Characterization of lacZ Complementation Deletions Using Membrane Receptor Dimerization

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ABSTRACT

We describe the screening of lacZ deletions in mammalian cells and the discovery of a novel pair of lacZ deletions that can undergo α -complementation only when they are fused to peptides that interact with each other. The two lacZ deletions, Δ N 11-75 and Δ C 82-1023, were first characterized by fusing to two small interacting peptides and were then further analyzed by fusing to three membrane receptors (G protein-coupled receptors α_{2c} AR and D2DR_L and receptor tyrosine kinase insulin receptor) that were known to form homodimers in the membrane. Histochemical and quantitative FACS[®] assays demonstrated that the novel deletions have much lower level of association with each other, thus offering a much lower background in monitoring membrane protein interactions compared to previously published lacZ deletions. Furthermore, our method has the exciting potential to monitor simultaneously membrane receptor dimerization and localization to the cell surface of living cells.

INTRODUCTION

The α complementation of the bacterial lacZ gene (encoding β -galactosidase, or β -gal) has been studied for 30 years in prokaryotes (7–9,17) and has recently been adapted for the use in eukaryotes (13,15). Two weakly complementing mutants of β -gal, $\Delta\alpha$ and $\Delta\omega$, were used to detect interactions between cytoplasmic protein (12,15) and membrane protein (2) in living mammalian cells. The β -gal mutants were fused to different target proteins, and the interaction of the non- β -gal portions of the chimeric proteins were found to be able to restore the complementation capacity of $\Delta\alpha$ and $\Delta\omega$. Thus, the reconstitution of the β -gal activity was taken as an indicator of the interaction between the target proteins.

While the two-hybrid system has been widely used for exploring interactions between soluble proteins, the study of interactions between different membrane proteins and membrane proteins and their ligands has lagged behind because of the lack of robust assay methodology. We have attempted to implement a plasmid-based assay to study the interactions between G protein-coupled receptor (GPCR) using $\Delta\alpha$ and $\Delta\omega$ instead of β -gal, but we observed significant spontaneous α -complementation from the co-expressed mutants even when they were not fused to any peptides. This background signal made it difficult for the method to be employed in high-throughput applications.

Here we screened for novel β -gal deletions in living mammalian cells that could reduce such background. We

discovered a pair of deletions, Δ N 11-75 and Δ C 82-1023, which showed significantly lower spontaneous complementation when co-expressed transiently in mammalian cells. This pair of deletions was first characterized using affinity peptides and the human insulin receptor (IR) as model systems to perform homodimerization studies. We then examined the dimerization state of the rat adrenergic receptor α_2 -receptor subtype α_{2c} AR and the rat D2 dopamine receptor (D2DR_L) using this pair of lacZ deletions. Here we found histochemical evidence that α_{2c} AR homodimerizes in vivo. Although different techniques have been used to examine rat D2DR_L dimerization (14,21), we are the first to report that D2DR_L forms homodimers in living mammalian cells. Our results suggest that the combination of a plasmid-based assay system with the novel lacZ deletions could provide a high-throughput system in which the interaction between different membrane receptors and its localization could be analyzed in a rapid and sensitive manner.

MATERIALS AND METHODS

Expression Plasmids Encoding for Δ N 11-30, Δ N 11-41, and Δ N 11-75

Δ N 11-41 was first cloned by the fusion of the *Bam*HI filled-in *Xho*I fragment from the previously described lacZ construct pWZL $\Delta\alpha$ into the *Xba*I filled-in *Xho*I site of the His-tagged pcDNA3.1 (Interactiva). To design the Δ N deletion mutants Δ N 11-30 and Δ N

