Light-Driven Activation of β_2-Adrenergic Receptor Signaling by a Chimeric Rhodopsin Containing the β_2-Adrenergic Receptor Cytoplasmic Loops†

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ABSTRACT: Structure−function studies of rhodopsin indicate that both intradiscal and transmembrane (TM) domains are required for retinal binding and subsequent light-induced structural changes in the cytoplasmic domain. Further, a hypothesis involving a common mechanism for activation of G-protein-coupled receptor (GPCR) has been proposed. To test this hypothesis, chimeric receptors were required in which the cytoplasmic domains of rhodopsin were replaced with those of the β_2-adrenergic receptor (β_2-AR). Their preparation required identification of the boundaries between the TM domain of rhodopsin and the cytoplasmic domain of the β_2-AR necessary for formation of the rhodopsin chromophore and its activation by light and subsequent optimal activation of β_2-AR signaling. Chimeric receptors were constructed in which the cytoplasmic loops of rhodopsin were replaced one at a time and in combination. In these replacements, size of the third cytoplasmic (EF) loop critically determined the extent of chromophore formation, its stability, and subsequent signal transduction specificity. All the EF loop replacements showed significant decreases in transducin activation, while only minor effects were observed by replacements of the CD and AB loops. Light-dependent activation of β_2-AR leading to Gαs signaling was observed only for the EF2 chimera, and its activation was further enhanced by replacements of the other loops. The results demonstrate coupling between light-induced conformational changes occurring in the transmembrane domain of rhodopsin and the cytoplasmic domain of the β_2-AR.

G-protein coupled receptors (GPCRs)[1] mediate signal transduction in response to a wide variety of extracellular stimuli (*1*). Upon binding of the signaling molecules such as hormones and neurotransmitters, GPCRs activate heterotrimeric GTP/GDP-binding proteins, which in turn activate or inhibit secondary effectors leading to physiological responses. While GPCRs recognize diverse extracellular signals, they all have a common structural topology, based on seven membrane-spanning helices and certain conserved sequences. This suggests that GPCRs may have a common activation mechanism (*2*). Support for this conclusion has come from biochemical studies that indicate similar movements of TM helices upon activation of GPCRs (*3*−*4*) and of chimeric receptors (*5*−*13*) containing signal accepting extracellular (EC) and/or transmembrane (TM) domains from one GPCR and signal-delivering, cytoplasmic (CP) domains from another GPCR. This approach has provided evidence for similar activation mechanism among GPCRs. However, further studies are necessary to identify critical regions required for optimal agonist binding and signal delivery.

Rhodopsin and adrenergic receptors provide prototypic systems for structural and functional studies of GPCRs (*14*). The dark state crystal structure of rhodopsin (*15*−*17*) has served as the model for studies of other GPCRs. Structural and functional analysis of rhodopsin and of β_2-AR by using site-directed spin labeling and/or cysteine-scanning mutagenesis followed by reactivity of the cysteines to cysteine-specific reagents indicated helical movements for activation of these GPCRs (*18*−*21*). Both receptors showed similar conformational changes in TM helices III and VI upon activation indicating a similar activation mechanism in them. This finding raises the possibility that transmission of the ligand-binding signal to the CP surface and the ensuing changes in the CP loops are similarly conserved. In this paper, we set out to test this hypothesis by preparing functional chimeras between rhodopsin and β_2-AR that retain rhodopsin chromophore but signal through AR-specific pathway. To determine the functional TM/CP boundaries of rhodopsin and the β_2-AR, chimeras were constructed and tested for chromophore formation and transducin and/or Gαs signaling specificity. The chimeras differed in the lengths of rhodopsin loop sequence replaced by the corresponding β_2-AR loop sequence. The results indicated that all the loop replacements affect chromophore formation and signal trans-

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[1] Abbreviations: GPCR, G-protein-coupled receptors; DM, *n*-dodecyl β-D-maltoside; G_T, transducin; G_S, stimulatory G-protein; TM, transmembrane; CP, cytoplasmic.

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duction specificity depending upon the extent of replacement of the loop. Replacement of the EF loop proved to be the most critical and optimization of this replacement permitted successful construction of chimeric receptors showing light-dependent synthesis of cyclic AMP (cAMP). This study provides further evidence for the hypothesis of a common activation mechanism among GPCRs, and importantly, defines the critical boundary regions necessary for efficient signal transduction.

EXPERIMENTAL PROCEDURES

Materials. Dodecyl maltoside (DM) was obtained from Anatrace (Maumee, OH) and anti-rhodopsin mAb, rho-1D4 (*22*) was purified from a myeloma cell line provided by R. S. Molday (University of British Columbia). GTP-γ^{35}-S and [2,8-^{3}H]-adenine were obtained from Dupont/NEN. 11-*cis*-Retinal was prepared from all-*trans*-retinal as described (*23*). Frozen bovine retinae were from W. L. Lawson Co. (Lincoln, NE). Assays for cyclic AMP (cAMP-^{3}H) using competition binding were carried out either with a system obtained from Amersham (Piscataway, NJ) or with a system including cAMP dependent protein kinase, dextran-coated charcoal and gelatin obtained from Sigma, and ^{3}H-cAMP from ARC, Inc. (Saint Louis, MO).

