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Identification of Novel Transplantable GPCR Recycling Motif for Drug Discovery

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Abstract

β_1 -adrenergic receptor (β_1 -AR) agonists and antagonists are widely used in the treatment of major cardiovascular diseases such as heart failure and hypertension. The β_1 -AR like other G protein-couple receptors (GPCR) is endocytosed in response to intense agonist activation. Recycling of the agonist-internalized β_1 -AR is dependent on its carboxy-terminal type-1 PSD-95/DLG/ZO1 (PDZ) and on phospho-serine³¹² in the third intracellular loop of the β_1 -AR. Progressive elongation of the β_1 -AR at its C-tail inactivated the PDZ-binding domain and inhibited the recycling of the β_1 -AR. However, fusing a twenty amino acid peptide derived from the multiple cloning region of the mammalian expression vector pCDNA3 to the C-tail of the β_1 -AR (β_1 -AR[+20]) produced a chimeric β_1 -AR that recycled rapidly and efficiently. The β_1 -AR[+20] recycled in a type-1 PDZ and phospho-Ser³¹²-independent manner, indicating that this peptide provided a general GPCR recycling signal. Fusing the enhanced yellow fluorescent protein (EYFP) down-stream of β_1 -AR[+20] generated a β_1 -AR-EYFP chimera that was expressed on the membrane and recycled efficiently after agonist-induced internalization. This construct trafficked in a PDZ-SNX27/retromer-independent manner. We also fused EYFP to the N-terminus of the β_1 -AR to created EYFP-WT β_1 -AR. This construct recycled in PDZ and SNX27/retromer dependent manner. These β_1 -AR-EYFP constructs would be useful for high throughput screening (HTS) programs to identify new entities that would interfere with the recycling of agonist internalized GPCR that traffic in PDZ-dependent vs. PDZ-independent roadmaps.

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Author contributions

SWB and SM conceived and designed the experiments. SWB and MMN created the various constructs by molecular and cellular techniques. MMN performed the experiments. SWB and SM performed the pixel measurement experiments and calculated GPCR trafficking kinetics. SM and SWB wrote the paper.

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Keywords

GPCR; β -adrenergic receptors; trafficking; PDZ; SNX27; confocal microscopy

1. Introduction

Activation of G protein-coupled receptors (GPCR) by agonists is transduced by their cognate G proteins into activation of appropriate effector enzymes involved in generating downstream second messengers [1]. Persistent activation of GPCRs also promotes the phosphorylation of the agonist-occupied GPCR by GPCR kinases (GRKs), which enhance the binding of β -arrestins to the phosphorylated GPCR. β -arrestins sterically hinder further GPCR-G protein coupling and activate a program for GPCR internalization [2–3]. The fate of the internalized GPCR varies depending upon the structure of GPCR and cell under study. For example, robust signaling outputs, as indexed by cyclic AMP biosensors, was generated by internal β_2 -adrenergic receptors (β_2 -AR) in HEK-293 cell and by the receptors for TSH and PTH in thyroid and bone cells, respectively [4–6]. These studies demonstrated that persistent cyclic AMP signaling by TSH and PTH receptors required internalization and that persistent signaling outputs were localized in early endosomes [4]. However, signaling outputs by the plasma membrane delineated GPCR appear to differ from those generated by internal GPCR. A case in point is β_2 -AR and M_1 -muscarinic acetylcholine (M_1 -AChR) receptor-mediated activation of mitogen activated protein kinase (MAPK), where by the internalization of these GPCR into endosomes was required for subsequent MAPK activation [7].

Trafficking proteins appear to be involved in regulating persistent signaling outputs of endosomal GPCR with physiologically relevant manifestations. For example, increasing the magnitude of PTH receptor internalization by overexpressing β -arrestin enhanced endosomal cyclic AMP generation in bone cells, while overexpressing of retromer proteins, such as VPS35 that accelerate PTH receptor trafficking out of early endosomes, terminated the generation of cyclic AMP by internalized PTH receptors [8]. Sustained endosomal PTH receptor signaling enhanced cortical bone turnover, indicating that modulating trafficking outcomes of the GPCR may be physiologically relevant.

Trafficking of the internalized GPCR from endosomes back to the cell membrane “*recycling*” is dependent on recycling motifs imprinted onto the GPCR backbone. A major GPCR recycling motif is a type-1 PDZ sequence corresponding to $(X_{-3}\text{-S/T-X}_{-1}\text{-}\emptyset)$, where X at positions $(P =) -1$ and -3 is any amino acid, $\emptyset$ at $P = 0$ is a hydrophobic residue while $P = -2$ is either a serine or a threonine), commonly found in the C-tail of recycling GPCRs [9–10]. In addition, non-PDZ recycling motifs were identified in μ -opioid and dopamine1-receptors [11–12]. Given the importance of endosomal trafficking in regulating signaling outputs of the agonist-internalized GPCR in general and pharmacological resensitization of GPCR in particular, the need to better understand how this pathway is controlled by GPCR trafficking regulators is warranted. These agents would preserve normal or membrane-delimited GPCR signaling, but modulate aberrant or sustained signaling by endosomal or other internalized GPCR.

To initiate this line of investigation, we used the β_1 -AR as a model to characterize the role of type-1 PDZ and other domains in regulating GPCR trafficking. Using new constructs that traffic from endosomes back to the cell membrane either in a PDZ/retromer-dependent or PDZ/retromer-independent pathway we determined that PDZ-dependent trafficking was also retromer dependent. Furthermore, we discovered that GPCR recycling would proceed via non-PDZ sequences in a retromer-independent manner. These β_1 -AR constructs were later fused to enhanced yellow fluorescent protein (EYFP) to create β_1 -AR probes suitable for high throughput screening. These constructs may be used to design agents that interfere with retromer-mediated GPCR trafficking versus agents that interfere with retromer independent GPCR trafficking. We believe that GPCR trafficking modulators would provide a new class of agents to regulate aberrant GPCR signaling in conditions associated with GPCR hyperactivation.