11-75, the coding region for the construct pWZL $\Delta\omega$ (15) was cloned by the insertion of the *XhoI-EcoRV* fragment from pWZL $\Delta\omega$ into the *PstI* filled-in *XhoI* site of Bluescript[®] SK⁽⁺⁾ (Stratagene, La Jolla, CA, USA). The deletion mutants ΔN 11-30 and ΔN 11-75 were constructed by PCR with the primers ΔN 11 reverse (Interactiva) and ΔN 30 forward (ΔN 11-30); ΔN 11 reverse and ΔN 75 forward (ΔN 11-75). (Table 1 summarizes all the sequences of the primers and other synthetic oligonucleotides.) The PCR products were digested with *DpnI*, ligated, and checked by DNA sequencing with T7 primer (Interactiva) using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA). Both deletion mutants were inserted in frame right after the His-tag of ΔN 11-41 by fusion of the *XhoI-Bsu36I* fragments from pBSK⁽⁺⁾ ΔN 11-30 and pBSK⁽⁺⁾ ΔN 11-75 into the *XhoI-Bsu36I* site of ΔN 11-41.

Expression Plasmids Coding for ΔC 46-1023, ΔC 82-1023, ΔC 148-1023, ΔC 377-1023, ΔC 653-1023, and ΔC 789-1023

To design the ΔC deletion mutants, the coding region for the construct pWZL $\Delta\omega$ was cloned first by the fusion of the *Bsp120I* filled-in *XhoI* fragment in frame into the *XbaI* filled-in *XhoI* site of pcDNA3.1/Hygro⁽⁺⁾ (Interactiva) ($\Delta\omega$ 89-1023). This construct was used as a template for PCR to design the deletion mutant ΔC 46-1023 with the primers ΔC 46 forward and ΔC 46 reverse (Table 1), which included three stop-codons at the 3' end. The PCR product was digested with *XhoI* and cloned into the *ApaI* filled-in *XhoI* site of pcDNA3.1/Hygro⁽⁺⁾. ΔC 46-1023 was digested with *XbaI* filled-in-*XhoI* to insert the *Bsu36I* filled-in-*XhoI* fragment from ΔC 789-1023 to design the construct $\Delta\omega$ 82-1023. ΔC 148-1023 was constructed by the insertion of the *XhoI-HpaI* fragment from $\Delta\omega$ 789-1023 into the *XbaI* filled-in *XhoI* site of ΔC 46-1023. ΔC 377-1023 was constructed by the insertion of the *XhoI-EcoRV* fragment from ΔC 789-1023 into the *XbaI* filled-in *XhoI* site of ΔC 46-1023. ΔC 653-1023 was constructed by the insertion of the *SacI* filled-in *XhoI* frag-

Table 1. Primers and Other Short Synthetic DNA Sequences

Name of the Oligonucleotides	Sequences of the Primer (5'→3')
ΔN 11 rev.	GACGGCCAGTGAATCCGTA
ΔN 30 forw.	CATCCCCCTTTTCGCCAGC
ΔN 75 forw.	GAGTGCGATCTTCCTGAGGCC
ARprim1	GTAAGGTACCCATGGGCTCCCTGCAGC
ARprim2	ATTCCTCGAGCACGATGCGCTTTCTGTCCC
D2prim1	CTCCTCATCTTTATCATCGTC
D2prim2	CTTTCTGCGGCTCATCGT
ΔC 46 forw.	CTGCAGATATCCAGCACAGTGG
ΔC 46 rev.	TCAGCTAGTTATCTAGAGCGGGCCTCTTCGCTATTAC
PepA(+)	CTATGAACGAAGCATATGTACATGACGGTCCTGTACGCTCACTGAACC
PepA(-)	GGTTCAGTGAGCGTACAGGACCGTCATGTACATATGCTTCGTTTCATAG
PepB(+)	ATGAAGGCACGAAAGGAAGCAGAACTGGCAGCAGCAACTGCAGAACAGC
PepB(-)	GCTGTTCTGCAGTTGCTGCTGCCAGTTCTGCTTCCTTTCGTCCTTCAT
Rev., reverse; forw., forward.	

ment from ΔC 789-1023 into the *XbaI* filled-in *XhoI* site of ΔC 46-1023.