Compositions of the buffers used for the purification of chimeras are as follows: buffer A, NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.8 mM, Na_2HPO_4 10 mM, pH 7.2; buffer B, buffer A plus phenylmethylsulfonylfluoride (PMSF) and 1% (wt/vol) DM; buffer C, buffer A plus 0.05%DM; buffer D, 2 mM NaH_2PO_4 pH 6.0 and 0.05% DM; and buffers E and F, buffer D containing 50 and 150 mM NaCl, respectively. Dowex 50W X-8 column was prepared by successive washings of the beads with 6 volumes of 0.1 N NaOH, H_2O, 1 N HCl, and H_2O. Neutral alumina was prepared by pouring 0.6 g of powder into the column followed by being washed with 10 mL of 0.1 M Tris HCl (pH 7.5).

Cloning of Chimeric Rhodopsin/β_2-AR Receptors. Construction of chimeric receptors was carried out based on the nucleotide sequence of the synthetic opsin (*24*) and β_2-AR (*25*) genes (Figure 1). The following considerations were taken into account in designing the chimeras. First, the conserved amino acids in the putative TM domain of opsin were retained. Second, the chimera with the largest loop replacement was constructed by replacing maximal numbers of amino acids after the last conserved residue by that of β_2-AR. In addition, several other constructs containing smaller replacements were designed to examine amino acid residues required to form a proper rhodopsin chromophore binding pocket and to adopt the cytoplasmic tertiary structure required for interaction with G-proteins. Restriction fragments used for cloning encoded the interhelical loops of hamster β_2-AR receptors and/or the flanking opsin sequences. For replacements of AB and CD loops, oligonucleotides containing the corresponding loop regions of AR were cloned into the *Bsp*Lu 11*l*-*Bgl*II, *Bsa*AI-*Aha*II sites of the opsin gene, respectively (*24*). Replacements of EF loop and C-terminal regions were carried out by the cloning of 0.15 kb *Mlu*I-*Hind*III and 0.2 kb *Pvu*I-*Not*I/*Sac*I-*Not*I fragments of AR together with the oligonucleotides containing flanking sequences into the *Ava*II-*Nde*I and *Apa*I-*Not*I or *Sac*I-*Not*I sites of the opsin genes, respectively. As indicated in Figure 1, two (C-terminus), three (AB), four (CD), and six (EF) chimeras with different sizes of loop replacements in the corresponding region were constructed. Each chimeric receptor was designated by the replaced cytoplasmic loop region followed by suffices 1, 2, 3, 4, 5, or 6 depending on the sizes of the replaced loops (Figure 1) from the longer to the shorter replacements, respectively. Chimeric receptors containing multiple loop replacements were obtained by shuffling the restriction fragments containing each loop replacements (*24*).

Expression and Purification of Rhodopsin. Wild-type opsin and chimeric receptor genes were transiently expressed either in COS-1 or HEK 293S cells using DEAE/Dextran or calcium phosphate, respectively, as described (*24*, *26*, *27*). Reconstituted rhodopsin pigments were purified (*24*, *26*) after incubation of transfected cells with 50 μM 11-*cis*-retinal at 4 °C for 3 h followed by being loaded onto 1D4-sepharose microcolumns preequilibrated with buffer B. Elution of chimeras was carried out by using the nonapeptide-rho-1D4 epitope in 2 mM sodium phosphate buffers (pH 6.0) containing 0, 50, or 150 mM NaCl, respectively, to assess the extent of protein folding (*28*). Isolation of stable opsin was carried out with five 15 cm culture plates of cells transfected using BTP buffer as described (*26*).

UV−vis absorption spectra were recorded with a Perkin-Elmer λ-6 UV−vis spectrophotometer, as described (*24*). The rates of Meta II decay and chromophore formation were measured by fluorescence assay as described (*29*).

Transducin Activation Assay by GTP-γ-S Binding. Rod outer segments (ROS) were prepared from bovine retinae by the method of Papermaster (*30*). Transducin (Gt) was purified from ROS using DE52 column chromatography as described (*31*). Transducin activation assays were carried out in a mixture containing 10 mM Tris HCl (pH 7.4), 100 mM NaCl, 0.5 mM $MgCl_2$, 0.015% DM, 2 nM rhodopsin, 0.25 μM Gt, and 3 μM GTP-γ-^{35}S (specific activity ~5000 cpm/pmol) as described (*32*).

Characterization of β_2-AR Function using cAMP Assay. For wild-type rhodopsin, incubation of transfected cells with 10 μM 11-*cis*-retinal for 1 h gave maximal reconstitution as increasing the retinal concentration to 50 μM gave little increase in chromophore formation. Several parameters required for optimizing the reconstitution such as the time required for preincubation with theophylline and 11-*cis*-retinal and the concentration of 11-*cis*-retinal were examined. Chimeras with single AB or CD loop replacements also showed no significant change in the extents of chromophore regeneration upon use of higher retinal concentration. However, chimeras with the EF loop replacement, and in particular, those combined with other loop replacements, regenerated the rhodopsin chromophore somewhat poorly as indicated by a further increase in chromophore formation upon increase of retinal concentration. Therefore, all analyses for cAMP formation were carried out in whole cells regenerated in the presence of 50 μM 11-*cis* retinal for 1 h. The presence of 5 mM theophylline or 10 μM isoproterenol did not affect the reconstitution of the chimera under the conditions tested.

