1*$^-$ cells transformed with wild-type or mutant cAR1 were washed, resuspended to 1×10^7 cells/ml in development buffer, plated at 1×10^6 cells/cm^2 on phosphate-buffered agar, and developed for 48 h. Immunoblot analysis of 0-h cells expressing mutants I-7 (*lane 1*), IV-8 (*lane 2*), I-14 (*lane 3*), IIIa-1 (*lane 4*), or wild-type cAR1 (*lane 5*).

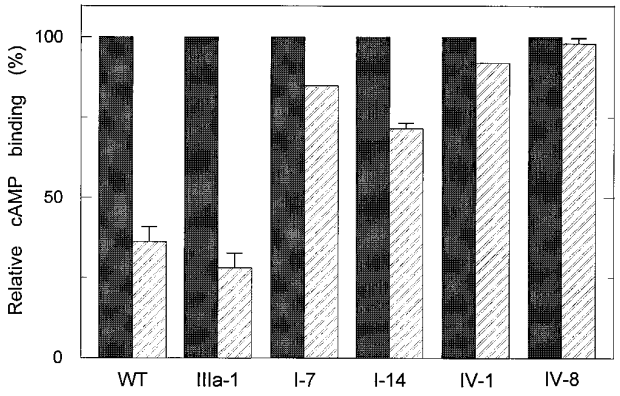

FIG. 5. **GTPγS inhibition of cAMP binding to wild-type cAR1, and mutants IIIa-1, I-7, I-14, IV-1, and IV-8.** Crude membranes from 6-h starved cells were prepared, and binding of 2 nM [^{3}H]cAMP was measured in triplicate in the presence (*striped bars*) or absence (*closed bars*) of 100 μM GTPγS. Results shown are expressed relative to samples not receiving GTPγS and are the average ±S.E. of data obtained in three separate experiments (wild-type, IIIa-1, I-14, and IV-8). Results for I-7 and IV-1 are the average of data obtained in two experiments, which varied by less than 5%. Absolute levels of [^{3}H]cAMP binding (in cpm/mg protein) to buffer-treated membranes was 16755 (wild-type cAR1), 2880 (mutant IIIa-1), 22812 (mutant I-7), 1379 (mutant I-14), 14954 (mutant IV-1), and 4783 (mutant IV-8).

the third intracellular loop. Mutant IV-7 also had a single mutation in this area as well as several other in the central and C-terminal side of the loop, including Tyr^{204} → Asp. The most defective signal transduction mutant, IV-8, bound cAMP (Fig. 3) but failed to elicit any response (Figs. 2, 4, and 5 and Table I). This mutant has two alterations in amino acids (Ser^{183} → Pro, Thr^{186} → Ser) adjacent to the fifth transmembrane helix. The proline substitution is likely the more important determinant of the IV-8 phenotype since mutant I-9, although not wtl, contains the same Thr^{186} → Ser substitution and was able to elicit all responses and rescue development. Introduction of a proline in the adjacent residue, Arg^{184}, also caused severe defects in signal transduction; mutant IV-4 underwent cAMP-dependent receptor phosphorylation, although it did not stimulate Ca^{2+} entry or rescue aggregation.

DISCUSSION

Our analysis suggests that the third intracellular loop of cAR1 can tolerate many amino acid substitutions, with the retention of G protein-dependent and -independent signaling responses. Agonist-induced phosphorylation was the least influenced response, whereas cAMP-dependent Ca^{2+} entry and coupling to G proteins were influenced in a greater number of mutants. However, despite the presence of biochemical deficiencies in certain of the mutants, many functioned sufficiently well to rescue the development of *car1*$^-$ cells.

Of the receptors with defective function, mutants IIIa-1 and IIIa-2 showed defects strictly in cAMP binding affinity, since they effectively elicited all responses but required high concentrations of cAMP to induce receptor phosphorylation (Table I and Figs. 2, 4, and 5). The EC_{50} of agonist-induced receptor phosphorylation of the other class IIIa and many class I mutants suggests that they also likely have defects in affinity. While impairment of cAMP-dependent cAR1 phosphorylation could be due to an inability of the receptor to interact with receptor kinases or undergo conformational changes to expose the phosphorylation domain, Scatchard analysis of IIIa-1 and I-14 supports the idea that at least certain of these mutants bind cAMP with reduced affinity relative to wild-type cAR1 (Fig. 3).