Design of the pepA- ΔN - and pepB- ΔC Constructs

For the expression of pepA (MNEAYVHDGPVRS LN) and pepB (MKARKEAELAAATAEQ), oligonucleotides were designed with the coding sequence pepA(+) and pepA(-), and pepB(+) and pepB(-) (Interactiva) (Table 1). The annealed oligonucleotide sequences expressing pepA and pepB were cloned into the blunt-ended *EcoRV* sites from His-tagged pcDNA3.1 and pcDNA3.1/Hygro⁽⁺⁾. pepA- ΔN 11-30, pepA- ΔN 11-41, and pepA- ΔN 11-75 were constructed by the fusion of the *ApaI-XhoI* fragments from the His-tagged pcDNA3.1 constructs ΔN 11-30, ΔN 11-41, and ΔN 11-75 into the *ApaI-XhoI* site of pepA-pcDNA3.1. To create the pepB- ΔC constructs, pepB- ΔC 46-1023 was first created by a three-fragment ligation: the vector ΔC 46-1023, which was cut with *HindIII-XhoI*, was ligated to both the *XmaI* filled-in *HindIII* fragment from HA-tagged pcDNA3.1/Hygro⁽⁺⁾, which encompasses the HA-tag coding

sequence, and to the *EcoRI* filled-in *XhoI* fragment encoding the pepB fusion from pepB-pcDNA3.1/Hygro⁽⁺⁾ that was cloned immediately after the HA-tag. For the design of pepB-fused ΔC 82-1023, - ΔC 148-1023, - ΔC 377-1023, - ΔC 653-1023, and - ΔC 789-1023, the vector pepB- ΔC 46-1023 was digested with *XbaI* filled-in *XhoI* to insert the fragments that were cut with the same restriction enzymes as described earlier for the design of the non pepB- ΔC constructs.

Construction of α_2c AR- ΔN 11-75, α_2c AR- ΔC 82-1023, D2DR_L- ΔN 11-75, D2DR_L- ΔC 82-1023, IR- ΔN 11-75, and IR- ΔC 82-1023

The coding region of rat α_2c AR was amplified from R α_2 pRc/CMV (19) by PCR with the primers ARprim1 and ARprim2 (Table 1). The PCR product was cloned in frame into the blunt-ended *KpnI-XhoI* site from ΔN 11-75, directly in front of the lacZ deletion mutant α_2c AR- ΔN 11-75, and verified by DNA sequencing. α_2c AR- ΔC 82-1023 was generated by insertion of the *HindIII-XhoI* fragment from α_2c AR- ΔN 11-75 into the *HindIII-XhoI* site of ΔC 82-

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1023, directly in front of the lacZ deletion mutant. The coding region for rat D2DR_L was amplified from pBSK(+) D2DR_L (5) by PCR with the primers D2prim1 and D2prim2 (Table 1). The PCR product was cloned in frame into the blunt-ended *KpnI-XhoI* site from ΔN 11-75, directly in front of the lacZ deletion mutant to design D2DR_L-ΔN 11-75, and verified by DNA sequencing. D2DR_L-ΔC 82-1023 was generated by the insertion of the *KpnI-XhoI* fragment from D2DR_L-ΔN 11-75 into the *KpnI-XhoI* site of ΔC 82-1023. IR-ΔN 11-75 and IR-ΔC 82-1023 were created by the insertion of the *HindIII-FspI* fragment from pcRCMV_{IR}(A) (10) in frame into the *XhoI* filled-in *HindIII* site of ΔN 11-75 and ΔC 82-1023.

