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Switching of Chemoattractant Receptors Programs Development and Morphogenesis in *Dictyostelium:* Receptor Subtypes Activate Common Responses at Different Agonist Concentrations[1]

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One of the common functional features among G-protein coupled receptors is the occurrence of multiple subtypes involved in similar signal transduction events. The cAMP chemoattractant receptor family of *Dictyostelium discoideum* is composed of four receptors (cAR1–cAR4), which are expressed sequentially throughout the developmental transition from a unicellular to a multicellular organism. The receptors differ in affinity for cAMP and in the sequences of their C-terminal domains. In this study, we constitutively expressed cAR1, cAR2, and cAR3 as well as a series of chimeric and mutant receptors and assessed the capacity of each to mediate chemotaxis, activation of adenylyl cyclase and actin polymerization, and rescue the developmental defect of *car1*$^-$/*car3*$^-$ cells. We found that various receptors and mutants sense different concentration ranges of cAMP but all can mediate identical responses during the aggregation stage of development. The responses displayed very similar kinetics, suggesting no major differences in regulatory properties attributable to the C-terminal domains. We speculate that switching of receptor subtypes during development enables the organism to respond to the changing concentrations of the chemoattractant and thereby program morphogenesis appropriately.

INTRODUCTION

The four cAMP chemoattractant receptors, cAR1–cAR4, of *Dictyostelium discoideum* are probably among the earliest progenitors of the superfamily of G-protein coupled receptors (GPCR), but they appear to share many features with their mammalian counterparts (Johnson *et al.*, 1992; Louis *et al.*, 1994; Strader, 1995). Frequently, GPCR are found in groups of several subtypes which bind the same ligands but with different affinities and specificities. In some instances, the subtypes appear to interact with identical sets of G-proteins and effectors, while in others receptors for the same agonist trigger different responses (Dolhman *et al.*, 1991; Kobilka, 1988). Analyses of chimeras and mutants have shown the importance of various segments of the intracellular loops in determining G-protein specificity and of the transmembrane helices or extracellular loops in determining binding specificity (Hausdorff *et al.*, 1990). The cytoplasmic tail domains, which often undergo agonist-induced phosphorylation, appear to play a regulatory role (Kurose and Lefkowitz, 1994). Often the subtypes differ in timing of expression during development and in pattern of expression in the organism (Koch, 1995).

In *D. discoideum* gene deletion and replacement can be used to explore parallel signaling pathways mediated by multiple components with similar properties. In addition to multiple chemoattractant receptors, there are eight G-protein α-subunits and a single $\beta\gamma$-complex (Lilly *et al.*, 1993; Wu *et al.*, 1995; Zhang *et al.*, manuscript in preparation). Free-living amoebae initiate a developmental program upon nutritional deprivation, utilizing cAMP as an intercellular as well as intracellular signal (Schaap *et al.*, 1984). The four cAMP receptors are expressed sequentially to coordinate many responses needed for the completion of multicellular development. Cells lacking cAR1 fail to aggregate (Sun and Devreotes, 1991) and deletion of either cAR2 or cAR4 causes developmental abnormalities expected from the

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temporal and spatial expression patterns of these genes (Saxe *et al.*, 1993; Louis *et al.*, 1994). Surprisingly, *car3*$^-$ cells are apparently normal suggesting that cAR3 significantly overlaps in function with cAR1 (Johnson *et al.*, 1993). Despite the high degree of identity (60%) among these receptors, each of the cARs has a distinct affinity for cAMP and a different pharmacological profile. Furthermore, the C-terminal cytoplasmic domains of the cARs, which have been shown in cAR1, cAR2, and cAR3 to be rapidly and extensively phosphorylated in response to agonist, are highly divergent suggesting that these receptors may be regulated differently (Saxe *et al.*, 1991).

Previous studies have shown that cAR1, cAR2, and cAR3 can activate similar arrays of G-protein-independent responses such as Ca^{2+} influx (Milne and Devreotes, 1993). It is not known, however, to what extent the receptors couple to the same G-protein-mediated responses, nor has it been shown that each receptor can function as a chemoattractant receptor. To elucidate the functions of each receptor and the significance of receptor switching during development, we tested the ability of the various subtypes to activate responses normally mediated by cAR1 by expressing cAR1, cAR2, or cAR3 in a *car1*$^-$/*car3*$^-$ cell line. We find that the major differences in the receptors are in affinity, rather than in coupling to downstream effectors. We further show that the affinity differences are determined by specific amino acid residues in the second extracellular loop. Finally, despite their completely divergent sequences, the C-terminal cytoplasmic domains do not appear to confer major differences in regulation of receptor-mediated responses.

MATERIALS AND METHODS

DNA Constructs

A full-length genomic cAR2 DNA, a generous gift from Dr. Karl Saxe III, was subcloned into the extrachromosomal vector pJK1. This vector contains an actin15 promoter which overexpressed the insert in growing and differentiating cells (Kim and Devreotes, 1994). The plasmid used to overexpress cAR3 was described previously (Johnson *et al.*, 1993). The cAR1 C-terminal truncation mutants were generated by *Exo*III-mediated 3′ deletion digestion of cAR1 cDNA as described before (Hereld *et al.*, 1994). At the C-terminal end of the truncated proteins, T365 has vector peptide WNSIS, T289 has GEEFD, T278 has FD, and T248 has GLNEFD (single-letter designation of amino acids) added. Generation and characterization of binding properties of N272 and 272C chimeras were described previously (Kim and Devreotes, 1994).

Site-Directed Mutagenesis

Oligonucleotide-directed mutagenesis was carried out as described earlier (Caterina *et al.*, 1994). The primers were 256-fold degenerate designed to generate multiple mutations in the domain. Single-stranded DNA was sequenced to identify the mutations and cDNA containing desired mutations were subcloned into pMC34 (Caterina *et al.*, 1994).

Cell Lines and Cultures

All of the plasmid constructs were transformed into the *car1*$^-$/*car3*$^-$ cell line which has both cAR1 and cAR3 genes deleted and has previously been shown to have no remaining cAMP-mediated responses (Insall *et al.*, 1994). Transformation and maintenance of cell lines were carried out as described elsewhere (Caterina *et al.*, 1994). Cells maintained on nutrient media were inoculated into shaking HL5 media with 20 μg/ml of G418 2 to 3 days prior to the experiments to optimize the culture condition.