2. Materials and methods

2.1 cDNA constructs

cDNA extensions to the carboxy-tail of β_1 -AR were generated by PCR using a forward primer (5' - CATGAAGCTTATGGACTACAAGGACGATGACGACAAGGGCGCGGGGGTGCTCGT CCTGGGCGCC-3') that introduced a Hind-III restriction site, followed by the FLAG sequence (DYKDDDDK) upstream of the N-terminal region of the β_1 -AR. The reverse primers contained the desired extensions to the carboxy-terminus at Val⁴⁷⁷ in the β_1 -AR, followed a stop codon (*) and a SalI restriction enzyme sequence. Primer oligo's were synthesized by Integrated DNA technologies (Coralville, Iowa). An example is the [+ 4] reverse sequence, 5' - CTGAGTCGACCTAGCAATCCAATGCCACCTTGGATTCCGAGGCGAAGCCGGGGCGG -3', which would generate a cDNA encompassing WT Flag-tagged β_1 -AR followed by ALDC* amino acids and flanked by HindIII/Sal I sites. The sequence for the reverse β_1 -AR cDNA oligomer used in generating the +20 β_1 -AR construct without a stop codon that was flanked by an EcoR1 site was 5' - TGCAGAATTCACCTTGGATTCCGAGGCGAAGCCGGGGCGGCACGGGTCCAGGCT-3'. Each forward and reverse primer pair was used to amplify the desired β_1 -AR cDNA using the Advantage-GC PCR kit (Takara-Clontech, Mountain View, CA). Each PCR product was cloned in the pCR4-TOPO-TA vector (Invitrogen, Grand Island, NY) and then completely sequenced using M13 forward and reverse primers as well as internal β_1 -AR sequencing primers. Each cDNA in the +4 to +16 series of β_1 -AR constructs was excised by HindIII and SalI and cloned into pCMV Script mammalian expression vector (Agilent technologies, Santa Clara, CA) and resequenced by automated sequencing. To generate the β_1 -AR[+20] constructs, a PCR fragment encoding the FLAG-tagged WT β_1 -AR without a stop codon was cloned into the Hind III-EcoR1 sites of pCDNA3.0 (Invitrogen, Grand Island, NY). This maneuver created a β_1 -AR[+20] construct which terminated using the stop codons of the mammalian expression pcDNA3 vector.

To generate the β_1 -AR[+20]-EYFP, EYFP cDNA was amplified from pEYFP-C1 plasmid (Takara-Clontech, Mountain View, CA) by PCR and cloned into pCR4-TOPO. Then the

EcoRI-flanked insert encoding EYFP was cloned downstream of the β_1 -AR [+20] coding region. To generate the C-terminal EYFP- β_1 -AR construct, the HindIII-EcoRI flanked cDNA encoding the WT β_1 -AR* was cloned in-frame into the pEYFP-C1 vector.

2.2 Antibodies, siRNA, reagents and cell lines

Anti-FLAG (M2) Cy3-labeled antibody was from Sigma (St. Louis MO). Anti-SAP97 antibody from Stressgen (Enzo Life Sciences, Farmingdale, NY), anti-SNX27 (ab77799) from Abcam (Cambridge MA). Anti β -actin antibody (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA). Myristoylated PKA inhibitory peptide, 14–22 amide myristoylated Myr-N-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH₂ (PKI) was purchased from Calbiochem (EMD-Millipore, Billerica MA). Isoproterenol, alprenolol, dithiothreitol, poly-L-lysine (P0899), leupeptin, aprotinin, chymostatin and phenyl methyl sulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). Flexi siRNA oligo sequence to human SNX27 5'-AACCAGGTAATTGCATTTGAA-3' and its inactive control 5'-CCATAATACAAG GTATAA-3' were purchased from Qiagen (Valencia CA). Human embryonic kidney-293 (HEK-293) cells (CRL-1573) were from ATCC (Manassas, VA). HEK-293 cells passages 40–46 were grown in DMEM supplemented with 10% fetal bovine serum.

2.3 Acid strip confocal recycling microscopy and dual microscopy protocols

For recycling assays, cells expressing FLAG-tagged wild-type (WT) β_1 -AR or the other constructs were plated on poly-L-lysine-coated glass coverslips and serum-starved at 37°C for 1 h in DMEM supplemented with 12.5 mM HEPES, pH 7.4. The receptors were labeled with Cy3-conjugated, anti-FLAG M2 IgG (4 μ g/ml) for 1 h at 37°C. In some experiments, diluents or 3 μ M mPKI were added during the antibody incubation step. This concentration of mPKI ensures selective inhibition of PKA without adversely affecting other kinases or phosphatases [13]. Cells were treated with 10 μ M isoproterenol for 30 min at 37°C to promote agonist-mediated receptor internalization. Then, the cells were chilled to stop endocytosis and exposed to 0.5 M NaCl, 0.2 M acetic acid (pH 3.5) for 4 min on ice to remove antibody bound to extracellular β_1 -AR [14–16]. Cells were then incubated in culture medium supplemented with 100 μ M of the β -antagonist alprenolol at 37°C for 15, 30 or 60 min to establish the recycling time. After each time period, coverslips were rinsed and fixed in PBS containing 4% paraformaldehyde and 4% sucrose (pH 7.4) for 10 min at room temperature. At the completion of recycling (i.e. the 60 min slide), the slide was exposed to a second acid wash to strip the Cy3-labeled antibody from the externalized receptor population and then fixed. Confocal fluorescence microscopy was performed on coded slides and images were acquired using Olympus FV1000 confocal microscope [17]. To calculate the recycling kinetics, a boundary was drawn around the inner circumference of cells using *image J* software (NIH.gov). Pixel intensities in the intracellular boundary (internalized receptors) of isoproterenol-treated cells were set arbitrarily as 100% and pixel intensities in the intracellular boundaries of alprenolol-treated cells were calculated as percent of this initial value. In confocal recycling assays, the time constants (τ) for receptor recycling were determined by fitting the percent internal receptor pixel data to a single exponential decay function of time from $y = y_0 + Ae^{-t/\tau}$, where y_0 and A are constants. Recycling kinetics are presented as means $\pm$ S.E., except where indicated. For comparison