DNA Construction of EGFP-ΔN 11-75, α_{2c}AR-EGFP-ΔN 11-75, D2DR_L-EGFP-ΔN 11-75, IR-EGFP-ΔN 11-75, Red2-ΔC 82-1023, α_{2c}AR-Red2-ΔC 82-1023, D2DR_L-Red2-ΔC 82-1023, and IR-Red2-ΔC 82-1023

EGFP-ΔN 11-75, α_{2c}AR-EGFP-ΔN 11-75, and D2DR_L-EGFP-ΔN 11-75 were constructed by the insertion of the *BglIII-Eco47III* fragment from pEGFP-C3 [enhanced GFP (EGFP); BD Biosciences Clontech, Palo Alto, CA, USA] into the blunt-ended *XhoI* site of ΔN 11-75, α_{2c}AR-ΔN 11-75, and D2DR_L-ΔN 11-75. IR-EGFP-ΔN 11-75 was created by the fusion of the *HindIII-FspI* fragment from pcRCMV-IR into the *NotI* filled-in *HindIII* site of EGFP-ΔN 11-75. Red2-ΔC 82-1023, α_{2c}AR-Red2-ΔC 82-1023, and D2DR_L-Red2-ΔC 82-1023 were constructed by the insertion of the *EcoRI-Eco47III* fragment from pDsRed2-C1 (BD Biosciences Clontech) into the blunt-ended *XhoI* site of ΔC 82-1023, α_{2c}AR-ΔC 82-1023, and D2DR_L-ΔC 82-1023. IR-Red2-ΔC 82-1023 was generated by a three-fragment ligation: the vector Red2-ΔC 82-1023, cut with *HindIII*, was ligated to both the *HindIII-FspI* fragment from pcRCMV-IR, encompassing the IR coding sequence, and to the *NotI* filled-in *HindIII* fragment encoding the Red2 sequence from Red2-ΔC 82-1023. All chimeric constructs were checked by DNA sequencing.

Cell Culture

Human embryonic kidney (HEK)-293 cells were grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS and 1% penicillin (10 000 U/mL)/streptomycin (10 000 mg/mL) at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded at a density of 2–3 × 10⁵ cells per 24-well plate (Greiner).

Transient transfection was performed the following day with 0.2 pmol cDNA (individually or in combination) by using TransFast™ Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. After 48 h, the cells were prepared for further experiments. All experiments were done in triplicate. The efficiency of the transfection for all constructs was found to be in similar range, as indicated by the co-transfection of a firefly luciferase plasmid.

Indigogenic X-Gal Histochemistry for β-gal

Cells were processed as described (12). Briefly, cells were fixed for 5 min in PBS plus 4% paraformaldehyde and rinsed in PBS before staining. Indigogenic X-gal staining was performed for 45 min to 4 h at 37°C in PBS, plus 1 mg/mL X-gal, 1 mM MgCl₂, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆.

Fluorescence Microscopy

Fluorescence microscopy was performed on a Leica DM RXA microscope using a Leica 40x/1.25 oil numerical aperture. HEK-293 cells expressing EGFP-ΔN 11-75, α_{2c}AR-EGFP-ΔN 11-75, D2DR_L-EGFP-ΔN 11-75, IR-EGFP-ΔN 11-75, Red2-ΔC 82-1023, α_{2c}AR-Red2-ΔC 82-1023, D2DR_L-Red2-ΔC 82-1023, and IR-Red2-ΔC 82-1023, individually or in combination, were grown on 24-well glass-bottomed culture plates. After 48 h, the cells were washed twice with PBS, and the subcellular distribution of chimeric- and non-chimeric EGFP and Red2 constructs were detected using single-line excitation at 488 and 558 nm. Co-localization studies of these constructs were performed using dual-line switching excitation (488 nm for

EGFP; 588 nm for Red2) and emission (500–550 nm GFP; 572–648 nm Cy3) filter sets, and images represented in false color using Adobe® Photoshop® 5.0 software.

