THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 24, Issue of June 11, pp. 25590–25597, 2004
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β-Arrestin-dependent Constitutive Internalization of the Human Chemokine Decoy Receptor D6*

Received for publication, January 13, 2004, and in revised form, April 2, 2004
Published, JBC Papers in Press, April 13, 2004, DOI 10.1074/jbc.M400363200

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Seven transmembrane receptors mediate diverse physiological responses including hormone action, olfaction, neurotransmission, and chemotaxis. Human D6 is a non-signaling seven-transmembrane receptor expressed on lymphatic endothelium interacting with most inflammatory CC-chemokines resulting in their rapid internalization. Here, we demonstrate that this scavenging activity is mediated by continuous internalization and constant surface expression of the receptor, a process involving the clathrin-coated pit-dependent pathway. D6 constitutively associates with the cytoplasmic adaptor β-arrestin, and this interaction is essential for D6 internalization. An acidic region, but not the putative phosphorylation sites in the cytoplasmic tail of D6, is critical for receptor interaction with β-arrestin and subsequent internalization. Neither the native D6 nor mutants uncoupled from β-arrestin activate any G-protein-mediated signaling pathways. Therefore, D6 may be considered a decoy receptor structurally adapted to perform chemokine scavenging.

Leukocyte recruitment into inflamed tissues is mediated by chemokines acting on a distinct subfamily of G-protein-coupled receptors (GPCR).[1] This includes 18 receptors with the ability to activate $G_{\alpha i}$-protein-dependent signaling events and cell migration (1–4). Two other chemokine binding molecules, with homology to chemokine receptors but defective in signaling function, called Duffy antigen and D6, have been identified (5–7) and recently classified as "silent" chemokine receptors (8). Evidence in *in vitro* models, gene-targeted mice, and individuals with erythrocyte-restricted deficiency suggests that the Duffy antigen silent receptor may facilitate chemokine transport across endothelial cells as well as acting as a chemokine buffering and scavenging system (5, 9–12). D6 is highly expressed in endothelial cells lining afferent lymphatic vessels (13) and supports rapid internalization and degradation of inflammatory CC-chemokines, acting as a chemokine scavenger (7). D6 was suggested to act as a gatekeeper on afferent lymphatic endothelium, preventing excessive transfer to lymph nodes of inflammatory chemokines and disruptive leukocyte recruitment (7). The molecular mechanisms underlying this scavenging function are unknown.

Upon agonist binding, most GPCRs activate a signaling cascade mediated by G-protein activation leading to receptor phosphorylation by a G-protein-coupled receptor kinase, which results in uncoupling of the receptor from G-proteins (14). This desensitization process is further facilitated by the association of the phosphorylated receptors with the cytoplasmic adaptor, β-arrestin (15). The receptor-β-arrestin complex associates with clathrin and accessory proteins involved in the formation of clathrin-coated pits, ultimately leading to receptor internalization (16, 17). While mediating the inhibition of G-protein functions, both G-protein-coupled receptor kinases and β-arrestins also activate G-protein-independent signaling events by direct association with a number of cytoplasmic molecules (18). Since D6 internalizes its ligands without transducing any apparent signals, we investigated the mechanisms of its internalization, and in particular, the role of receptor phosphorylation and β-arrestin interaction in this process. The results showed that D6 undergoes constitutive internalization in a β-arrestin-dependent and receptor phosphorylation-independent manner. Thus, D6 is a unique member of the seven-transmembrane receptor family with distinct molecular and trafficking properties and scavenging function.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Site-directed Mutagenesis—The human D6 receptor-encoding D6/pcDNA3, the human leukotriene B_4 receptor 1-encoding BLT1/pDSRed, and the green fluorescence protein (GFP)-tagged β-arrestin 1-encoding expression vectors have been described previously (7, 19).[2] pEGFP-Endo vector (encodes a fusion protein consisting of the enhanced green fluorescent protein (EGFP), c-Myc, and Rho B) was pur-

* This work was supported by Grants PRIN 1999, 2000, and 2001 from the Italian Ministry of the University (to A. M.) and grants from the National Institutes of Health (Grants AI-43184, AI-52381, and TW-01198) and Kentucky Lung Cancer Research Board (to B. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GPCR, G-protein-coupled receptor; CCL, CC chemokine ligand; CCR, CC chemokine receptor; MEF, mouse embryo fibroblast; RFP, red fluorescence protein; DsRed, red fluorescent protein from *Discosoma* sp.; GFP, green fluorescence protein; EGFP, enhanced GFP; BLT1, leukotriene B_4 receptor 1; HA, hemagglutinin; RBL, rat basophilic leukemia; CHO, Chinese hamster ovary.

[2] V. R. Jala and B. Haribabu, manuscript in preparation.

