Targeting of G Protein-coupled Receptors to the Basolateral Surface of Polarized Renal Epithelial Cells Involves Multiple, Non-contiguous Structural Signals

(Received for publication, April 9, 1998, and in revised form, July 6, 1998)

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Truncations and chimeras of the α2A-adrenergic receptor (α2AAR) were evaluated to identify membrane domains responsible for its direct basolateral targeting in Madin-Darby canine kidney cells. An α2AAR truncation, encoding transmembrane (TM) regions 1–5, was first delivered basolaterally, but within minutes appeared apically, and at steady-state was primarily lateral in its immunocytochemical localization. A TM 1–5 truncation with the third intracellular loop revealed more intense lateral localization than for the TM 1–5 structure, consistent with the role of the third intracellular loop in α2AAR stabilization. Addition of TM 6–7 of A1 adenosine receptor (A1AdoR) to α2AARTM1–5 creates a chimera, α2AARTM1–5/A1AdoRTM6–7, which was first delivered apically, resulting either from loss of α2AAR sorting information in TM 6–7 or acquisition of apical trafficking signals within A1AdoRTM6–7. Evidence that α2AARTM6–7 imparts basolateral targeting information is revealed by the significant basolateral localization of the A1AdoRTM1–5/α2AARTM6–7 and A1AdoRTM1–5/α2AARTM6–7+3 chimeras, in contrast to the dominant apical localization of A1AdoR. These results reveal that sequences within TM 1–5 and within TM 6–7 of the α2AAR confer basolateral targeting, providing the first evidence that α2AAR basolateral localization is not conferred by a single region but by non-contiguous membrane-embedded or proximal sequences.

The coordinated cellular functions evoked by endogenous and exogenous ligands depend on the availability of appropriate receptors at the particular surface domains where the ligand has access. In polarized cells, the non-random localization is essential for vectorial functions of the cells; in its absence, disease ensues (1, 2). We are interested in elucidating mechanisms and structural regions within G protein-coupled receptors (GPCR) responsible for their polarized expression in renal epithelia.

We previously demonstrated that the α2A-adrenergic recep-
tor subtype (α2AAR) is predominantly localized (>85%) to the basolateral surface of Madin-Darby canine kidney (MDCKII) cells, a polarized model system for renal epithelia that accurately reflects α2AAR localization in vivo (3). Mutagenesis studies demonstrated that direct basolateral targeting was uncomplicated by elimination of glycosylation, deletion of the third cytoplasmic loop, truncation of the carboxyl terminus, uncoupling from G proteins by pertussis toxin, or modification of tyrosine-based endocytosis motifs (3, 4). Subsequently, similar observations were made for the thyrotropin-releasing hormone receptor, again demonstrating that regions in GPCR involved in targeting are distinct from those involved in coupling to G proteins or in ligand-induced endocytosis (5). Removal of the third cytoplasmic loop of the α2AAR did, however, interfere with the retention of the receptor once at the basolateral surface, shortening the half-life on that surface from 10–12 to 4–6 h (4). These data collectively suggest that the transmembrane regions of the α2AAR are predominantly involved in targeting, whereas the third cytoplasmic loop of the α2AAR is involved in retention at the basolateral surface.

The goal of the present studies was to delineate further the membrane-embedded sequences involved in the basolateral targeting of α2AAR. Previous studies have demonstrated that truncation and especially chimeric receptor strategies can reveal the roles of membrane-embedded or proximal sequences in GPCR while stabilizing the overall tertiary structure of these heptahelical molecules. For example, chimeras between the α2AAR and β2AR have revealed that the seventh transmembrane (TM) domain is necessary for antagonist binding specificity (6). Chimeras also have revealed regions that confer G protein selectivity of varying receptor families (7–9), differing agonist binding properties (10), and sites for receptor-dependent sequestration and down-regulation (11, 12). Previous studies with receptor truncations and chimeras also have led to the interpretation that TM 1–5 and TM 6–7 of GPCR can operate as independent domains and that both truncations and chimeras involving these regions can be synthesized, folded, and properly delivered to the membrane surface (6, 13).

We therefore addressed the role of the basolateral targeting information of the α2AAR using deletion, truncation, and chimera strategies. We selected the A1 adenosine receptor (A1AdoR) as the chimeric partner for the α2AAR, as we had previously demonstrated that the A1AdoR is apically enriched (~70%) in both Madin Darby canine kidney II (MDCKII) and porcine renal LLC-PK1 polarized epithelial cells and achieves this apical enrichment by direct targeting to that surface (14). Our study of the delivery and steady-state localization of deletions and truncations in the α2AAR and chimeras with the A1AdoR has revealed the following: 1) there is basolateral targeting information for the α2AAR in TM 1–5 of the receptor, 2) there is also basolateral targeting information in TM 6–7 of the α2AAR, and 3) targeting information for other GPCR, such...
Non-contiguous Basolateral Targeting Determinants of the α2AaAR