FACS® Analysis

After one wash with PBS, HEK-293 cells were trypsinized, pelleted, resuspended in staining medium (4% FCS, 10 mM HEPES, pH 7.3, and PBS), and adjusted to 5–10 × 10⁶ cells/mL. The cell suspension (100 μL) was transferred to a Falcon 2058 tube. After the cells had been allowed to equilibrate in a water bath at 37°C for 10 min, an equal volume of pre-warmed 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR, USA) was rapidly mixed with the cell suspension. After 1 min of incubation at 37°C, the FDG loading was stopped by the addition of 2 mL ice-cold staining media containing 1 μg/mL propidium iodide (PI; Sigma), and the tube was placed on ice. The cells were left on ice for 2–3 h to allow for the enzymatic conversion of FDG to fluorescein. These samples were run on a FACScan® Cell Analyzer (BD Biosciences, San Jose, CA, USA).

Colorimetric β-gal Assay

HEK-293 cells were trypsinized and resuspended in medium (4% FCS, 10 mM HEPES, pH 7.3, PBS). Cells (2 × 10⁵) were incubated with 2 mM FDG for 1 min at 37°C. The FDG loading was stopped by the addition of 2 mL ice-cold medium, and the cells were placed on ice for 3 h. After pelleting the cells, the enzymatic conversion of FDG to fluorescein was determined photometrically at 485 nm.

RESULTS AND DISCUSSION

Design and Screening of a Novel Pair of lacZ Deletions

The transient co-transfection of the previously described weakly complementing Δα 11-41 and Δω 789-1023 deletion mutants (2,12,15) generated a high-background β-gal activity in HEK-293 and NIH-3T3 cells in our ex-

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periments (data not shown). Therefore, we constructed plasmids expressing several novel amino (N)- and carboxyl (C)-terminal-deleted versions (called ΔN and ΔC) of lacZ (Figure 1A). Our goal was to find pairs of inactive β-gal deletion mutants that were capable of complementing one another *in trans* and assembling to form an active enzymatic complex only in the presence of interacting peptides. Chimeric proteins were formed between the lacZ deletions and a pair of small interacting peptides, pepA and pepB, in the first instance. The two peptides were recently isolated from a library using a λ repres-

sor-reconstitution assay (22) and were chosen for this study because their small size could minimize the risk of influencing the protein structure of the tested lacZ deletions and avoid nonspecific β-gal activity. PepA (MNEAY-VHDGPVRS LN) was fused to the ΔN constructs (Figure 1B), and pepB (MKARKEAELAAATAEQ) was fused to the designed ΔC constructs.

All ΔN and ΔC constructs were transiently co-transfected pairwise into HEK-293 cells to screen for β-gal deletion mutants that displayed a low level of spontaneous complementation when co-expressed as non-chimeric proteins.

The degree of spontaneous complementation was quantified by counting the numbers of cells stained blue in the presence of X-gal. No blue-stained cells were detected for the deletion mutants ΔN 11-75 and ΔC 46-1023 when co-expressed with or without the chimeric peptides (Figure 1C, right panel, background) because the lack of 29 amino acids prevented the formation of an active enzyme complex. The previously described weakly complementing deletion mutants Δα 11-41 and Δω 789-1023 showed a high level of β-gal activity with more than 400 X-gal-positive cells in this assay, even with no

Figure 1. Schematic representation of various deletion mutants derived from wild-type *E. coli* LacZ and the effects of their transient transfection in HEK-293 cells. (A and B) Black bars show native lacZ sequences; black line, deleted region in our ΔN constructs. The indicated numbers represent the positions of the amino acids. The chimeric ΔN and ΔC constructs are shown as gray areas that describe the protein pepA. The striped area represents the protein pepB. (C) Scheme for the results of histochemical assays of the induction of β-gal activity upon chimeric complementation. Equimolar amounts of ΔN and ΔC expression vectors or pepA-ΔN and pepB-ΔC constructs were co-transfected pairwise, and after 48 h, the cells were stained with X-gal and visualized under a microscope. Average numbers of blue-stained cells in five view fields were quantitatively determined as followed: xxxx (more than 300 blue cells); xxx (between 200 and 300); xx (between 100 and 200); x (between 10 and 100); and - (less than 10).