between two groups of data, Student's unpaired *t*-test was used to determine significance, while multiple groups were compared by one-way (ANOVA) with Newman-Keuls post-tests using Prism software (GraphPad, San Diego). Results were considered significant at *p* <0.05.

2.4 Co-Immunoprecipitations

Co-immunoprecipitations between SAP97 and the various FLAG- β_1 -AR constructs were performed as follows. Cells expressing the appropriate construct were lysed in lysis buffer composed of: 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.2% triton X-100, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml chymostatin and 1 mM phenyl methyl sulfonyl fluoride for 1 hour at 4°C. Then insoluble cellular debris was removed by centrifugation at 14,000 g_{av} for 15 min at 4°C. After equalizing protein concentrations across all samples, lysates were added to ~ 5 μ l M2 anti-FLAG-IgG agarose beads. Control experiments were performed by incubating lysates with pre-immune IgG conjugated to agarose beads at the same concentration for 4 h at 4°C. The immune complexes were washed five times in lysis buffer and eluted from the beads with 2x-Laemmli sample buffer containing 20 mM dithiothreitol. Affinity isolated proteins and lysate inputs were separated by SDS-polyacrylamide gel electrophoresis under denaturing conditions and electroblotted to nitrocellulose (Protran BA85, 0.45 μ m membrane (GE healthcare, Pittsburgh, PA). Identical gels were run and transferred for separate detection of FLAG, β_1 -AR or SAP97 by Western blotting.

3. Results

3.1 Determining if an internal type-1 PDZ would support the recycling of the human β_1 -AR

The addition of a single alanine residue after the type-1 PDZ at position +1 in the human β_1 -AR inactivated the PDZ-binding domain and inhibited the recycling of GPCR following their internalization by their respective agonists [18–19]. These data provide strong evidence for a primary role for the carboxy-terminal PDZ-interacting sequence in regulating the trafficking of internalized GPCR. However, there are reports that β_2 -AR-GFP chimeras would internalize in response to agonist and endosomal receptors would recycle, even though the GFP is inserted downstream of the PDZ-binding domain in the C-terminus of β_1 -AR and β_2 -AR [20–21]. Also, there are reports that internal PDZ-binding domains were active in supporting the recycling of some GPCR [22]. Moreover, substituting the β_1 -AR PDZ-binding sequence with non-PDZ sequences derived from recycling GPCRs such as the μ -opioid receptor or dopamine₁-receptor supported the recycling of these β_1 -AR chimeras in a PDZ-independent manner [18]. To find out if progressive elongation of the β_1 -AR would somehow facilitate its recycling, we fused stretches of 4, 6, 12 and 16 amino acids downstream of the β_1 -AR and determined their trafficking profiles by confocal microscopy (Fig. 1A). Transient expressions of the wild-type (WT) β_1 -AR with an intact type-1 PDZ or the WT β_1 -AR fusions with +4 to +16 amino acids into HEK-293 cells were used to determine the effect of β -agonists on internalization and recycling of the β_1 -AR (Fig 1A). Extending the C-tail of the β_1 -AR by 4, 8, 12 and 16 amino acids did not interfere with the membranous distribution of the β_1 -AR (Fig 1B, *images a, f, k, p, and u*). Exposing these cells to isoproterenol for 30 min resulted in rapid endocytosis of membranous β_1 -AR into

intracellular endosomal vesicles (Fig. 1B, *images b, g, l, q and v*). The effect of progressive elongation of the C-tail of the β_1 -AR on recycling of internal β_1 -AR was initiated by removing isoproterenol followed by the addition of the β -adrenergic receptor antagonist alprenolol (10 μ M) to block further internalization, so that the recycling of internal β_1 -AR could be unambiguously determined. In cells expressing the WT β_1 -AR, the agonist-internalized β_1 -AR recycled back to the cell membrane within 60 min from the removal of isoproterenol (Fig. 1B *images c–e*). If externalized WT β_1 -AR were inserted properly into the cell membrane, then Cy3-conjugated anti-FLAG IgG bound to the N-terminal FLAG epitope of the β_1 -AR would be oriented extracellularly. In this case, a second acid wash would strip Cy3-anti-FLAG IgG from the externalized β_1 -AR population [14–16]. Exposing cells in which the WT β_1 -AR was internalized and then allowed to recycle to an acid wash, stripped >90% of the Cy3 pixels, indicating that recycled WT β_1 -AR were externalized (Fig. 1A *image e'*). Progressively elongated β_1 -AR chimera with [+4–+16] amino acids resulted in a non-recycling phenotype of this receptor and the acid strip procedure indicated that >85% of Cy3 pixels were retained intracellularly (Fig. 1B, *images f'–y'*). These results indicate that progressive elongation of the β_1 -AR by the computer-generated sequences shown in Fig. 1A inactivated the type-1 PDZ and created a non-recycling phenotype.