How might alterations in the amino acid sequence of the third intracellular loop domain influence the ability of mutant IIIa-1 to bind cAMP? Studies of rhodopsin and the β-adrenergic receptor indicate that ligand binding occurs within the membrane bilayer in a pocket arising from tight interactions between the seven transmembrane helices (60). Since ligand-induced changes in the spatial arrangement of helices induce conformational changes in the intracellular domains required to couple to downstream proteins, it is plausible that at least certain amino acid changes in the third intracellular loop of cAR1 might trigger conformational changes, which alter the cAMP binding pocket within the membrane. In addition, mutant I-14 replaces Lys^{207} with Asn near the cytoplasmic border of the sixth transmembrane helix and likely also disrupts the relative orientation of the helices. This is probably not due to replacement of the positively charged Lys^{207} residue or due to changes in the size of the amino acid side chain since mutant wtl-9 (Lys^{207} → Ile) did not influence cAMP binding affinity. Rather, insertion of Asn may impair helix packing through its ability to form an additional hydrogen bond through its amide group (61). It remains to be determined if amino acid substitutions in the third intracellular loop introduce conformational changes in the receptor directly or whether they alter interactions with cAR1-binding proteins that modulate binding affin-

ity. Regardless of the mechanism, this study, together with the mapping of mutations within the transmembrane and extracellular domains of cAR1 that modulate cAMP binding affinity (62, 77), suggests that ligand binding to cAR1 is complex, requiring multiple intracellular and extracellular domains.

A number of receptors with defects in signal transduction were also identified. General activation mutants (class IV) were defective in all G protein-dependent and G protein-independent signal transduction (Table I). One of the most severely impaired receptors isolated in this or in a companion study (77), IV-8, failed to elicit any response (Figs. 2, 4, and 5), despite its ability to bind cAMP comparatively well under physiological conditions (Fig. 3). This mutation thus uncouples ligand binding from all subsequent downstream signaling events. In contrast, class I receptors showed more selective defects (Table I). For example, receptors I-1 and I-2 were specifically impaired in coupling to G proteins, possibly due to a reduced ability to bind the G protein or to activate it once it is bound. Similarly, receptors unable to activate cAMP-dependent Ca^{2+} influx (I-3, I-4, I-5, and I-6) may be impaired in their ability to bind or activate the yet unidentified downstream effector(s) which trigger Ca^{2+} entry. It is less likely that cAR1 itself mediates Ca^{2+} entry since there do not appear to be sufficient numbers of acidic amino acid residues in the transmembrane region to form an effective Ca^{2+} binding domain. Although very few of these mutants were isolated, they provide important biochemical evidence complementary to earlier genetic analysis (36, 37) that G protein-dependent signaling through cAR1 can be dissociated from G protein-independent Ca^{2+} signal transduction. It remains to be determined if these mutants influence other G protein-independent events triggered by cAR1, namely, the activation of a MAP kinase (38) and regulation of gene expression events occurring during development (39, 40).

Surprisingly, even though the loop was heavily mutagenized, the specific defect in G protein-dependent signaling seen in I-1 and I-2 was rare and incomplete. In general, functional cARs could mediate all responses, whereas mutants lacking one response were defective in all others. Moreover, many of the mutant cARs retained function despite extensive changes in amino acid sequence, which in many instances, introduced or removed acidic or basic amino acid residues (mutants wtl-1, wtl-3, wtl-5, wtl-6, wtl-7, wtl-8, IIIa-1, I-3, I-4, I-5, I-6, I-8, I-9, I-10, I-12, I-13, and I-15). Interestingly, wtl-8 disrupts a motif containing basic amino acids that is conserved in a large number of seven-helix receptors (2) and thought to be important for interactions between the receptor and G proteins (24). These results are consistent with several models. One possibility is that the loop is required for G protein coupling but the alteration of single or several amino acids was not severe enough to alter the binding affinity of the receptor for G proteins. A second possibility is that the loop may not be needed for specific interactions and that other domains of cAR1 couple to G proteins, as has been suggested for the *N*-formyl peptide receptor (23, 63). If this were so, why do certain mutations in the third loop block all functions? We propose that domains within the third intracellular loop may act as a hinge. Agonist binding may remove a constraint on the wild-type receptor that holds it in a resting conformation, permitting the generation of intermediate states that interact with G proteins, the components involved in G protein-independent signaling and the receptor kinases required for desensitization. The third intracellular loop will likely be essential for the general activation of many, if not all, seven-helix receptors, since activation mutants and constitutively active mutants have been mapped to this region (22, 30, 64–66).

