Molecular Pharmacology Fast Forward. Published on October 18, 2004 as doi:10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

The composition of the beta-2 adrenergic receptor oligomer affects its membrane trafficking after ligand-induced endocytosis

Tracy T. Cao[+*], Anne Brelot[Ψ¶*] and Mark von Zastrow[ΨÚ∞]

Departments of [+]Biochemistry and Biophysics, [Ψ]Psychiatry, and

ÚCellular & Molecular Pharmacology

Program in Cell Biology

University of California, San Francisco

San Francisco, CA 94143-0984

Running Title: Post-endocytic sorting of GPCR oligomers

∞ corresponding author:
Room N212E Genentech Hall
University of California, San Francisco
600 16th Street
San Francisco, CA 94143-2140
phone (415) 476-7855, fax (415) 514-4146
email zastrow@itsa.ucsf.edu

¶ present address:
Institut Cochin, U567
Departement de biologie cellulaire
22, rue Mechain
75014 Paris, FRANCE

* these authors contributed equally to the results presented

Text pages: 29
Figures: 4
Tables: 0
Abstract: 239 words
Introduction: 547 words
Discussion: 1425 words
References: 51

Abbreviations: GPCR, G protein-coupled receptor; B2AR, beta-2 adrenergic receptor; DOR, delta opioid receptor (DOP-1).

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The beta-2 adrenergic receptor (B2AR) is well known to form oligomeric complexes *in vivo* but the functional significance of this process is not fully understood. The present results identify an effect of oligomerization of the human B2AR on the membrane trafficking of receptors after agonist-induced endocytosis in stably transfected HEK293 cells. A sequence present in the cytoplasmic tail of the B2AR has been shown previously to be required for efficient recycling of internalized receptors. Mutation of this sequence was observed to inhibit recycling, not only of the receptor in receptor containing the mutation, but also of the co-expressed wild type B2AR. Co-expression of recycling-defective mutant B2ARs also enhanced proteolytic degradation of the wild type B2AR after agonist-induced endocytosis, consistent with trafficking of both receptors to lysosomes in an oligomeric complex. Co-expression of the delta opioid receptor (DOR) at similar levels produced a much smaller effect on endocytic trafficking of the B2AR, even though DOR traverses a similar membrane pathway as recycling-defective mutant B2ARs following agonist-induced endocytosis. Biochemical studies confirmed that B2AR / B2AR-ala 'homomeric' complexes form more readily than DOR / B2AR heteromers in expression-matched cell clones, and support the hypothesis that B2AR / B2AR-ala complexes are not disrupted by agonist. These results suggest that a significant fraction of B2ARs exist in oligomeric complexes after ligand-induced endocytosis, and that the composition of the oligomeric complex influences the sorting of endocytosed receptors between functionally distinct recycling and degradative membrane pathways.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

There is a growing appreciation that many G protein-coupled receptors (GPCRs) exist in oligomeric complexes (Bouvier, 2001; Milligan, 2001; Zeng and Wess, 2000). For some GPCRs, notably family C receptors such as the GABA-B receptor (Kuner et al., 1999; White et al., 1998) but also certain family A GPCRs (Lee et al., 2000)(Salahpour et al., 2004), oligomerization is critical for surface expression of functionally active receptors. For other GPCRs oligomerization is not known to be essential for functional activity and, particularly for many family A GPCRs, the physiological significance of this process is not clear.

The beta-2 adrenergic receptor (B2AR) is a family A GPCR that has been shown, in extensive biochemical and biophysical studies, to form homo-dimers and larger oligomers in biological membranes, and to do so at physiologically relevant levels of expression (Angers et al., 2000; Mercier et al., 2002; Salahpour et al., 2003). Chemical disruption of B2AR oligomers inhibits receptor activity in an isolated membrane fraction (Hebert et al., 1996), overexpression of the wild type B2AR can rescue activity of a functionally impaired mutant receptor (Hebert et al., 1998), and oligomerization is required for efficient delivery of newly synthesized receptors from the biosynthetic pathway to plasma membrane(Salahpour et al., 2004). To our knowledge, no functional consequence of oligomerization has been demonstrated at later stages in the "life cycle" of the B2AR.

The B2AR undergoes rapid endocytosis via clathrin-coated pits following ligand-induced activation in

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

the plasma membrane (Goodman et al., 1996; von Zastrow and Kobilka, 1994; Zhang et al., 1996). This process can mediate distinct functional effects, which are determined in large part by the specificity with which receptors are 'sorted' between divergent downstream membrane pathways after endocytosis. Recycling of B2ARs to the plasma membrane promotes functional resensitization of receptor signaling, whereas trafficking of internalized receptors to lysosomes is one mechanism promoting proteolytic down-regulation (Gagnon et al., 1998; Lefkowitz et al., 1998; Pippig et al., 1995; Tsao and von Zastrow, 2000a). The sorting of internalized receptors between these distinct membrane pathways is controlled by a sequence present in the carboxyl-terminal cytoplasmic domain of the B2AR. Mutation of this sequence does not detectably affect receptor-mediated activation of heterotrimeric G proteins or agonist-induced endocytosis of receptors, but specifically disrupts protein interaction(s) with the cytoplasmic tail that control the post-endocytic sorting mechanism (Cao et al., 1999; Cong et al., 2001; Gage et al., 2001).

Despite considerable progress in identifying specific post-translational modifications and cytoplasmic protein interactions that determine the endocytic trafficking fate of the B2AR (Ferguson et al., 1998; Goodman et al., 1998; Shenoy et al., 2001; Whistler et al., 2002), to our knowledge the possibility that interactions between receptors themselves might influence later stage(s) of endocytic membrane traffic has not been investigated. Nevertheless, there is considerable evidence that various GPCRs can endocytose in homo- or hetero- oligomeric complexes (He et al., 2002; Jordan et al., 2001; Overton and Blumer, 2000; Terrillon et al., 2004; Yesilaltay and Jenness, 2000), and biophysical studies suggest that

B2AR homo-oligomers are not disrupted after agonist activation (Mercier et al., 2002; Salahpour et al., 2003). The present results provide cell biological evidence for the existence of B2AR oligomers in the endocytic pathway, and they suggest that the composition of B2AR oligomers influences the sorting of internalized receptors between distinct recycling and degradative fates.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

cDNA constructs, cell culture and transfection

Amino-terminally HA and FLAG-tagged versions of the human beta-2 adrenergic receptor (B2AR, (Kobilka et al., 1987)) and mouse delta opioid receptor (DOP-1 or DOR, (Evans et al., 1992)) were described previously (Cao et al., 1998; Chu et al., 1997; von Zastrow and Kobilka, 1992). Mutant versions of the B2AR in which the distal carboxyl-terminal cytoplasmic domain was truncated after Gly 364 (B2AR-T364) or in which a single alanine residue or a 10-residue EE-epitope tag sequence were added to the full-length carboxyl terminus (B2AR-ala and B2AR-EE, respectively) have been described previously (Cao et al., 1999). All receptor cDNAs were cloned into pcDNA 3.0 or 3.1 (Invitrogen) for mammalian expression. Human embryonal kidney (HEK) 293 cells (ATCC) were grown in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (UCSF Cell Culture Facility). Stably transfected cells expressing epitope-tagged B2AR and/or DOR were generated by calcium phosphate co-precipitation of the appropriate constructs (Okayama and Berg, 1983) followed by culture of cells for several weeks at limiting dilution in 200 μg/ml G418 (Geneticin, Life Technologies) and/or 40 μg/ml Zeocin (Invitrogen). Cell clones were isolated and amplified in separate flasks.

Selection of stably transfected cell clones by radioligand binding and fluorescence flow cytometry

Initial screening of antibiotic-resistant cell clones was conducted by single-point radioligand binding in

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

whole cells using 5 nM [^{3}H] dihydroalprenolol or diprenorphine (Amersham) to detect adrenergic or opioid receptors, respectively, and 1 μM alprenolol or naloxone (Research Biochemicals) to define nonspecific binding, as described previously (Tsao and von Zastrow, 2000b). Singly transfected cell clones, expressing either FLAG- B2AR or DOR constructs separately, were selected for further study if they bound 0.5 – 1 pmol of the appropriate radioligand / mg cell protein in this assay. Doubly transfected FLAG-B2AR / HA-DOR clones were selected in a similar manner, except that specific binding of 0.5 – 1 pmol / mg was required for both radioligands.