Previous studies from our group have shown that recycling of the β_1 -AR is dependent on SAP97 binding to the type-1 PDZ of the β_1 -AR [17–18]. SAP97 is a PDZ-ligand binding protein that binds simultaneously to the type-1 PDZ in the β_1 -AR and to a complex composed of A-kinase anchoring protein 79/150 (AKAP5) and protein kinase A [17]. Assembly of the SAP97-AKAP5/PKA complex at the β_1 -AR PDZ-binding domain has been shown to be a priori for the recycling of the agonist-internalized β_1 -AR [17], [23–24]. Therefore, to directly determine if progressive elongation of the C-tail of the β_1 -AR interfered with the activity of the type-1 PDZ, we conducted co-immunoprecipitation (co-IP) experiments between the various β_1 -AR[plus] constructs of Fig. 1A and endogenous SAP97 (Fig. 1C). Extracts from HEK-293 cells transiently expressing one of the FLAG tagged β_1 -AR constructs described in Fig. 1A and SAP97 were prepared. About 4% of the crude lysates were subjected to Western blotting and probed with anti-SAP97 antibody to insure that equivalent amounts of endogenous SAP97 was contained in cell lysates (Fig. 1C, *top left hand panel*). Equal amounts of input cell lysates were incubated with anti-FLAG IgG resin, followed by washing and eluting the resin. Resin eluates were divided into two portions. One portion representing about 4% of the eluate by volume was subjected to Western blotting and probed with anti-FLAG antibody to verify that equal amounts of each chimeric β_1 -AR construct was bound and eluted from the affinity resin (Fig. 1C, *bottom right hand panel*). The rest of the FLAG IP's representing about 90% of resin eluates, were probed with anti-SAP97 antibody to index SAP97 co-IP'd with each β_1 -AR construct (Fig. 1C, *right hand panel*). The results indicate that only the WT β_1 -AR co-IP'd SAP97 (Fig. 1C *right panel, lane 1*). Therefore, failure of the β_1 -AR chimeras that were described in Fig. 1 to recycle was due to inactivation of their PDZ-binding domains.

3.2 Discovery of a 20 amino acid recycling peptide in the multiple cloning domain of pCDA3.0

During the course of our GPCR trafficking studies, we discovered that cloning the WT β_1 -AR cDNA without a stop codon into the multiple cloning region of pCDA3.0 generated a β_1 -AR construct that internalized normally in response to the agonist isoproterenol and then recycled. This was intriguing because based upon the result of Fig. 1, it would appear that inserting either short or long amino acid sequences downstream of the type-1 PDZ would inhibit the recycling of the GPCR. To investigate this matter further, we cloned the cDNA of the β_1 -AR w/o stop codon into other mammalian expression vectors such as pCMV script and pCI and determined that these WT β_1 -AR constructs did not recycle (data not shown). Therefore, we conducted more extensive confocal recycling experiments on the WT β_1 -AR w/o stop codon construct in pCDA 3.0 (Fig. 2). Structurally, this construct contained a 20 amino acid peptide linker between C-terminal valine (Val⁴⁷⁷) of the β_1 -AR and the stop codon of pCDA3.0 and was named WT β_1 -AR[+20]. In HEK-293 cells expressing this construct, we determined that WT β_1 -AR[+20] was expressed at the membrane and was internalized efficiently in response to isoproterenol (Fig. 2B, *images f, g*). Internal WT β_1 -AR[+20] recycled efficiently in cells exposed to the β -adrenergic antagonist alprenolol (Fig. 2B, *images h-j*). These receptors were inserted properly into the membrane because an acid wash stripped the Cy-3 label from externalized WT β_1 -AR[+20] (Fig. 2B, *image j'*). The time for recycling of 50% ($t_{0.5}$) of internal WT β_1 -AR[+20] from the endosome back to the cell membrane was $\sim 24 \pm 7$ min, which was not significantly different from the $t_{0.5}$ of 21 ± 6 min for recycling of the WT β_1 -AR (Fig. 2C).

3.3 The 20-mer peptide facilitates GPCR recycling independently of its type-1 PDZ

These findings could be interpreted two ways. One interpretation would be that the presumably “*blocked*” type-1 PDZ actively supported the recycling of WT β_1 -AR[+20]. Another would be that the recycling of WT β_1 -AR[+20] was independent of the β_1 -AR PDZ-binding sequence, but dependent on the 20 amino acid linker. To determine which of these two scenarios was involved, we inactivated the β_1 -AR PDZ-binding domain by mutating its “ESKV” sequence into alanine to create the β_1 -AR PDZ[+20] construct described in Fig. 2A. In confocal recycling assays β_1 -AR PDZ[+20] construct was expressed on the surface of HEK-293 cells and was internalized in response to isoproterenol (Fig. 2B, *image k and l*). After substituting isoproterenol with alprenolol, internal β_1 -AR PDZ[+20] recycled rather efficiently ($t_{0.5} = 26 \pm 8$ min) and was inserted properly in the membrane as indexed by an acid wash that stripped externalized β_1 -AR PDZ[+20] (Fig. 2B, *images m-o'*). Therefore, it appears that we have uncovered a novel GPCR recycling sequence encoded by the multiple cloning domain of pCDA3.0.

We exploited this salient discovery and generated two fluorescently-tagged β_1 -AR constructs, one in which enhanced-YFP was inserted downstream of the 20 amino acid linker to create (WT β_1 -AR [+20]-EYFP) and the other originally described in Gardner et al. [17], in which EYFP was inserted up-stream of the WT β_1 -AR with a stop codon, to create (EYFP/WT β_1 -AR) as illustrated in Fig. 2A. Confocal microscopy revealed that these constructs were expressed on the cell membrane (Fig. 2B, *images p, u*) and were efficiently internalized in response to isoproterenol (Fig. 2B, *images q, v*). After substituting

isoproterenol with alprenolol, the two YFP labeled β_1 -AR-chimeras recycled rather efficiently with normal kinetics ($t_{0.5} \sim 24 \pm 7$ min) back to the plasma membrane (Fig. 2A, *images r-t, and w-y*). Please note that EYFP is covalently attached to the β_1 -AR and cannot be displaced by the mild acid stripping procedure.