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chimeric peptides. Only a novel pair of lacZ deletions, Δ N 11-75 and Δ C 82-1023, were found to fulfill our selection criteria: the lowest background when co-expressed as non-fusion proteins and high signal when fused with interacting peptides. For Δ N 11-75 and Δ C 82-1023, fewer than 10 blue cells were observed when the lacZ deletions were not fused to the peptides, whereas 30 times more blue cells were found when the lacZ deletions were attached to the pepA and pepB (Figure 1C, left panel). Note that all deletions could find at least one complementation partner. This proved that none of the deletions was deficient in accumulation (Figure 1C).

Induction of β -gal Activity on Co-Expressed IR when Fused to the Deletions Δ N 11-75 and Δ C 82-1023

We first chose IR to test the applicability of our novel lacZ deletions in the study of membrane protein interaction because it is known that IR, a tyrosine kinase, is internalized as a dimer at the cell surface (6,16,18). HEK-293 cells were co-transfected with equimolar amounts of IR- Δ N 11-75 and IR- Δ C 82-1023 simultaneously or individually, and then X-gal staining was used to visualize the reconstitution of the β -gal activity. The results showed a high proportion of blue-stained cells when both chimeric receptors were co-transfected, indicating that they interacted with each other (Figure 2A). In comparison, only a few cells were X-gal-positive when both constructs Δ N 11-75 and Δ C 82-1023 were co-expressed as non-fusion proteins (Figure 2B). In cells that expressed only one of the two chimeric constructs, no β -gal activity could be detected (Figure 2, C and D). These results were corroborated by the FACS analysis of the cell populations co-expressed with IR-fused Δ N 11-75 and Δ C 82-1023 construct pair and, as negative controls, the non-fused proteins Δ N 11-75 and Δ C 82-1023 pair (Figure 2E). IR-fused and non-fused $\Delta\alpha$ 11-41 and $\Delta\omega$ 789-1023 construct pairs were also included for a comparison (Figure 2F). The assay clearly showed a much higher β -gal activity for the IR-fused Δ N 11-75 and Δ C 82-1023 construct pair compared to the non-fused proteins Δ N 11-75 and Δ C 82-1023 pair, in

which the peak overlapped completely with the peak generated by untransfected cells (data not shown). The β -gal activity was not significantly different between the cells that were co-transfected IR-fused and non-fused $\Delta\alpha$ 11-41 and $\Delta\omega$ 789-1023 construct pairs.

To verify the localization of the fusion proteins, IR- Δ N 11-75 was further fused to the EGFP derived from *Aequorea victoria*, whereas IR- Δ C 82-1023 was further fused to the red fluo-

rescent protein (Red2) derived from *Discosoma* sp. The constructs were transfected into HEK-293 cells individually or simultaneously, and the localization of the fusion proteins was visualized by fluorescence microscopy. The results demonstrated that both IR-EGFP- Δ N 11-75 (Figure 2G) and IR-Red2- Δ C 82-1023 (Figure 2H) showed a non-ambiguous distribution to the cell membrane, although the receptors were also found intracellularly as clusters