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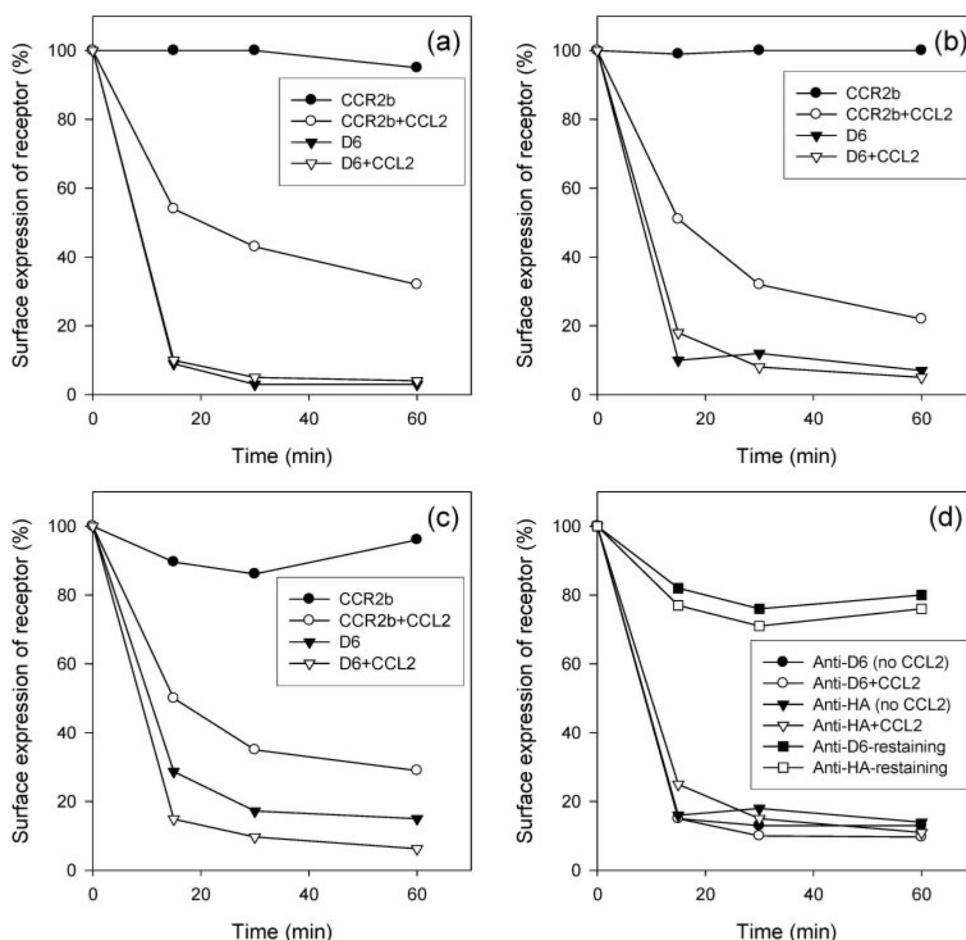

FIG. 1. **Constitutive internalization and constant surface expression of D6.** *a* and *b*, internalization of D6 and CCR2b in L1.2 transfectants. *a*, D6/L1.2 and CCR2b/L1.2 cells were stained with anti-HA primary antibody followed by incubation at 37 °C with or without 500 ng/ml CCL2 for the indicated times and then stained with the secondary antibody. *b*, D6/L1.2 and CCR2b/L1.2 cells were stained with anti-D6 primary antibody followed by incubation at 37 °C with or without 500 ng/ml CCL2 for the indicated times and then stained with the secondary antibody. *c*, internalization of D6 and CCR2b in CHO-K1 transfectants. D6/CHO-K1 and CCR2b/CHO-K1 cells were stained with anti-HA monoclonal antibody, and internalization was analyzed as described above. *d*, D6 expression in L1.2 transfectants. D6/L1.2 cells were stained with anti-HA or anti-D6 primary antibody and incubated at 37 °C with or without 500 ng/ml CCL2 for the indicated times. After transfer at 4 °C, cells were stained again or not with the primary antibody and then with the secondary antibody. The symbols are as follows: anti-D6, −CCL2, no restaining (●); anti-D6, +CCL2, no restaining (○); anti-HA, −CCL2, no restaining (▼); anti-HA, +CCL2, no restaining (▽); anti-D6, −CCL2, restaining (■); anti-HA, −CCL2, restaining (□). Data in each panel are representative of at least three independent experiments.