3.4 The 20-mer peptide facilitates β_1 -AR recycling in a PKA independent manner

As described before, two separate domains regulated the recycling of endosomal β_1 -AR, one is the type 1-PDZ in the C-tail, and the other is a PKA substrate serine at position 312 in the 3rd intracellular loop of the β_1 -AR [17]. Cross-talk between these two separate domains is mediated by the pool of PKA bound to the β_1 -AR PDZ-interacting domain via SAP97-AKAP79/PKA complex [17]. In this scenario, the PDZ-bound pool of PKA acts as a recycling “switch” by phosphorylating Ser³¹² in the 3rd IC of the β_1 -AR upon the activation of the β_1 -AR [17, 25]. To find out if PKA was involved in regulating the recycling of WT β_1 -AR[+20] or its PDZ counterpart, we repeated the recycling assays in the presence of the cell-permeant PKA inhibitor myristoylated PKA inhibitory peptide (mPKI). On the one hand, mPKI inhibited the recycling of the WT β_1 -AR (Fig. 3A, *compare images c-e with h-j*), because mPKI inhibited PKA-mediated phosphorylation of Ser³¹², which is required for trafficking of the β_1 -AR [17], [24], and [26]. On the other hand, mPKI did not inhibit the recycling of the agonist-internalized WT β_1 -AR[+20] construct (Fig. 3A *images m-o*) or β_1 -AR PDZ[+20] construct (Fig. 3A *images r-t*), indicating that the recycling of these constructs was not PKA dependent.

3.4 The 20-mer peptide facilitates β_1 -AR recycling in a phospho-Ser³¹² independent manner

After determining that the 20-mer peptide supported the recycling of WT β_1 -AR in a type-1 PDZ- and PKA-independent fashion, we sought to determine if the status of Ser³¹² in the 3rd IC of the β_1 -AR influenced the function of the peptide. In Gardner *et al.* [17], we determined that phospho-Ser³¹² was the ultimate recycling signal for the β_1 -AR because inactivation of Ser³¹² by mutagenizing it to alanine ((S312A) β_1 -AR) inhibited the recycling of the β_1 -AR, while mutagenesis of Ser³¹² to the phospho-serine mimic aspartic acid ((S312D) β_1 -AR) supported the recycling of the β_1 -AR even when the PDZ-interacting sequence of the β_1 -AR and/or PKA were inactivated [17]. Therefore, if Ser³¹² was “inadvertently” phosphorylated as a consequence of the 20-mer peptide, it would theoretically promote the recycling of the WT β_1 -AR[+20] or its PDZ counterpart. To address this point, we compared the effect of this peptide on trafficking of β_1 -AR constructs in which Ser³¹² was inactivated by mutating it to alanine to that of β_1 -AR constructs in which Ser³¹² was activated through its mutagenesis to the phospho-serine mimic aspartic acid (S312D). The effect of the 20-mer peptide on recycling of β_1 -AR constructs with an inactivated Ser³¹² was examined first by comparing the recycling kinetics of (S312A) β_1 -AR vs. (S312A) β_1 -AR[+20] (Fig. 4, *images a-f*). (S312A) β_1 -AR and (S312A) β_1 -AR[+20] were expressed on the cell membrane and were internalized in response to isoproterenol (Fig. 4A, *images a-b and f-g*). As reported earlier, the (S312A) β_1 -AR construct did not recycle and its pixels were retained intracellularly, as evidenced by the acid wash procedure (Fig. 4A, *images c-e*). Fusing the 20-mer peptide to (S312A) β_1 -AR rescued its recycling and the majority of its internal pixels were externalized (Fig. 4A, *images h-j*). However, recycling kinetics of ((S312A) β_1 -

AR [+20]) were slower than the WT β_1 -AR (35 ± 8 min vs. 21 ± 6 min $P < 0.05$). Unlike, (S312A) β_1 -AR, the (S312D) β_1 -AR mutant recycled efficiently and rapidly (Fig. 4A, *images m-o'*). Fusing the 20-mer peptide to the (S312D)WT β_1 -AR construct did not alter its recycling phenotype (Fig. 4A, *images u-y'*) or its rate of recycling ($t_{0.5}$ 20 ± 7 min for (S312D) β_1 -AR vs. 21 ± 7 min for (S312D) β_1 -AR[+20]). The recycling of (S312D) β_1 -AR or (S312D) β_1 -AR [+20] was not inhibited by mPKI (Fig. 4A and B). However, global inhibition of PKA by mPKI significantly slowed the recycling kinetics of these constructs relative to their counterparts in untreated cells. We speculate that mPKI-mediated inhibition of PKA perturbed numerous PKA-regulated signaling and trafficking events. We confirmed these findings in the EYFP-tagged constructs derived from these two types of β_1 -AR. Where by, WT β_1 -AR [+20]-EYFP was internalized in response to isoproterenol even in the presence of mPKI (Fig. 3A, *images u* and *v*) and internal WT β_1 -AR [+20]-EYFP recycled efficiently with a $t_{0.5}$ of 30 ± 9 min (Fig. 3A, *images w-y* and Fig. 3B). As expected, the recycling of EYFP-WT β_1 -AR, which recycles in a PDZ-dependent manner, was inhibited by mPKI (Fig. 3A, *images b1-d1* and Fig. 3B). These data reveal that we have created two EYFP-tagged β_1 -AR constructs with different trafficking roadmaps.