Nonnutrient Agar Development Assay

Growth-stage cells were washed once in sterile DB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.8) to remove nutrients and 10^7 cells were plated on 35-mm 1% agar plates in DB, allowed to settle for 10 min, drained of excess buffer, and left to develop for 10–48 h (Klein *et al.*, 1988). In the cases specified, cells were first developed in suspended culture as described below for adenylyl cyclase to induce development and then plated on DB agar plates as above.

Affinity Measurements and Loss-of-Ligand Binding

Three methods previously described were used to determine the affinity of various receptors (Caterina *et al.*, 1995). cAMP-induced electrophoretic mobility shift assays were performed as before (Kim and Devreotes, 1994). Loss-of-ligand binding was induced by treating washed cells with 10^{-5} M cAMP for 15 min at 22°C. cAMP was removed by washing five times in ice-cold phosphate buffer. Binding was measured with 16 nM [^{3}H]cAMP in phosphate buffer as previously described.

Chemotaxis Assay, Adenylyl Cyclase, and Actin Polymerization Assays

The small-drop chemotaxis assays were performed as described before on developed cells (van Haastert *et al.*, 1982). Cells grown in shaking culture were washed with DB and developed with 50–500 nM cAMP pulses for 6 h as described (Caterina *et al.*, 1994). For adenylyl cyclase assay, cells were resuspended to 8×10^7/ml, transferred to 22°C, and stimulated with 10^{-5}–10^{-3} M cAMP. The amount of cAMP synthesized in 1 min was assessed as before (Theibert and Devreotes, 1986). For actin polymerization, developed cells were resuspended at 3×10^7 cells/ml and kept on ice. Cells were transferred to room temperature and shaken for 10 min to equilibrate to room temperature. At time 0, 10^{-7} M cAMP and 10 mM DTT were added to induce actin polymerization and the reactions were stopped by taking aliquots of cells at various times as described (Condellis *et al.*, 1991; Kim *et al.*, 1997).

Immunoblots

Immunoblotting was performed using ACA-, cAR1-, cAR2-, or cAR3-specific rabbit polyclonal antiserum described before (Parent and Devreotes, 1995; Vaughan and Devreotes, 1988; Johnson *et al.*, 1993; Saxe *et al.*, 1993). Primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham) and a chemiluminescence detection kit (DuPont).

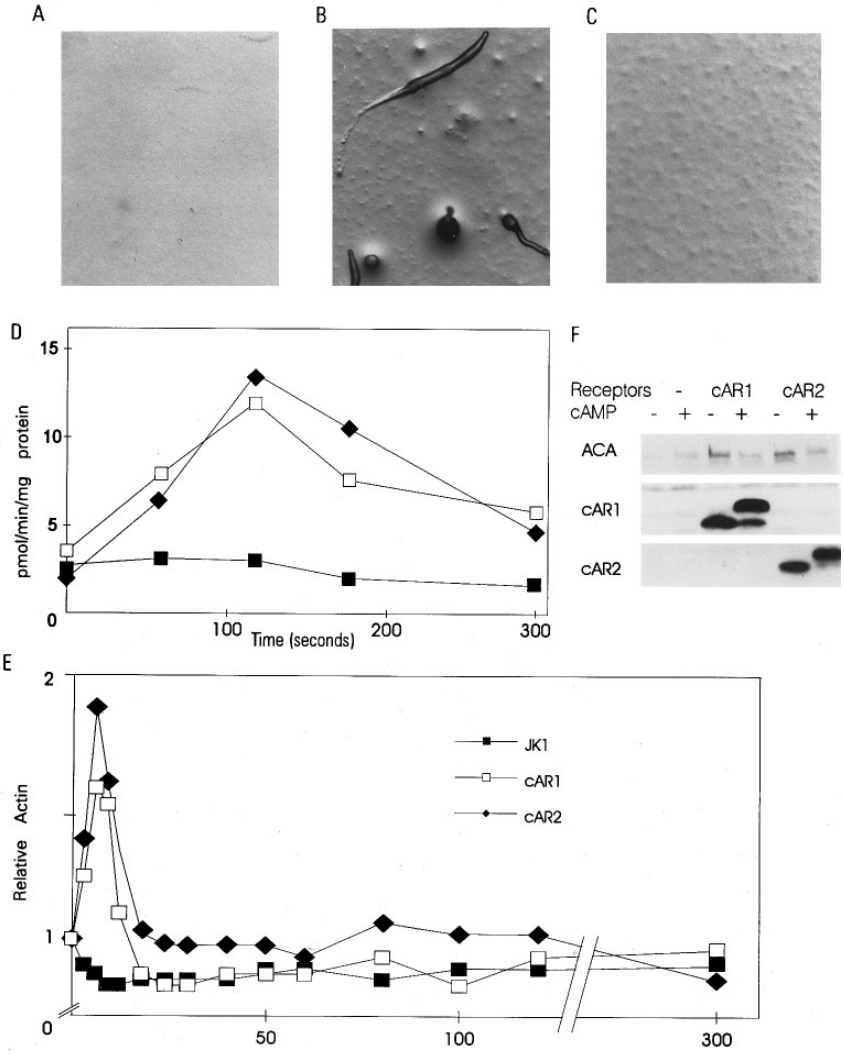

FIG. 1. Characterization of cAR1 and cAR2 expressing *car1*$^-$/*car3*$^-$ cells. The *car1*$^-$/*car3*$^-$ cells were transformed with (A) an empty vector (pJK1), (B) pJK1 containing wild-type cAR1, or (C) pJK1 containing a wild-type cAR2. Growth-stage cells were washed with DB, and 10^7 cells were plated on nutrient-free agar and incubated for 48 h at 22°C; the magnification is 12.5×. cAMP-mediated activation of adenylyl cyclase with 10^{-3} M cAMP (D) and actin polymerization (E) was measured as described under Materials and Methods. Vector control transformants (■); cAR1 transformants (□); cAR2 transformants (◆). (F) Aliquots of cells were lysed into sample buffer before and 5 min after the addition of 1 mM cAMP (from experiment in D). Immunoblot analysis was carried out with antisera to ACA, cAR1, or cAR2.

