

Mutation of the Third Intracellular Loop of the cAMP Receptor, *cAR1*, of *Dictyostelium* Yields Mutants Impaired in Multiple Signaling Pathways*

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Seven-membrane span receptors transduce a wide range of signals across the plasma membrane. One member of this family, the cAMP receptor, *cAR1*, of *Dictyostelium*, mediates some responses (e.g. adenylyl cyclase activation, multicellular aggregation) which require G-proteins and others (e.g. Ca^{2+} influx, loss of ligand binding, *cAR1* phosphorylation) which appear to be G-protein-independent. In this study, we randomly mutagenized the NH_2 -terminal eight amino acids of the third intracellular loop of *cAR1* and examined the ability of these mutants to exhibit the three G-protein-independent responses listed above. Most mutants (classes I, II) exhibited wild-type or mildly defective responses. Several mutants (class III), however, were severely impaired in all three processes but not in cAMP binding. Furthermore, these mutants failed to couple productively with G-proteins and could not replace *cAR1* in a *cAR1*[−] cell. For these reasons, we propose that class III mutations interfere with the formation of an "active" conformation of the receptor.

Seven-membrane span, G-protein-coupled receptors transduce intercellular signals in organisms as diverse as mammals (1), yeast (2), and the slime mold, *Dictyostelium* (3). When deprived of nutrients, *Dictyostelium* amoebae initiate a developmental program. Cyclic adenosine 3',5'-monophosphate (cAMP)¹ is secreted from central points and diffuses to nearby cells. cAMP binding to cell surface receptors (cARs) on the responding cells causes them to orient their migration toward the centers and to synthesize and secrete additional cAMP. These events mediate the transition from a unicellular growth phase to a multicellular, sporogenous phase (4, 5).

Four highly homologous cARs have been cloned (3, 6).² The first of these to appear, *cAR1*, mediates the chemotactic, cell

signaling, and gene expression changes which occur within the initial hours of development. Cells lacking *cAR1* fail to aggregate and differentiate (3, 7).

cAR1 occupancy triggers the activation of effector enzymes (e.g. adenylyl cyclase), transmembrane ion fluxes, and morphological changes (reviewed in Ref. 8). As with many G-protein-coupled receptors, these events are closely followed by adaptation; within several minutes of cAMP application, all of these responses return to basal levels (9, reviewed in Ref. 8). Some of these changes, such as adenylyl cyclase adaptation, are correlated with rapid, occupancy-dependent alterations in *cAR1*, including the phosphorylation of serine residues on the cytoplasmic carboxyl domain³ (10, 11) and a reduction in the number of receptors capable of binding cAMP (12, 13). Similar alterations in the mammalian β -adrenergic receptor appear to regulate desensitization of catecholamine-activated adenylyl cyclase (14, reviewed in Ref. 15).

Many, but not all, responses of this family of receptors result from the activation of heterotrimeric guanine nucleotide binding regulatory proteins. In *Dictyostelium*, eight G-protein α -subunits have been identified (16–18).⁴ One of these, $G_{\alpha 2}$, is essential for many of the *cAR1*-mediated responses listed above (19, 20). However, several *cAR1*-dependent responses, including Ca^{2+} influx, *cAR1* phosphorylation, and *cAR1* loss of ligand binding (LLB), can occur in the absence of this or any other of the G-protein α -subunits examined thus far (21–23). These responses appear to be G-protein-independent (22).^{5, 6}

The triggering of activation and desensitization pathways requires the specific detection of occupied receptors by other signal transducing proteins. The existence of an active receptor conformation has been postulated on spectroscopic and/or biochemical grounds (24, 25). Moreover, studies involving mutant adrenergic receptors suggest that ligand binding and the activation isomerization of receptors are separate but interrelated processes (26, 27).

Many mutant seven membrane-span receptors possessing substitutions or deletions in their third intracellular loops fail to activate G-proteins (28–30). These mutations are thought to perturb contact sites between receptors and G-proteins. However, certain of these mutations also interfere with LLB (30), which can occur without heterotrimeric G-proteins (31). Therefore, another explanation is that these mutant receptors may be deficient in the attainment of an activated conformation, causing a failure in all responses.

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¹ The abbreviations used are: cAMP, adenosine, 3',5'-cyclic monophosphate; cAR, cAMP receptor; cGMP, guanosine 3',5'-cyclic monophosphate; LLB, loss of ligand binding; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; BSA, bovine serum albumin; kb, kilobases; rpm, revolutions/min.

² J. M. Louis, J. T. Ginsberg, and A. R. Kimmel, manuscript in preparation.

³ D. Hereld, R. Vaughan, J. Y. Kim, J. Borleis, and P. N. Devreotes, manuscript submitted.

⁴ M. Brandon, S. Vogelmaier, and P. N. Devreotes, manuscript in preparation.

⁵ L. Wu, and P. N. Devreotes, manuscript in preparation.

⁶ J. L. S. Milne, L. Wu, and P. N. Devreotes, manuscript in preparation.

In this study, we examined the role of the cAR1 third intracellular loop in cAMP-mediated activation and desensitization events by randomly mutagenizing a portion of this domain. Many of the mutant receptors exhibited minor deficits in the functions examined. However, we identified a class of mutants which bind ligand with high affinity, but which are severely compromised in both G-protein-dependent and G-protein-independent functions. Because of the pleiotropic nature of this phenotype, we propose that these receptors may be specifically impaired in activation isomerization.

MATERIALS AND METHODS

Cells and Cell Culture—AX-3 cells were grown in shaking culture in HL5 (32). Transformed cell lines were maintained on Petri dishes in HL5 with G418 (20 µg/ml, Sigma) or in HL5 alone (JB-4 cells only). Unless otherwise indicated, cells were grown for 1–2 days in shaking culture, harvested at $<5 \times 10^6$ /ml, and washed once in PB (5 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5 mM KH_2PO_4 , pH 6.1). Development in shaking culture with 300 nM cAMP pulses and on developmental buffer plates was initiated as described (5). Bacterial plate development was initiated by spotting 1×10^5 cells on a lawn of *Klebsiella aerogenes*.

Construction of a *car1* Cell Line—A G418-sensitive, *car1*[−] *Dictyostelium* cell line was created from AX-3 cells in two steps. First, a uracil auxotroph, DH-1, was produced by deletion of the *pyr5–6* locus (33). A *ClaI*-*EcoRI* fragment was removed from p188.50 (34) and replaced with a PCR fragment extending from nucleotide 1797 of the *pyr 5–6* 3′-noncoding region (35) to the *EcoRI* site of p188.50 to generate pRG24. This plasmid was linearized and electroporated into AX-3 cells (see below). 5-Fluoroorotic acid-resistant (100 µg/ml, Sigma) cells were selected clonally in FM medium (36) supplemented with uracil (20 µg/ml, Sigma). Deletion of the *pyr 5–6* locus was confirmed by Southern blot analysis.

Second, the two endogenous *cAR1* genes were deleted from DH-1 cells by homologous recombination. A 2.0-kb fragment containing sequence immediately upstream of the *cAR1* coding region (37) and a fragment containing the final 0.4 kb of coding sequence and 0.1 kb of 3′-untranslated region of the *cAR1* cDNA were adjacently subcloned into pBluescriptSK. A 3.7-kb *ClaI* fragment from pDU3B1 (33) was subcloned between these inserts to create pMC25. This vector was linearized and transformed into DH-1 cells by electroporation, and uracil prototrophs were selected clonally. The deletion of both *cAR1* genes was demonstrated by Southern blot analysis. Like previously reported *car1*-cell lines (7), these cells (JB-4) fail to express cAR1 protein or to aggregate upon starvation.