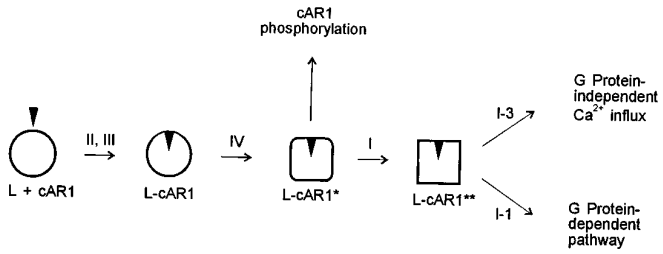

FIG. 6. **Model of putative cAR1 intermediates involved in cAMP-dependent signal transduction.** Classes of mutants which block different steps in the activation pathway are indicated. The affinity mutants of classes II and III and the class I mutant which blocks the formation of L-cAR** are discussed in a companion study (77). *L* = cAMP agonist. Additional details are provided in the text.

In light of these findings, the interpretations from earlier mutagenesis studies of other seven-helix receptors implicating the involvement of the N-terminal and C-terminal domains of the loop for the activation of G proteins need to be reassessed. All of those studies analyzed only G protein-dependent signal transduction and do not preclude the possibility that the mutations impair the ability of the receptor to undergo a general activation step. This would, of course, block subsequent steps in the recognition and activation of G proteins. Recent biochemical evidence suggests that certain mammalian G protein-coupled receptors may also activate G protein-independent signals (67–69) other than receptor desensitization (70–72); these may provide useful systems to address this issue.

The results of the third loop mutagenesis and the random mutagenesis of cAR1 (77) suggest that binding of agonist causes a series of conformational changes in the receptor during the activation process. A model depicting these steps is illustrated in Fig. 6. We propose that cAMP binding to cAR1 leads to an activated state of the receptor, L-cAR1*, which is able to interact with a receptor kinase. Additional conformational change(s) lead to the formation of L-cAR1** enabling the receptor to interact with G proteins or with components required for Ca^{2+} entry. Mutants of class II most likely limit access of cAMP (77), while class III mutants may influence interactions of the agonist with the binding site. The reduction in affinity for cAMP does not prevent the formation of active receptor intermediates, since saturating concentrations of cAMP restores downstream signaling events in most of these mutants. In contrast, the general activation mutants of class IV effectively bind cAMP, but show markedly reduced or absent responses. These signaling defects were not overcome by high concentrations of cAMP, suggesting that the receptors are unable to undergo conformational changes required for the generation or stabilization of any active cAR1 intermediates. In class I mutants, cAMP elicits essentially wild-type phosphorylation responses yet activates poorly G protein-dependent events or G protein-independent influx of Ca^{2+}. These data suggest that there may be a hierarchy among signaling functions; generation of the L-cAR1* intermediate is sufficient for receptor phosphorylation, while L-cAR1** likely is required for Ca^{2+} influx and coupling to G proteins. A few class I mutants, such as I-1 and I-3, were specifically defective either in coupling to G proteins or in cAMP-dependent Ca^{2+} influx. These receptors may attain the L-cAR1** conformation but fail to interact or activate the G proteins or the factor(s) required for ion fluxes, respectively. Alternatively, these mutations potentially could block the formation of yet additional cAR1 intermediates essential for one or both of the responses.

Our model implies that the receptor must go through an intermediate, L-cAR1*, which is able to be phosphorylated before it forms L-cAR1**, which then mediates other signal

transduction events. All of our data are consistent with this model. However, it is conceivable that there are mutants which attain the conformation required for coupling to G proteins and G protein-independent events, but which do not attain the conformation required to interact with receptor kinase. Our experimental designs may have precluded the identification of this particular type of mutant. The third loop mutagenesis targeted a very small region of the receptor, which may not be involved in receptor phosphorylation, whereas the general mutagenesis focused on the characterization of aggregation-deficient clones. In fact, deletion of all of the sites within cAR1 which undergo agonist-induced phosphorylation does not impair aggregation[4] (41).

Our identification of these mutant classes, together with recently emerging information of rhodopsin (73, 74), suggests that the activation of G protein-coupled receptors may be more complex than previously envisioned and may involve multiple intermediates. Given the advances in determining the structure of other seven-helix receptors (75, 76) and progress in the purification of cAR1,[5] these mutants will provide an important tool for structural determination of cAR1 intermediates during the activation process.

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[4] J. Y. Kim and D. Hereld, unpublished results.
[5] X. Xiao and P. N Devreotes, unpublished results.