RESULTS

Overexpression of cAR1, cAR2, and cAR3 in car1$^-$*/car3*$^-$ *Cells*

To test whether cAR2 and cAR1 are functionally interchangeable in spite of the 1000-fold lower affinity of cAR2 (Johnson *et al.*, 1992; Kim and Devreotes, 1994; Milne and Devreotes, 1993), we overexpressed cAR1 or cAR2 in *car1*$^-$/*car3*$^-$ cells. Wild-type cAR1 was able to rescue the developmental defect of *car1*$^-$/*car3*$^-$ (Figs. 1A and 1B). This confirmed previous observations that deletion of cAR3 does not yield a visible morphological phenotype (Johnson *et al.*, 1993). Under the same conditions cAR2 did not reverse the aggregation minus phenotype of *car1*$^-$/*car3*$^-$ (Fig. 1C). However, when examined at a biochemical level, cAR2 like cAR1 was able to rescue many cAMP-mediated responses such as activation of adenylyl cyclase, actin polymerization, and receptor phosphorylation. At high stimulus concentrations needed to saturate the low-affinity receptors, the responses mediated by cAR2 were indistinguishable from those mediated by cAR1 (Figs. 1D–1F). These observations furthermore indicate that cAR2 supported the induction by cAMP of the genes required for these responses. The cells

transformed with empty vector did not display any of these responses; most significantly, cAR2 mediated chemotaxis (Figs. 2A–2C). The EC_{50}s for chemotaxis and for cAMP-mediated phosphorylation of cAR2 were shifted nearly three orders of magnitude to the right as has been reported previously for Ca^{2+} influx (Figs. 2C and 2D) (Milne and Devreotes, 1993). These results clearly illustrated that cAR2 was a chemoattractant receptor and was capable of generating the responses normally carried out by cAR1.

Next we overexpressed cAR3 in the *car1*$^-$/*car3*$^-$ cells. cAR3 has an intermediate affinity and is expressed between cAR1 and cAR2 during development (Johnson *et al.*, 1992, 1993). In contrast to our previous report, this receptor was able to induce development (Fig. 3A); the discrepancy probably can be attributed to a defect in the *car1*$^-$/*car3*$^-$ cell line we used previously (Johnson *et al.*, 1993). The cells expressing cAR3 produced larger aggregation territories than those expressing cAR1, suggesting that the lower affinity receptor is producing fewer oscillation centers. After 10–12 h of development, the large streams broke into small areas (Fig. 3A), resulting in the formation of small mounds. These progressed with morphogenesis and produced small, but normally proportioned, fruiting bodies. In the cAR3-expressing cells, cAMP stimulated both adenylyl cyclase and actin polymerization (Figs. 3B and 3C). The kinetic profile of the adenylyl cyclase activation was normal. The time course of actin polymerization seemed to be slightly slower and demonstrated a more prominent second peak of actin polymerization (60 s). These slight differences are often observed among actin responses in wild-type cells and do not indicate a significant difference between the two cARs (Borleis and Devreotes, unpublished data). As shown in the inset each cell line expressed equivalent amounts of the adenylyl cyclase (ACA) and equal or greater than wild-type amounts of the appropriate receptor. We have previously shown that cAR1, cAR2, and cAR3 are phosphorylated in response to agonist occupancy. Unlike cAR1 and cAR2, cAR3 does not undergo a decrease in electrophoretic mobility on SDS–PAGE upon phosphorylation.

Major Determinants of Affinity Differences among the Receptors

Previously we established that a portion of the second extracellular loop (residues 145 to 157 based on the cAR1 sequence) is the major determinant of the affinity difference between cAR1 and cAR2 (Kim and Devreotes, 1994). Since the markedly lower affinity of cAR2 and the intermediate affinity of cAR3 appear to be major differences among the receptors, we proceeded to further study the basis of the affinity differences. We carried out site-directed mutagenesis on cAR1 to substitute the residues in this domain with the corresponding residues from cAR2 (N148G, V154D, S155N, F156Y, and T157D). Substitution of these five amino acid residues (Fig. 4A) reduced the apparent affinity of cAR1 by 100- to 1000-fold. Both the EC_{50} of the agonist-induced electrophoretic mobility shift and binding

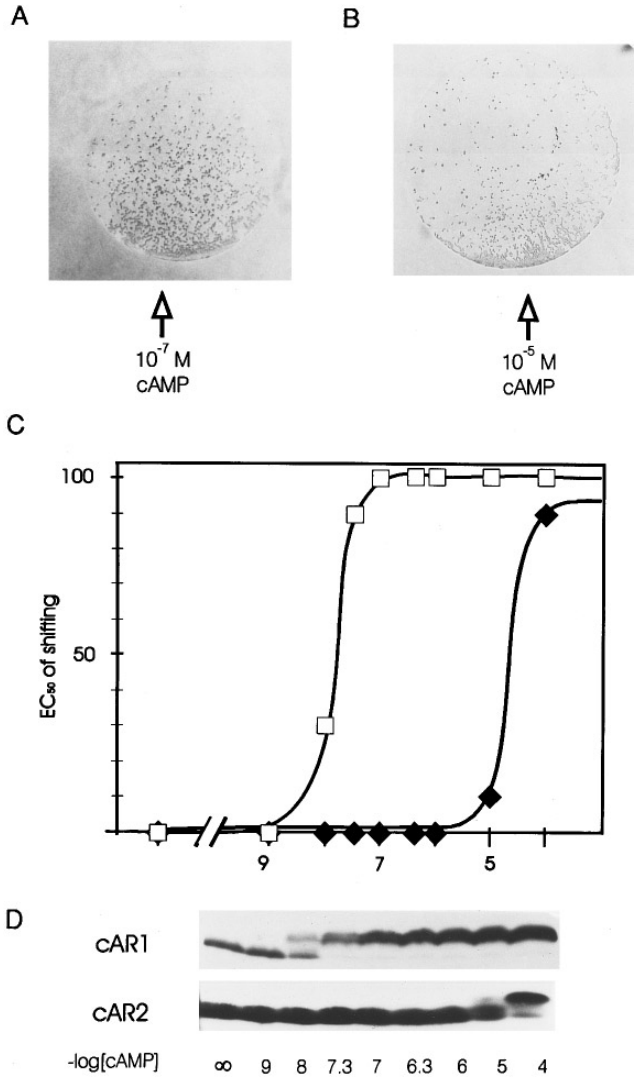

FIG. 2. Chemotactic responses and characterization of the dose response of cAR1 and cAR2. (A and B) cAR1 or cAR2 expressing *car1*$^-$/*car3*$^-$ cells were developed for 6 h, with 500 nM cAMP stimuli added at 6-min intervals, to induce competency for chemotaxis toward cAMP. Cells were diluted to 10^5/ml in PB and spotted on 0.8% agarose containing 3 mM caffeine. Different doses of cAMP solutions were spotted 1–2 mm away from the drops containing cells. The plates were kept in a humid chamber at 22°C. Photographs were taken after 15 min of migration. (C) Growth-stage cells were washed, treated with 3 mM caffeine for 20 min to synchronize the receptor population to an unphosphorylated form, and then treated with varying concentrations of cAMP (0, 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 10 μM, or 100 μM) for 15 min in the presence of 10 mM dithiothreitol. Cells were lysed in sample buffer, subjected to SDS–PAGE, and immunoblotted for cAR1 or cAR2. The resulting autoradiographs were then scanned and quantitated to generate the curves shown. Values are shown as the fractions of receptor in the slower mobility form and represent the means of at least two independent experiments. cAR1 transformants (□); cAR2 transformants (◆). A representative autoradiograph for each cell line is shown in D.