Doubly transfected cell clones co-expressing differentially epitope-tagged adrenergic receptor mutants (FLAG-B2AR / HA-B2AR-ala, FLAG-B2AR / HA-B2AR-T364, FLAG-B2AR / HA-B2AR-EE and FLAG-B2AR-EE / HA-B2AR) were selected by a two-stage process. This was done because the HA and FLAG epitope tags used do not detectably affect B2AR ligand binding properties (Guan et al., 1992; von Zastrow and Kobilka, 1992) and therefore differentially tagged receptors cannot be distinguished by radioligand binding assay. Antibiotic-resistant cell clones were first selected for total [^{3}H] dihydroalprenolol binding (regardless of epitope tag) of $\leq$3 pmol /mg. Clones that satisfied this criterion were subjected to secondary screening using a previously described flow cytometric assay to specifically detect either FLAG or HA –tagged receptors using specific monoclonal antibodies (Chu et al., 1997; von Zastrow and Kobilka, 1992)(Keith et al., 1998). Singly transfected cell clones (expressing HA-B2AR or FLAG-B2AR separately at 0.5 – 1 pmol/mg, as estimated by saturation radioligand

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

binding) were analyzed alongside doubly transfected cell clones, to provide an internal reference for the desired expression level of the corresponding tagged receptors. Doubly transfected cell clones that expressed surface FLAG and HA immunoreactivity within 30% of that detected (in the same experiment) from the corresponding FLAG and HA – B2AR singly transfected "reference" cells were selected for further study.

Total B2AR and/or DOR expression level was then re-assayed in selected cell clones by multi-point saturation radioligand binding assay (Tsao and von Zastrow, 2000b), and confirmed to be <3 pmol/mg in all cases. Actual receptor number per cell was estimated for each cell clone by dividing B_{max} determinations derived from radioligand binding assay by cell number determined using a hemacytometer (Fisher Scientific), assuming one radioligand binding site per receptor. Total B2AR expression estimates (from [^{3}H] dihydroalprenolol B_{max} determinations) ranged from 42,000 $\pm$ 3,700 receptors / cell to 139,000 $\pm$ 3,900 receptors per cell across B2AR-transfected cell clones. Total DOR expression (from [^{3}H] diprenorphine B_{max} determinations) ranged from 61,000 $\pm$ 4,300 to 210,000 $\pm$ 14,000 receptors per cell across DOR-transfected cell clones. Endogenous [^{3}H] dihydroalprenolol binding in untransfected HEK293 cells was estimated at $\leq$1000 receptors / cell and [^{3}H] diprenorphine binding was undetectable.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

Cell surface biotinylation and assay of agonist-induced proteolysis

Cells were grown on poly-L-lysine (Sigma) coated 6 cm tissue culture dishes (Falcon), and cell surface biotinylation was conducted by incubating monolayers with 300 µg/ml sulfo-NHS-biotin (Pierce) in phosphate-buffered saline, pH 7.4 (PBS) at 4ÚC for 30 minutes. Unreacted sulfo-NHS-biotin was removed by 3 washes with ice-cold Tris-buffered saline pH 7.5 (TBS). One dish was left at 4ÚC to determine total surface biotinylated proteins, and the other dishes were incubated with normal culture media at 37ÚC for 4 hours in the absence or presence of a saturating concentration of agonist (10 µM isoproterenol for B2AR singly transfected and B2AR / B2AR-ala co-transfected cells, or 10 µM isoproterenol + 10 µM etorphine for B2AR / DOR co-transfected cells). Cells were then chilled on ice, washed 3 times with PBS and extracted with 1 ml/dish ice-cold TX-100 extraction buffer (0.5% (v/v) TX-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotinin, 2 µg/ml PMSF). Extracts were clarified by centrifugation in a microcentrifuge (12,000 x g for 10 minutes) prior to immunoprecipitation of receptors.

Receptors were immunoprecipitated from cell extracts using 3 µg anti-HA (HA.11) or 3 µg anti-FLAG (M2) monoclonal antibodies (Sigma), 6 µg rabbit anti-mouse linker antibody (Jackson ImmunoResearch) and 30 µl of protein A Sepharose beads (Pharmacia Biotech) as described previously (Cao et al., 1998). To minimize nonspecific binding, immunoprecipitates were spun through a 1 M sucrose cushion in a "high-stringency" wash buffer (HSB: 0.1% SDS, 0.5% TX-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM NaCl, 25 mM KCl) (Mays et al., 1995), then washed sequentially three

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

times with 1 M NaCl in HSB followed by three washes with a low-salt wash buffer (0.5% TX-100 in 10 mM Tris-HCl, pH 7.5). Washed beads were extracted with SDS sample buffer containing 20 mM DTT and eluted proteins were resolved by SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and blocked for 30 minutes in 5% dry milk, 0.1% Tween-20 in TBS. Biotinylated proteins were detected using horseradish peroxidase (VectaStain ABC, Vector Laboratories) followed by enzyme-linked chemiluminescence (ECL system, Amersham Life Sciences). Band intensities were quantified by densitometry (Alpha Innotech) of films (Kodak XAR) exposed in the linear range.

Visualization of receptor trafficking by fluorescence microscopy

Cells were grown on poly-L-lysine coated coverslips and incubated with 10 µg/ml anti-HA (HA.11) and 10 µg/ml anti-FLAG (M1) monoclonal antibodies (Sigma), and 10 µM isoproterenol at 37ÚC for 30 minutes to drive internalization of labeled B2ARs. Coverslips were then washed with ice-cold PBS to remove residual agonist and either immediately fixed, to determine total internalized receptors, or incubated for the indicated time periods at 37ÚC with culture media containing 10 µM of the adrenergic antagonist alprenolol (Sigma). At the indicated time point cells were then fixed in 4% formaldehyde (Sigma) dissolved freshly in PBS, pH 7.4 for 10 minutes and then quenched with 3 washes of TBS + 1 mM $CaCl_2$. Specimens were permeabilized with 0.1% TX-100, 3% bovine serum albumin (Sigma) in TBS + 1 mM $CaCl_2$, and incubated with 1:1000 rabbit anti-mouse IgG_{2b} antibody (Zymed) to

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

specifically label M1-bound FLAG-B2AR. A fluorescein-conjugated goat anti-mouse IgG_1 (Boehringer Mannheim) followed by Texas Red-conjugated goat anti-rabbit (1:1000 dilution, Jackson ImmunoResearch) was used to specifically detect labeled HA-B2AR-ala and FLAG-B2AR, respectively, as described previously for specific detection of co-expressed dopamine receptors (Vickery and von Zastrow, 1999). Epifluorescence microscopy was carried out using an inverted Nikon microscope, a Nikon 60X NA1.4 objective and standard filter sets (Omega Optical) that have been previously established to prevent detectable bleedthrough between fluorescein and Texas Red channels (Vickery and von Zastrow, 1999). Images were collected using a cooled (10ÚC) charge-coupled device (CCD) camera (Princeton Instruments). Illumination intensity and exposure time were adjusted to prevent pixel saturation, and 12-bit images were collected and processed uniformly using IPLab Spectrum software (Scanalytics) running on a Macintosh computer.

Fluorescence flow cytometric assay of receptor recycling

Cells were grown on 6-well tissue culture dishes (not coated with poly-L-lysine) and incubated with 5 µg/ml M1 antibody for 30 minutes to label FLAG-tagged receptors present in the plasma membrane, then incubated in the absence (’untreated’ condition) or presence of 10 µM isoproterenol for 30 minutes at 37ÚC. Cells were chilled to 4ÚC and untreated cells were washed with PBS containing 1mM $CaCl_2$ whereas agonist-treated cells were washed with calcium-free PBS supplemented with 1mM EDTA to dissociate antibodies from receptors remaining in the plasma membrane. Agonist-treated dishes were

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

either left at 4ÚC ('iso' condition), or washed and incubated in media containing excess (10 µM) of the adrenergic antagonist alprenolol ('iso -> alp' condition) for 15, 30, or 60 minutes at 37ÚC. Cells were collected from dishes by gentle mechanical dissociation at 4C and approximately 2 x 10^5 cells were incubated in suspension for 1 h at 4 °C in 100 µl of phosphate-buffered saline, 2% fetal bovine serum containing a phycoerythrin-conjugated goat anti-mouse secondary antibody (1:70, Dako) then fixed in 1.5% paraformaldehyde freshly dissolved in PBS. Cells were analyzed for PE fluorescence using a FACScan flow cytometer (BD Biosciences). Mean fluorescence intensities were determined from fluorescence histograms (representing analysis of 10,000 cells per data point) using CellQuest software. Mean fluorescence values were used to compute the fractional recycling of antibody-labeled receptors after agonist washout as follows:

$$\% \text{ recycling} = \frac{\text{Mean PE ('iso -> alp') – Mean PE ('iso')}}{\text{Mean PE ('untreated') – Mean PE ('iso')}} \times 100$$

Analysis of receptor recycling using fluorescence ratio imaging

Recycling of FLAG antibody-labeled mutant B2ARs was quantified in individual cells by a previously established method using fluorescence ratio imaging (Tanowitz and von Zastrow, 2003). Briefly, stably transfected cell clones plated on glass coverslips were incubated with 5 µg/ml Alexa488-conjugated M1 anti-FLAG antibody (prepared by standard methods using Alexa-fluor 488 N-hydroxysuccinimide ester, Molecular Probes) to selectively label FLAG-tagged receptors present in the plasma membrane at the beginning of the experiment. Then cells were incubated (at 37 °C for 30 min) in the presence of 10