Activation of β_2-AR-specific signaling was monitored by measuring cAMP levels using the following competition binding assay. Transfected cells were incubated with 1 mL of 1X PBS containing 5 mM theophylline and 50 μM 11-

(A) Replacements of rhodopsin loops by β_2-AR

I) AB Loop

	58 … 72
opsin	T LYV TV QHKKLR TP L
AB1	**V I TA IA KFERLQ TV T**
AB2	T LYV **IA KFERLQ TV** L
AB3	T LYV TV **KFERLQ** TP L

II) CD Loop

	137 … 152
opsin	VVV CK P MSNFRFG E N H
CD1	**IAI TSPFKYQSLLT KN K**
CD2	VVV **TSPFKYQSLLT KN K**
CD3	VVV **TSPFKYQSLLT KN** H
CD4	VVV **TSPFKYQSLLT** E N H

II) EF Loop

	224 … 254
opsin	G Q L V FTV K E A A A Q Q Q E S A T T QKA EKE V T R M V
EF1	**S RV FQVAK RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKFC LKEHKA L KTLG**
EF2	G **RV FQVAK RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKFC LKEHKA L** R M V
EF3	G QLVFTV K **RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKFC LKEHKA L** R M V
EF4	G **RV FQVAK RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKF** QKA EKE V T R M V
EF5	G QLVFTV K **RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKF** QKA EKE V T R M V
EF6	G QLVFTV K E **RQLQK IDKSEGRFHS PNLGQVEQDG RSGHGLRR SSKF** QKA EKE V T R M V

III)C-terminus

	307 … 348
opsin	I MMNKQ FR N C M V T T L C C G K N P L G D D E A S T T V S K TETSQVAPA
Ct1	**CRSPD FR IAF QELL CLRRSS (---50 a.a.--) TVPSLSLDSQ GRNCSTNDSP L** TETSQVAPA
Ct2	I MM NKQFR **IAF QELL CLRRSS (---50 a.a.--) TVPSLSLDSQ GRNCSTNDSP L** TETSQVAPA

(B)

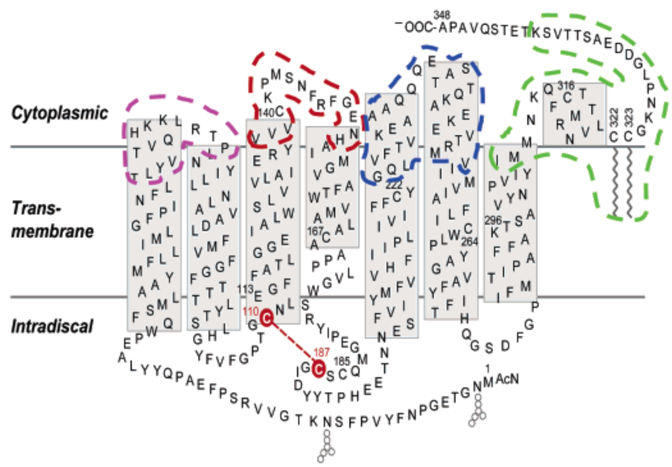

FIGURE 1: (A) Replacement of rhodopsin sequences in cytoplasmic loops by analogous sequences from β_2-AR. Alignments of the bovine opsin and hamster β_2-AR sequences around TM/CP boundaries are based on structural information of rhodopsin and its sequence homology to β_2-AR. Nomenclature of the chimeras and their replaced amino acid sequences are shown. The lettering indicates the loop replacement with the longest given the designation 1 (e.g., EF1). (B) Secondary structure model of bovine rhodopsin. Cytoplasmic loop regions replaced in this study are indicated.

cis retinal at 4 °C for 1 h. The cells were then illuminated for 2 min or not illuminated and then left in the dark for 30 min at 20 °C. After the cells were incubated in boiling water for 3 min followed by centrifugation at 13 000*g* for 2 min, the cAMP level in the supernatant was determined by competitive binding to cAMP dependent kinase in the presence of ^{3}H-cAMP. All the assay tubes, prepared in triplicate, were mixed with 50 μL of 10 mM Tris. HCl (pH 8.0) and 1 mM EDTA containing ^{3}H-cAMP together with 100 μL of the cAMP dependent kinase followed by incubation at 4 °C for 2 h. After treatment with 100 μL of a 1% charcoal suspension followed by centrifugation at 13 000*g* for 3 min at 4 °C, radioactivity in the supernatant was measured by scintillation counting. The cAMP levels in HEK293S cells expressing chimeric receptors were also measured by an assay involving pre-labeling of cAMP (*33*) in cells grown in the presence of ^{3}H-adenine. Cells, regenerated as described previously, were incubated with 1% perchloric acid at 4 °C for 30 min followed by centrifugation at 13 000*g* for 2 min. The supernatant was applied to Dowex column (0.4 × 4 cm) followed by an alumina column as described (*33*).

RESULTS

Construction and analysis of chimeric receptors consisting of the TM domain of rhodopsin and cytoplasmic domains of the β_2-AR was carried out to determine the boundaries of the functional domains of these two prototypical GPCRs and to address the hypothesis that conformational changes required for GPCR activation are conserved. A protein with light-induced Gαs signaling would address the latter provided the β_2-AR loops are fused correctly to contain optimum sequences required for ligand binding and proper conformational changes to present sequences in the CP domain in the spatial orientation necessary for interaction with the Gαs protein. Two (C-terminus), three (AB), four (CD), and