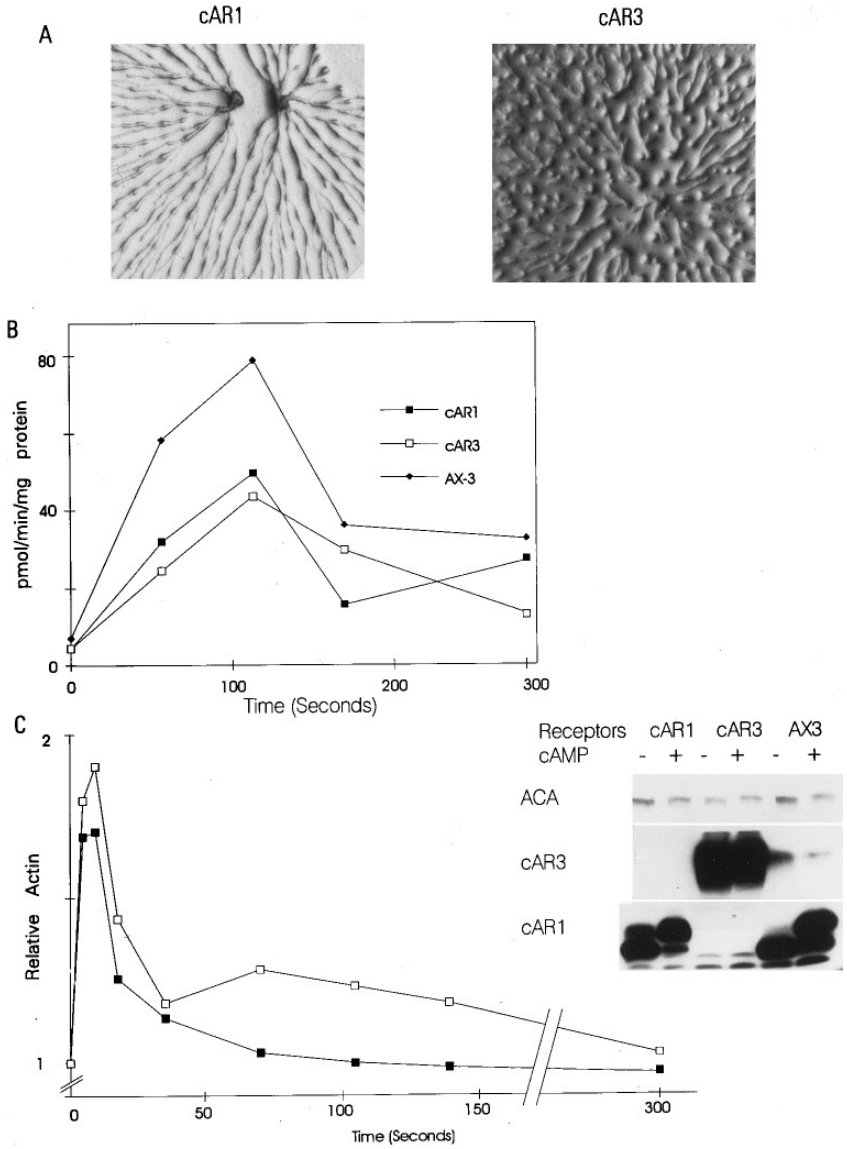

FIG. 3. Characterization of cAR3-expressing cells. Growth-stage transformants were washed with DB, plated on nutrient-free DB agar, and incubated for 8 h in a humidified chamber until they reached the late streaming stage. (A). Cells transformed with a plasmid encoding wild-type cAR1 (panel on the left) or cAR3-expressing cells (panel on the right). Pictures are magnified five times the actual image. (B and C) cAMP-mediated activation of adenylyl cyclase and actin polymerization were measured as in Fig. 1; cells were stimulated with 100 μM cAMP. Wild-type cAR1 expressing cells (■); cAR3 expressing cells (□); and wild-type cell line, AX3, (◆). (Inset) Aliquots of cells were immunoblotted for appropriate expression of receptors and of adenylyl cyclase.

affinity in phosphate buffer were drastically reduced (Figs. 4B and 4D), but the ''intrinsic'' affinity measured in ammonium sulfate was not significantly reduced (Fig. 4C). Note that the apparent affinity is slightly higher than that of a three-part chimera, 1/2/1, where both the major and minor affinity determinants of cAR1 and cAR2 were swapped. This mutant receptor was able to mediate activation of both ACA (Fig. 4E) and actin polymerization (not shown), but it supported development only when cells were plated at very high density (not shown). We also generated a mutant receptor with an intermediate affinity, similar to that of cAR3, by substituting three residues, (N148G, V154D, and S155N) in this domain including the introduction of one rather than two negative charges (Figs. 4A and 4B). This receptor supported development under standard conditions (Fig. 4F).