The numbers below are from one experiment per condition.

| Structure  | Clone | +Triton X-100 | ~Triton X-100 |
|------------|-------|--------------|--------------|
| α2AAR      | 3     | 0.14         | 0.12         |
| A2AAdoR    | 17    | 0.22         | 0.25         |
| α2A3A3A3    | 3     | 0.55         | 2.14         |
| α2A3A3A3    | 66    | 2.07         | 3.35         |
| α2AARTM1–5 | 140   | 1.10         | 4.12         |
| α2AARTM1–5 | 169   | 6.17         | 16.0         |
| α2AARTM1–5 | 15    | 4.20         | 15.36        |
| α2AARTM1–5 | 2     | 6.30         | 35.0         |
| α2AAR/A2AdoR| 38    | 1.24         | 4.05         |
| α2AAR/A2AdoR| 57    | 3.21         | 9.20         |
| α2AAR/A2AdoR| 26    | 7.27         | 7.42         |
| A2AAdoRα2A3A3| 55    | 2.11         | 8.53         |
| A2AAdoRα2A3A3| 59    | 6.30         | 12.5         |
| A2AAdoRα2A3A3| 25    | 9.45         | 21.42        |
| A2AAdoRα2A3A3| 14    | 3.25         | 7.55         |

The numbers below are from one experiment per condition.

Non-contiguous Basolateral Targeting Determinants of the α2AaAR

For polarity experiments, MDCKII cells were seeded at a density of 3 × 10^4 cells/cm^2. chambers, 0.4-μm pore size, Costar, Cambridge, MA) and cultured for 5–8 days with medium changes every day. Prior to each functional or immunocytochemical experiment, the integrity of the monolayer was assessed by adding [3H]methoxyinulin to the apical medium and monitoring the leak of [3H]methoxyinulin from the apical compartment to the basolateral compartment. Samples were obtained by collecting the basolateral medium in a β-counter (Packard Tricarb) after a 1-h incubation at 37 °C. Chambers with greater than 3% leak per hour were discarded.

**Metabolic Labeling/Biotinylation Strategy for Determining Surface Delivery of the Chimeric Receptors**—The amount of newly synthesized chimeric receptor delivered to the apical versus basolateral surface was quantified by biotinylating one surface or the other of metabolically labeled MDCKII cells in Transwells. For immunostaining of MDCKII cells on coverslips, cells were plated at confluence on glass coverslips and cultured for 2–3 days prior to staining. Cells were fixed and stained with a 1:50 dilution of 12Ca5 primary antibody as described (14). Upon rinsing the cells after the incubation of the primary antibody, a 1:200 dilution of the secondary Cy5-conjugated donkey anti-mouse IgG was added to the cells in phosphate-buffered saline containing 2% bovine serum albumin and incubated for 1 h at room temperature in the dark. The cells then were mounted on glass slides with Aqua-PolyMount (Polysciences Inc., Warrington, PA).

For immunostaining of cells grown in Transwell culture, the cells on the polycarbonate filter were fixed and stained via the same protocol as those grown on the coverslips, except that the filter was excised from the Transwell support prior to the first antibody incubation. Samples were visualized by confocal microscopy on a Zeiss Axiovert 135 Micro Systems LSM (Germany). The samples were first visualized in the xy plane, and then in the xz plane. Also, z sections were taken at 1-μm thickness from the most apical portion of the cell down to the most lateral, rendering 10–12 sections. The images were down-loaded onto a Silicon Graphics Iris Indigo workstation for analysis using Showcase software, with analysis performed by D. A1AdoR/A1AdoR 57 3.21 9.20
A2AR/A2AdoR 26 7.27 7.42
A2AAdoRα2A3A3| 55    | 2.11         | 8.53         |
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A2AAdoRα2A3A3| 25    | 9.45         | 21.42        |
A2AAdoRα2A3A3| 14    | 3.25         | 7.55         |

as the apically directed A2AdoR, is similarly distributed throughout the receptor molecule.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]-Express protein labeling mixture (1200 Ci/mmol), [3H]methoxyinulin (125.6 nCi/μg), and α-[35S]-DATP (1389 Ci/μmol) were purchased from NEN Life Science Products. Biotin hydrazide and streptavidin-agarose were purchased from Pierce. The protein A-purified antibody for 12Ca5 monoclonal antibody was purchased from Babco. Cy3-conjugated donkey anti-mouse IgG was purchased from Jackson ImmunoResearch.