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

μM isoproterenol to promote internalization of receptors. Cells were then quickly washed three times at 4ÚC in PBS lacking Ca 2+ or Mg 2+ and supplemented with 0.04% EDTA, to dissociate FLAG antibody bound to residual surface receptors remaining in the plasma membrane, thereby leaving antibody bound only to the internalized pool of receptors. EDTA-stripped cells were then incubated (at 37 °C for 45 min) in the presence of 10 μM alprenolol to prevent receptor activation by possible residual agonist, then cells were fixed without permeabilizing by exposure to 4% paraformaldehyde freshly dissolved in PBS at 4ÚC for 20 minutes. Cells were washed in PBS, blocked using PBS containing 3% BSA (without detergent), and surface-accessible FLAG antibody was detected by incubation at 4ÚC for 30 minutes with 5 μg/ml Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) followed by 3 washes in PBS to remove unbound antibody. In each experiment, and for each receptor construct examined, two parallel control coverslips were included, one in which cells were fixed after a 30-min incubation in the absence of agonist and without an EDTA stripping step (100% surface control) and one in which cells were fixed immediately after the EDTA-mediated stripping step (0% surface control). Cells were examined by epifluorescence microscopy using appropriate filter sets to selectively detect Alexa488 or Cy3, images were collected under non-saturating conditions using the 12-bit CCD camera, and staining intensities of each fluorochrome in individual cells were integrated using IPLab software. The percentage of FLAG-labeled receptors recycled in individual cells (20 – 30 per coverslip) following agonist washout was then calculated from the red/green ratios determined from the control conditions according to the following formula: (E-Z)/(C-Z)x100, where E= the mean ratio for the experimental coverslip, Z= the mean ratio for the 0% surface control, and C= the mean ratio for the

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

100% surface control.

Co-immunoprecipitation of receptors

Cell monolayers maintained in 6 cm tissue culture dishes (60 – 80% confluence) were incubated for 15 minutes at 37°C in the absence or presence of 10 μM isoproterenol (together with 10 μM etorphine for cells co-expressing B2AR and DOR), then rapidly chilled to 4ÚC and washed 3 times in ice-cold PBS. Monolayers were extracted with 1.0 ml / dish NDM buffer (0.3% n-dodecyl-ß-D-maltoside [Sigma] in TBS + 1 mM $CaCl_2$, supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml freshly prepared PMSF and supplemented with 5 mM freshly prepared iodoacetamide). Extracts were clarified by centrifugation and receptors were immunoprecipitated by adding 0.3 μg HA.11 or 0.3 μgM1 antibody and 30 μl protein A Sepharose. HA.11 immunoprecipitations were supplemented with 0.4 μg rabbit anti-mouse linker antibody (Jackson ImmunoResearch). Antibody incubations were conducted for 3 hours at 4ÚC with slow inversion of tubes. Immunoprecipitates were collected by sedimentation using a microcentrifuge, then resuspended and washed 5 times with 1.0 ml NDM buffer (without added iodoacetamide). Washed beads were eluted with SDS sample buffer under non-reducing conditions (to mimimize dissociation of IgG chains in the gel) and resolved by SDS-PAGE using 10% acrylamide (BioRad). Proteins were transferred to nitrocellulose membrane, blocked with 5% dry milk, 0.1% Tween-20 in TBS supplemented with 1mM $CaCl_2$ and probed with 3 μg/ml HA.11 (anti-HA) or 3 μg/ml M1 (anti-FLAG). In some experiments the wild type B2AR was also detected using a 1:1000

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

dilution of a previously described antiserum (86.72) generated against a peptide corresponding to the extreme C terminus of the receptor (von Zastrow and Kobilka, 1992). As this antibody recognizes the wild type B2AR tail better than the B2AR-ala mutant tail (not shown), this reagent was used only for detection of receptors containing the wild type B2AR tail. Blots were washed and incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG (1:1000, Jackson ImmunoResearch) and immunoreactive receptors were detected by enzyme-linked chemiluminescence.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

A FLAG epitope-tagged version of the wild type B2AR (FLAG-B2AR) was not detectably proteolyzed after exposure of stably transfected 293 cells to a saturating concentration of agonist (10 μM isoproterenol) for 4 hours after surface biotinylation in stably transfected HEK293 cells (Figure 1 A, left panel), whereas adding an alanine residue to the carboxyl-terminus resulted in extensive proteolysis of receptors under these conditions (Figure 1 A, right panel). These results are consistent with previous studies indicating that the wild type B2AR can endocytose and recycle repeatedly in the continuous presence of agonist (Kurz and Perkins, 1992; Tsao and von Zastrow, 2000b; von Zastrow and Kobilka, 1992) and that disruption of a specific 'recycling signal' present in distal cytoplasmic tail inhibits recycling and promotes proteolytic degradation of internalized receptors (Cao et al., 1999; Cong et al., 2001; Gage et al., 2001). Significantly different results were obtained in doubly transfected cells co-expressing FLAG-tagged B2AR and HA-tagged B2AR-ala mutant receptors together. In these cells, both the 'wild type' FLAG-B2AR and recycling-defective HA-B2AR-ala mutant receptors were extensively proteolyzed within 4 hours after agonist addition to the culture medium (Figure 1B, left and right panels). Quantification of these results confirmed, in multiple experiments conducted on independently isolated cell clones (n=3), that co-expression of the HA-B2AR-ala mutant receptor significantly enhanced agonist-induced proteolysis of the FLAG-B2AR (Figure 1 C). Agonist-induced proteolysis of both receptors was strongly (~75%) inhibited when agonist incubations were conducted (as described previously (Tsao and von Zastrow, 2000b)) with chloroquine (200 μM) or ammonium chloride (50 mM) added to the culture medium (not shown), consistent with previous studies

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

demonstrating lysosomal trafficking of wild type B2AR and B2AR-ala mutant receptors in HEK293 cells (Cao et al., 1999; Gagnon et al., 1998). Together these observations suggested that co-expression of the B2AR-ala mutant receptor may affect membrane trafficking of the wild type B2AR after agonist-induced endocytosis.

To further investigate this possibility, fluorescence microscopy was used to visualize the distribution of antibody-labeled receptors in stably transfected HEK293 cells expressing these receptors separately or in combination. In singly transfected cells (Figure 2 A, top panels), the FLAG-B2AR was observed to redistribute from the plasma membrane to numerous endocytic vesicles within 30 minutes after addition of agonist (10 μM isoproterenol) to the culture medium (panels a and b). A nearly complete return of receptor immunoreactivity to the plasma membrane was evident within ~30 minutes after agonist washout (panels c – e), consistent with efficient recycling of this receptor shown previously in HEK293 cells under similar conditions (Cao et al., 1999; Tsao and von Zastrow, 2000b). The FLAG-B2AR-ala mutant receptor (Figure 2 A, bottom panels) also internalized rapidly after agonist addition (panels f and g) but, consistent with inhibited recycling shown previously (Cao et al., 1999), remained in numerous endocytic vesicles after agonist washout (panel j). In doubly transfected cells (Figure 2 B), in which FLAG-B2AR and HA-B2AR-ala mutant receptors were expressed together at similar levels (see *Materials and Methods*), both receptors colocalized in the plasma membrane of untreated cells and translocated to an overlapping population of endocytic vesicles following agonist addition to the culture medium (panels b and g), consistent with previous studies indicating that both receptors undergo

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

agonist-induced endocytosis via a similar membrane pathway (Cao et al., 1999). However, in marked contrast to results obtained in singly transfected cells, FLAG-B2AR did not recycle completely to the plasma membrane after agonist washout in doubly transfected cells. Numerous endocytic membrane structures containing antibody-labeled FLAG-B2AR were observed in cells at all time points after agonist washout (compare Figure 2 B and 2 A, panels c – e), and internalized FLAG-B2AR colocalized extensively with HA-B2AR-ala mutant receptors in these intracellular membranes (Figure 2 B, panels e and j).

We next examined the effects of HA-B2AR-ala co-expression on FLAG-B2AR recycling using a flow cytometric assay in which recycling of antibody-labeled receptors was detected by accessibility to a fluorochrome-conjugated secondary antibody in non-permeabilized cells (see *Materials and Methods*). In singly transfected cells FLAG-B2AR recycled efficiently within 60 minutes after agonist washout, as indicated by the nearly complete recovery of antibody-labeled receptors accessible at the surface of non-permeabilized cells (Figure 3A, left histogram). As expected, the FLAG-tagged B2AR-ala mutant receptor recycled to a much smaller extent, indicated by a pronounced left-shift in the histogram representing recycled receptors (Figure 3A, middle panel; compare distributions indicated by arrows in left and middle panels). In cells co-transfected with FLAG-B2AR and HA-B2AR-ala, the histogram representing recycled FLAG-B2AR (Figure 3A, right panel, arrow) was significantly left-shifted relative to that characteristic of the FLAG-B2AR in singly transfected cells (left panel), although recycling of wild type receptors in doubly transfected cells still seemed to be greater than that of the