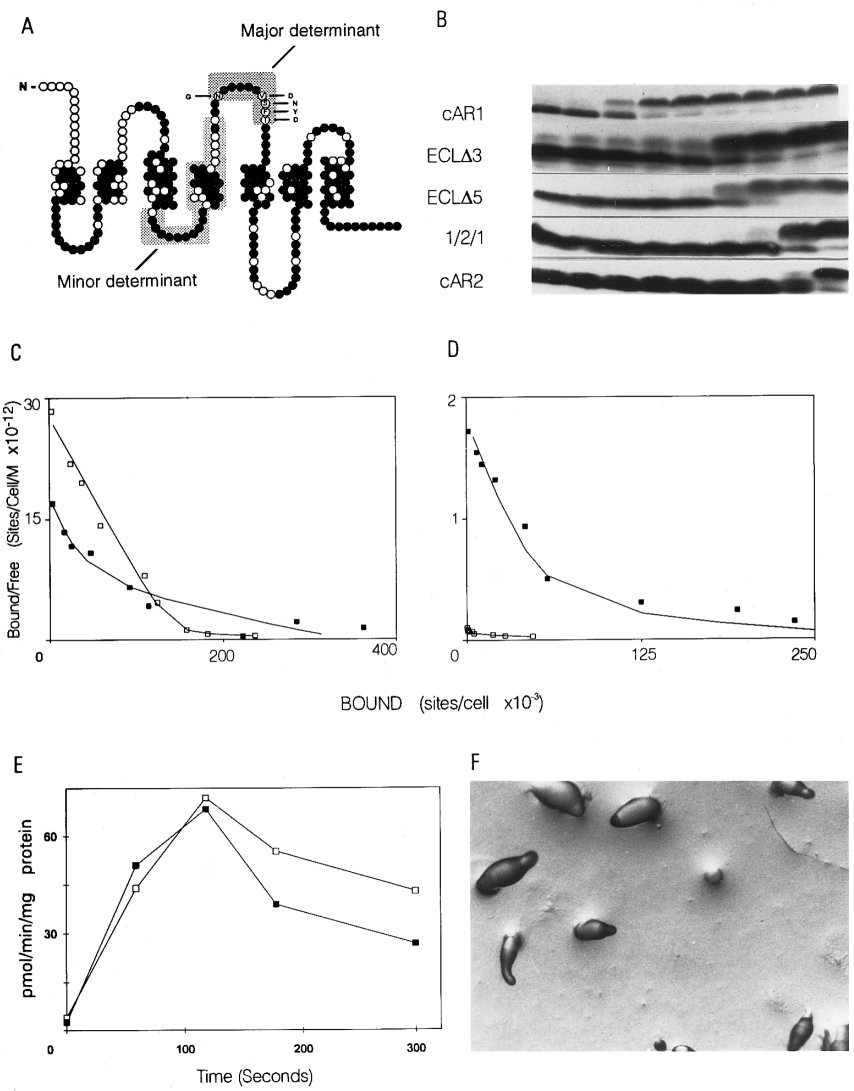

FIG. 4. Characterization of mutants in the second extracellular loop. (A) Schematic diagram of the major affinity determinant domain and the residues replaced. The conventional single-letter designations of amino acids were used; the letters inside of the circles are those of cAR1, while the letters outside are of cAR2. (B) Agonist-induced electrophoretic mobility shift of the mutant receptors. The cAMP doses used were identical to those shown in Fig. 2. ECLIIΔ3 (N148G, V154D, and S155N) is the mutant where three residues were changed and ECLIIΔ5 (N148G, V154D, S155N, F156Y, and T156D) has all five residues substituted to those of cAR2. The mutant 1/2/1 (120–159) is a three-part chimera previously characterized where both the major and minor determinants (residues 120–168) were replaced with cAR2. (C and D) Scatchard analyses of [^{3}H]cAMP binding to cells expressing WT-cAR1 or ECLIIΔ5 in ammonium sulfate (C) and phosphate buffer (D). [^{3}H]cAMP binding was measured by competition with unlabeled cAMP at a cAMP concentration range of 10^{-9} to 2×10^{-6} M for phosphate buffer binding (PB) and of 10^{-10} to 2×10^{-7} M for ammonium sulfate. Bound cAMP was separated from unbound cAMP by spinning through silicon oil in the case of PB binding. For AS binding analysis, cells were centrifuged and washed once with 3 M ammonium sulfate. Data are means of triplicate samples from an experiment which is representative of at least two independent experiments. (E). ''Activation trap'' assay of ACA activity measurement was performed as for Figs. 1 and 3. Cells expressing wild-type cAR1 (■) or ECLIIΔ5 (□) were tested. (F). DB developmental phenotype of ECLIIΔ3 expressing cells after 36 h of development.

Function of the Cytoplasmic C-Terminal Domain

cAR3, many cAR1/cAR2 chimeras, and cAR2 replaced most of the functions of cAR1, generating equivalent responses with similar kinetics. This observation led us to speculate that the highly divergent cytoplasmic C-terminal domains, which are believed to be important in regulation of desensitization of G-protein coupled receptors, might be dispensable for most receptor functions. To test this, we constructed a series of C-terminal truncations of cAR1.