Plasmids—Plasmid pMC33 was prepared by inserting a *Bam*HI fragment from pDH20 containing a *cAR1* cDNA³ into m13mp19 replicative form DNA (Sigma). In extrachromosomal expression plasmid pMC36, a *cAR1* cDNA is flanked by an Actin15 promoter, which drives constitutive transcription in both growth-phase and vegetative cells, and a 2H3 terminator (38). pMC36 was made as follows. First, a DNA fragment containing nucleotides 97–1312 of the *cAR1* cDNA(3) (but lacking the first two codons) was cloned into the *Bgl*II site of pJK1³ to make pMC34. Second, the *Bgl*III-*Bst*XI fragment of pMC34 was replaced with a *Bgl*III-*Bst*XI fragment from pMC33 to restore the initial *cAR1* codons and thereby create pMC36.

Oligonucleotide-directed Mutagenesis—A partially degenerate antisense primer bearing the following sequence was synthesized encoding nucleotides 636–659 of the *cAR1* cDNA: 5′-TTATGAATa′aca′aca′tag′g′-ta′taa′cgt′gat′gtTAAACCAA-3′. Capital letters indicate a homogenous position, and lower case letters represent a degenerate position. Positions without a prime contain 93% of the indicated nucleotide and 2.3% of each of the other nucleotides. Symbols with primes represent the following: a′ = 92% A, 8% G; t′ = 92% T, 4% A, 4% G; g′ = 92% G, 8% A. pMC33 was randomly mutagenized using this degenerate primer as described (39). A *Bam*HI-*Bst*XI fragment from the resulting mutagenized replicative form DNA was then subcloned into pMC34 to generate a library of mutant plasmids.

Electroporation, Plasmid Rescue, and Sequence Analysis—AX-3 or JB4 cells were electroporated using a Bio-Rad Gene Pulser (40) and selected clonally in HL-5 with G418 (20 µg/ml). For plasmid rescue, total DNA from 4×10^7 *Dictyostelium* cells was prepared as described (37), and the entire preparation used to transform competent MC1061 bacteria. Rescued plasmids were isolated using standard procedures and sequenced with Sequenase (U. S. Biochemical Corp.).

[³²P]cAMP Synthesis—[³²P]cAMP was synthesized enzymatically as

described (41). Briefly, 100 µCi (10 µl) of [³²P]cAMP was incubated (30 min, 37 °C) with 1.2 µg of *Bacillus anthracis* EF toxin (the generous gift of Dr. Stephen Leppa), in 20 mM Tris-HCl, 12.5 mM CaCl_2 , 2.5 µg/ml bovine brain calmodulin (Sigma), 5 mM MgCl_2 , 1 mM EDTA, 0.1 mg/ml bovine serum albumin, pH 7.5. Conversion efficiency was assessed by thin layer chromatography on polyethyleneimine cellulose (42). cAMP was then purified to radioactive homogeneity using a Dowex-50 column (43). The concentration of the radiolabeled cAMP was determined by scintillation counting.

Filter Plate cAMP Binding Assays—[³²P]cAMP binding was measured in the presence of ammonium sulfate, which stabilizes cAMP binding to cAR1 (44). To facilitate screening, we performed these assays in 96-well plates. Cells (4.5×10^6), harvested from shaking culture, were washed once in PB and loaded into triplicate wells of a 96-well polyvinylidene difluoride-bottomed plate (0.65 µm, Millipore). The buffer was removed by manifold filtration and replaced with 50 µl of PB, 10 mM dithiothreitol (DTT). Each well received 250 µl of ice-cold 98% saturated ammonium sulfate containing 5×10^{-10} M [³²P]cAMP with or without 10^{-4} M unlabeled cAMP. After 5 min, wells were filtered and washed three times with 250 µl of ammonium sulfate. Filters were air-dried and subjected to autoradiography. Results were linear over a 10-fold range of cell densities (data not shown).

Ca²⁺ Influx—Unless otherwise indicated, vegetative amoebae grown axenically (5×10^6 /ml) were assayed for chemoattractant-induced ⁴⁵Ca²⁺ uptake in the presence of 10 µM CaCl_2 and 0.5 mM CoCl_2 as described (22). Receptor-induced Ca²⁺ entry is equal to the amount of Ca²⁺ accumulated by chemoattractant-treated cells minus the amount of Ca²⁺ accumulated by resting cells. Certain strains (A3, A42, A53, and A81) were grown on plates in association with bacteria in experiments to measure folate-induced Ca²⁺ entry. Protein was measured as described (45), using bovine serum albumin as standard.

Immunoblotting—Whole cells, solubilized in Laemmli buffer (46), were subjected to SDS-PAGE on 10% low bis-polyacrylamide gels, electrophoretically transferred to PVDF membranes, blocked with 3% BSA as described (47), and blotted with anti-cAR1 serum CR4 (3), anti-G_{α2} (48), or anti-G_β (49). Proteins were detected using ¹²⁵I-Protein A and autoradiography except for Fig. 10, where an enhanced chemiluminescence kit (Amersham Corp.) was used as described by the manufacturer. Autoradiographs of immunoblots were digitized using an Eagle-Eye photographic system (Stratagene). The images were analyzed using the program NIH Image.

Loss of Ligand Binding—Washed, growth-stage cells were resuspended to 3×10^7 /ml and divided into 2 aliquots. LLB was assayed essentially as described (13). Cells were shaken (20 min, 200 rpm, 22 °C), and DTT was added to 10 mM. One sample then received cAMP at 10^{-5} M or 10^{-6} M, as indicated, while the other received an identical volume of H₂O. At the designated times, cells were diluted 15-fold in ice-cold PB, pelleted (3 min, 2000 rpm, SS34 rotor) and washed three times with ice-cold PB. Cells were resuspended to 5×10^7 /ml in ice-cold PB and kept on ice. Binding assays were performed in PB at 16 nM [³H]cAMP and 10 mM DTT (44).

Phosphorylation—cAR1 phosphorylation was performed as described (11). Phosphorylation was halted by the rapid solubilization of cells in Laemmli buffer. Samples were stored at −20 °C until immunoblotting. Control experiments indicated that phosphorylation stopped upon the addition of Laemmli buffer and remained stable for at least 2 h on ice.

Scatchard Analysis—Washed, growth-stage cells were resuspended in PB to 1×10^8 /ml, and kept on ice. [³²P]cAMP binding in PB was performed in triplicate in the presence of 10^{-9} M [³²P]cAMP and varying concentrations of unlabeled cAMP (0 – 10^{-6} M) (44). Scatchard plots were generated, and the data analyzed by linear regression.

[³²P]cAMP Uptake—Washed growth-stage cells were resuspended in PB to 3×10^7 /ml, shaken (200 rpm, 22 °C, 15 min), and DTT added to 10 mM. Cells were then shaken in the presence of 10^{-9} M [³²P]cAMP and 2×10^{-6} M unlabeled cAMP. Nonspecific uptake was assessed by adding 10^{-4} M unlabeled cAMP to parallel samples. At the indicated times, cells were diluted 15-fold in ice-cold PB, 10^{-4} M cAMP, immediately pelleted (5 min, 4000 rpm, HS-4 rotor), and washed three more times in PB. The pellets were solubilized in 300 µl of 1% SDS, 4 ml of scintillation fluid were added, and radioactivity was assessed. [³²P]cAMP binding assays were performed in parallel to allow normalization of uptake to total receptor number.