recycling-defective FLAG-B2AR-ala mutant receptor observed either in singly transfected cells (middle panel) or doubly transfected cells (see below). These observations were verified across multiple experiments, and fractional recovery of surface receptor immunoreactivity was calculated from mean fluorescence intensity determinations (see *Materials and Methods*) at several time points after agonist washout. This analysis confirmed that co-expression of the HA-B2AR-ala mutant receptor significantly inhibited recycling of FLAG-B2AR in doubly transfected cells. However, the observed inhibition was not complete and was most pronounced at later time points after agonist washout (Figure 3B). This inhibitory effect on FLAG-B2AR recycling was further verified using a different recycling assay (Tanowitz and von Zastrow, 2003) based on quantitative fluorescence microscopy (Figure 3C, bars 1 – 3 from left). Two other recycling-defective mutant versions of the B2AR, in which the recycling signal was either removed by truncation (HA-B2AR-T364) or disrupted by fusion of a carboxyl-terminal epitope tag sequence (HA-B2AR-EE) (Cao et al., 1999), were also tested and found to produce significant inhibition of FLAG-B2AR recycling as the B2AR-ala mutant receptor (Figure 3C, bars 4 and 5 from left). To investigate the converse possibility, that the wild type B2AR may enhance recycling of a recycling defective mutant receptor, we generated expression-matched stably transfected HEK293 cells expressing a FLAG-tagged version of the recycling-defective B2AR-EE mutant receptor by itself, or together with an HA-tagged version of wild type B2AR. As expected the FLAG-B2AR-EE recycled poorly when examined in singly transfected HEK293 cells (Figure 3C, bar 6 from left). This mutant receptor also recycled poorly in doubly transfected cells co-expressing an HA-tagged 'wild type' receptor (Figure 3C, bar 7). Examination of mean recycling values averaged over

multiple experiments (Figure 3 C, compare bars 6 and 7) failed to detect a statistically significant enhancement of FLAG-B2AR-EE recycling by co-expression of the (recycling-competent) HA-B2AR, in contrast to the highly significant converse effect detected by the same assay. Thus the major effect observed in doubly transfected cells was inhibited recycling of the wild type B2AR. These results are consistent with the previous data (Figure 1) indicating that both wild type and tail-mutant receptors exhibit pronounced agonist-induced proteolysis when co-expressed.

To investigate the specificity of the observed *in trans* effects on endocytic trafficking, we focused on the delta opioid receptor (DOR), a distinct GPCR that internalizes by an indistinguishable early endocytic pathway as the B2AR (Keith et al., 1996; Tsao and von Zastrow, 2000b; Zhang et al., 1999). Unlike the wild type B2AR, but similar to the B2AR-ala mutant receptor, DOR recycles inefficiently after agonist-induced endocytosis in HEK293 cells and traffics preferentially to lysosomes (Tsao and von Zastrow, 2000b). DOR is capable of forming mixed oligomers with the B2AR and this process has been reported to influence agonist-induced endocytosis of receptors (Jordan et al., 2001), although relatively high levels of receptor expression (>250,000 receptors / cell) are required for detectable B2AR / DOR hetero-oligomer formation in HEK293 cells (Ramsay et al., 2002). Other studies, using significantly lower levels of receptor expression, observed differential agonist regulation of co-expressed adrenergic and opioid receptor endocytosis in the same (HEK293) cell background (He et al., 2002; Klein et al., 2001; Tsao and von Zastrow, 2000b). Thus we generated stably transfected cell clones expressing epitope-tagged DOR at levels closely similar to those of mutant B2AR constructs examined above. We

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

confirmed by fluorescence microscopy that adrenergic (10 μM isoproterenol) and opioid (10 μM etorphine) agonist selectively promoted endocytosis of FLAG-B2AR or HA-DOR, respectively (not shown), as observed previously (Tsao and von Zastrow, 2000b). Then the surface biotinylation assay was used to assay proteolysis of differentially tagged B2AR and DOR constructs in singly and doubly transfected cells in the presence of both agonists, a condition which is sufficient to promote rapid endocytosis of both receptors (Klein et al., 2001; Tsao and von Zastrow, 2000b). Surface biotinylated FLAG-B2AR was not detectably proteolyzed in doubly transfected cells over a 4 hour time course, as indicated by the uniform recovery in anti-FLAG immunoprecipitates (Figure 4 A, left panel), whereas HA-DOR was proteolyzed almost completely in the same cells (Figure 4A, right panel). These results are fully consistent with the differential endocytic trafficking of receptors established previously in singly transfected cells (Tsao and von Zastrow, 2000b), and are in marked contrast to the pronounced *in trans* enhancement of FLAG-B2AR proteolysis produced by co-expression of HA-B2AR-ala (Figure 1). Together these observations suggest that DOR has a significantly less pronounced effect on endocytic trafficking of the wild type B2AR than do recycling-defective mutant B2ARs, at least when evaluated at similar "moderate" levels of receptor expression.

We next investigated whether the selective effect of B2AR-ala relative to DOR on B2AR membrane traffic was associated with a biochemical difference in the ability of these receptors to form oligomeric complexes in intact cells. To accomplish this we immunoprecipitated receptors, from the same doubly transfected cell clones as used for trafficking assays, and assayed for co-immunoprecipitation of the

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

other receptor by immunoblotting. FLAG-B2AR / HA-B2AR-ala complexes were readily detected using either anti-FLAG or anti-HA immunoprecipitation (Figure 4 B). In most experiments a major fraction (>50%) of co-immunoprecipitated receptor immunoreactivity resolved after solubilization in sample buffer with an electrophoretic mobility corresponding to the complex-glycosylated monomeric receptor (~55 – 75kD, indicated by the bracket marked 'B2AR' in Figure 4 B). In some experiments receptor species resolving at higher apparent molecular mass were also observed (not shown), consistent with previous studies indicating that HA-B2AR-ala and FLAG-B2AR may associate in these cells both in dimeric and higher order oligomeric complexes (Salahpour et al., 2003). Amounts of HA-B2AR-ala / FLAG-B2AR complexes recovered from extracts were not significantly affected by agonist pretreatment of cells (compare lanes marked '-' and '+' in each panel), consistent with previous studies indicating that B2AR dimers and oligomers form constitutively and are not disrupted by agonist activation (Mercier et al., 2002; Salahpour et al., 2003). In contrast HA-DOR / FLAG-B2AR complexes were detected at much lower levels, similar to non-specific background levels, at the electrophoretic mobility corresponding to SDS-solubilized monomeric or larger forms (see legend), using identical conditions of anti-HA or anti-FLAG immunoprecipitation from the respective (expression-matched) doubly transfected cell clones and after incubation of cells either in the absence ('-') or presence ('+') of 10 μM each of isoproterenol and etorphine (Figure 4 C, left and right panels). These results are consistent with previous biophysical studies regarding B2AR / DOR hetero-oligomer formation in vivo (Ramsay et al., 2002) and demonstrate preferential formation of B2AR / B2AR-ala receptor complexes in the same cells in which a specific effect on endocytic trafficking of receptors was

observed.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

The present studies demonstrate that recycling-defective mutant versions of the B2AR, when co-expressed at similar moderate levels with the wild type B2AR, function in *trans* to inhibit recycling of the wild type allele. Inhibited recycling of the B2AR was associated with increased agonist-induced proteolysis following surface biotinylation. This proteolysis was sensitive to inhibitors of lysosome function and the internalized pool of B2AR colocalized extensively with the recycling-defective B2AR-ala mutant receptor, which has been shown previously to traffic rapidly to lysosomes in HEK293 cells (Cao et al., 1999). Co-expression of DOR at comparable levels produced a less pronounced effect on B2AR post-endocytic trafficking, even though DOR traffics to lysosomes efficiently and via a similar endocytic pathway as the recycling-defective mutant versions of the B2AR studied (Cao et al., 1999; Gage et al., 2001; Tsao and von Zastrow, 2000b). B2AR / B2AR-ala complexes were readily detected by co-immunoprecipitation in cells in which post-endocytic trafficking effects were observed, whereas B2AR / DOR complexes were not detected in comparable amount using the same methods in expression-matched cell clones. Taken together, these results indicate that recycling-defective mutant versions of the B2AR produce a *trans*-dominant effect on post-endocytic membrane trafficking of the wild type B2AR, and suggest that this effect is likely to represent a specific functional consequence B2AR oligomer formation occurring in intact cells. Despite compelling evidence supporting the existence of B2AR dimers and oligomers (Hebert et al., 1996; Mercier et al., 2002; Ramsay et al., 2002; Salahpour et al., 2003), and recent data indicating that oligomer formation is important for receptor export from the biosynthetic pathway (Salahpour et al., 2004), we believe the present results are the first

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

to identify an effect of B2AR oligomerization at later stages of receptor membrane traffic.