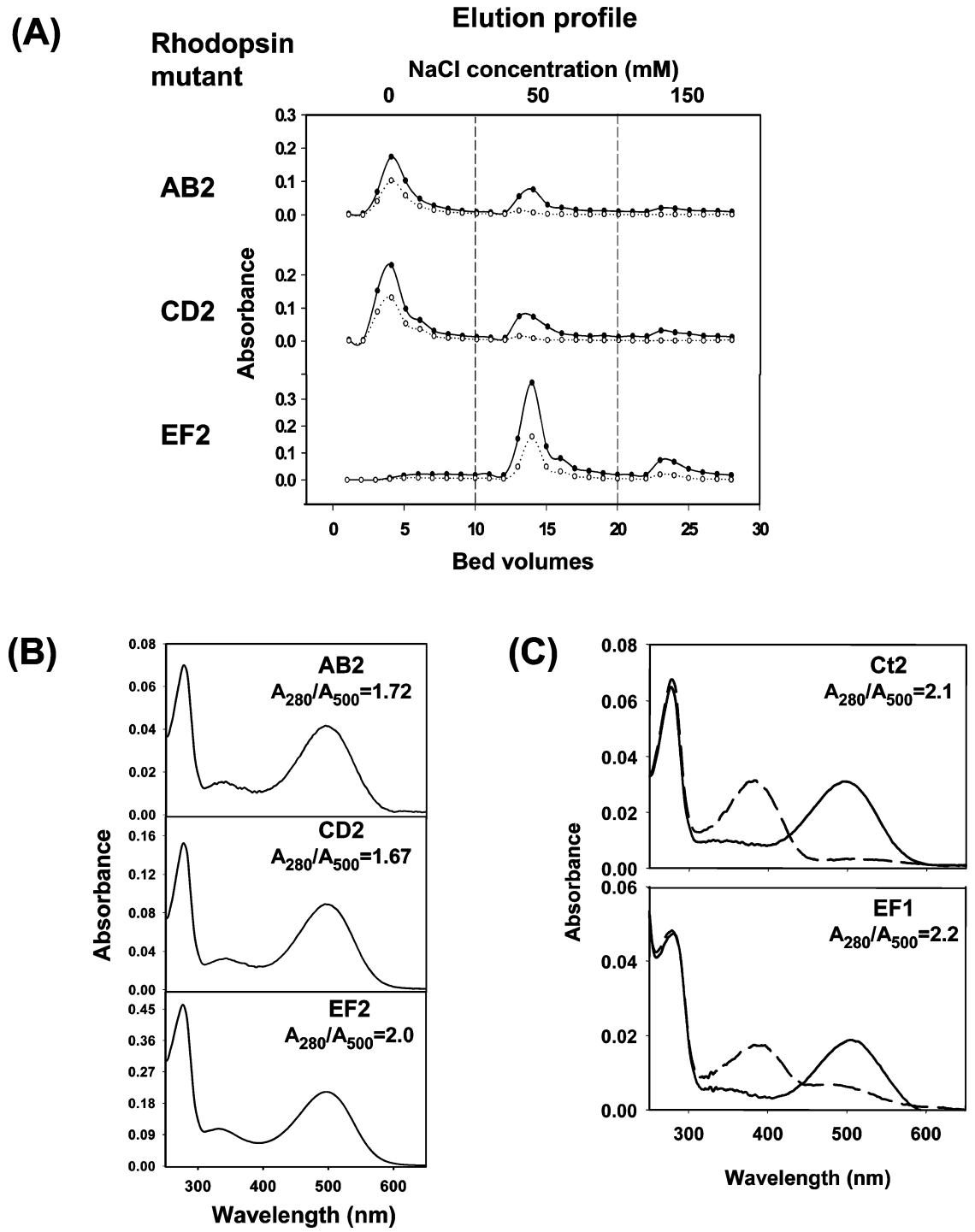

FIGURE 2: Purification and spectral characterization of chimeric receptors. (A) Elution profiles of a representative collection of chimeric receptors from rho-1D4 sepharose column. Ratios of A_{280} (●)/A_{500} (○) observed for peak fractions are indicated. (B) UV/vis absorbance spectra of three representative chimeras, AB2, CD2 and EF2, containing an intermediate size of loop replacement. (C) UV/Vis absorbance spectra and photobleaching behavior of two representative chimeras, EF1 and Ct2. While chimeras with single loop replacements, AB2 and CD2, showed a ratio similar to that of wild-type rhodopsin (∼1.7), chimera EF1, EF2, and Ct2 showed ratios of 2.2, 2.0, and 2.1, respectively.

six (EF) chimeras with different sizes of loop replacements (Figure 1) in the corresponding region were constructed as described in Experimental Procedures. Chimeric receptors containing multiple loop replacements were also prepared by restriction fragment shuffling.

Position and Size of the Loop Replacements Determine Chromophore Formation and Expression. Expression of chimeras constructed in pMT4-based vectors (*24*) was carried out in COS-1 cells (*24*, *26*) as well as in HEK293S cell system using cotransfection with plasmid pRSVTAg containing the gene for T-antigen (*27*). Upon reconstitution with 11-*cis*-retinal, all chimeras with single loop replacements showed chromophore formation with absorption maxima at close to 500 nm (Figure 2A,B). Photobleaching of the regenerated chimeras resulted in formation of species with absorption maxima of 380 nm similar to that of wild-type rhodopsin (representative data shown for EF1 and Ct2, Figure 2C). While chimeras with single loop replacements were