**Construction of Mutants**—To create the structures utilized in this study, polymerase chain reaction or subcloning was used, according to standard recombinant DNA strategies. The wild-type and mutant receptors were all amino-terminally epitope-tagged as described previously (3, 4, 14); the first 9 amino acids after the initiating methionine encode a hemagglutinin (HA) epitope recognized by the commercially available monoclonal antibody 12CA5 (Babco). The structures developed and amino acids that corresponded to the junctions within chimeras are noted in the legend to Fig. 1A. Positive mutations were verified using dyeoxy-DNA sequencing (Sequenase 2.0, Sequenase Kit, U.S. Biochemical Corp.) utilizing pCMV4 DNA polymerase with α-[35S]-DATP. Before making permanent cell lines in MDCKII cells, all of the resulting pCMV4 truncation and chimeric constructs were transiently transfected into COSM6 cells, and membranes from these transfectants were assayed for binding and feasibility for immunodetection with 12Ca5 antibody.

**Development of Permanent Transformants of MDCKII Cells**—The pCMV4 plasmids containing the epitope-tagged receptors of interest were transfected as described previously (3). G418-resistant colonies were screened for chimeric receptor expression by immunodetection. Untransfected MDCKII cells displayed no detectable staining pattern. Table I lists the camera exposure time (minutes) for all of the epitope-tagged clones evaluated in this study. Camera exposure time under the microscope (using auto-exposure) was used as an indirect measure of the level of expression of the non-binding mutant and chimeric receptors relative to the “parent” wild-type receptors, for which the receptor expression was assessed by adding [3H]methoxyinulin to the apical medium and monitoring the leak of [3H]methoxyinulin from the apical compartment to the basolateral compartment. Samples were obtained by collecting the basolateral medium in a β-counter (Packard Tricarb) after a 1-h incubation at 37 °C. Chambers with greater than 3% leak per hour were discarded.

**Metabolic Labeling/Biotinylation Strategy for Determining Surface Delivery of the Chimeric Receptors**—The amount of newly synthesized chimeric receptor delivered to the apical versus basolateral surface was quantified by biotinylating one surface or the other of metabolically labeled MDCKII cells in Transwells. For immunostaining of MDCKII cells on coverslips, cells were plated at confluence on glass coverslips and cultured for 2–3 days prior to staining. Cells were fixed and stained with a 1:50 dilution of 12Ca5 primary antibody as described (14). Upon rinsing the cells after the incubation of the primary antibody, a 1:200 dilution of the secondary Cy5-conjugated donkey anti-mouse IgG was added to the cells in phosphate-buffered saline containing 2% bovine serum albumin and incubated for 1 h at room temperature in the dark. The cells then were mounted on glass slides with Aqua-PolyMount (Polysciences Inc., Warrington, PA).

**Immunolocalization of the Deletion, Truncation, and Chimeric Receptors**—Primary and secondary immunostaining protocols were optimized using cells plated on coverslips before evaluating cells polarized by culture in Transwells. For immunostaining of MDCKII cells on coverslips, cells were plated at confluence on glass coverslips and cultured for 2–3 days prior to staining. Cells were fixed and stained with a 1:50 dilution of 12Ca5 primary antibody as described (14). Upon rinsing the cells after the incubation of the primary antibody, a 1:200 dilution of the secondary Cy5-conjugated donkey anti-mouse IgG was added to the cells in phosphate-buffered saline containing 2% bovine serum albumin and incubated for 1 h at room temperature in the dark. The cells then were mounted on glass slides with Aqua-PolyMount (Polysciences Inc., Warrington, PA).

In order to quantify how much epitope-tagged receptor was on the surface of the cells compared with inside, we used the “NIH Image” program to manually quantitate the pixel intensity (pixel values 0–255) in the cytoplasm versus at the plasma membrane. To assess what fraction of the total receptor was apical plasma membrane-associated receptor, the z scans from total receptor immunofluorescence (obtained from staining in the absence of Triton X-100) were compared with those from just plasma membrane immunofluorescence (obtained from staining in the absence of Triton X-100). For every value obtained, 15–20 cells were “counted,” thus constituting one experiment. The surface:intracellular ratio of immunoreactivity was calculated by manually outlining the cytoplasm and then the plasma membrane staining and by using Microsoft Excel for the calculations, as described previously (15). This was performed three times for one clone and one to two times for another clone of the same wild-type or mutant structure in order to verify that estimates of surface:intracellular expression were reflective of a receptor structure, and not simply of one cell line. These findings are summarized in Fig. 1B.

**Assessment of Receptor Binding of Wild-type α2AAR and Mutant Structures**—Various cDNAs encoding α2AAR and receptor truncations or chimeras were transfected into COSM6 cells as described previously (16) and evaluated for [3H]yohimbine binding 60 h after transfection (4). For each cDNA construction, 10 μg was transfected; in cells co-transfected with 2 cDNAs, a total of 20 μg of cDNA was added to the cells. A positive control (wild-type α2AAR) and negative control (either vector alone or no DNA) were included in every experiment. Specific [3H]Yohimbine binding was that competed for by 10 μM phentolamine.