Figure 2. Dimerization studies of the human IR when fused to the lacZ deletions Δ N 11-75 and Δ C 82-1023 in HEK-293 cells. Reconstitution of β -gal activity on chimeric complementation was monitored by X-gal staining. The enzymatic reactions with X-gal were stopped after 127 min and visualized by microscopy. The blue-stained cells are an indicator that the receptor forms a dimer within the cells (A). No β -gal activity was detected for the non-fused co-expressed lacZ deletions Δ N 11-75 and Δ C 82-1023 (B) or for the separately expressed chimeric receptor pairs (C and D). (E and F) FACS analysis of β -gal activity upon chimeric complementation. The blue cell frequency and the level of β -gal activity were expressed as a blue line for Δ N 11-75 and Δ C 82-1023, a red line for the chimeric receptor pair IR- Δ N 11-75 and IR- Δ C 82-1023 (E), and a black line for $\Delta\alpha$ 11-41 and $\Delta\omega$ 789-1023 (F). The vertical axis represents the relative cell number, and the horizontal axis represents the intensity of β -gal fluorescence on a logarithmic scale. The localization of the chimeric proteins was monitored by fluorescence microscopy. Representative cells show the membrane distribution of the co-expressed membrane receptor IR-EGFP- Δ N 11-75- (G) and IR-Red2- Δ C 82-1023 (H), and for the co-expressed lacZ deletions EGFP- Δ N 11-75 (I) and Red2- Δ C 82-1023 (J).

that were possibly associated with intracellular compartments or vesicles. This was in clear contrast to the cytoplasmic distribution observed for the lacZ deletions EGFP-ΔN 11-75 (Figure 2I) and Red2-ΔC 82-1023 (Figure 2J). It is worth mentioning that the insertion of EGFP and Red2 into the receptor constructs did not lead to a destruction of the enzymatic β-gal complex because the addition of X-gal still resulted in blue-stained cells (data not shown). This feature allows us to analyze simultaneously both the dimerization and localization in a single cell.

α_{2c}AR and D2DR_L Dimerization Monitored by Using β-Gal Complementation

We further characterized the assay system by using it to investigate the protein-protein interactions of GPCRs using α_{2c}AR and D2DR_L as target genes. HEK-293 cells were co-transfected with equimolar amounts of α_{2c}AR-ΔN 11-75 and α_{2c}AR-ΔC 82-1023 or with D2DR_L-ΔN 11-75 and D2DR_L-ΔC 82-1023 simultaneously or individually, with non-fusion ΔN 11-75 and ΔC 82-1023 as negative controls. Only a few cells were stained blue in the presence of X-gal when both non-fusion ΔN 11-75 and ΔC 82-1023 constructs were co-expressed (Figure 3B). Fusion to α_{2c}AR elevated the number of blue-stained cells significantly (Figure 3A). Similar but moderate increases were also observed for D2DR_L fusions. The enzymatic reactions for D2DR_L fusions were carried out for a longer time due to the weaker signal obtained from these constructs, but this did not change the major conclusions about either the homodimerization of the D2DR_L or the applicability of the ΔN 11-75 and ΔC 82-1023 mutants in such assays. No β-gal activity could be detected when only one of the two chimeric constructs was expressed (Figure 3, C and D). FACS analysis and colorimetric β-gal activity assay provided a more quantitative confirmation to this conclusion. Cell populations co-expressed with α_{2c}AR- or D2DR_L-fused ΔN 11-75 and ΔC 82-1023 construct pairs showed a β-gal activity that was elevated from 10 (D2DR_L) to 100 times (α_{2c}AR) (Figure 4A). The colorimetric

measurement of β-gal activity confirmed that the enzyme activity was elevated 10-fold (D2DR_L), 35 times (IR), and 125 times (α_{2c}AR), respectively, by the homodimerization of the fusion proteins compared to the non-fused ΔN 11-75 and ΔC 82-1023 pairs. No β-gal activity could be detected when only one of the two chimeric IR, α_{2c}AR, and D2DR_L constructs were expressed (Figure 4B).