GTP Inhibition of Binding—Cells developed in shaking culture were diluted 10-fold in PB, shaken (200 rpm, 20 min 22 °C), washed once in PB, resuspended to 4×10^7 /ml, and shaken (200 rpm, 10 min, 0 °C). Cells were then lysed through 5-µm filters (Millipore) and the lysates pelleted (5 min, 10,000 rpm, SS34 rotor). The pellets were resuspended in PB to a density of 6×10^7 cell equivalents/ml and kept on ice. Binding

of 2 nM [^3H]cAMP binding was then assessed by spinning through silicone oil as described (50).

RESULTS

Construction of a Mutant Receptor Library—We performed oligonucleotide-directed mutagenesis of cAR1 cDNA in m13 (pMC33) to synthesize a randomly mutagenized population of cAR1 cDNAs. A degenerate primer was designed to introduce 1–4 random nucleotide mutations into the region encoding the amino-terminal portion of the cAR1 third intracellular loop (Thr¹⁸²–Val¹⁸⁹). The resulting cDNA pool was then subcloned into a segregating, extrachromosomal expression vector (pMC34), and transformed into *Dictyostelium*. Several hundred independent clones were obtained.

Vegetative amoebae contain only a few thousand cAMP-binding sites/cell, and these levels increase 20–100-fold upon transformation with a cAR-containing plasmid (3). A 96-well assay was developed to rapidly measure this increase in the mutant clones ("Materials and Methods"). Under these conditions,

wild-type cAR1-transformed cells bind 15–20-fold as much cAMP as vector-transformed control cells. Most of the mutants we analyzed displayed cAMP binding levels comparable to that of wild-type cAR1. Fig. 1 contains data from 22 randomly chosen clones; 15 displayed high affinity cAMP binding. Immunoblots confirmed the cAR1 expression (data not shown).

Plasmids were rescued from 22 clones exhibiting significant cAMP binding and the relevant portion of the cAR1 cDNA sequenced. Seven contained the wild-type sequence, while the rest contained one of 15 mutant sequences (Table I). The mutations were randomly distributed through the targeted region, although there was a paucity of mutations in the first two codons. No mutations were observed within 30 base pairs upstream or downstream of this region. Complete sequencing of three cDNAs (A42, A53, and A81) also revealed no additional mutations. Previous studies indicate that constitutive overexpression of cAR1 does not impair development. A mutant receptor, however, might impair development if, for instance, it bound tightly but nonproductively to a G-protein or a kinase. However, despite a significant extent of mutagenesis in the overexpressed receptors, all 15 clones showed normal aggregation and formation of fruiting bodies when starved on bacterial plates (data not shown). We concluded that our receptor population contained no dominant negative mutants.

Next, we assessed the capacity of the mutant receptors to mediate three biochemical processes: cAMP-induced influx of extracellular Ca^{2+} , LLB, and phosphorylation. The constitutive expression of exogenous cARs allows these responses to be studied in vegetative cells, in the virtual absence of endogenous wild-type cAR1 (3, 22, 38).

cAMP-mediated Ca^{2+} Uptake—cAMP triggers Ca^{2+} influx in growth-stage cells expressing exogenous cAR1 but not in cells transformed with a control plasmid (9). As illustrated in Table I, AX3 cells expressing wild-type cAR1 displayed cAMP-induced Ca^{2+} influx. The ratio of Ca^{2+} uptake into cAMP-treated cells versus that into resting cells was about 2-fold, as reported previously (9). Mutant receptors showed differential ability to mediate cAMP-induced Ca^{2+} influx. Certain mutants (A2, A7, A22, A40, A46, A55, and A63) showed an unimpaired or rela-

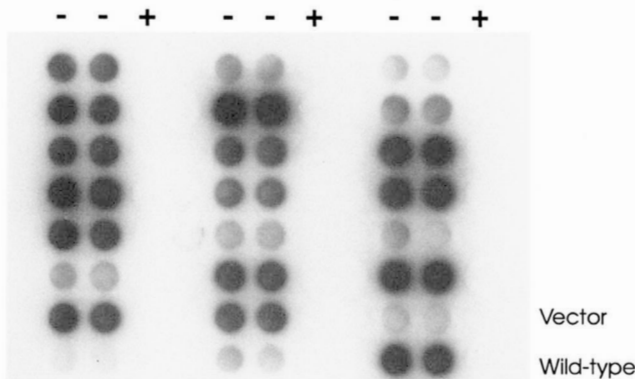


FIG. 1. [^{32}P]cAMP binding to 22 random cAR1 mutants. Growth-stage AX-3 cells expressing mutant cARs were loaded in triplicate into 96-well filter-bottomed tissue culture plates and cAMP binding at 5×10^{-10} M [^{32}P]cAMP measured in the absence (–) or presence (+) of 10^{-4} M unlabeled cAMP as described under "Materials and Methods." Wells containing control vector-transformed or wild-type cAR1-transformed cells are labeled at lower right.

TABLE I
cAMP-stimulated Ca^{2+} influx and loss of ligand binding by cells expressing wild-type and mutant cARs

The amino acid sequence within the targeted region is indicated for each cAR1 mutant. A blank space indicates a wild-type residue at that position. Ca^{2+} entry into growth-stage cells was measured for 30 s in the absence or presence of cAMP as described under "Materials and Methods." Values shown are the ratio of Ca^{2+} uptake into cAMP-treated cells/ Ca^{2+} uptake into resting cells and are the means \pm S.E. of the indicated number of experiments. Loss of ligand binding was assessed in growth-stage cells after treatment with 1 μM or 10 μM cAMP for 15 min, as described under "Materials and Methods." % Lost is defined as $100 - ((\text{cAMP binding in stimulated cells}/\text{cAMP binding in unstimulated cells}) \times 100)$. Values shown are the mean \pm S.E. of the indicated number of experiments.

Class	Cell line									Ca ²⁺ Influx		Loss of ligand binding			
	Sequence									Ratio	n	1 μM cAMP		10 μM cAMP	
												% Lost	n	% Lost	n
	Wild-type	T	S	R	Y	T	Y	V	V	2.04 ± 0.10	20	70 ± 4	9	77 ± 2	8
I	A60					A	D			1.10	1	69 ± 5	2	76	1
	A63				C			G		1.81 ± 0.09	2	55 ± 2	2	76	1
	A46						H			1.40 ± 0.02	9	78 ± 4	2	72	1
	A55		A	P					F	1.35 ± 0.09	3	49	1	73 ± 13	2
	A62			C				A	A	1.30 ± 0.10	2	61 ± 7	2	70	1
	A22							F	L	1.81 ± 0.11	3	60 ± 13	2	66	1
	A7	R			F			A		1.66 ± 0.12	3	61 ± 8	2	66	1
II	A2							D		1.80 ± 0.34	3	47	1	62	1
	A40	I		S			D			1.50 ± 0.15	3	59	1	57	1
	A5	S					H			1.13 ± 0.02	6	62	1	53	1
	A16				H	S				1.30 ± 0.09	4	58	1	49	1
III	A3			L	N		S			1.04 ± 0.02	4	33 ± 7	3	37 ± 6	3
	A53	S						T	R	1.12 ± 0.08	4	18 ± 6	4	32	1
	A81				N	N	H	D		1.05 ± 0.03	3	24 ± 2	2	27 ± 6	2
	A42							D		1.02 ± 0.02	7	28	1	23 ± 1	3