**RESULTS**

**Immunolocalization of the Deletion and Truncation Mutant α2AAR in MDCKII Cells**—Because introduction of the HA epitope into the amino terminus does not appear to alter the
Non-contiguous Basolateral Targeting Determinants of the α2A AR

A

Wild-Type Epitope-Tagged GPCR

α2A AR

A1 AdoR

Truncated, Deleted, and Chimeric Epitope-Tagged GPCR

α2A AR TM1-5

α2A AR TM1-5+13

α2A ARΔ13

A1 AdoR TM1-5/α2A AR TM6-7

A1 AdoR TM1-5/α2A AR TM6-7+13

A1 AdoR TM1-5/α2A AR TM6-7

B

Fig. 1
localization of α₂AR or the A₁AdoR in MDCKII cells (3, 4, 14, 17), we evaluated clones of MDCKII cells permanently expressing the hemagglutinin epitope-tagged versions of receptors mutated by deletion and truncation. As seen in Fig. 2A, confocal microscopy images confirm that the steady-state localization of the wild-type α₂AR is lateral. Even though previous surface biotinylation strategies confirm that this lateral localization is at the surface (3, 4, 14), the signal is enhanced in the presence of Triton X-100, suggesting that the epitope is more accessible to antibody recognition under these conditions. Quantification of the fluorescence signal for the wild-type α₂AR in the presence of Triton X-100 reveals a surface:intracellular value of 3.17 ± 0.09 (Fig. 1B). As shown in Fig. 2B, the deletion of the large third intracellular loop of the α₂AR (α₂AR Δi3) does not perturb its lateral localization; this structure also manifests a surface:intracellular staining ratio similar to wild-type α₂AR, 3.16 ± 0.4 (Fig. 1B). In contrast, truncation of the α₂AR to a structure including only TM 1–5 and the third intracellular loop, α₂ARTM1–5+Δi3, results in a staining profile that is largely basolateral (Fig. 2C) but also has enriched intracellular staining, consistent with reduced surface:intracellular staining ratio for the α₂ARTM1–5+Δi3 structure of 1.5± 0.03 (Fig. 1B). When we examined the localization of a truncated α₂AR that includes only TM 1–5 and lacks the third intracellular loop (α₂ARTM1–5), we detected only marginal basolateral localization at steady state and a large population of detectable truncated receptor inside the cell, consistent with the calculated surface:intracellular staining ratio of 1.07 ± 0.06 (Fig. 1B). The greater intracellular accumulation of α₂ARTM1–5 versus α₂ARTM1–5+Δi3 confirms previous findings that the third cytoplasmic loop (i3) contributes to stabilization on the basolateral surface (4). These data suggest that the TM 1–5 domain has some information that permits lateral localization (Fig. 2, C and D); however, TM 6–7 must also impart some of the lateral localization information, since in the absence of this domain, exclusive surface basolateral localization characteristic of the wild-type α₂AR and α₂ARTM1Δi3 mutant structure is lost.