3.5 The 20-mer peptide imprints a general trafficking signal onto the GPCR

A major finding concerning the mechanism of PDZ-dependent GPCR trafficking from endosomes back to the membrane is its dependence on sorting nexin 27 (SNX27) [27]. SNX27 contains a PX domain that binds to phosphatidylinositol 3-phosphate enriched on the cytoplasmic surface of early endosomes and a PDZ-binding domain that is apparently essential for PDZ-directed recycling of several GPCR [28–29]. Therefore, we investigated if siRNA-mediated down-regulation of cellular SNX27 expression was involved in differential regulation of endosome to membrane trafficking of these β_1 -AR chimeras in HEK-293 cells (Fig. 5). Transient transfection of a highly-specific siRNA to human SNX27 resulted in >90% down regulation of the SNX27 protein in HEK-293 cells and this down-regulation was specific to SNX27 because the levels of SNX27 in cells exposed to the scrambled siRNA sequence were no different than those in control cells (Fig. 5A). As reported earlier, scrambled siRNA oligo did not alter the membranous distribution of WT β_1 -AR or their internalization in response to isoproterenol and subsequent recycling upon the removal of isoproterenol (Fig. 5B, *images a-e*). Down-regulation of SNX27 did not alter the membranous distribution or internalization of WT β_1 -AR in response to isoproterenol (Fig. 5B, *images f, g*). However, endosome to membrane recycling of WT β_1 -AR was completely inhibited by SNX27 siRNA as determined by the acid wash procedure (Fig. 5B, compare *image e'* to *j'*). Similarly, SNX27 siRNA inhibited endosome to membrane trafficking of the EYFP- β_1 -AR chimera (Fig. 5B, *images w-y*). Therefore, SNX27 inhibited the recycling of β_1 -AR chimera that trafficked in a type-1 PDZ- and PKA-dependent manner. In the next series of experiments we determined the effect of cellular SNX27 on endosome to membrane trafficking of WT β_1 -AR [+20] in pcDNA 3.0 or its fluorescent counter part WT β_1 -AR [+20]-EYFP (Fig. 5B, *images k-o'* and *p-t*). siRNA-mediated down regulation of SNX27 did not alter the membranous distribution of these receptor or their internalization to endosomes in response to isoproterenol. These receptors trafficked from endosome back to the cell membrane even in the presence of SNX27 siRNA, indicating that their trafficking itineraries were independent of the type-1 PDZ or other retromer-associated proteins such as

SNX27. The $t_{0.5}$ for trafficking of WT β_1 -AR [+20]-EYFP in the presence in SNX27 siRNA or its scrambled control were 30 ± 8 vs. 25 ± 7 min, respectively ($P > 0.05$).

4. Discussion

GPCR signaling continues unabated even after the GPCR is internalized to endosomes in response to intense GPCR activation by their cognate agonists. GPCR internalization is linked to their activation and proceeds via a GRK- β -arrestin pathway that promotes their endocytosis via clathrin-coated pits to early endosomes (EE). The fate of endosomal GPCR depends on information encoded within the GPCR protein backbone [30]. GPCR with type-1 PDZ at their extreme C-tail, traffic efficiently from EE to recycling endosomes, which subsequently fuse with the cell membrane [10], [31]. This mode of transport is referred to as “*sequence-dependent*” trafficking and requires a series of PDZ-binding proteins, such as SNX27 and others to facilitate endosome to membrane transport of the GPCR [27–28] and [32–33]. Inactivation of the GPCR PDZ-binding domain either by mutating the type-1 PDZ sequence (PDZ) or by inserting one or more alanines downstream of the PDZ-binding region inhibited the trafficking of the GPCR and promoted its degradation in lysosomes [18], [31]. There are however, another class of recycling GPCR such as μ -opioid receptors and dopamine₁-receptors that traffic from endosome to the membrane in a PDZ-independent manner after their internalization by opiates or dopamine, respectively [11–12]. These receptors traffic by means of “*recycling sequences*” because transplanting these sequences onto trafficking-defective GPCR restored their endosome to membrane recycling [18], [32]. There is yet another group of receptors, such as transferrin receptors (Tfr) and LDL receptors that undergo constitutive internalization and recycling without an identifiable “*recycling sequence*” within their protein backbone. This class of receptors recycles by “*bulk flow*” using ultrathin tubules that constitutively bud out of EE and then fuse directly or indirectly with the cell membrane [32], [34].

During the course of our GPCR trafficking studies, we discovered that deletion of the stop codon from the WT β_1 -AR would generate a β_1 -AR chimera in the pcDNA3.0 mammalian expression vector that would internalize in response to the agonist isoproterenol and then recycle. This construct contained a 20 amino acid linker peptide in between Val⁴⁷⁷ in the β_1 -AR and the stop codon of pcDNA3.0, which was dissimilar to the “*recycling sequences*” of the μ -opioid receptor and dopamine₁-receptor, indicating broad sequence diversity of these trafficking domains [11–12] and [18]. Therefore, we investigated if this sequence was a transplantable GPCR recycling motif and sought to determine its mechanism of action. Given the diversity of known “*recycling sequences*”, we initially determined if a set of synthetic progressively elongated peptides would endow a recycling signal onto the β_1 -AR (Fig. 1). The set shown in Fig. 1 and two other 16-mer peptides (data not shown) could not provide a “*recycling signal*” for the β_1 -AR, suggesting that such sequences were inherently rare and could not be guessed by computer aided design algorithms.