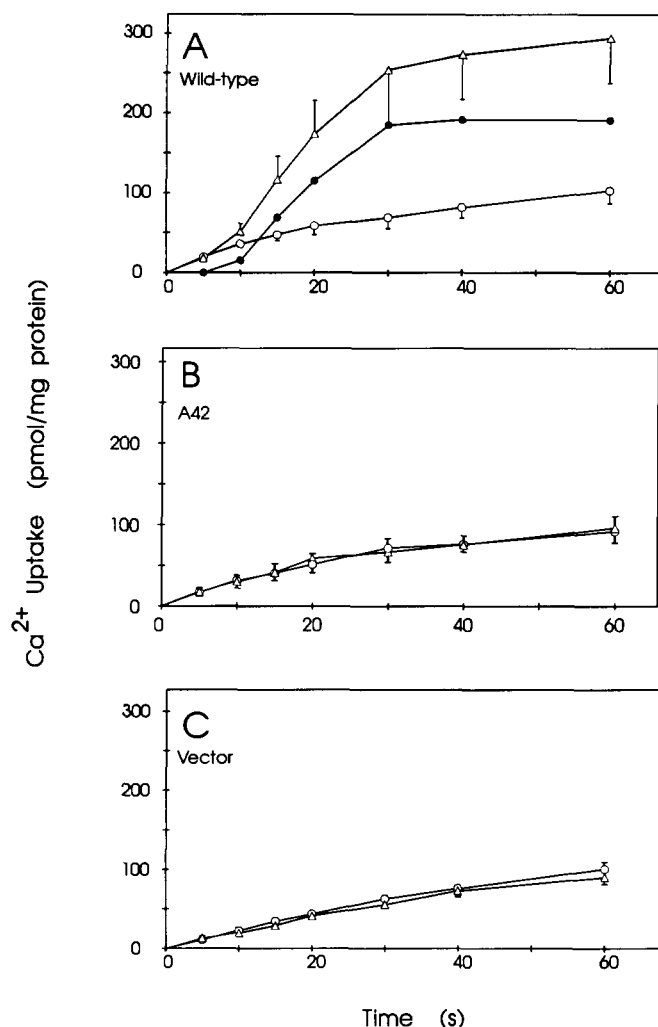


FIG. 2. Ca^{2+} uptake into *car1*⁻ cells expressing A, wild-type cAR1; B, mutant A42 or; C, control vector. Growth-stage cells were assayed for Ca^{2+} entry in the presence of $100 \mu\text{M}$ Ca^{2+} as described under "Materials and Methods." Values shown are for Ca^{2+} uptake into resting (open circles) and cAMP-stimulated cells (open triangles) and for cAMP-induced Ca^{2+} (filled circles). Results shown in each panel represent the means \pm S.E. of data from three independent experiments.

tively unimpaired Ca^{2+} response, despite the fact that many contained multiple amino acid replacements. The remaining mutants did not trigger a cAMP-induced Ca^{2+} response. Mutant A42 is noteworthy since it possesses a single amino acid replacement and is very similar to responder mutant A2.

Additional experiments were performed to confirm the Ca^{2+} null phenotype. Null mutants (A3 and A42) failed to show stimulated uptake when the Ca^{2+} uptake reaction was followed for longer times (60 s) in the presence of higher concentrations ($100 \mu\text{M}$) of Ca^{2+} (Fig. 2 and data not shown). In addition, cAMP-induced uptake into mutants A3, A42, or A53 was not restored when the cells (a) were depleted of intracellular Ca^{2+} prior to assay (51) or (b) were stimulated with cAMP in the presence of EGTA 10 s before the addition of $^{45}\text{Ca}^{2+}$, a procedure which lowers basal Ca^{2+} uptake (9, data not shown). Finally, the folate-induced Ca^{2+} response of A3, A42, A53, and A81 was comparable to the wild-type response, suggesting that non-cAMP receptor component(s) of the Ca^{2+} -signaling pathway were functional in these cells (data not shown). To further test this idea, wild-type cAR1 and mutant A42 were expressed in *car1*⁻ cells ("Materials and Methods"). Growth-stage cells expressing wild-type cAR1 exhibited a cAMP-induced Ca^{2+} up-

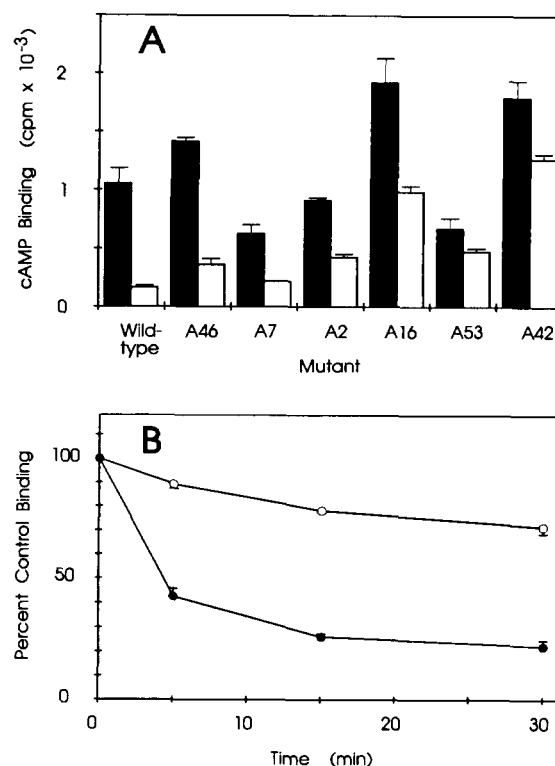


FIG. 3. Loss of ligand binding in cAR1 mutants. A, [³H]cAMP binding in PB was measured in growth-stage AX-3 cells expressing wild-type or mutant cAR1 after pretreatment without (shaded bars) or with (open bars) 10^{-6} M cAMP as described under "Materials and Methods." Specific binding is plotted as the mean \pm S.E. of triplicate determinations from a representative experiment. Specific binding by control vector-transformed cells was typically less than 60 counts/min. B, kinetics of loss of ligand binding in growth-stage AX-3 cells expressing wild-type cAR1 (filled circles) or mutant A42 (open circles). [³H]cAMP binding in PB was measured after pretreatment of cells in the absence (0 min) or presence (5, 15, 30 min) of 10^{-6} M cAMP as described under "Materials and Methods." The percentage of control (0 min) binding remaining after the indicated period of cAMP treatment is shown as the mean \pm S.E. of two independent experiments performed in triplicate.

take comparable to other cAR1-expressing strains (compare Fig. 2A with Figs. 2 and 7 in Ref. 22). However, cells containing similar levels of A42 (see below) or vector control cells failed to show stimulated uptake (Fig. 2, B and C). Comparable levels of basal Ca^{2+} uptake were observed in all three cell lines (Fig. 2).

Loss of Ligand Binding—cAMP pretreatment of *Dictyostelium* amoebae leads to a rapid reduction of up to 80% of surface cAMP-binding sites within 7 min (12, 13, 38). While the mechanism of LLB is unclear, it depends upon cAR1 occupancy. LLB was examined in cAR1 mutant-expressing clones following pretreatment for 15 min with 1 or $10 \mu\text{M}$ cAMP. As shown in Fig. 3A and Table I, wild-type cAR1 displayed a 77% LLB. The mutant clones displayed similar levels of cAMP binding, but showed a continuum of impairment in LLB.

We divided these mutants into three classes, based on the degree of impairment. Class I was relatively unimpaired (66–76% LLB). The retention of nearly full activity in mutants with three replacements (A7, A55, A62) was striking. Mutants of Class II were moderately impaired (49–62% LLB). Class III mutants were severely impaired (23–37% LLB). Even prolonged cAMP treatment failed to induce significant LLB in mutant A42 (Fig. 3B) or mutants A53, A3, or A81 (data not shown), suggesting a fundamental defect in this process. It was noted, furthermore, that those mutants which displayed the greatest impairment in LLB also displayed the most pronounced reduction in stimulated Ca^{2+} influx.