expressed to a level similar to that of wild-type opsin genes, chimeras EF1 with the largest replacement in the EF loop region and Ct2 containing a replacement in C-terminus showed 3−5-fold lower level of expression as analyzed by difference spectra (data not shown). Extent of rhodopsin chromophore formation in EF1 and EF2 was reduced to 30 and 70%, respectively, of that of wild-type rhodopsin under the same conditions. Chimeras with multiple loop replacements also showed a lower extent of chromophore formation as exemplified by CD2/EF2 (58%) and AB2/CD2/EF2 (38%). Purified chimeras with shorter loop replacements showed a lower A_{280}/A_{500} ratio, close to that of wild-type rhodopsin indicating better folding of the opsin. However, chimeras with the larger size loop replacement showed an increase in the A_{280}/A_{500} ratio. The highest A_{280}/A_{500} ratio was found in the EF1 mutant (2.2), which has the largest replacement in the EF loop (Figure 1). The A_{280}/A_{500} ratio decreased as the size of the replacement decreased to EF2 of ratio 2.0 and EF5 of ratio 1.7. Gradual increase in protein misfolding was also detected as more loop replacements were introduced in the chimeras as shown by the ratios of 1.8 with the AB2/CD2 and 3.3 with AB2/CD2/EF2. A slightly improved ratio of 2.9 in AB2/CD2/EF5 was observed when the replacements AB2/CD2 were combined with the shorter replacement in the EF loop, EF5. The results indicate that the size and extent of replacement of the loops affects the folding of the chromophore.

Upon differential salt elution of chimeras from a microcapillary rho 1D4-Sepharose column, chimeras with single loop replacement in the AB or CD loop (Figure 2) and C-terminal regions showed elution profiles similar to wild-type rhodopsin and eluted under low salt condition. However, all the chimeras with the EF loop replacement required higher (50 mM) salt concentration for elution. This effect could be associated with misfolding of rhodopsin or with affinity changes to the 1D4 column matrix as a result of proximity of the β_2 EF loop sequences to the 1D4 epitope. The elution profile of the chimeras with the EF loop replacement appeared to be pH-dependent as these eluted predominantly at low salt under low pH condition and progressively required higher salt for elution when the pH was increased to 5.0 and 6.0 (data not shown). Under the same conditions, the elution of the wild-type rhodopsin remained unaffected in this pH range. The additive effect of loop replacement on the elution profile was also detected as all the chimeras containing multiple loop replacements including the EF loop region required high salt for elution (*28*).

Stability of Opsin and Rhodopsin in the Rhodopsin/β_2-AR Chimera. To investigate the effects of size of loop replacements on the stability of apoprotein, purified opsin-AR chimeras were incubated in 1% DMPC/1% CHAPS micelles for various times (*34*) prior to the addition of retinal to regenerate the rhodopsin chromophore. The extent of denaturation of chimeric opsin is indicated by decreases in the yield of rhodopsin chromophore formation. Wild-type opsin and the chimera EF6 containing the shortest EF loop replacement is stable enough to bind retinal to similar extents even after 40 h of incubation (Figure 3). However, the ability of the other mutants to bind chromophore gradually decreased as the lengths of EF loop replacements increased, indicating that the length of loop replacement affects the stability of the chimeric opsin.

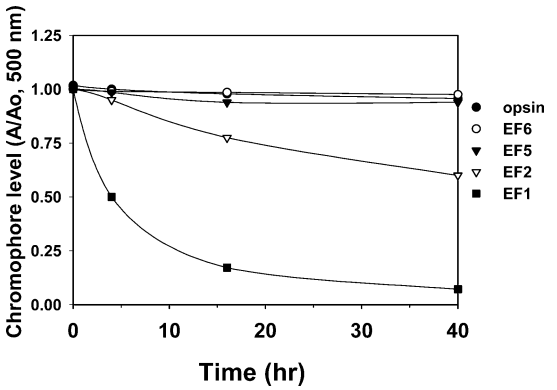

FIGURE 3: Relative stability of chimeric opsins in DMPC/CHAPS micelles. Chimeric opsin purified in 1% DMPC/1% CHAPS was kept for the indicated times at 20 °C. Chromophore was generated by the addition of 11-*cis*-retinal (2:1 molar ratio of retinal/opsin) and the level of chromophore formation measured after 20 h incubation in the dark at 20 °C. The level of chromophore formation was normalized to that of freshly purified chimeric opsin.

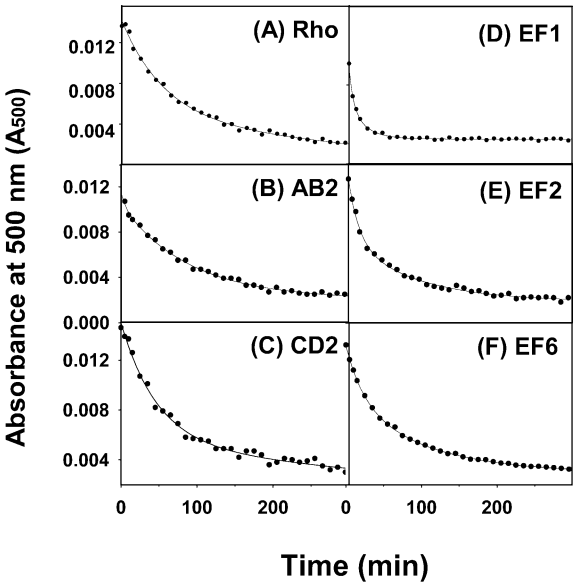

FIGURE 4: Thermal decay profile of the wild-type rhodopsin (A) and chimeras AB2 (B), CD2 (C), EF1 (D), EF2 (E), and EF6 (F) each containing a single loop replacement in the AB, CD, and EF loop regions, respectively. Absorbance was measured at 500 nm as a function of time at 55 °C.