Fusing the 20-mer peptide in frame with β_1 -AR constructs containing active or inactive type-1 PDZs resulted in β_1 -AR chimera that trafficked with normal internalization and recycling kinetics (Fig. 2B). Moreover, the WT β_1 -AR[+20] construct did not bind to the PDZ-binding domain of SAP97. These results provided strong evidence that the 20-mer

peptide provided a “*recycling signal*” that functioned independently of the PDZ-interacting domain. However, trafficking of the β_1 -AR is mediated by means other than its type-1 PDZ. As outlined earlier, trafficking of the β_1 -AR is mediated by cross talk between the PDZ-binding domain in the C-tail and the PKA-substrate Ser³¹² in the 3rd IC. Cross talk is mediated by the pool of PKA anchored at the β_1 -AR PDZ-binding domain, which phosphorylates Ser³¹² to imprint a recycling signal onto the β_1 -AR backbone. Proof for this concept was obtained from several approaches. Where by, mutagenesis of Ser³¹² to alanine, which prevented its phosphorylation by PKA or inhibition of PKA with H-89 or mPKI also prevented the recycling of the β_1 -AR even in the presence of the active PDZ-interacting domain. Meanwhile, mutagenesis of Ser³¹² to the phospho-serine mimic aspartic acid (S312D) promoted the recycling of the β_1 -AR even when its type-1 PDZ was inactivated or when PKA was inhibited [17]. Thus, the next logical step was to determine if the mechanism of the 20-mer peptide involved one of these players.

The involvement of PKA was examined first. mPKI inhibited the recycling of the WT β_1 -AR, but had no effect on endosome to membrane translocation of WT β_1 -AR[+20], indicating that PKA activity was not involved in the trafficking mechanism of the 20-mer peptide (Fig. 3). The involvement of Ser³¹² was examined using β_1 -AR mutants with inactivated (S312A) or activated Ser³¹² (S312D). Inactivation of Ser³¹² in the context of the WT β_1 -AR inhibited its recycling, but did not alter the recycling kinetics of [S312A] β_1 -AR[+20]. These results and those obtained with [S312D] β_1 -AR indicated that the 20-mer peptide provided a general trafficking signal that drove the recycling of the β_1 -AR[+20] chimera in a PDZ, PKA- and Ser³¹²-independent manner. These findings indicate that the 20-mer peptide was the sole provider of the endosome to membrane trafficking signal.

In Nooh *et al.* [18], we described several roadmaps used by trafficking GPCR in their translocation from the endosome to the membrane. The first was the type-1 PDZ regulated or “*sequence-dependent trafficking*” pathway exemplified by β_2 -AR and other GPCR [31]. The second was exemplified by the β_1 -AR, which traffics via PDZ-dependent or by phospho-Ser³¹²-dependent, but PDZ-independent pathways [17–18]. The third was exemplified by μ -opioid and dopamine-1 receptors, which traffic in a PDZ-independent manner, via a transplantable recycling sequence embedded in the receptor backbone. The fourth was exemplified by Tfr and LDL receptors, which recycle by “*bulk*” sorting that is largely independent of specific cytoplasmic sequences [32], [34].

To better define the trafficking pathway for the WT β_1 -AR[+20] chimera we investigated the role of the retromer-WASH complex associated protein SNX27 in this pathway [28] and [35–37]. SNX27 is an EE-associated protein that regulates an aspect of endosome-to-cell surface recycling of proteins [38]. SNX27 contains a PDZ-binding domain that is involved in regulating the trafficking of endosomal GPCR by its association with the type-1 PDZ present in the C-tail of these proteins [27], [39]. While SNX27 does not bind to the core “*cargo selection trimer*” of the retromer, it is intimately involved in targeting the retromer to EE and in mediating PDZ-dependent trafficking of many GPCR including β_1 -AR and β_2 -AR [27], [29]. Therefore, we investigated if SNX27 was involved in endosome-to-cell surface recycling of WT β_1 -AR versus WT β_1 -AR[+20] chimera. Since SNX27 siRNA inhibited the recycling of the WT β_1 -AR, but not the WT β_1 -AR[+20], we conclude that the versatile

“*recycling peptide*” that we discovered transplanted a trafficking signal onto the GPCR. Confocal examination of the trafficking roadmap of WT β_1 -AR[+20] indicates that it recycled by a retromer-independent pathway that is worthy of intense future investigation.

To better understand the mechanistic underpinnings of this novel pathway we created fluorescent homologues of the WT β_1 -AR, such as EYFP-WT β_1 -AR that would traffic in PDZ- and retromer-dependent manner and WT β_1 -AR[+20]-EYFP that would traffic in PDZ- and retromer-independent manner. High throughput screening (HTS) of agents that might inhibit or accelerate the trafficking of GPCR via the retromer-independent pathway might uncover a novel basis for some diseases and hopefully a basic chemical platform for regulating this mode of trafficking. At this time there are several compounds that have been described as retromer inhibitors and retromer activators [40–41]. Invariably, conditions that reduced or inhibited its activity were associated with increased likelihood of neurodegenerative disorders such as alzheimer’s disease due to hyperstimulation of lysosomes and increased degradation of proteins that relied on the retromer for recycling [40], [42]; while activation of the retromer is thought to be beneficial in reversing damage associated with neurodegenerative disorders [41] and [43]. Screening for agents that affect trafficking by the retromer-independent pathway might reveal a novel physiological trafficking pathway involved in recycling of a large number of useful GPCR and hopefully identify novel pharmacological modifiers of GPCR-mediated signaling events, such as those involved in aberrant signaling by cardiac β_1 -AR in heart failure and other circulatory disorders.