FIG. 4. cAMP-induced phosphorylation of cAR1 mutants. Growth-stage AX-3 cells containing control vector pMC34 or expressing wild-type or mutant cAR1 were stimulated for 15 min without (–) or with (+) 10^{-5} M cAMP for 15 min, solubilized in Laemmli buffer, and immunoblotted with cAR1 antiserum as described under “Materials and Methods.” The lower and upper bands in each panel are basal and phosphorylated cAR1, respectively. Wild-type cAR1 is loaded in both panels for direct comparison with the mutants. The class designations are described under “Results.”

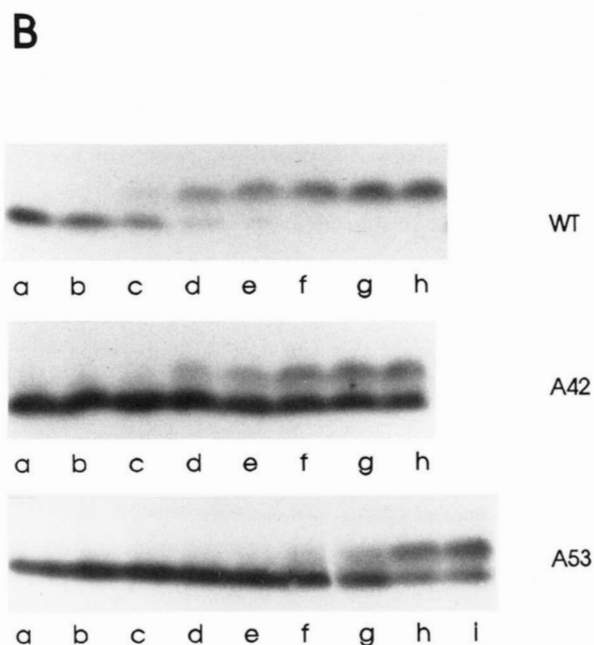
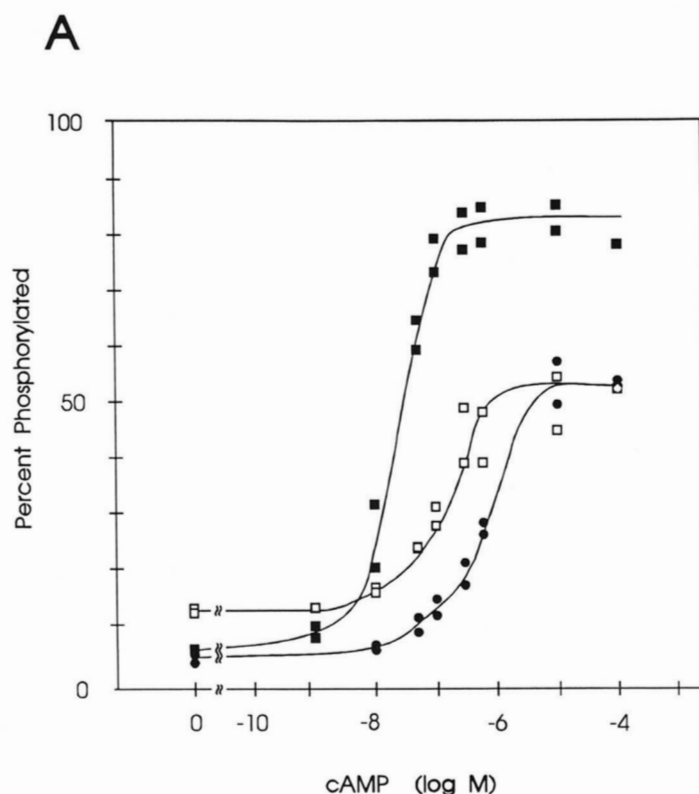
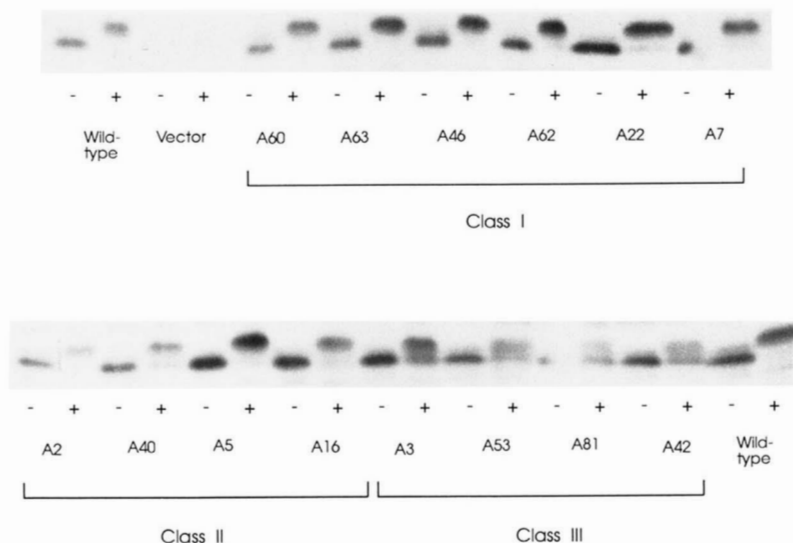


FIG. 5. Dose dependence of cAMP-induced phosphorylation of cAR1 mutants. Growth-phase AX-3 cells expressing wild-type cAR1 (filled squares), mutant A42 (open squares), or mutant A53 (filled circles) were stimulated with the indicated dose of cAMP for 30 min, solubilized in Laemmli buffer, and immunoblotted with cAR1 antiserum as described under “Materials and Methods.” A, percentage of receptor in the phosphorylated (upper band) form was calculated by image analysis of autoradiographs as described under “Materials and Methods.” Results shown are from two independent experiments. B, representative autoradiographs used to derive values in panel A. a–i represent the following doses, respectively: 0, 10^{-9} M, 5×10^{-9} M, 10^{-8} M, 5×10^{-8} M, 10^{-7} M, 3×10^{-7} M, 6×10^{-7} M, 10^{-5} M, 10^{-4} M.

Phosphorylation—The relative abilities of the mutants to undergo cAMP-induced phosphorylation were compared by monitoring by immunoblot a change in the apparent molecular mass of the receptor from 40 to 43 kDa (Fig. 4). This electrophoretic mobility shift is the result of serine phosphorylation within the sequence S²⁹⁹PYSSSRGTS³⁰⁸ in the cytoplasmic carboxyl terminus of cAR1.³ For wild-type cAR1, nearly all of the 40 kDa band shifted to 43 kDa (Fig. 4, top left, bottom right). While many of the mutants exhibited a nearly wild-type response (Fig. 4, top right, bottom left), class III mutants were markedly impaired (Fig. 4, bottom right). The apparent increase in molecular mass of the mutant receptors was identical to that of

wild-type cAR1, but the fraction of phosphorylated molecules at steady-state was reduced.