To examine the thermal stability of the various chimeras, we monitored the rhodopsin chromophore (A_{500}) at 55 °C as a function of time (Figure 4). The thermal decay profiles of the 500 nm chromophore with time fit best with a double exponential function with two components (Table 1). Wild-type rhodopsin has two components, a fast ($t_{1/2}$ = 38.9 min) and a slow ($t_{1/2}$ = 450 min) component, which correspond to 56.8 and 43.2% of the total composition. Chimera AB2 also showed two components, although the relative composition of each component is quite different from that of wild-type rhodopsin as indicated by 10% of the faster component and the remaining slow component. Chimera CD2 showed a decay behavior similar to that of wild-type rhodopsin although the rates are faster. All chimeras with EF loop replacements also showed decay with two components. Chimera EF6 with the shortest replacement in the EF loop showed a further decreased stability than chimera AB2 and CD2 probably due to its larger size of loop replacement.

Table 1: Thermal Stability of Rhodopsin Chromophore in Chimeras[a]

	component 1		component 2	
mutants	$T1/2$ (min)	(%)	$T1/2$ (min)	(%)
Rho (wt)	38.9	56.8	450.0	43.2
AB2	4.2	12.6	63.0	87.3
CD2	32.5	63.6	410.0	36.4
EF1	3.6	51.7	14.6	48.3
EF2	9.6	50.5	63.0	49.5
EF5	15.7	39.6	69.3	60.4

[a] Thermal stability was followed using the purified rhodopsin chromophore regenerated with 11-*cis*-retinal as described in the Experimental Procedures. The amount of chromophore (A_{500} nm) remaining after its incubation at 55 °C for specific time intervals matched well with a double exponential decay profile.

Chimeras EF1 and EF2 were even more unstable, the order of stability being EF6 > EF2 > EF1, indicating a decrease in stability with an increase in the size of the EF loop replacement. The results indicate that all the loop replacements affect the stability of the chromophore as shown by their reduced $t_{1/2}$ even in the shortest loop replacement and correlation between chromophore instability and size of loop replacement.

Light Activation of Rhodopsin-Specific Signaling by Chimeric Receptors. Effects of loop replacements on rhodopsin-specific signaling were studied by monitoring the activation of transducin (G_T). Like wild-type rhodopsin, the chimeras showed little activation of G_T in the dark. Upon illumination, chimeras with shorter replacements in the AB loop (AB2 and AB3) or in the C-terminal regions (Ct2) showed G_T activation comparable to that of wild type (Figure 5). The chimeras containing replacements in the CD loop showed moderate G_T activation, while chimeras containing EF loop replacements showed drastically reduced G_T activation. Chimeras EF1 and EF2 containing longer EF loop replacements showed no light-dependent G_T activation despite exhibiting chromophore formation and photobleaching behaviors similar to that of wild-type rhodopsin. Low level but distinct light-dependent G_T activation was observed in chimeras EF5 and EF6 that contained shorter replacements in the EF loop. The results suggest that amino acid residues present in EF6 but absent in EF2 are important for G_T activation.

Light-Dependent AR-Specific Signaling by Chimeric Receptors. To test whether chimeras forming rhodopsin chromophore can elicit β_2-AR-specific signaling upon stimulation by light, accumulation of cAMP in the cells was used as an assay for activation of the β_2-AR-specific signaling through Gαs. Analysis of cAMP level was carried out in COS-1 cells as well as in HEK 293 cell system, which has a lower level of endogenous β_2-AR. After reconstitution with 50 μM 11-*cis*-retinal, the cells were divided into two equal fractions. Cells were illuminated or not for 1 min and incubated in the dark for 30 min, and the cAMP levels in cells were measured. Chimeras with shorter, single loop replacements in the AB, CD, and C-terminal regions showed no light-dependent accumulation of cAMP (Figure 6A). However, among chimeras with the EF loop replacement, the chimera EF2 containing an intermediate size (52 amino acid) replacement in the region showed light-dependent cAMP production. These results indicate a unique role of the EF loop in