We also co-transfected fusion constructs for different target proteins to study how different membrane proteins interact with each other. A weak but significant level of β-gal reconstitution was observed when α_{2c}AR-ΔC 82-1023 and IR-ΔN 11-75 were co-expressed, in comparison to that of the non-fusion ΔC 82-1023 and ΔN 11-75 control (Figure 3E). This demonstrated that the contact between α_{2c}AR and IR was strong enough to mediate the α-complementation between the lacZ

mutants. The same was found between D2DR_L and IR, although to a lesser extent (Figure 3E). At this stage, we were uncertain whether the heterodimeric interactions between the three receptors had functional consequences. Comparison of the number of blue-stained cells suggested that the receptors IR, α_{2c}AR, and D2DR_L would predominantly form homodimers rather than heterodimers with each other under the experimental conditions described here. It further demonstrated that the homodimeric interactions of IR, α_{2c}AR, and D2DR_L were specific and to a large extent at least and not consequences of transmembrane protein overexpression.

Several lines of evidence suggested that constitutive oligomerization is a general feature of GPCRs (4). Such basal oligomerization has been reported for the β₂-adrenergic (1,11), the dopamine D₂ (20), and the insulin (3)

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receptor. We treated the cells co-expressing the fusion receptors with their cognate ligands insulin, brimonidine, and dopamine, but we did not observe a difference in the induction of β -gal activity. The result suggested that the ligands did not change the constitutive dimerization state of IR, $\alpha_2\text{cAR}$, and D2DR_L under the described experi-

mental conditions (data not shown).

In summary, we present results that a pair of novel lacZ complementation deletions can be used for monitoring interactions between proteins to which they are fused. We demonstrated proof of the principle using membrane pro-

tein interaction as a model and provided convincing evidence that the assay could distinguish such interactions from background signals. The application of this method on a large scale could allow the construction of an interaction map between all membrane

Figure 3. The GPCRs $\alpha_2\text{cAR}$ and D2DR_L homodimerize in vivo shown by the β -gal complementation system using histochemical assays. The enzymatic reactions were stopped after 95 min for the $\alpha_2\text{cAR}$ experiments, and after 260 min for the D2DR_L experiments. The monitored β -gal activity is shown for $\alpha_2\text{cAR}$ and D2DR_L (A), for the co-expressed lacZ deletions $\Delta\text{N 11-75}$ and $\Delta\text{C 82-1023}$ (B), and for the separately expressed chimeric receptor pairs (C and D). To control for the specificity of the interactions, $\alpha_2\text{cAR}$ - $\Delta\text{C 82-1023}$ was co-expressed with the distantly related IR fused to $\Delta\text{N 11-75}$, and D2DR_L- $\Delta\text{N 11-75}$ was co-expressed with IR- $\Delta\text{C 82-1023}$ (E).

Figure 4. Dimerization of chimeric receptors $\alpha_2\text{cAR}$ and D2DR_L in HEK-293 cells was examined by FACS analysis and colorimetric measurement. (A) The graphs represent the level of β -gal activity expressing both $\Delta\text{N 11-75}$ and $\Delta\text{C 82-1023}$ (blue curve), the chimeric receptor pairs $\alpha_2\text{cAR}$ - $\Delta\text{N 11-75}$ and $\alpha_2\text{cAR}$ - $\Delta\text{C 82-1023}$, and D2DR_L- $\Delta\text{N 11-75}$ and D2DR_L- $\Delta\text{C 82-1023}$ (red curves). (B) The β -gal activity was measured for the co-expressed chimeric receptors IR, $\alpha_2\text{cAR}$, and D2DR_L fused to the $\Delta\text{N 11-75}$ and $\Delta\text{C 82-1023}$ pair. As controls, the non-transfected cells, non-fusion $\Delta\text{N 11-75}$ and $\Delta\text{C 82-1023}$ pair and individually expressed chimeric receptor constructs, were used. The D2DR_L comparison was depicted as an inset to highlight the different activity levels between the complementing pair and individual constructs.

proteins. The system has been validated with membrane proteins that dimerize constitutively so far, but it also has the potential to be extended for monitoring ligand-induced interactions. The discovery of the novel peptides Δ C 82-1023 and Δ N 11-75 that can undergo complementation extended our knowledge about the interesting phenomena. The novel peptides could serve as useful tools for exploring the molecular events in the complementation process.

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