The dose dependence and rate of the ligand-induced phosphorylation of several representative mutant receptors were examined next. cAMP elicited half-maximal phosphorylation of wild-type cAR1 at 25 nM (Fig. 5). Mutant A42 showed a decreased sensitivity to cAMP (EC_{50} = 178 nM), while that of mutant A53 was even further reduced (EC_{50} = 630 nM). The responses of both mutants reached a plateau at 10 μ M cAMP, at a fractional level of phosphorylation considerably below that of wild-type. Prolonged cAMP treatment (10^{-4} M, 2 h) failed to further increase the phosphorylation of these two mutant re-

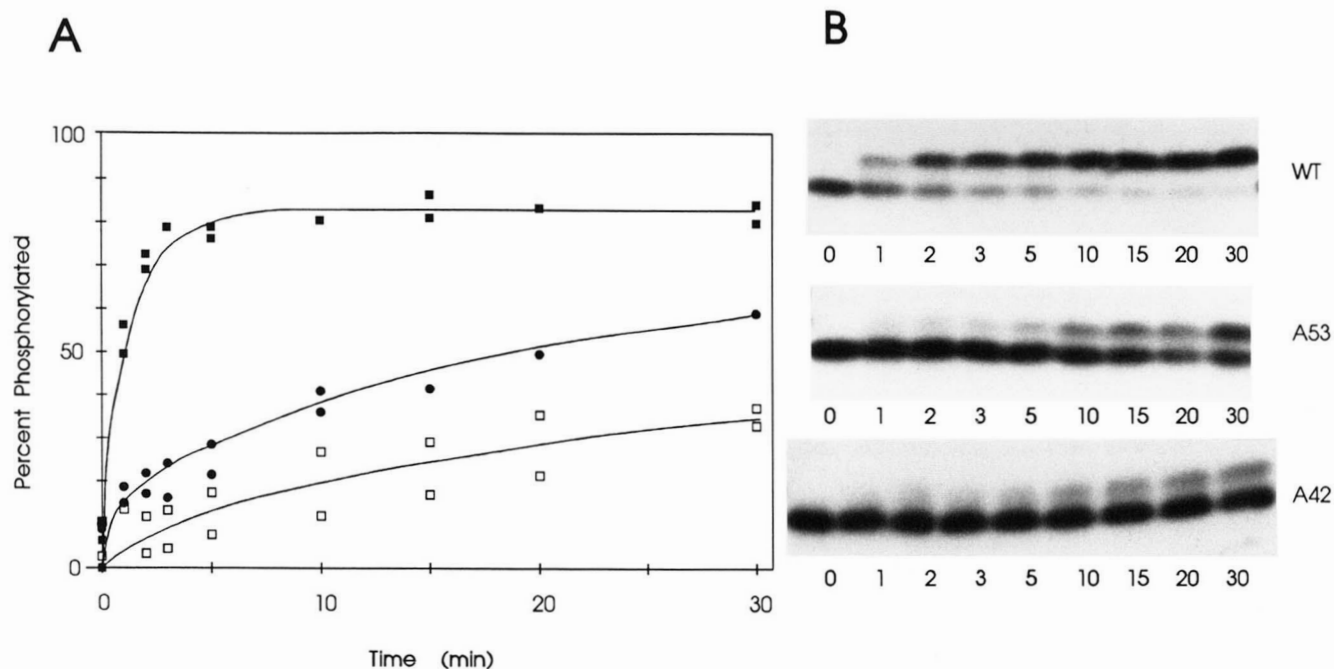


Fig. 6. Kinetics of cAMP-induced phosphorylation in cAR1 mutants. Growth-stage AX-3 cells expressing wild-type cAR1 (filled squares), mutant A53 (filled circles), or mutant A42 (open squares) were stimulated for the indicated times with 10^{-4} M cAMP, solubilized in Laemmli buffer, and immunoblotted with cAR1 antiserum as described under "Materials and Methods." A, the percentage of phosphorylated (upper band) cAR1 was quantitated by image analysis of autoradiographs as described under "Materials and Methods." Results shown are from two independent experiments. B, representative autoradiographs used to calculate the values in panel A. cAMP treatment time (min) is indicated below each lane.

ceptors (data not shown).

The rates of phosphorylation were also altered in these mutants (Fig. 6). Wild-type cAR1 was phosphorylated with a half-time of 1–2 min. In contrast, phosphorylation of the class III mutants was much slower: mutants A42 and A53 were phosphorylated with half-times of 8 and 6 min, respectively; mutants A3 and A81 were also phosphorylated much more slowly and required more cAMP than wild-type cAR1 (data not shown).

cAMP Binding—cAMP binding assays were performed to assess whether the impaired responsiveness of the class III mutants resulted from incorrect receptor targeting or a reduction in cAMP binding affinity. As previously reported (52), cells expressing wild-type cAR1 displayed two classes of binding sites. About 97% of sites had an affinity of 213 nM, while the remainder had an affinity for cAMP of 13 nM (Fig. 7). Mutants A42 and A53 each exhibited only one class of sites with affinities of 117 and 280 nM, respectively. In these cell lines, the maximal levels of cAMP binding were also similar (wild-type, $1.60 \pm 0.29 \times 10^5$ sites/cell; A42, $1.24 \pm 0.15 \times 10^5$ sites/cell; A53, $1.74 \pm 0.05 \times 10^5$ sites/cell), as were cAR1 expression levels (Fig. 4). These results suggest that class III mutants are targeted correctly to the plasma membrane and that the biochemical abnormalities of cells expressing these mutants are not due to an inability to bind cAMP.

cAMP Uptake—cAR1 mediates the formation of a slowly dissociating pool of cell-associated cAMP. This pool can be distinguished from simple receptor-bound cAMP by its extremely slow rates of formation and disappearance (53, 54). The relative abilities of wild-type and mutant cAR1 to carry out this function were evaluated. When cells expressing wild-type cAR1 were incubated for 30 min with [32 P]cAMP, some radiolabel slowly became associated with the cells and remained so, despite repeated washing (Fig. 8). cAMP "uptake" was cAR1 dependent, since vector control cells failed to exhibit this phenomenon (Fig. 8, legend). Surprisingly, cells expressing mutant A42 also accumulated a slowly dissociating pool of ligand. In fact,

both the rate and extent of this process were at least as high as for cells expressing wild-type cAR1.

Rescue of *car1*[−] Cells and G-protein Coupling—To assess the physiological consequences of third intracellular loop mutagenesis, wild-type cAR1 and mutants A42 and A53 were overexpressed in a *car1*[−] cell line (JB4). *car1*[−] cells fail to aggregate upon nutrient depletion, a response which is restored upon transformation with wild-type cAR1 (Fig. 9). Expression of equivalent levels of mutant A42 and A53 in the *car1*[−] cells failed to restore the wild-type phenotype. These receptors are thus defective in some function crucial for development.

Expression of mutant receptors in the *car1*[−] cells allowed us to examine receptor G-protein coupling. The interaction of cAR1 with the G-protein G₂ results in the formation of a high-affinity form of receptor (48, 50), which can be eliminated *in vitro* by the addition of GTP. The *car1*[−] cells transformed with the mutant cAR1s were starved to induce expression of G_{α2}, cell lysates were prepared and binding at 2 nM [3 H]cAMP measured in the presence and absence of GTP. GTP reduced cAMP binding in membranes containing wild-type cAR1 by 70%. In contrast, this compound had little effect on cAMP binding by membranes containing mutant A42 (Fig. 10). Vector control membranes exhibited no specific cAMP binding. Immunoblots of the lysates revealed comparable levels of G_{α2} and G_β proteins in all three cell lines (Fig. 10). Preliminary experiments have yielded similar results for mutant A53 (data not shown). These observations suggest that in addition to their other defects, class III mutants do not interact productively with G-proteins.

DISCUSSION

The third intracellular loops of several G-protein-coupled receptors have been implicated as sites of interaction with both G-proteins and receptor kinases. Mutations within this region have resulted in receptors which no longer interact with G-proteins (28–30, 55, 56), which are deficient in ligand-mediated loss of ligand binding (30), or which exhibit ligand-independent signaling activity (26, 27). The juxtamembranous segments of

this domain may be especially important (26, 29). However, the assignment of specific functions to specific portions of this loop has been complicated by at least three factors. First, receptors possess loops of widely varying sizes. Second, amino acid replacements at the same position in two different receptors can have opposite effects on signaling (56). Third, many analyses have involved either large deletions or a relatively small number of point mutations.