The presence of B2AR oligomers in the endocytic pathway is consistent with previous studies of several other GPCRs (He et al., 2002; Jordan et al., 2001; Overton and Blumer, 2000; Terrillon et al., 2004; Yesilaltay and Jenness, 2000) and with the ability of B2AR oligomers to remain intact after agonist activation (Mercier et al., 2002; Salahpour et al., 2003) (also evident in Figure 4 B). It is interesting to note that hetero-dimerization of V1 and V2 vasopressin receptors was shown recently to produce *in trans* effects on receptor endocytosis and recycling, although possible effect(s) on lysosomal traffic of receptor proteolysis were not investigated (Terrillon et al., 2004). In the case of hetero-dimerization of vasopressin receptors, the observed differences in recycling were proposed to be mediated by differences in endosome localization of arrestins elicited by activation of these distinct class A and class B GPCRs (Oakley et al., 2000; Terrillon et al., 2004). To our knowledge, the present results are the first to provide evidence for a significant role of homo- (as opposed to hetero-) oligomerization in determining the post-endocytic membrane trafficking (as opposed to initial endocytosis) of any GPCR. We also note that the B2AR, as well as the B2AR-ala mutant receptor (not shown), does not promote detectable endosome recruitment of arrestins, in contrast to the V2 vasopressin receptor (Oakley et al., 2000)(Klein et al., 2001; Terrillon et al., 2004). Thus we also believe that the present results provide the first evidence for *in trans* post-endocytic sorting effects that are not associated with differential endosome association of arrestins.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

A limitation of the present studies is that they were conducted using heterologous expression of recombinant receptors. Although we have taken care to examine receptor trafficking effects in cells expressing receptors at moderate levels, which are significantly below levels at which nonspecific receptor interactions have been observed previously using energy transfer assays (Mercier et al., 2002; Ramsay et al., 2002; Salahpour et al., 2003), these levels of receptor expression may still exceed those occurring in native tissues (Mercier et al., 2002). A second caveat is that, because we have focused entirely on trafficking of the B2AR and the specificity of endocytic sorting effects in expression-matched cell clones, we do not presently know to what degree the present results can be generalized to other GPCRs. Nevertheless, it is increasingly evident that many GPCRs can form dimers or larger oligomeric complexes when expressed at moderate levels (Bouvier, 2001; Milligan, 2001). Thus, taken together with the recent functional studies of vasopressin receptors (Terrillon et al., 2004), it seems likely that specific homo and hetero –oligomerization events could be of rather widespread importance in determining the post-endocytic fate of GPCRs. A third limitation is that we have focused entirely on the novel question of oligomerization effects on post-endocytic membrane traffic of the B2AR, in contrast to effects of oligomerization on initial endocytosis as shown previously for a number of other GPCRs (He et al., 2002; Jordan et al., 2001; Overton and Blumer, 2000; Yesilaltay and Jenness, 2000). This could be addressed in future studies using endocytosis-defective mutant receptors, as was done previously to demonstrate endocytosis of GPCR oligomers in yeast (Overton and Blumer, 2000; Yesilaltay and Jenness, 2000). A fourth caveat is that our results do not define the stoichiometry of B2AR oligomerization relevant to post-endocytic membrane traffic. While our co-

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

immunoprecipitation experiments readily detected complexes containing both FLAG-B2AR and HA-B2AR-ala mutant receptors, they do not define the stoichiometry. Recycling assays indicated that the inhibitory effect of B2AR-ala on recycling of co-expressed B2AR was not complete (Figure 2), and recycling at the earliest time point was not detectably affected (Figure 2 B). The existence of B2AR in multiple forms, including monomers and various oligomeric complexes, is consistent with previous biochemical and biophysical studies (Mercier et al., 2002; Salahpour et al., 2003). It will be interesting in future experiments to define the specific oligomeric form(s) of the receptor protein relevant to post-endocytic sorting.

Our results also provide cell biological support for the existence of considerable specificity in GPCR oligomer formation. While previous studies indicate that B2AR / DOR complexes can form in intact cells (Jordan et al., 2001; Ramsay et al., 2002) and influence receptor endocytosis (Jordan et al., 2001), we observed relatively little effect of DOR co-expression on B2AR endocytic trafficking in the present work. A clue to this apparent discrepancy is that biophysical studies indicate that detectable B2AR / DOR hetero-oligomer formation requires expression levels in excess of 250,000 receptors / cell for both receptors (Ramsay et al., 2002), much higher than required to detect B2AR / B2AR homo-oligomers (Mercier et al., 2002; Ramsay et al., 2002) and also substantially higher than expression levels achieved in any of our doubly transfected cell clones (see *Materials and Methods*). Thus we believe that, while it is possible to generate both B2AR / B2AR and B2AR / DOR complexes in intact cells at sufficiently high levels of receptor expression, B2AR/ B2AR homo-oligomers are highly favored at moderate levels

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

that are more likely to be physiologically relevant (Mercier et al., 2002). This hypothesis is consistent with previous studies of differential receptor endocytosis (Chu et al., 1997; He et al., 2002; Tsao and von Zastrow, 2000b) and with the present co-immunoprecipitation studies. So far we have been unable to generate stably co-transfected cell clones expressing both FLAG-B2AR and HA-DOR at >250,000 receptors / cell using the present system, but we have observed evidence for impaired recycling of FLAG-B2AR (in immunocytochemical experiments similar to those shown in Figure 2) when HA-DOR was expressed by transient transfection at very high levels (≥5-fold over those tested in the present stably transfected cells, as estimated by relative fluorescence intensity), suggesting that B2AR / DOR hetero-oligomers can indeed occur influence endocytic traffic under some conditions (not shown). We also note that, while DOR undergoes rapid proteolysis in HEK293 cells and certain neuronal cell types when activated either by peptide or alkaloid agonists (Law and Loh, 1999; Tsao and von Zastrow, 2000b), a recent study suggests that post-endocytic sorting of this receptor can be differentially regulated by distinct agonists in SK-N-BE neuroblastoma cells (Marie et al., 2003). It will be interesting to investigate this phenomenon in future studies, and to determine if this additional complexity of GPCR regulation observed in certain cell types may reflect the existence of ligand-specific conformational states or oligomeric complexes.

In conclusion, the present results provide evidence that the composition of the B2AR oligomer influences the sorting of internalized receptors between distinct, and functionally important, downstream membrane pathways. While our studies are presently limited to a single type of GPCR and to a

transfected cell model system, they provide strong evidence for a role of oligomerization in determining the post-endocytic membrane trafficking of a specific GPCR, and they identify a novel functional consequence of B2AR oligomerization occurring in intact cells.

Acknowledgements

We thank Catherine de Coupade for conducting some of the radioligand binding assays, Paul Dazin and Nigel Killeen for advice and assistance with flow cytometry assays, Heather Deacon for advice on statistical analysis and Michael Tanowitz for advice on quantitative fluorescence microscopy.

REFERENCES

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M and Bouvier M (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci U S A* **97**:3684-3689.

Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* **2**:274-286.

Cao TC, Mays RW and von Zastrow M (1998) Regulated endocytosis of G protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem.* **273**:24592-24602.

Cao TT, Deacon HW, Reczek D, Bretscher A and von Zastrow M (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. *Nature* **401**:286-290.

Chu P, Murray S, Lissin D and von Zastrow M (1997) Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. *J Biol Chem* **272**:27124-27130.

Cong M, Perry SJ, Hu LA, Hanson PI, Claing A and Lefkowitz RJ (2001) Binding of the beta2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling. *J Biol Chem* **276**:45145-45152.

Evans CJ, Keith DJ, Morrison H, Magendzo K and Edwards RH (1992) Cloning of a delta opioid receptor by functional expression. *Science* **258**:1952-1955.

Ferguson SS, Zhang J, Barak LS and Caron MG (1998) Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci* **62**:1561-1565.

Gage RM, Kim KA, Cao TT and von Zastrow M (2001) A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. *J Biol Chem* **276**:44712-44720.

Gagnon AW, Kallal L and Benovic JL (1998) Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. *J Biol Chem* **273**:6976-6981.

Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH and Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2- adrenergic receptor. *Nature* **383**:447-450.

Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH and Benovic JL (1998) Role of arrestins in G-protein-coupled receptor endocytosis. *Adv Pharmacol* **42**:429-433.

Guan XM, Kobilka TS and Kobilka BK (1992) Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide. *J Biol Chem* **267**:21995-21998.

He L, Fong J, von Zastrow M and Whistler JL (2002) Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* **108**:271-282.

Hebert TE, Loisel TP, Adam L, Ethier N, Onge SS and Bouvier M (1998) Functional rescue of a constitutively desensitized beta2AR through receptor dimerization. *Biochem J* **330**:287-293.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, Barret C and Bouvier M (1996) A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* **271**:16384-16392.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R and Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc Natl Acad Sci U S A* **98**:343-348.

Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet AG, Stewart PL, Evans CJ and von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**:377-84.

Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271**:19021-4.

Klein U, Muller C, Chu P, Birnbaumer M and von Zastrow M (2001) Heterologous inhibition of G protein-coupled receptor endocytosis mediated by receptor-specific trafficking of beta-arrestins. *J Biol Chem* **276**:17442-7.

Kobilka BK, Dixon RA, Frielle T, Dohlman HG, Bolanowski MA, Sigal IS, Yang-Feng TL, Francke U, Caron MG and Lefkowitz RJ (1987) cDNA for the human beta 2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci U S A* **84**:46-50.

Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A and Kornau HC (1999) Role of heteromer formation in GABAB receptor function. *Science* **283**:74-7.

Kurz JB and Perkins JP (1992) Isoproterenol-initiated beta-adrenergic receptor diacytosis in cultured cells. *Mol Pharmacol* **41**:375-81.