Transmembrane Domains 6 and 7 of the α₂AR Also Contain Basolateral Targeting Information—To evaluate further the role of TM 6–7 of the α₂AR in conferring surface localization information, two independent approaches were used. First, we determined if TM 6–7 of the α₂AR could redirect apical A₁AdoR localization. Thus, the front half of the A₁AdoR (TM 1–5) was joined to the back half of the α₂AR (TM 6–7) in the presence or absence of the third intracellular loop of the α₂AR. As seen in Fig. 3B, A₁AdoRTM1–5/α₂ARTM6–7+Δi3 chimera has a steady-state localization which is largely lateral: the surface:intracellular value is 1.07 ± 0.06, which was significantly lower than the wild-type and loop deletion mutant (α₂ARTM1–5 Δi3). The pixel intensity of the immunofluorescence for the stained cells was quantified by means of the program NIH Image as described under “Experimental Procedures.” Immunocytochemical experiments were performed at least three times for one clone in a mutant type, and 2–3 clones were examined for each mutant receptor (data not shown). A nonparametric analysis of variance followed by a Dunn’s multiple comparisons test was performed to determine which surface to intracellular ratios were significantly different from each other (* p < 0.05). The reference point was the wild-type α₂AR, which demonstrated a surface:intracellular value of 3.17 ± 0.08 (n = 3). The loop deletion mutant, α₂AR Δi3, had a value similar to wild-type: 3.2 ± 0.46 (n = 3). The truncation mutant, α₂ARTM1–5, had the lowest surface:intracellular value, 1.07 ± 0.06, which was significantly lower than the wild-type and loop deletion mutant (n = 3; p < 0.05). The other truncation mutant, α₂ARTM1–5+Δi3, had a surface:intracellular value of 1.5 ± 0.03 (n = 4). The three chimeras had values as follows: A₁AdoRTM1–5/α₂ARTM6–7+Δi3 had a ratio of 1.83 ± 0.28 (n = 3); A₁AdoRTM1–5/α₂ARTM6–7 was 1.21 ± 0.09 (n = 3); and α₂ARTM1–5/A₁AdoRTM6–7 was 1.4 ± 0.05 (n = 3).
FIG. 2  Partial structures of the α2AAR are localized to the basolateral domain of MDCKII cells. The amino-terminally epitope-tagged wild-type α2AAR (A) was stained in the presence of Triton X-100 to permeabilize the cells and gain access to any epitope associated with an intracellular receptor population in the absence of Triton X-100, which should stain only the cell-surface receptor pool. Comparison of immunofluorescence in the absence and presence of Triton X-100 was also performed for the mutant receptor with the third loop deletion, α2AARΔi3 (B), a truncation mutant without the transmembrane domains 6–7, α2AARTM1–5+i3 (C), and a truncation mutant encoding TM1–5, α2AARTM1–5 (D). The confocal gallery of the Z sections in each panel gives a complete representation of the localization of these structures at steady state when stained in the presence of Triton X-100. Each square of the nine-member composite gallery represents a 1-μm Z section through the cell. Section 1 represents the upper (apical) portion of the cells and section 9 represents the lower (basal) portion of the cells.
Fig. 3. Chimeras containing structures from both the A1AdoR and the α2aAR lose the preferential apical localization characteristic of wild-type A1AdoR. A, the steady-state localizations of the wild-type α2aAR and A1AdoR in the presence of Triton X-100. The steady-state localizations of A1AdoRTM1–5/α2aARTM6–7+i3 (B), A1AdoRTM1–5/α2aARTM6–7 (C), and α2aARTM1–5/A1AdoRTM6–7 (D) are shown in the presence and absence of permeabilization for the immunocytochemical procedure; the surface:intracellular values were 1.83 ± 0.28, 1.21 ± 0.1, and 1.4 ± 0.057, respectively. The galleries of z sections are from experiments in the presence of Triton X-100.

GPCR (19, 20, 22).

Similar co-expression studies were performed for the receptor chimeras (Fig. 4B). Independently expressed chimeras revealed virtually no capability to bind [3H]yohimbine. However, co-expression of α2aARTM1–5/A1AdoRTM6–7 and A1AdoRTM1–5/α2aARTM6–7+i3 also led to readily detectable...
A  Binding Rescue with $\alpha_{2A}$AR Truncations

B  Binding Rescue with $\alpha_{2A}$AR Chimeras
A  \(\alpha_{2A}\)-AR\(\Delta i3\): direct basolateral targeting

\[
\begin{array}{c|c|c|c|c|c}
\hline
\text{Pulse (min):} & 90 & 120 \\
\text{Surface:} & A & B & A & B \\
\hline
\end{array}
\]

B  \(\alpha_{2A}\)-ARTM1–5: basolateral > apical ----> apical > basolateral

\[
\begin{array}{c|c|c|c|c|c|c|c}
\hline
\text{Pulse (min):} & 20 & 30 & 45 & 60 & 90 \\
\text{Surface:} & A & B & A & B & A \\
\hline
\end{array}
\]

C  \(\alpha_{2A}\)-AR/A\(_1\)-AdoR: apical > basolateral ----> basolateral > apical

\[
\begin{array}{c|c|c|c|c|c|c|c}
\hline
\text{Pulse (min):} & 30 & 45 & 60 & 90 \\
\text{Surface:} & A & B & A & B \\
\hline
\end{array}
\]

\[^{3}\text{H}\]Yohimbine binding (n = 4). Similarly, \[^{3}\text{H}\]Yohimbine binding could be detected by co-expression of \(\alpha_{2A}\)-AdoRTM1–5/\(\alpha_{2A}\)ARTM6–7 or \(\alpha_{2A}\)-ARTM1–5 and especially by the co-expression of \(\alpha_{2A}\)-AdoRTM1–5/\(\alpha_{2A}\)ARTM6–7 and \(\alpha_{2A}\)-ARTM1–5+i3, consistent with the data in Fig. 2C and previous findings (4) suggesting that the third intracellular loop affords a longer retention of \(\alpha_{2A}\)AR on the basolateral surface. Finally, the \(\alpha_{2A}\)ARTM1–5/\(\alpha_{2A}\)-AdoRTM6–7 chimera can also restore significant \[^{3}\text{H}\]Yohimbine binding to the non-binding D113N \(\alpha_{2A}\)AR mutation. Overall, the rescue of binding for all structures evaluated ranged from 3–19%, which is in the range of rescue reported by others for GPCR (19, 20, 22). Our interpretation of these findings is that the independently expressed receptor domains retain a structure that resembles, functionally, that of the native receptor, thus warranting assessment of surface delivery of these truncations and chimeric structures.