To determine if the NH_2 -terminal portion of the third intracellular loop of cAR1 plays a role in cAMP-mediated signal transduction, we randomly mutagenized it and expressed the mutant versions of cAR1 from a segregating, extrachromosomal expression vector. This strategy allowed us to assess the functional properties of many receptors individually. The plasmids encoding these receptors were easily rescued and sequenced within the targeted domain. A similar strategy has yielded several interesting mutations within the analogous domain of the yeast STE2 pheromone receptor (55).

Many of the mutants we analyzed (class I and II), even some with multiple nonconservative amino acid replacements, exhibited normal or only slightly impaired ligand-dependent responses. In fact, none of the amino acids in the targeted region were essential for cAMP-induced Ca^{2+} influx or LLB. Another class of mutants (class III) was very defective in several signaling properties, despite exhibiting normal cAMP binding. Two class III mutants, A42 and A53, contained replacements at Val¹⁸⁹. While replacement of hydrophobic residues at this position were well tolerated (mutants A62 and A22), its replacement with a charged amino acid in A42 (and possibly A53) markedly decreased function. Strikingly, while in A42, the single replacement of Val¹⁸⁹ with Asp yielded a class III receptor, the identical replacement of Val¹⁸⁸ in A2 was not nearly as disruptive. It may be noteworthy that the former valine is completely conserved among the four cARs cloned to date, while the latter is not² (3, 57, 58). The other class III mutants, A3 and A81, share two characteristics. In both cases, Tyr¹⁸⁵ is replaced with an Asn and several nearby residues are mutated, as well. We are now determining if the Tyr to Asn mutation alone is sufficient to confer a class III phenotype.

Class III mutants are deficient in coupling to multiple downstream effectors. These mutants are impaired in cAMP-mediated Ca^{2+} influx, LLB, receptor phosphorylation and G-protein coupling, and they fail to rescue the developmental phenotype of *car1*⁻ cells. These defects most likely represent a failure to couple to at least two independent signaling pathways, as Ca^{2+} influx, LLB, and receptor phosphorylation are G-protein-independent (22).^{5,6}

There are several possible explanations for the multiple defects in class III mutants. First, they might be defective in targeting to the plasma membrane. However, these mutants bind cAMP with affinity and surface B_{max} values similar to those of wild-type cAR1. Second, the effectors of these multiple pathways might interact with a common contact site on cAR1, which is altered in the mutants. A third, more intriguing, possibility is that class III mutants fail to efficiently achieve an occupancy-induced activation isomerization.

It has been proposed that conversion of a receptor to an active conformational state is an event distinct from ligand binding (27). Recent studies of "constitutively active" mutants of catecholamine receptors support this notion (26, 27). These mutants exhibit an elevated level of signaling in the absence of ligand and a higher affinity for agonists but not antagonists. These characteristics are accounted for in a model wherein agonist, but not antagonist, binding promotes the isomerization of a wild-type receptor to a higher affinity, "active" conformation. This conformational change also confers a greater propensity to interact with G-proteins. We propose that an

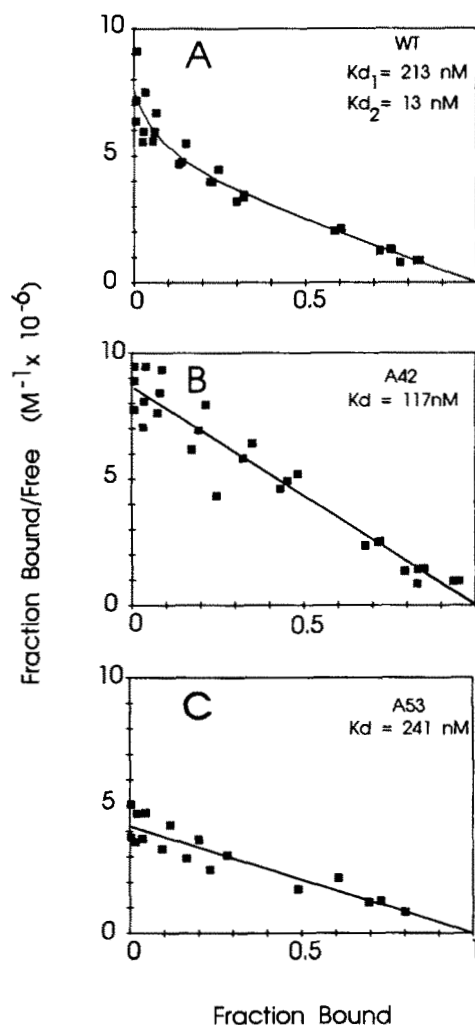


FIG. 7. Scatchard plots of cAMP binding to cAR1 mutants. [³²P]cAMP binding assays were performed on growth-stage AX-3 cells expressing wild-type cAR1 (A), mutant A42 (B), or mutant A53 (C) and Scatchard plots generated as described under "Materials and Methods." A fitted line and the dissociation constant(s) (K_d) for each cell line are indicated in each panel. Both low- (K_{d1}) and high- (K_{d2}) affinities were calculated for wild-type cAR1. Means of triplicate determinations from each of two to three independent experiments are shown. For each experiment, the data have been normalized to the calculated B_{max} from that experiment to facilitate the comparison of affinities between the three cell lines. Absolute B_{max} values are given under "Results."

activated receptor interacts more avidly with receptor kinase, as well. According to this scheme, it should be possible to isolate mutants which can bind ligand and yet cannot efficiently switch to the activated state. Such mutants are predicted to be defective in multiple ligand-induced responses and display phenotypes similar to cAR1 class III mutants. A reduction in their rates of activation isomerization could account for their reduced rates of phosphorylation.

Since binding affinity is dependent upon the distribution between active and inactive forms of the receptor, a mutant which cannot undergo this activation is expected to exhibit a lower binding affinity. The anticipated affinity difference should be small, however, for a receptor like cAR1, which is relatively inactive in the absence of ligand.

We did not observe large enough differences in affinity between wild-type cAR1 and mutants A42 or A53 to account for their functional defects. We did, however, note two dose-related changes in these mutants. First, as previously reported (11), we found that wild-type cAR1 exhibits an EC_{50} for phosphorylation which is 10-fold lower than its binding affinity. In contrast,

FIG. 8. [32 P]cAMP uptake by wild-type cAR1 and mutant A42. [32 P]cAMP uptake by growth-stage AX-3 cells containing control vector (data not shown) or expressing wild-type cAR1 (filled circles) or mutant A42 (open circles) was measured and normalized to total cAMP binding levels as described under "Materials and Methods." Values shown are the mean values of triplicate determinations from two independent experiments. Mean absolute values (moles cAMP uptake/ 5×10^6 cells) were as follows: wild-type, 1.11×10^{-14} ; A42, 1.45×10^{-14} ; vector, 0.05×10^{-14} .