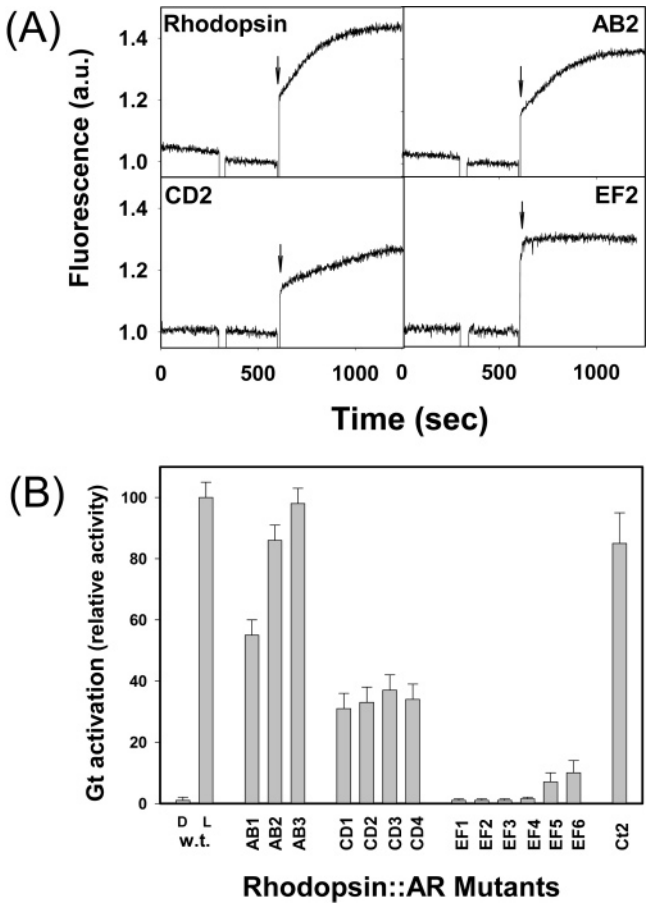

FIGURE 5: Effects of loop replacements on rhodopsin-specific signaling. (A) Transducin activation was measured by following the binding of GTP-γ-S to $G_{T(\alpha)}$ using a fluorescence based assay at 20 °C (see Experimental Procedures). Initial rate of G_T activation was calculated from the initial rate of fluorescence increase upon addition of GTP-γ-S (A, arrows). (B) Relative rate of G_T activations exhibited by wild-type rhodopsin in the dark (D) and after the light activation (L) was set as 1 and 100, respectively. Initial rates of G_T activation by chimeras measured after the light activation are shown.

recognition of the specific G-protein (Gs) and of an optimal sequence requirement for producing agonist-dependent conformational changes.

To test the effects of combined loop replacements on β_2-AR-signaling, a series of chimeras containing combinations of such loop replacements was analyzed (Figure 6B). While the chimera AB2/CD2 alone showed no significant light-induced increase in cAMP production, chimera CD2/EF2 showed a slightly higher level of light-dependent accumulation of cAMP than that of chimera EF2 with a single loop replacement. In addition, the chimera containing a combination of AB2/CD2 together with EF2 but not with EF1 and EF5 showed a further light-dependent cAMP production level even higher than that shown by EF2 alone or CD2/EF2 loop replacements. The cAMP level was further increased upon addition of C-terminus replacement (Ct2), although the chimera showed 5-fold lower level of chromophore formation. No light-dependent β_2-AR signaling was observed in the triple loop-replaced chimera AB1/CD1/EF1 despite a higher proportion of β_2-AR sequence in the chimera. This suggested an optimum sequence requirement for obtaining light-dependent AR-signaling. To compare the degree of stimulation of cAMP production by the chimeras to that of

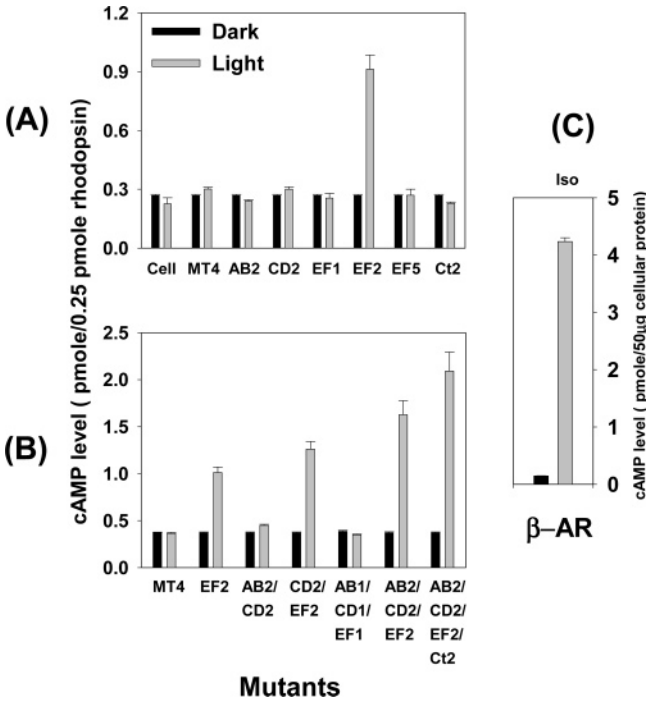

FIGURE 6: Comparison of cAMP produced by chimeras containing a single loop replacement (A) and chimeras with multiple loop replacement (B). Assay was carried out using portions of the transfected HEK293S cellular extracts equivalent to 50 μg of total protein. cAMP content in the samples was normalized to the amount of 500 nm chromophore formed in the chimera AB2/CD2/EF2 and compared to that of an equivalent portion of the cells transfected with β_2-AR with or without 10 μM isoproterenol treatment (C).

the fully activated β_2-AR expressed under the same condition, a cAMP assay was carried out using HEK293S cells transfected with wild type β_2-AR. Upon stimulation with isoproterenol, the net increase in cAMP production was calculated by subtracting that of the control without stimulation. cAMP produced by the chimera AB2/CD2/EF2/Ct2 upon light stimulation was approximately 50% of that produced by β_2-AR stimulated by isoproterenol. A similar level of light-dependent stimulation of cAMP production (a net increase of 4.3 pmol upon isoproterenol activation of wild-type β_2-AR and 2.0 pmol of light-dependent-accumulation of the chimera AB2/CD2/EF2/Ct2) was also obtained using an assay for cAMP involving the prelabeling of cAMP in transfected HEK293S cells grown in media containing ^{3}H-adenine.