Law PY and Loh HH (1999) Regulation of opioid receptor activities. *J Pharmacol Exp Ther* **289**:607-24.

Lee SP, O'Dowd BF, Ng GY, Varghese G, Akil H, Mansour A, Nguyen T and George SR (2000) Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. *Mol Pharmacol* **58**:120-8.

Lefkowitz RJ, Pitcher J, Krueger K and Daaka Y (1998) Mechanisms of beta-adrenergic receptor desensitization and resensitization. *Adv Pharmacol* **42**:416-20.

Marie N, Lecoq I, Jauzac P and Allouche S (2003) Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists. *J Biol Chem* **278**:22795-804.

Mays RW, Siemers KA, Fritz BA, Lowe AW, van Meer G and Nelson WJ (1995) Hierarchy of mechanisms involved in generating Na/K-ATPase polarity in MDCK epithelial cells. *J Cell Biol* **130**:1105-15.

Mercier JF, Salahpour A, Angers S, Breit A and Bouvier M (2002) Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem* **277**:44925-31.

Milligan G (2001) Oligomerisation of G-protein-coupled receptors. *J Cell Sci* **114**:1265-71.

Oakley RH, Laporte SA, Holt JA, Caron MG and Barak LS (2000) Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* **275**:17201-10.

Okayama H and Berg P (1983) A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol Cell Biol* **3**:280-9.

Overton MC and Blumer KJ (2000) G-protein-coupled receptors function as oligomers in vivo. *Curr Biol* **10**:341-4.

Pippig S, Andexinger S and Lohse MJ (1995) Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666-76.

Ramsay D, Kellett E, McVey M, Rees S and Milligan G (2002) Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem J* **365**:429-40.

Salahpour A, Angers S, Mercier JF, Lagace M, Marullo S and Bouvier M (2004) Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting. *J Biol Chem* **279**:33390-7.

Salahpour A, Bonin H, Bhalla S, Petaja-Repo U and Bouvier M (2003) Biochemical characterization of beta2-adrenergic receptor dimers and oligomers. *Biol Chem* **384**:117-23.

Shenoy SK, McDonald PH, Kohout TA and Lefkowitz RJ (2001) Regulation of receptor fate by ubiquitination of activated beta 2- adrenergic receptor and beta-arrestin. *Science* **294**:1307-13.

Tanowitz M and von Zastrow M (2003) A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J Biol Chem.* **278**:45978-45986.

Terrillon S, Barberis C and Bouvier M (2004) Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. *Proc Natl Acad Sci U S A* **101**:1548-53.

Tsao P and von Zastrow M (2000a) Downregulation of G protein-coupled receptors. *Curr Opin Neurobiol* **10**:365-9.

Tsao PI and von Zastrow M (2000b) Type-specific sorting of G protein-coupled receptors after endocytosis. *J Biol Chem* **275**:11130-11140.

Vickery RG and von Zastrow M (1999) Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol* **144**:31-43.

von Zastrow M and Kobilka BK (1992) Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J Biol Chem* **267**:3530-8.

von Zastrow M and Kobilka BK (1994) Antagonist -dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J Biol Chem* **269**:18448-52.

Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray S and von Zastrow M (2002)

Modulation of Post-Endocytic Sorting of G Protein-Coupled Receptors. *Science* **297**:615-620.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM and Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* **396**:679-82.

Yesilaltay A and Jenness DD (2000) Homo-oligomeric complexes of the yeast alpha-factor pheromone receptor are functional units of endocytosis. *Mol Biol Cell* **11**:2873-84.

Zeng F and Wess J (2000) Molecular aspects of muscarinic receptor dimerization. *Neuropsychopharmacol* **23**:S19-31.

Zhang J, Ferguson S, Barak LS, Menard L and Caron MG (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem* **271**:18302-5.

Zhang J, Ferguson SS, Law PY, Barak LS and Caron MG (1999) Agonist-specific regulation of delta-opioid receptor trafficking by G protein-coupled receptor kinase and beta-arrestin. *J Rec Sig Trans Res* **19**:301-13.

FOOTNOTE

These studies were supported by research grants from the NIH (MvZ). TTC was supported for part of these studies by an NIH institutional predoctoral training grant and was awarded the UCSF Chancellor's Fund fellowship. AB was awarded an EMBO postdoctoral fellowship.

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

Figure 1. Effect of B2AR-ala mutant receptor co-expression on agonist-induced proteolysis of the wild type B2AR. **A.** Cells that were singly transfected with FLAG-B2AR (left panel) or HA-B2AR-ala were analyzed. 'Total' indicates biotinylated receptors recovered from cell extracts that were maintained on ice immediately after biotinylation. 'iso' indicates recovery from cells that were biotinylated and then incubated for 37ÚC for 4 hours in the absence of agonist prior to lysis. '+iso' indicates recovery from biotinylated cells after incubation in the presence of 10 μM isoproterenol for 4 hours prior to lysis. **B.** The equivalent experiment conducted using FLAG-B2AR / HA-B2AR-ala co-expressing cells. Results shown are representative of 3 independent cell clones for each receptor combination. **C.** The recovery of biotinylated receptors after agonist exposure of cells for 4 hours was estimated across multiple experiments by scanning densitometry of streptavidin-HRP blots. Bars 1 – 3 (from left) show recovery of the indicated biotinylated receptors isolated from singly transfected cells, and bars 4 – 5 show receptor recovery from doubly transfected (FLAG-B2AR / HA-B2AR-ala) cells. Error bars represent standard deviation among individual determinations (three cell clones each). Co-expression of HA-B2AR-ala produced a significant reduction in FLAG-B2AR (compare bar 4 to bar 1, * indicates $p<0.01$ by non-paired Student's t-test).

Figure 2. Effect on B2AR endocytic trafficking visualized by fluorescence microscopy. Cells expressing differentially epitope-tagged B2ARs were labeled at the cell surface with the appropriate

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

anti-HA or FLAG monoclonal, and receptor localization was visualized under the indicated conditions by epifluorescence microscopy. **A.** Receptor localization in singly transfected cells. **B.** Receptor localization in FLAG-B2AR / HA-B2AR-ala doubly transfected cells. The results shown are representative of five experiments using three clones of each singly and doubly transfected cell population.

Figure 3. Quantitative analysis of recycling effects. **A.** Fluorescence histograms representing surface-accessible FLAG-tagged receptor immunoreactivity in singly transfected cells expressing FLAG-B2AR (left panel), singly transfected cells expressing FLAG-B2AR-ala (middle panel), and doubly transfected cells co-expressing FLAG-B2AR and HA-B2AR-ala (right panel). Results are representative of three clones. Total B2AR expression levels in the example shown were 47,300 $\pm$ 3,900 receptors / cell (FLAG-B2AR); 64,200 $\pm$ 2,100 receptors / cell (FLAG-B2AR-ala); and 116,000 $\pm$ 2,800 receptors / cell (FLAG-B2AR / HA-B2AR-ala). **B.** Fractional recovery of antibody-labeled FLAG-B2AR to the plasma membrane after agonist washout calculated from flow cytometric data. Points represent mean surface receptor recovery at the indicated time points after agonist washout, calculated from mean fluorescence histograms (10,000 cells each, n=3 experiments). Error bars represent the standard error of the means across these independent determinations. **C.** Quantification of FLAG-tagged B2AR recycling by fluorescence ratio imaging performed 45 minutes after agonist washout. Bars represent the mean percentage of FLAG-tagged receptor recycling determined in three independent experiments. The transfected receptor complement is indicated below each bar. Error bars

Molecular Pharmacology Fast Forward. Published on October 18, 2004 as DOI: 10.1124/mol.104.003608
This article has not been copyedited and formatted. The final version may differ from this version.

represent the standard error of the mean across the experiments (n = 3 – 5 for each combination).

Figure 4. Examination of B2AR and DOR trafficking in doubly transfected cells and detection of receptor oligomers by co-immunoprecipitation. **A.** Doubly transfected HEK293 cells co-expressing FLAG-B2AR and HA-DOR were surface biotinylated and receptor recovery in cell extracts was determined at the indicated time points after addition of 10 μM isoproterenol and 10 μM etorphine to the culture medium. The results shown are representative of 3 separate experiments. **B.** Co-immunoprecipitation analysis of FLAG-B2AR / HA-B2AR-ala doubly transfected cells. *Left panel.* HA-B2AR-ala mutant receptors were immunoprecipitated from FLAG-B2AR / HA-B2AR-ala doubly transfected cells using HA.11 anti-HA monoclonal antibody for immunoprecipitation and blotting for FLAG-B2AR. '–' and '+' refer to preincubation (15 minutes) with 10 μM isoproterenol prior to cell lysis. 'Con' represents the nonspecific signal obtained from immunoprecipitates prepared from singly transfected cells that express FLAG-B2AR but not HA-B2AR-ala. *Right panel.* The converse experiment using anti-FLAG immunoprecipitation followed by anti-HA immunoblot. 'Con' represents the nonspecific signal detected in the control immunoprecipitate prepared from HA-B2AR-ala singly transfected cells. For both panels 'lysate' represents the signal of the blotted receptor (FLAG-B2AR or HA-B2AR-ala, respectively) detected in cell extracts corresponding to 5% of the input to the immunoprecipitation. **C.** Co-immunoprecipitation analysis of FLAG-B2AR / HA-DOR doubly transfected cells. *Left panel:* Expression-matched FLAG-B2AR / HA-DOR doubly transfected cells were immunoprecipitated with anti-HA monoclonal antibody and blotted for FLAG-B2AR. '-' and

'+' refer to preincubation (15 minutes) with both 10 μM isoproterenol and 10 μM etorphine prior to cell lysis. 'Con' represents negative control signal from FLAG-B2AR singly transfected cells. The additional band resolving near the top of the gel is a cross-reacting immunoglobulin species not related to the receptor (present also in the 'Con' lane). *Right panel:* The corresponding analysis performed using anti-FLAG immunoprecipitation followed by anti-HA immunoblot. For both panels 'lysate' represents the signal of the blotted receptor (FLAG-B2AR or HA-DOR, respectively) detected in cell extracts corresponding to 5% of the input to the immunoprecipitation.