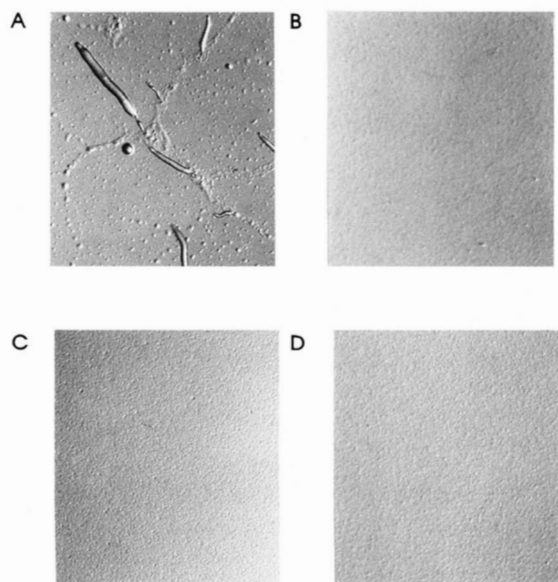
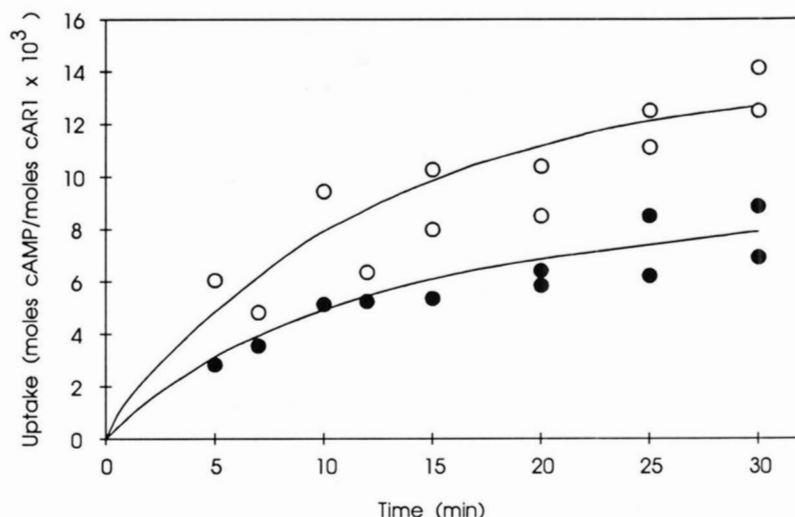


FIG. 9. Rescue of the aggregation-deficient phenotype of *car1*⁻ cells by wild-type and mutant cARs. Growth-stage *car1*⁻ cells containing control vector (B and E, lane 2), or expressing wild-type cAR1 (A and E, lane 1), mutant A42 (C and E, lane 3), or mutant A53 (D and E, lane 4) are shown at 25 h (A) or 43 h (B–D) after the initiation of starvation on DB plates as described under "Materials and Methods." The cells in panel A went on to form mature fruiting bodies, while the others remained as a monolayer for three more days. E, an aliquot of each cell type was solubilized in Laemmli buffer at the initiation of starvation and immunoblotted with cAR1 antiserum as described under "Materials and Methods" (arrow indicates position of cAR1.)

the phosphorylation EC₅₀ values of mutants A42 and A53 were similar to their binding affinities. Second, a small percentage of wild-type cAR1 molecules typically display very high affinity binding sites, whereas no such sites were observed for mutants A42 or A53. These differences may reflect the inability of these mutants to isomerize.

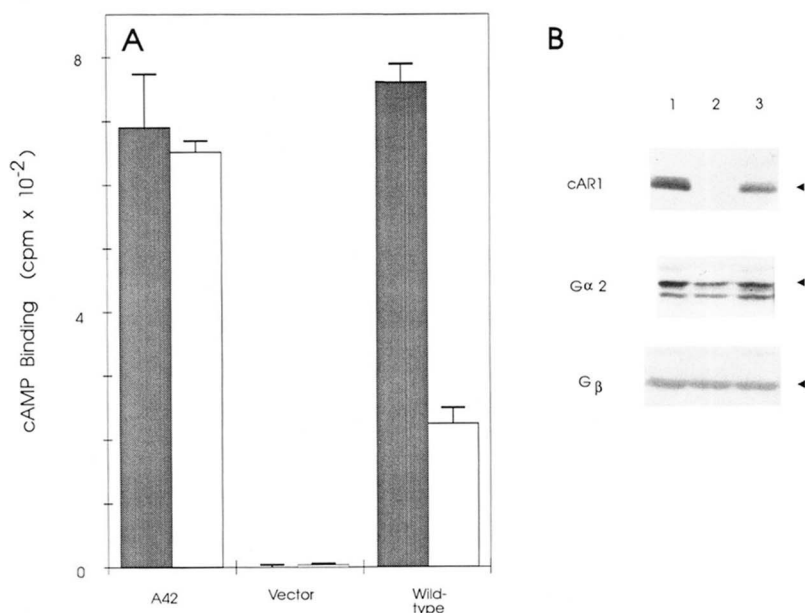
While the phosphorylation and loss of binding responses are only partially defective in class III mutants, Ca²⁺ influx is nearly completely absent. One possible explanation for this difference is that the threshold level of activated receptor required for Ca²⁺ influx is higher than that for phosphorylation or LLB. Alternatively, these three responses may lie on divergent pathways, since phosphorylation and loss of binding occur over a relatively long time period, and are cumulative (11, 13), whereas Ca²⁺ influx is more rapid and transient, and may be turned off in a receptor-autonomous fashion (9).

Upon prolonged incubation of *Dictyostelium* amoebae with radiolabeled cAMP, there is the formation of a time- and cAR1-dependent "slowly dissociating" pool of cell-associated ligand, which can be detected after extensive washing of cells (53). This pool does not appear to be tightly associated with the cAR1 protein (54). Interestingly, the ability of a class III mutant (A42) to bring about the formation of this "slowly-dissociating" pool was at least as robust as that of wild-type cAR1. It has

been hypothesized that the "slowly dissociating pool" is a result of receptor-mediated endocytosis, and that the latter may also be responsible for cAMP-mediated LLB (53). However, the retention of this function, despite a drastic impairment in LLB, shows that these phenomena are distinct, as previously suggested (54). Moreover, this finding suggests that cAMP "uptake" may not depend upon an activating isomerization of cAR1.

In studies involving random mutagenesis of the third intracellular loop of the yeast STE2 receptor, many of the mutated receptors failed to exhibit ligand binding (55). In contrast, most of the cAR1 mutants generated in this study bound cAMP with high affinity. This difference might be due to the fact that while the STE2 third intracellular loop is predicted to contain only 12 amino acids, the cAR1 loop is predicted to contain 24 amino acids. This added length may allow cAR1 to structurally accommodate more amino acid replacements than the STE2 protein. The STE2 receptor mutants which were capable of ligand binding exhibited a similar phenotype to the cAR1 class III mutants; both classes of mutants exhibited multiple signaling defects despite their high binding affinities. As in cAR1, this phenotype could be produced in the STE2 protein by replacement of a hydrophobic residue with a chemically dissimilar amino acid. These findings support the contention (55) that

FIG. 10. GTP inhibition of cAMP binding by wild-type cAR1 and mutant A42. **A**, *car1*⁻ cells containing control vector or expressing wild-type cAR1 or mutant A42 were starved in shaking culture with cAMP pulses for 6 h as described under "Materials and Methods." Membranes were prepared and cAMP binding measured in the absence (filled bars) or presence (open bars) of 100 μ M GTP as described under "Materials and Methods." Values shown are the mean \pm S.E. of triplicate determinations from a single experiment, and are representative of three identical experiments. **B**, membranes were solubilized in Laemmli buffer and immunoblotted with cAR1 antiserum, G α ₂ antiserum, and G β antiserum, using enhanced chemiluminescence as described under "Materials and Methods." The arrows indicate the positions of each protein.



hydrophobic residues may be as critical as charged or polar residues for third intracellular loop structure and function in G-protein-coupled receptors.

In summary, our results suggest that residues within the NH₂-terminal portion of the *Dictyostelium* cAR1 third intracellular loop are involved in multiple cAMP-mediated signal transduction events, perhaps through their involvement in an activation isomerization which occurs upon ligand binding. While the specific roles played by these residues is unclear, continued mutational analysis of these and other amino acids within this domain should further our understanding of G-protein-coupled receptor activation and deactivation. The failure of class III mutants to restore aggregation competence to *car1*⁻ cells or to exhibit a complete mobility shift on SDS-PAGE should greatly facilitate these analyses.

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