Assessment of the Delivery of Mutant \(\alpha_{2A}\)AR to the Apical Versus the Basolateral Cell Surface—To examine how these mutant receptors achieved their steady-state localization in polarized MDCKII cells, we assessed appearance of metabolically labeled receptors to either the apical or basolateral surface. Both the wild-type \(\alpha_{2A}\)AR (3) and the \(\alpha_{2A}\)AR\(\Delta i3\) deletion mutant (4) achieve their steady-state localization on the basolateral surface by means of direct delivery to that membrane domain, as described previously and confirmed here for the \(\alpha_{2A}\)AR\(\Delta i3\) at pulse times of 90 and 120 min (Fig. 5A). As seen in Fig. 5B, delivery studies revealed that the truncation of the \(\alpha_{2A}\)AR to \(\alpha_{2A}\)-ARTM1–5 yielded a structure that was first delivered preferentially, but not exclusively, basolaterally; however, within a very short window of time, i.e. about 15–30 min, metabolically labeled receptor could also be detected on the apical surface. The lack of exclusive surface targeting of the \(\alpha_{2A}\)-ARTM1–5 truncation is in marked contrast to the wild-type \(\alpha_{2A}\)AR (3), suggesting, as do the steady-state localization data in Fig. 2D, that basolateral delivery cannot rely solely on this single domain. The key observation is that exclusive basolateral targeting characteristic of the wild-type \(\alpha_{2A}\)AR and \(\alpha_{2A}\)AR\(\Delta i3\) structures is not afforded by a structure encoding only TM 1–5 of the \(\alpha_{2A}\)AR. The changing relative distribution of this truncation mutant on the apical vs. the basolateral surfaces as a function of metabolic labeling times could be due to rapid turnover, recycling, and/or re-routing. For example, pulse-chase experiments revealed that the half-life of this structure on the apical surface was ~40 min and that on the basolateral surface ~20 min (data not shown), in contrast to the basolateral half-lives of 10–12 h for the wild-type \(\alpha_{2A}\)AR or 4–6 h for the \(\alpha_{2A}\)AR\(\Delta i3\) structure (3, 4).

Delivery data for chimeric structures also underscores the inability of a single domain of GPCR to achieve exclusive targeting to a given surface. For example, the \(\alpha_{2A}\)ARTM1–5/
A,AdoRTM6–7 chimera, when evaluated at early time points of metabolic labeling, was first detected on the apical surface (Fig. 5C). However, within a short window of time, this structure also appears on the basolateral surface and is ultimately enriched on that surface. One interpretation of these findings is that the initial basolateral signal of the truncated receptor, α₂AARTM1–5, can be overridden by addition of TM 6–7 of the apically targeted A,AdoR. Pulse-chase experiments revealed that this chimera was lost from both the apical and basolateral surfaces at a similar rate, with an estimated half-life of 1.5–2.0 h (data not shown). The longer surface half-life of the α₂AARTM1–5/Α,AdoRTM6–7 chimera when compared with that of the α₂AARTM1–5 mutant, estimated at 20–40 min, is consistent with the interpretation (from the data in Fig. 3D) that the basolateral targeting information present in TM 1–5 of the α₂AR needs to be stabilized by the TM 6–7 domain, which could serve as a hydrophobic anchor in GPCR, in order to have a longer retention on the basolateral surface. Again, we cannot definitively ascribe the changes in relative apical versus basolateral distribution of receptor chimeras with time of metabolic labeling to recycling or re-routing, but we can conclude that a single domain (i.e. TM 1–5 or TM 6–7) cannot alone account for exclusive delivery to a single surface.

**DISCUSSION**

A number of previous studies have led to the identification of basolateral targeting motifs for single transmembrane spanning receptors in polarized renal epithelial cells. For example, Thomas and Roth (23) have shown that the basolateral sorting signal for vesicular stomatitis virus G lies within the cytoplasmic domain and is encoded by the alphabetic sequence YAX. By making chimeras with transferrin receptor, Odorizzi and Trowbridge (24) demonstrated that a dihydrophobic motif in the cytoplasmic tail of the major histocompatibility complex class II invariant chain targets it basolaterally in MDCK cells. The molecular basis for basolateral targeting of these and other single transmembrane proteins has been reviewed previously (23, 25–27).