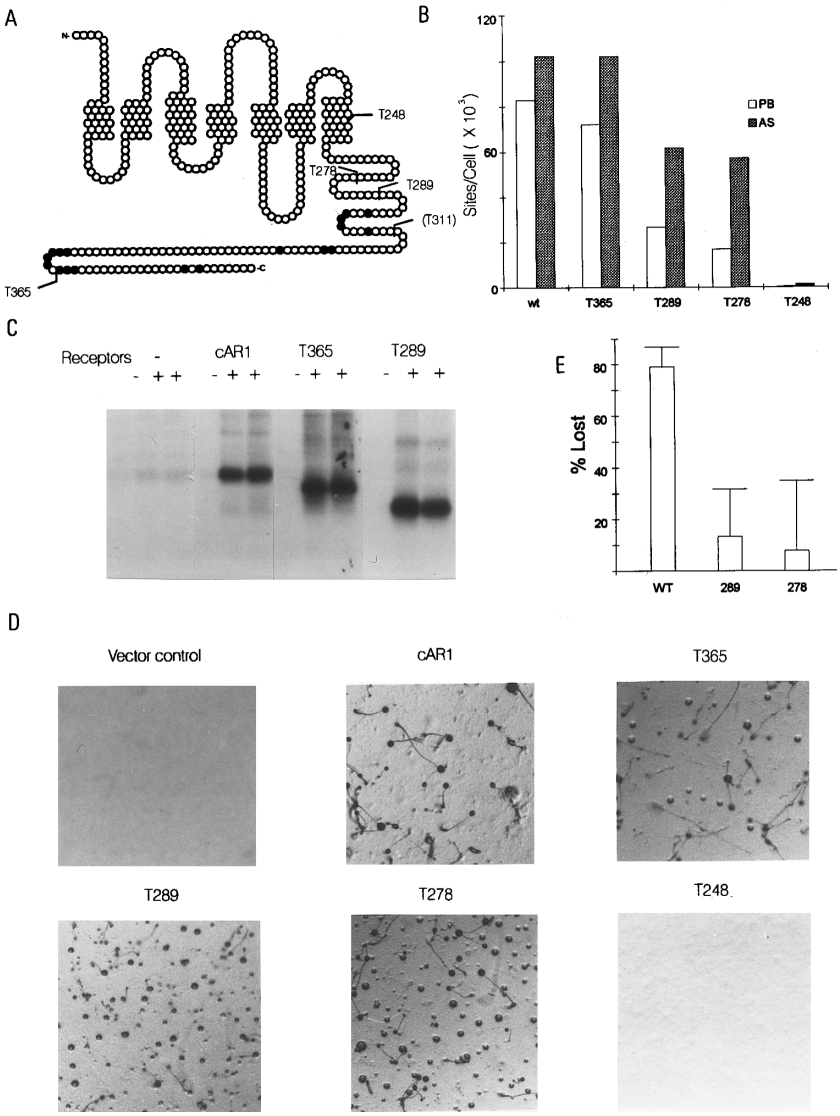

FIG. 5. Characterization of the series of C-terminal truncation mutants. (A) Schematic diagram of the topological model of cAR1 demonstrating the sites of truncations. Filled circles represent serine residues. (B) Binding properties of the truncated receptors in PB and AS. Open bars show the binding of 25 nM [^{3}H]cAMP in phosphate buffer; shaded bars represent binding of 25 nM in [^{3}H]cAMP in the presence of 3 M ammonium sulfate. (C) Photoaffinity labeling of the truncated receptors performed with [^{32}P]8-N_3-azido-cAMP as described under Materials and Methods. Cold cAMP (1 mM) was added to compete specific binding in the lanes marked with −. (D) Photos of development on nonnutrient agar for each of the cell lines. (E) Measurement of the loss-of-ligand binding on cells expressing T289 and T278 as described under Materials and Methods. T365 was not included since it showed a wild-type phenotype under these conditions.

When these receptors were expressed in the *car1*$^-$/*car3*$^-$ cells (Fig. 5A), it was clear that the majority of the C-terminal sequences are not needed for most of the functions of cAR1 (Figs. 5B–5D). The mutant T278, containing only 18 amino acids distal to the end of TMVII, was expressed poorly, but it bound cAMP in both ammonium sulfate and phosphate buffer and restored normal development (Figs. 5B and 5D). The truncated receptors T365 and T289 also restored normal development. We could also detect the truncated products by immunoblots and by photoaffinity labeling with [^{32}P]8-N_3-cAMP (Fig. 5C). Only the mutant T248, which truncates the receptor within TMVII (Fig. 5A), failed to bind cAMP and was unable to substitute for wild-type receptor (Figs. 5B and 5D).

T278 and T289 lack most of the cytoplasmic C-terminal domain, including all of the serine residues characterized to be the targets for ligand-mediated phosphorylation (Fig. 5A). As we anticipated based on the behavior of the pre-

viously characterized mutant cm1234 where all of the cytoplasmic serines were substituted with either alanine or glycine, both T278 and T289 failed to undergo loss-of-ligand binding (Fig. 5E) confirming the role of the C-terminal cytoplasmic domain in the loss-of-ligand binding process. Interestingly, despite the removal of most of the C-terminal sequences, these truncated receptors did not display additional abnormalities. Not only were they able to completely rescue the developmental program, but also the activations of adenylyl cyclase and actin polymerization and chemotactic responses mediated by these receptors were similar to those supported by the intact receptor (not shown).

However, studies of chimeras suggest that an inappropriate interaction of the C-terminal domain with the rest of the receptor can influence affinity. In our previous studies of cAR1 and cAR2 chimeras we made a puzzling observation in two reciprocal chimeras, N272 and 272C, that deviated from the general trend. The junctions of these chimeras reside in a highly conserved domain following TMVII and exiting to the C-terminal cytoplasmic tail. For the N272 chimera, the EC_{50} value of the cAMP-mediated electrophoretic mobility shift increased from the 30 nM of the surrounding chimeras (N302 and N228) to 1 μM, while for 272C it decreased from the 20 μM of the surrounding chimeras to 1 μM (Kim and Devreotes, 1994). When the growth-stage cells were plated directly on nutrient-free agar, neither of the chimeric receptors were able to induce development (Fig. 6A). However, when the cells were developed in a suspended culture by application of exogenous cAMP every 6 min and then plated, N272 was able to rescue development while 272C was not (Fig. 6B). Both chimeric receptors displayed equivalent capacities for activation of both ACA and actin polymerization (data not shown). We carried out a careful analysis of the affinities of the chimeras: In Scatchard analyses of [^{3}H]cAMP binding, the chimeras displayed lower affinity than cAR1 under physiological conditions, while all the receptors displayed high intrinsic affinity when measured in ammonium sulfate.(Figs. 7B and 7C). In the electrophoretic mobility shift, the EC_{50} of N272 was slightly lower than that of 272C (Figs. 6C and 6D). Consistently in chemotactic responses, the apparent affinities of both chimeras were intermediate between cAR1 and cAR2 and that of N272 was about fivefold higher than that of 272C, suggesting that the affinity of 272C is too low to rescue development (Fig. 7A).

DISCUSSION

In the present study, we examined the biochemical and phenotypic responses mediated by cAMP receptor subtypes of *D. discoideum* to explore the roles and extents of redundancy of a family of GPCRs. For the cAR subtypes tested (cAR1, cAR2, and cAR3), we found complete functional redundancy in activating the same array of biochemical responses in the aggregation stage of development. Previously the cAMP receptors cAR2, cAR3, and cAR4 were verified to bind cAMP and mediate some cAMP-induced responses (Johnson *et al.*, 1992; Milne and Devreotes, 1993; Louis *et al.*, 1994). However, it was never shown that these subtypes indeed function as chemoattractant receptors *in vivo*. In this study, we demonstrate clearly that cAR2 and cAR3 can mediate all of the responses mediated by cAR1 including chemotaxis toward cAMP. We anticipate that cAR4 will display the same functional properties. Thus, it is likely that each receptor does serve as a chemoattractant receptor at a specific stage in the program of multicellular development. Different functions are achieved by different affinities, timing, and pattern of expression. We propose that switching of receptors with different properties programs the orderly sequence of morphogenetic changes occurring throughout development.