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FIGURE 1. Effect of random amino acid insertions into the +1 position of the β_1 -AR on its trafficking

A, sequence of the peptides inserted into position [+1] down stream of Valine⁴⁷⁷ in the β_1 -AR. B, HEK-293 cells expressing the FLAG-tagged WT β_1 -AR (images a–e') and β_1 -AR constructs in which 4 (images f–j'), 8 (images k–o'), 12 (images p–t') or 16 amino acid (images u–y') peptides were inserted into the +1 position were plated on poly-L-lysine coated glass slides. Cells were prelabeled for 1 h with Cy3-anti-FLAG antibody and fixed (a, f, k, p and u). The rest of the slides were exposed to 10 μ M isoproterenol for 30 min and then fixed (b, g, l, q and v). The rest of the slides were subjected to recycling conditions for the indicated time period and then fixed. Slides incubated with alprenolol for 1 h were exposed to acid wash and then fixed (e', j', o', t', and y'). The distribution of fluorescent pixels was obtained using confocal microscopy and the colors shown are pseudo colors. These images were derived from three separate experiments with 20 aggregate images per condition. Each image closely represents the average data for each time set. Each *scale bar*

represents 5 μ m. C, HEK-293 cells were transiently transfected with FLAG-tagged WT β_1 -AR or β_1 -AR peptide fusions described in figures 1A and 2A. The cells were lysed, and about 4% of crude cell lysate (input) was subjected to Western blotting (IB) with anti-SAP97 IgG to verify that equal amounts of SAP97 were loaded onto the resin (*Top, left hand panels*). Then equal amounts of the remaining cell lysate proteins were incubated with anti-FLAG IgG resin, followed by washing and eluting the resin with Laemmli sample buffer. The eluates were divided into two portions. One portion representing 4% of the eluate by volume was probed with anti-FLAG antibody to verify that equal amounts of each β_1 -AR construct were bound and eluted from the affinity resin (*Bottom, left hand panels*). The rest of the FLAG IP's representing about 90% of resin eluates, were probed with anti-SAP97 antibody (*right hand panels*). The data are representative of $n = 4$ separate Western blots with similar results. A/W, *acid wash*.

Figure 2. Discovery that the multiple cloning sequence in pcDNA3.0 encodes a non-PDZ sorting sequence for endosome to cell membrane trafficking

A, sequence of the 20-mer peptide corresponding to the multiple cloning region of pcDNA3.0 and its location relative to the WT β_1 -AR or β_1 -AR PDZ and EYFP coding sequences. (*) indicates the position of the stop codon in each construct. B, HEK-293 cells expressing the β_1 -AR chimera described in Fig. 2A were plated on poly-L-lysine coated glass slides. The cells in *images a-o'* were subjected to internalization and recycling as described in the legend of Fig. 1A. The cells in *images p-y* were subjected to internalization and recycling without prior labeling with Cy3-conjugated anti-FLAG IgG. C, pixels inside a 300-nm boundary in isoproterenol exposed cells (*b, g, l, q* and *v*) were set arbitrarily to 100% to indicate 100% internalization. Pixels outside the 300-nm boundary in alprenolol-treated cells were estimated and their ratios relative to internal pixels in isoproterenol treated cells were expressed as % for each time period. These images were derived from three

separate experiments with 20 aggregate images per condition. Cumulative ratios from 20 independent images for each condition were calculated as mean $\pm$ S.E. and plotted on a *logarithmic Y-axis* to calculate the $t_{0.5}$ for recycling as described in *Materials and Methods*. The means $\pm$ SE for each time period were compared among the groups by one- or two-way ANOVA with Newman-Keuls post-tests. Statistical results are expressed as *NS* to indicate no significant differences. Each *scale bar* represents 5 μm .

FIGURE 3. Effect inhibiting the activity of protein kinase A on trafficking of β_1 -AR [+20] chimera

A, HEK-293 cells expressing the constructs described in Fig. 2A were pre-exposed to buffer or to 3 μ M of myristoylated PKI for 30 min during the Cy3-anti-FLAG incubation period. The recycling of these β_1 -AR chimeras was determined as described in the legend of Fig. 2B. These images were derived from four separate experiments with 20 aggregate images per condition. **B**. The mean $\pm$ S.E. for recycling was derived from 20 separate images per each timepoint and plotted as a logarithmic Y-axis to calculate the $t_{0.5}$ for recycling and were compared among the groups by ANOVA with Newman-Keuls post-tests and expressed as (*), (**), and (***) to indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Each *scale bar* represents 5 μ m.

FIGURE 4. Effect of the status of Ser³¹² in the β₁-AR on trafficking of β₁-AR by the 20-mer peptide

A, Single point mutant of the WT β₁-AR in which the serine at position 312 was mutated either to alanine (S312A) or to aspartic acid (S312D) were created. cDNAs encoding the β₁-AR without a stop codon and flanked with Hind III-EcoR1 were cloned into pCDNA 3.0 to extend the sequence of (S312A) β₁-AR or (S312D) β₁-AR by 20 amino acids. HEK-293 cells expressing these constructs were pre-exposed to buffer or to 3 μM of mPKI for 30 min during the Cy3-anti-FLAG incubation period. The recycling of these β₁-AR chimeras was determined as described in the legend of Fig. 2B. These images were derived from three separate experiments with 20 aggregate images per condition. **B**, Pixel intensities from 20 separate images for each timepoint were determined and calculated as mean ± S.E. for each time period and then compared among the groups by ANOVA with Newman-Keuls post-tests. Each *scale bar* represents 5 μm.

FIGURE 5. Effect of siRNA-mediated down regulation of SNX27 on trafficking of β_1 -AR [+20] chimera

A, HEK-293 cells transiently transfected with scrambled siRNA oligo or with SNX27 siRNA oligo were lysed and probed by western blotting for expression of human SNX27 and β -actin, simultaneously. *B*, HEK-293 cells were transiently transfected with the β_1 -AR chimera described in Fig. 2A and with either the scrambled siRNA oligo (*a–e*) or SNX27 siRNA oligo (*f–y*). These cells were subjected to internalization and recycling as described in the legend of Fig. 2B. These images were derived from four separate experiments with 20 aggregate images per condition. *C*, Pixel intensities from 20 separate images for each timepoint were determined and calculated as mean $\pm$ S.E. for each time period and then compared among the groups by ANOVA with Newman-Keuls post-tests. Each *scale bar* represents 5 μ m.