DISCUSSION

The aim of this study was to test the hypothesis that GPCRs share a common activation mechanism by employing a chimeric receptor approach between two well-characterized GPCRs, rhodopsin and the β_2-AR. We first sought to construct a photoreceptor that signals through G_S upon its activation by light. A further aim was to identify the optimum sequences required for ligand binding and proper conformational changes in the TM and CP domains required for interaction with G-proteins by constructing chimeras composed of various lengths of TM and CP domains of rhodopsin and β_2-AR (Figure 1). Expression and analysis of chimeras including chromophore formation and signal transduction were first tested in COS-1 cells. Light-dependent G_S signaling was also carried out in HEK293S cells that are known to contain a low level of endogenous β_2-AR. All single loop replacements in the AB and CD loops and a shorter replacement in the EF loop region showed a level of expression similar to that of wild-type rhodopsin. However, a larger replacement in the EF loop or C-terminal region resulted in reduced level of expression. The rate and extent of rhodopsin chromophore formation in chimeras is dependent on the size and the number of the exchanged replacements. Comparison of the A_{280}/A_{500} ratio of rhodopsin chromophore indicate that the chimeras with a longer loop replacement showed a higher level of misfolding and instability of opsin and rhodopsin chromophore as compared to that of the shorter replacement. Altered elution profile from the rho 1D4-Sepharose column was also exhibited by the chimeras with the EF loop replacement. This is most likely caused by charged amino acid residues in the EF loop region of β_2-AR, which might interfere with the folding of the protein or affect interaction with the rhodopsin 1D4 antibody column. In addition, chimeras with the shorter loop replacement were less thermally stable than wild-type rhodopsin. Taken together, these results suggest coupling of CP and TM domains and the stabilizing role of CP loops in opsin and rhodopsin.

Analysis of rhodopsin- and β_2-AR-specific signaling was carried out by activation of G_T and G_S, respectively. The most drastic effects on both G_T and G_S activation was exerted by chimeras with EF loop replacement. In contrast, moderate or minor effects were observed in chimeras with the CD loop replacement or with AB and C-terminus replacements, respectively. The results confirm the critical role of the EF loop on signaling, and these finding are thus consistent with previous observations on GPCRs by using mutagenesis (*35*, *36*), peptide competition (*37*), and analysis of GPCRs and chimeric GPCRs (*6*−*11*). In particular, our results are in agreement with those of Yamashita et al. (*5*). For example, distinct roles of the second and third CP loops of rhodopsin were suggested from the study of rhodopsin mutants in which CP loops were replaced by those of other GPCRs (*5*). The result indicated that replacement of, in particular, the N-terminal part of the second CP loop reduced G_T activation. Our result also showed a reduced level of G_T activation in chimera CD1-CD4 that may be due to the replacement of the N-terminal part of CD loop sequence including E134, V138, and C140. A low level but clear light-dependent G_T activation was observed in the chimeras EF5 and EF6 that contained mainly opsin sequences but not in the chimeras EF1 and EF2 containing more β_2-AR sequences. Our results indicate that the more amino acid residues from the EF loop region of rhodopsin are retained in the chimeras, the higher the G_T activation, thus implicating the importance of EF loop region on G_T activation. For light-dependent β_2-AR signaling, neither the chimera EF1 containing the largest number of amino acid residues from the EF loop of β_2-AR nor the chimera EF5 and EF6 containing more opsin residues (hence less of β_2-AR sequence) showed a light-dependent cAMP accumulation. Instead, only the chimera EF2 containing an intermediate size of replacement showed light-dependent β_2-AR signaling. Thus, there is an optimum sequence requirement in GPCRs for both signal acceptance and for efficient transmission of the signal to the CP domain. This approach not only provides useful information for finding functional

boundaries in GPCRs but also can be an important tool for verifying segment information deduced from computational analysis of GPCRs (*38*).

Further increases in the level of light-dependent cAMP accumulation were observed upon combination of EF2 together with other loop replacements. A synergistic effect of multiple loop replacements, in particular between CD and EF loop, on G-protein activation suggests the involvement of these two loops on G-protein activation, a finding that is entirely consistent with peptide inhibition experiments (*37*). Maximum light-dependent cAMP production was observed in chimera AB2/CD2/EF2/Ct2 (with all loop replacements) reaching a level of 50% of that obtained with β_2-AR in the presence of isoproterenol. While this level of cAMP synthesis seen with a chimeric protein is impressive, there are several possible reasons as to why it is less than 100%. One possibility is reduced stability of the active Meta II rhodopsin. Also, as the major conformational changes required for activation are conserved, there may be receptor specific conformational changes required for full β_2-AR activation. It is also possible that further refinement of the boundary sequences may improve cAMP production. Finally, internalization, degradation, and desensitization of the activated receptor may reduce G_S signaling by the chimeric protein.

The chimeric receptor approach has been used to examine the role of the structural domains that determine the binding and specificity for ligand binding and activation of G-proteins. This approach has also been used for identification of signal transduction pathway of orphan receptors where either ligand or target G-protein and effectors were unknown (*10*, *11*). Our chimeric receptor approach showing a light-dependent AR signaling not only provides supporting evidence for the common activation mechanism in GPCRs but also serves as a tool that can be useful for studies of GPCR signaling. In particular, chimera EF2 seemed to contain an optimum connection in efficiently transducing conformational changes of TM to that of CP domain upon receptor stimulation. Therefore, by combining an optimum sequence corresponding to the replacement in EF2 together with the corresponding region of another receptor, it should be possible to study the ligand or G-protein signaling pathway specific for orphan GPCR sequences.

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