We envision a very simple mechanism to respond chemotactically to extreme changes in environment. As the organism develops into an increasingly compact structure, the extracellular cAMP concentration is likely to increase (Bonner, 1974). When the local concentration of cAMP exceeds the affinity of a given receptor subtype, these receptors are saturated and a cell carrying only this receptor can no longer sense gradients. At this juncture, the cells must change the signal, the receptors, or perhaps even the effectors activated. *D. discoideum* seems to have evolved a very simple mechanism: The only component exchanged is the receptor. By switching to a lower affinity receptor, the cells can then sense gradients of higher mean concentration and the system is set to respond again. By maintaining the same downstream effectors, the cells can respond in the same manner. We also speculate that the abrupt changes in affinity bring about morphogenetic changes by changing the chemotactic behavior of subsets of cells.

The failure of cAR2 to rescue development does not originate from differences in coupling to downstream effectors. In fact, we demonstrated that the intracellular loops of cAR2, in the proper context, can rescue development. We expressed a set of previously characterized cAR1/cAR2 (N173) and cAR2/cAR1 (148C) chimeras (Kim and Devreotes, 1994). Both N173 and 148C retain high affinity but introduce long stretches of cAR2 sequences in either the N-terminal or C-terminal halves of the receptor. In the case of 148C, the third intracellular loop which is believed to be essential in specific coupling to G-proteins is derived entirely from cAR2, while in N173 both the first and the second intracellular loops are provided by cAR2. Nevertheless, both of these receptors rescue the developmental program.

Studies of chimeras between cAR1 and cAR2 suggested that the failure of cAR2 to replace cAR1 in the rescue of *car1*$^-$/*car3*$^-$ cells is merely due to its extremely low affinity. It has been shown previously that many defects in early aggregation can be overcome by applying 50–100 nM cAMP every 6 min, thus mimicking the oscillations that occur normally in suspensions of wild-type cells (Theibert *et al.*, 1986). This protocol, with 500 nM stimuli, was able to induce cAR2-expressing cells to differentiate. In these competent cells all cAMP-induced responses were wild-type in

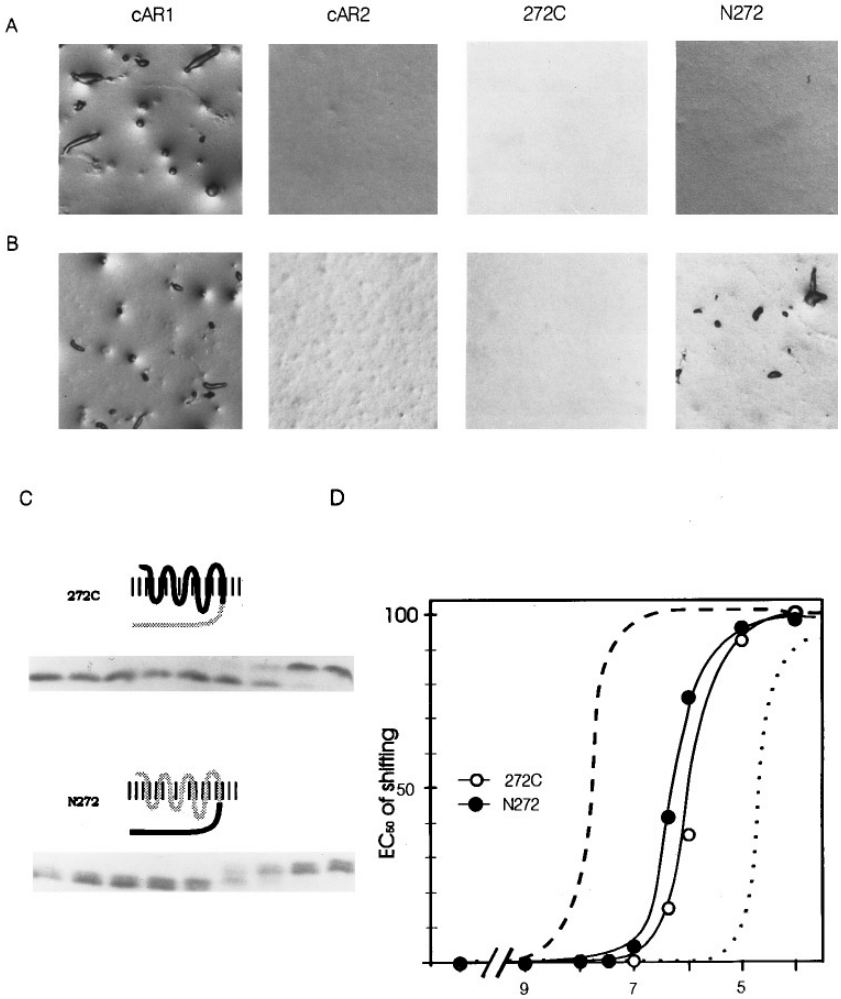

FIG. 6. Developmental phenotype and EC_{50} measurement of the mutants 272C and N272. Cells transformed with the specified plasmids were either harvested from the growth stage and plated directly on nonnutrient agar (A) or developed as described under Materials and Methods for 6 h and then plated (B). (C, D) EC_{50} measurement of the agonist-mediated receptor mobility shift of the chimeric receptors. (C) Representative Western blots of 272C and N272 are shown. As in Fig. 2, dashed and dotted lines are replotted data for cAR1 and cAR2, respectively. Open circles are 272C and filled circles are N272.

magnitude and kinetics. However, the cells still were unable to aggregate and proceed through development after they were plated on agar. Presumably the affinity of cAR2 was too low to mediate long-range cell–cell signaling. cAR2 has an affinity of about 20 μM for cAMP and is normally expressed after cells have aggregated into a tightly packed multicellular structure. The amount of cAMP synthesized by dispersed cells plated on agar may be too low to allow cell–cell signaling through cAR2. Comparison of several related receptors allowed us to define the lowest affinity required to induce development under standard conditions as about 1 μM (that displayed by N272).

We also produced cAR1 mutants with intermediate affinity by substituting three amino acid residues (N148G, V154D, and S155N) and with low affinity by substituting all five residues (N147G, V154D, S155N, F156Y, and T156D) in the major affinity determinant domain. The net charge of this domain is +1 in cAR1, +0 in cAR3, and −2 in cAR2 (Saxe *et al.*, 1991). These two mutants (ECLIIΔ3 and ECLIIΔ5), generated by site-directed mutagenesis, seem to confirm the hypothesis that changes in the charge of this domain are important for affinity under physiological conditions. Further support for this hypothesis is derived from another mutant, R160S, isolated in a random mutagenesis study of cAR1 (Kim *et al.*, 1997a). This substitution reduces the net charge of the second extracellular loop by removing a positive charge. R160S exhibited a similar affinity disparity between phosphate buffer and ammonium sulfate binding as did cAR2 and the cAR1 mutants presented here. This property is specific for mutations in the second extracellular loop.