Figure 1

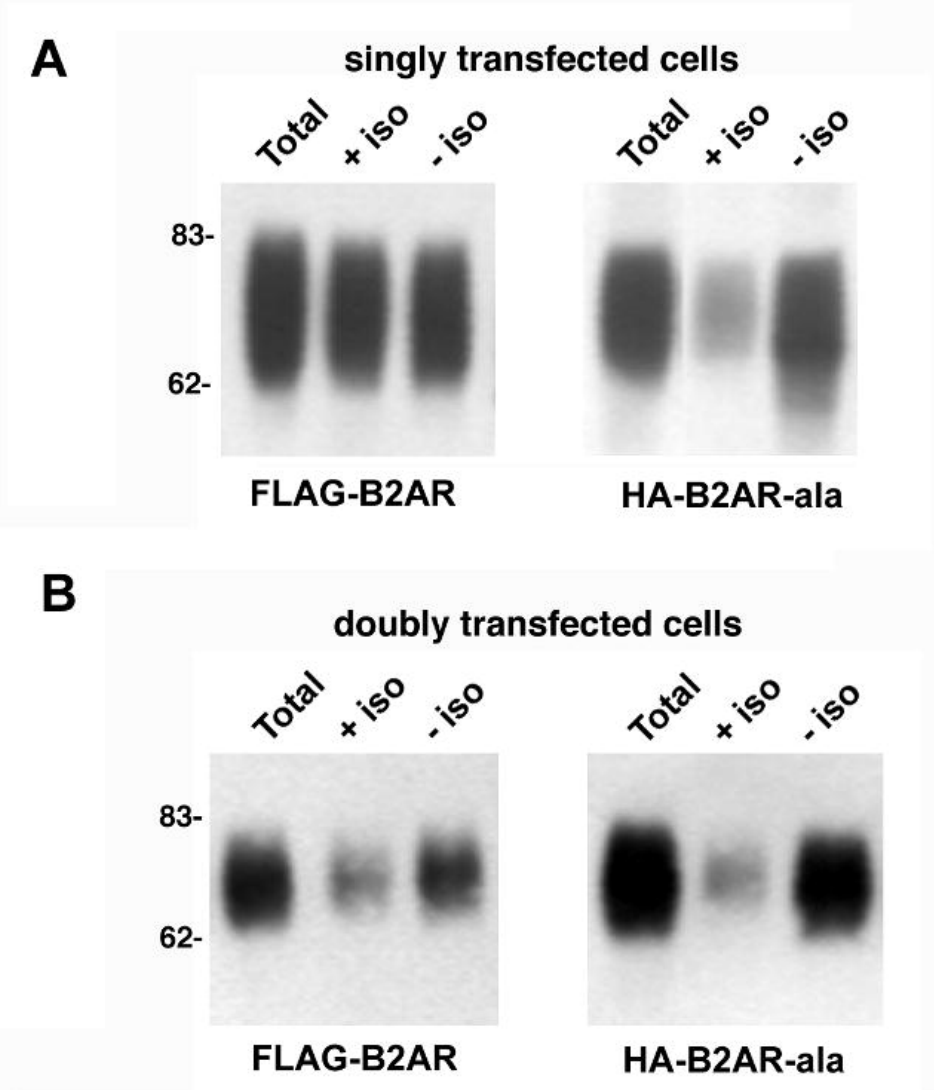

A
singly transfected cells
Total
+ iso
- iso
Total
+ iso
- iso
83-
62-
FLAG-B2AR
HA-B2AR-ala
B
doubly transfected cells
Total
+ iso
- iso
Total
+ iso
- iso
83-
62-
FLAG-B2AR
HA-B2AR-ala
C
singly transfected
doubly transfected
% recovery of biotinylated receptors
100
80
60
40
20
0
*
FLAG-B2AR
HA-B2AR
HA-B2AR-ala
FLAG-B2AR
HA-B2AR-ala

Figure 2

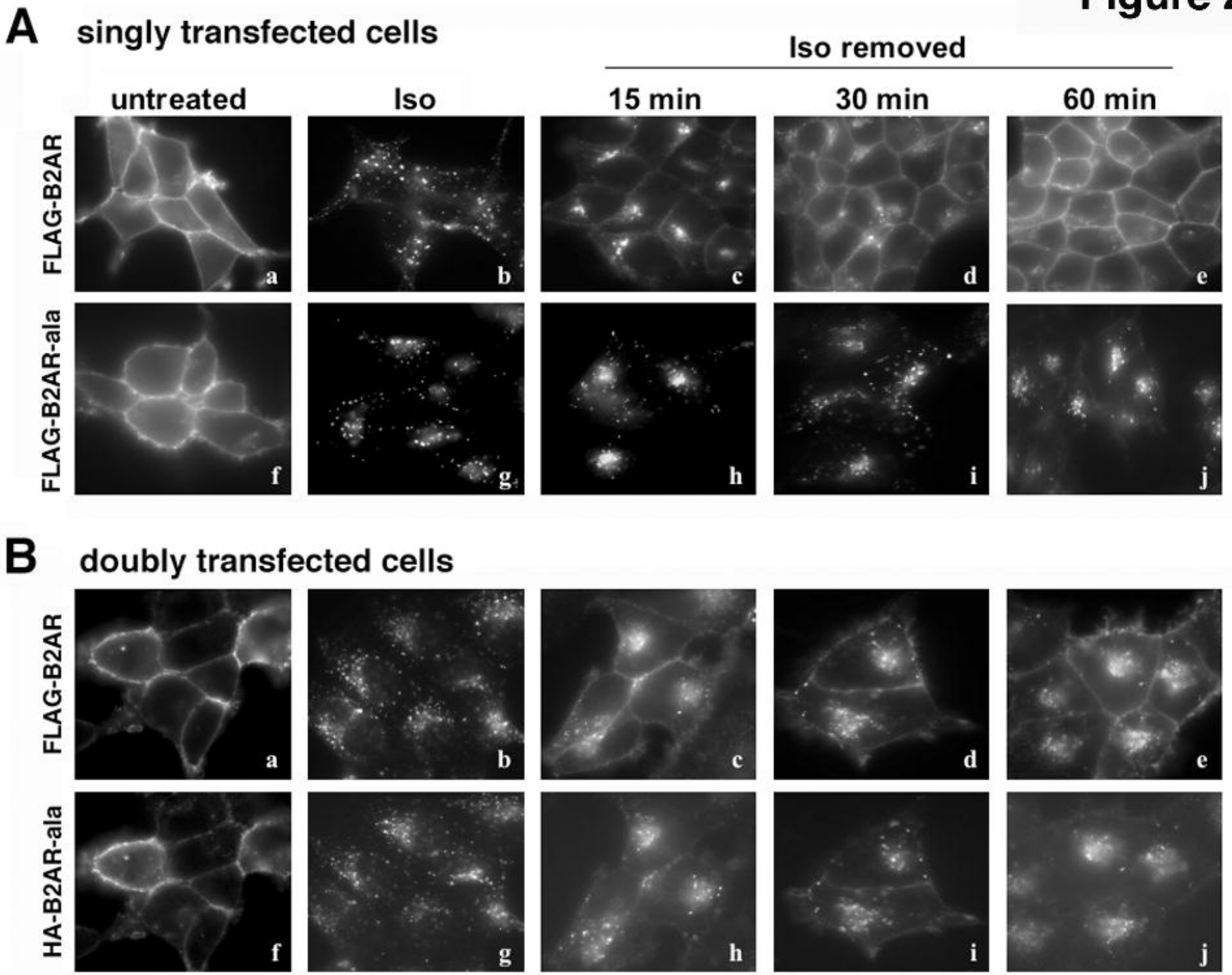

A
singly transfected cells
Iso removed
untreated
Iso
15 min
30 min
60 min
FLAG-B2AR
FLAG-B2AR-ala
a
b
c
d
e
f
g
h
i
j
B
doubly transfected cells
FLAG-B2AR
HA-B2AR-ala
a
b
c
d
e
f
g
h
i
j