The basolateral targeting motifs for multi-spanning proteins have been more difficult to deduce, as deletion or insertion mutants, useful in delineating motifs in single membrane-spanning proteins, can create confounding results in polytopic membrane proteins. Consequently, chimeras between proteins of similar structural families but with distinct trafficking itineraries have yielded the most informative insights. For example, chimeras between the 10 transmembrane-spanning ATP-powered Ca²⁺ pump in the plasma membrane and the closely related pump in the sarcoplasmic reticulum revealed that the first transmembrane domain of sarco(endo)plasmic reticulum was sufficient to target chimeras between these two proteins to the endoplasmic reticulum (28). In contrast to the role of transmembrane-spanning domains in the Ca²⁺-ATPase targeting, chimeras between the apically localized GLUT 5 and the basolaterally localized GLUT 1 isoform of glucose transporters revealed that the intracellular loops of these 12 transmembrane-spanning transporters confer apical versus basolateral localization in polarized Caco-2 intestinal cells (29). In a related molecular family, Perego et al. (30) demonstrated that basolateral sorting information lies in the cytoplasmic tail of the betaine transporter, whereas apical sorting information for the closely related γ-amino butyric acid transporter (GAT-1) does not and may be embedded in the bilayer or in endofacial domains, or both. These examples emphasize that there are no demonstrated consensus motifs or unifying mechanisms that confer basolateral targeting of polytopic membrane proteins in polarized epithelial cells.

The present studies were undertaken to establish targeting motifs that confer basolateral targeting of seven transmembrane-spanning GPCR, using the directly targeted α₂AR as a model molecule. Previous studies in our laboratory have provided evidence that basolateral targeting of the α₂AR relies on membrane-embedded or proximal sequences (4). The present studies exploit receptor truncations and chimeras to explore the membrane-spanning regions involved in basolateral targeting of the α₂AR. The ability of α₂AR truncations and chimeras to restore binding of the α₂AR antagonist, [³H]yohimbine, to a binding-defective D113Nα₂AR mutant suggests that these independently expressed domains possess a structure that, at least functionally, resembles these domains within the native receptor. Furthermore, the observation that multiple independent clonal cell lines for each structure examined revealed indistinguishable findings for receptor delivery and steady-state localization adds further confidence to our interpretations and argues that the truncations and chimeras evaluated are providing informative insights into subdomains of the α₂AR critical for direct basolateral delivery.

The findings presented here, and summarized in Fig. 6, indicate that there is basolateral targeting information in TM1–5 of the α₂AR that is stabilized by juxtaposition of the third intracellular loop (Fig. 2C). However, TM 1–5 of the α₂AR does not contain all of the necessary basolateral targeting information. This conclusion is based on the observation that the truncated α₂AARTM1–5 is not exclusively delivered to the basolateral surface like the wild-type α₂AR. The basolateral signals missing in α₂AARTM1–5 must lie within TM 6–7, since the mutant lacking the third cytoplasmic loop (α₂ARΔ3) does not show any signs of apical delivery. Chimeric structures of the α₂AR with the A,AdoR also support the importance of the TM 6–7 domain of the α₂AR in conferring basolateral localization information; the absence of TM 6–7 in the α₂AARTM1–5/Α,AdoRTM6–7 chimera leads to initial apical delivery (Fig. 5C), and introduction of TM 6–7 of the α₂ARTM onto TM 1–5 of the A,AdoR to create the Α,AdoRTM1–5/α₂AARTM6–7+i3 and the A,AdoRTM1–5/α₂AARTM6–7 chimeras results in structures with demonstrably lateral staining patterns (Fig. 3, B and C). Our interpretation of these collective findings is that information conferring basolateral localization of the α₂AR exists in both the TM 1–5 and TM 6–7 domains.

An enigmatic observation in our studies is that the α₂AARTM1–5 truncation is localized basolaterally and intracellularly at steady state, although the kinetic profile reveals targeting to both surfaces, with an apical t₁/₂ of ~40 min and a basolateral t₁/₂ of ~15–30 min (Fig. 5B). Predictions from these kinetic data would lead to an expectation of greater apical than basolateral localization at steady state, if the steady-state localization solely reflected direct delivery. There are two possible explanations for these unexpected findings. The first possibility is that the truncated receptor is quickly removed from both surfaces but that removal from the apical surface is followed by degradation or intracellular retention, whereas removal from the basolateral surface is followed by recycling back to the basolateral surface. We know from our own studies with the α₂AR, for example, that its short half-life on the apical surface (t₁/₂ 15–30 min) is not paralleled by detectable apical localization at steady state via confocal microscopy, suggesting that removal from the apical surface is followed by degradation (17). Thus, it may be a common trafficking itinerary for GPCR that apical delivery is followed by removal and degradation, and not recycling, in contrast to findings for molecules on the basolateral surface. A second possible explanation of our findings that no apical localization of the α₂AARTM1–5 is seen at steady-state in confocal images is that, after removal from the apical surface, the receptor transcytoses to the basolateral sur-
face, where it then “stays” by virtue of recycling back and forth to the basolateral domain. The independent recycling properties of proteins internalized from polarized surfaces via a distinct subset of exocytotic vesicles in MDCK cells has already been demonstrated for single transmembrane-spanning proteins (31). Thus, either of these two scenarios is possible. Existent tools for study of the \( \alpha_2 \)AR do not permit us to distinguish between these two possible explanations.