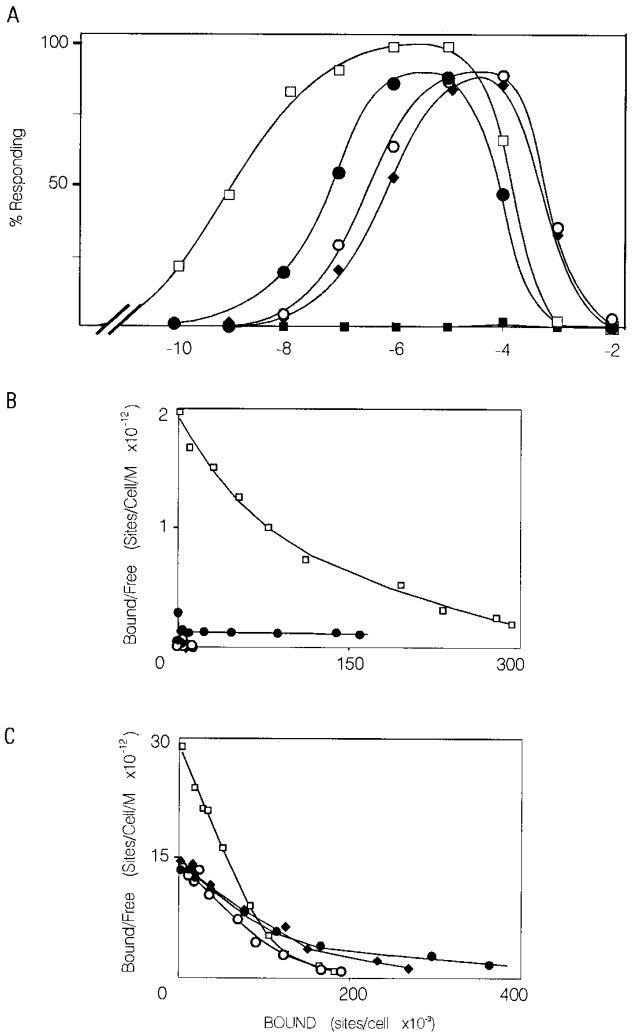

FIG. 7. Affinity characterization of the mutants 272C and N272. (A) The chemotaxis assay of five different cell lines, performed as described in the legend to Fig. 2. In each set at least 15 drops of each cell line in response to each dose were measured. The data presented are the averages of five independent experiments performed on different days. (B and C) Scatchard analysis of [^{3}H]cAMP binding of the mutant receptors in phosphate buffer (B) and ammonium sulfate (C). Vector control transformants (■); wild-type cAR1 transformants (□); cAR2-expressing cells (♦); 272C-expressing cells (○); and N272-expressing cells (●).

The discovery of an increasing number of responses mediated by GPCR signaling pathways raises important questions of specificity. This problem is amplified by the multiplicity of receptor subtypes. There are at least 10 subtypes of adrenergic receptors and multiple subtypes of dopamine, serotonin, and muscarinic acetylcholine receptors (Teeter *et al.*, 1994; Savarese and Fraser, 1992). The α_2- and β_2-adrenergic receptors share high similarities (70% homology) in their TM domains and exhibit similar ligand binding properties. However, they couple to distinct G-proteins (G_i and G_s), resulting in the opposed responses to similar ligands (Kobilka *et al.*, 1988). In muscarinic acetylcholine receptor subtypes, differential regulation of phosphoinositide hydrolysis and adenylyl cyclase inhibition by different subtypes was established (Hawes *et al.*, 1995). However, in many cases receptor subtypes display similar ligand binding properties and stimulate the same effectors: for example, both β_1- and β_2-adrenergic receptors activate adenylyl cyclase (Dohlman *et al.*, 1991). In multicellular organisms such apparent redundancy of receptor functions can be explained by the tissue and developmental stage-specific expression of the subtypes as shown for some β-adrenergic receptors (Koch *et al.*, 1995). It is also possible that redundant receptor subtypes undergo differential desensitization (Lohse *et al.*, 1992; Kim *et al.*, 1993) as shown in the case of three α_2-adrenergic receptor subtypes (Kurose and Lefkowitz, 1994).

In mammalian morphogenesis, for example, in neurogenesis during axonal migration, there are various attractants and/or repellents used to guide the migration (Devreotes, 1994). As the axons are converging to establish connections, the local concentration of axons gets denser. This can result in a higher local concentration of chemoattractant and/or repellents, causing saturation of the respective receptors. It will be interesting to see whether morphological development is achieved by switching receptor subtypes with different affinities but redundant effector coupling as shown here for *D. discoideum*.

Many studies have focused on the characterization of the domains and responses specific to a given receptor type and many specificity determinants have been characterized (Munch *et al.*, 1991). For ligand binding, studies of rhodopsin and β-adrenergic receptors have pointed to the transmembrane domains as ligand binding sites. However, these receptors may be exceptions to the rule. For example, a subfamily of receptors that bind large peptides tends to have large N-terminal domains where most of the high-affinity ligand binding is localized. In addition, chemokine receptors, adenosine receptors, and the cARs utilize the extracellular loops as major determinants of affinity (Murphy, 1994).

The capacity of the receptors to substitute for each other predicts that the overall sequences of the cytoplasmic tails of cARs do not play an essential role in development. These domains seem to perform fairly subtle function(s) since all of the truncated receptors exhibiting cAMP binding were able to mediate multiple responses including activation of ACA and actin polymerization (data not shown) and were able to rescue development. However, an appropriate interaction of the cytoplasmic tail with the rest of the receptor may be important. Mismatches within the region exiting from TMVII to the cytoplasm did produce receptors with unexpected affinities. We do not fully understand the role of this domain: Our present results suggest that, despite the unexpected affinity displayed by these receptors, they can activate downstream effectors with apparently normal kinetics. Within TMVII and the junctional area exiting into the cytoplasm there are very few amino acid substitutions

among the subtypes. Since the TMVII and domains immediately following serve an important function (Bouvier *et al.*, 1995) the domain swap might misalign the sequences affecting signal transduction.

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