Figure 3

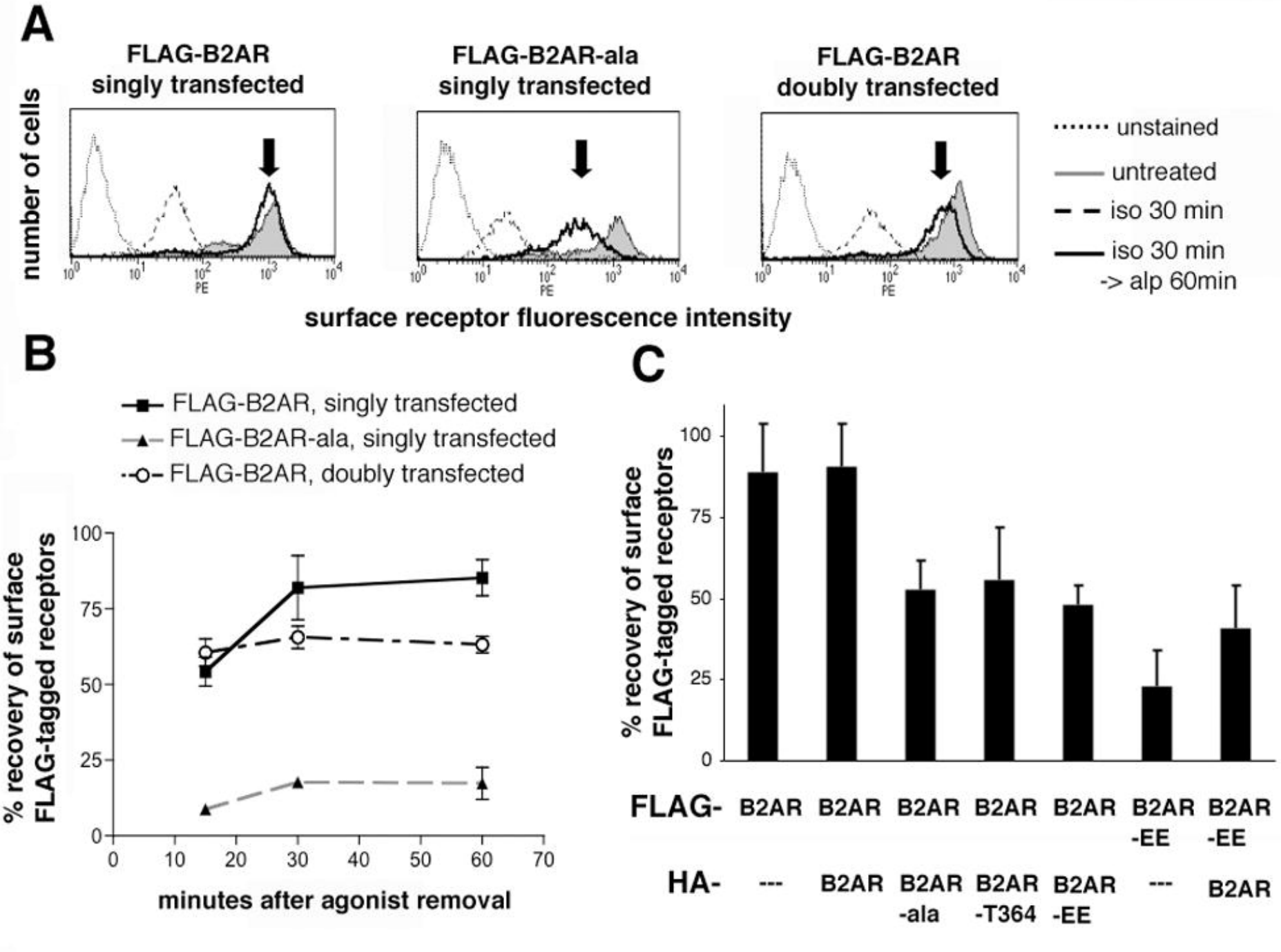
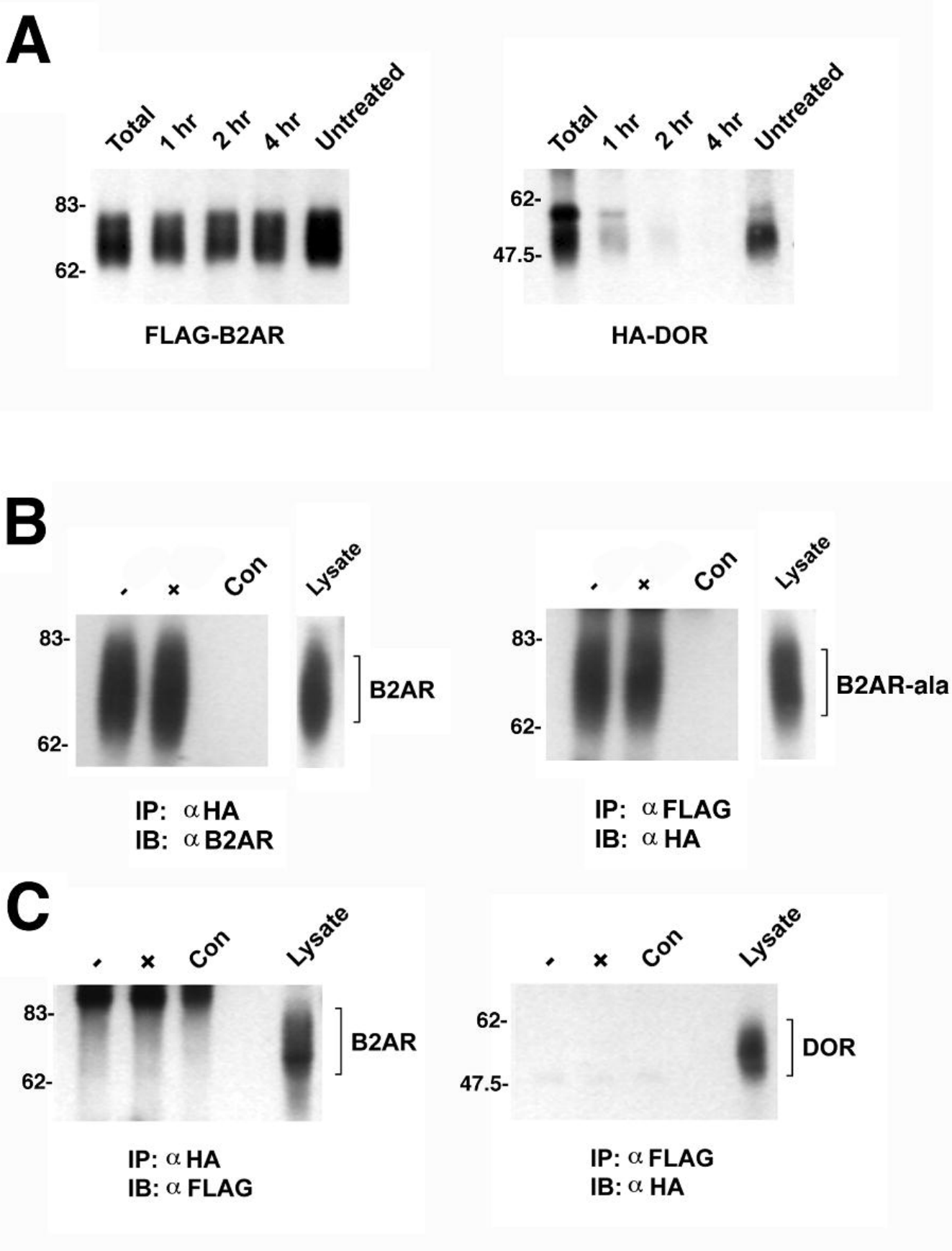

A
FLAG-B2AR
singly transfected
FLAG-B2AR-ala
singly transfected
FLAG-B2AR
doubly transfected
number of cells
PE
unstained
untreated
iso 30 min
iso 30 min
-> alp 60min
surface receptor fluorescence intensity
B
FLAG-B2AR, singly transfected
FLAG-B2AR-ala, singly transfected
FLAG-B2AR, doubly transfected
% recovery of surface
FLAG-tagged receptors
minutes after agonist removal
C
% recovery of surface
FLAG-tagged receptors
FLAG- B2AR B2AR B2AR B2AR B2AR B2AR B2AR
-EE -EE
HA- --- B2AR B2AR B2AR B2AR --- B2AR
-ala -T364 -EE

Figure 4


A
Total
1 hr
2 hr
4 hr
Untreated
83-
62-
FLAG-B2AR
Total
1 hr
2 hr
4 hr
Untreated
62-
47.5-
HA-DOR
B
-
+
Con
Lysate
83-
62-
B2AR
IP: αHA
IB: αB2AR
-
+
Con
Lysate
83-
62-
B2AR-ala
IP: αFLAG
IB: αHA
C
-
+
Con
Lysate
83-
62-
B2AR
IP: αHA
IB: αFLAG
-
+
Con
Lysate
62-
47.5-
DOR
IP: αFLAG
IB: αHA