Assigning the regions of the receptor that govern receptor targeting requires an appreciation that trafficking itineraries are likely controlled by both negative and positive signals. For example, interpretations of our chimeric receptor studies must be qualified by the realization that the addition of TM 6–7 of the A1AdoR to TM 1–5 of the \( \alpha_2 \)AR could lead to changes in delivery or steady-state localization either because of the addition of a new targeting signal to the chimera or because of a loss of a targeting signal inherent in the parent molecule. Thus, if \( \alpha_2 \)AdoRTM1–5/\( \alpha_2 \)ARTM6–7 and \( \alpha_2 \)AdoRTM1–5/A1AdoRTM6–7 have lateral localization due to the presence of a basolateral targeting signal in TM6–7 of the \( \alpha_2 \)AR, then this signal is able to “override” any signal that might be expressing apical targeting in TM1–5 of A1AdoR. In this case, the interpretation implies hierarchical targeting signals. Alternatively, it may be that the absence of TM6–7 of the A1AdoR, which may encode apical targeting, results in chimeras with a more lateral phenotype because the lateral surface is encoded by “default.” However, the \( \alpha_2 \)ARTM1–5/\( \alpha_2 \)ARTM6–7 is not exclusively apical. Adding TM6–7 of the A1AdoR to \( \alpha_2 \)ARTM1–5 gives the receptor a more apical phenotype, suggesting that for another GPCR, the A1AdoR, the apical targeting information is non-contiguous, since the \( \alpha_2 \)ARTM1–5/\( \alpha_2 \)ARTM6–7 and \( \alpha_2 \)AdoRTM1–5/\( \alpha_2 \)ARTM6–7 both express some apical localization as well.

The possibility that several regions of a polytopic protein can impart targeting information has been demonstrated previously. Verhey et al. (32) have demonstrated that signals in both the amino and carboxyl terminus of GLUT 1 (localized intracellularly and at the cell membrane) and GLUT 4 (localized only intracellularly) confer targeting information to the two isoforms. Marks et al. (33) demonstrated the presence of two distinct and saturable protein targeting processes, one tyrosine-based and the other dileucine-based, in the cytoplasmic region of transmembrane proteins (e.g., the \( \beta \) chain of HLA-DM/H-2M\( \beta \)) in HeLa cells. In a third example, Alanso et al. (34)
showed that multiple sequences are responsible for the apical localization of the nonglycosylated type I membrane protein, CD3-e, in MDCK cells. Multiple targeting signals have also been demonstrated in some single transmembrane-spanning proteins, both for basolaterally targeted (23–25, 35–37) and apically targeted proteins (38–40).

The present studies also provide insights into the stabilization of the α2AR on the basolateral surface. Multiple lines of evidence confirm that the third intracellular loop stabilizes lateral retention of GPCR. Findings summarized in Figs. 1B and Fig. 2, C and D demonstrate that higher surface/intracellular expression is seen for truncations that include the third intracellular loop of the α2AR. Similarly, in receptor chimeras, association of the third intracellular loop with the last two transmembrane regions of the α2AR(α2AR/α2AR+13) stabilizes whatever basolateral inclination those domains of the α2AR possess (Fig. 3B versus 3C). In addition and not previously appreciated, we noted that the TM 6–7 domain of GPCR may also serve as a hydrophobic stabilizing anchor for receptors on polarized surfaces. This conclusion is based on the observation that the surface half-life of α2ARTM1–5 (20–40 min) is dramatically shorter than that of the α2ARTM1–5/ A1AdRTM6–7 chimera, which has an estimated half-life of 1.5–2 h.

The present data reveal that basolateral targeting of the α2AR, and likely other GPCR, is encoded by structural information within both TM 1–5 and TM 6–7 domains. These findings provide the first molecular insights for adrenergic receptors which demonstrate that targeting to the basolateral surface of polarized epithelia involves multiple, non-contiguous structural information. It is probable that simple sequence motifs for surface targeting will not be identifiable for GPCR, in contrast to tyrosine and dileucine motifs for basolateral sorting of single transmembrane-spanning proteins. Ingenious strategies to reveal “surfaces” created by non-contiguous elements that serve as recognition motifs for targeting machinery will be required to fully understand the targeting of GPCR to basolateral versus apical surfaces of polarized epithelial cells.

Acknowledgments—We thank members of the Limbird laboratory for helpful discussions and assistance during these experiments. We also thank Drs. Leigh B. MacMillan and Jeremy G. Richman for their critical reading of this manuscript. We appreciate the helpful advice of Dr. Peter J. Dempsey (Vanderbilt University, TN). We are also grateful to Dr. John Scott (Vollum Institute, OR) for suggestions with this project at national meetings. Thanks also to Matthew H. Wilson (Vanderbilt University, TN) for the contribution of the D113Nα2AR mutant construction for use in the co-expression experiments.

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