chased from BD Biosciences (catalog number 6935-1). Tagged and truncated receptor variants were obtained using specific oligonucleotide primers and a PCR-based approach, as described previously (20). In brief, the HA-D6/pcDNA3 and HA-CCR2b/pcDNA3 plasmids were constructed by inserting the hemagglutinin (HA) epitope at the N terminus after the first codon of the receptor using D6/pcDNA3 and the human CCR2b receptor encoding CCR2b/p CEP4 expression vector (kindly provided by Dr. J. E. Pease, Imperial College, London, UK) as templates, respectively. The HA-tagged D6 deletion mutants of D6 (D6-Δ1, stop at 356Q; D6-Δ2, stop at 351S; D6-Δ3, stop at 342A; D6-Δ4, stop at 318R) were obtained using HA-D6/pcDNA3 as template and the HA-D6-FP (5′-ATGAGATCAA GCT-TGGATCC GGTATGGGCT ACCCA-3′) as the forward primer along with the following reverse primers for each deletion mutant: D6-Δ1-RP, 5′-C-ATGATCACC GGTGCTTGGG CAGTAAGTAT GCTGCTCTC-3′; D6-Δ2-RP, 5′-CATGATCACC GGTGCGCTGC TCTCAGAACA GCTGGATAAT G-3′; D6-Δ3-RP, 5′-CATGATCACC GGTGCGGCCT GGGCAGTGCC AG-GTGCCAGG T-3′; D6-Δ4-RP, 5′-CATGATCACC GGTGCGCGGT GACT-GGAGAA GGCATACAGG-3′. To obtain red fluorescent protein (RFP)-tagged variants, the RFP open reading frame in the pDsRed2-N1 vector was cloned in frame at the C terminus of the receptor after the removal of the stop codon. All receptor expression vectors used in the study were HA-tagged at the N-terminal. All constructs were sequenced to confirm the reading frame and to exclude the presence of nonspecific mutations.

Cell Culture and Transfection—Rat basophilic leukemia (RBL)-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The generation of β-arrestin-deficient mouse fibroblasts was described previously (21). Mouse embryonic fibroblasts (wild type or β-arrestin$^{-/-}$) were maintained in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the transient transfections were performed by electroporation. For each transfection, 25 μg of plasmid DNA coding for the specific receptor and 15 μg of β-arrestin 1-GFP were used. Cells were cultured in complete growth medium for 24 h after transfection and used for microscope experiments. The mouse L1.2 lymphoma cell line was grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 10 mM HEPES, pH 7.4, 50 μM 2-mercaptoethanol and transfected by electroporation as described previously (7). After electroporation with linearized vectors and selection with 500 μg/ml G418, resistant cells were cloned by limiting dilution. Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's medium-F-12 (Invitrogen) supplemented with 10% fetal calf serum. Cells were transfected with standard calcium phosphate procedure, selected with 500 μg/ml G418, and cloned by limiting dilution.

Analysis of Receptor Internalization—Stable CHO-K1 transfectants expressing the HA-tagged receptor of interest were trypsinized, washed twice, and resuspended at 5×10^5/ml in RPMI 1640 supplemented with 1% bovine serum albumin and 25 mM HEPES. Cells were incubated with the appropriate primary antibody at 4 °C for 1 h followed by an incubation step at 37 °C for the indicated time periods in the presence or absence of 500 ng/ml CCL2. Cells were then transferred to ice, washed twice in ice-cold buffer RPMI 1640 supplemented with 1% bovine serum albumin, 25 mM HEPES, and 0.02% sodium azide, and

incubated at 4 °C with 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for the anti-D6 monoclonal antibody and anti-HA antibody or streptavidin-fluorescein isothiocyanate for the anti-CCR2 antibody, respectively. Cells were then washed once and resuspended at 5×10^5/ml in ice-cold buffer RPMI 1640 supplemented with 1% bovine serum albumin, 25 mM HEPES, and 0.02% sodium azide and analyzed in a FACSCalibur™ flow cytometer (BD Biosciences). The percent of receptor internalization was calculated from the mean channel fluorescence values of cells incubated for the indicated time period at 37 °C *versus* the mean channel fluorescence of cells not incubated at 37 °C. p values were calculated by the Mann-Whitney U test. To determine the level of surface receptors, after the incubation step at 37 °C, matched samples were stained again at 4 °C with the appropriate primary antibody before detection with the secondary antibody.

Fluorescence Microscopy—24 h after transfection, RBL-2H3 cells or mouse embryonic fibroblasts (wild type or β-arrestin$^{-/-}$) were transferred on glass bottom dishes and incubated for 1 h at 37 °C. After medium replacement with RPMI 1640 without phenol red, cells were observed under oil immersion using a ×60 objective lens. Fluorescence images were collected using a TE-FM epi-fluorescence system attached to a Nikon inverted microscope Eclipse TE300 at 37 °C at different time intervals in the presence or absence of ligand. Confocal images were collected using the spinning disc fluorescence confocal system (CARV confocal cell imaging module; ATTO Biosciences) attached to the TE-FM epi-fluorescence system. All fluorescence images were captured by a CoolSNAP HQ digital B/W CCD camera (Roper Scientific). A LAMDA 10-2 optical filter changer (Sutter Instrument) was used to capture simultaneous images in different fluorescence wavelength (filter sets S480/20x, S525/40m and S565/25x, S620/60m for GFP and RFP, respectively; EGFP/DsRed, Chroma Technology). Data were analyzed and images were pseudocolored using the MetaMorph 4.6r5 software (Universal Imaging). All images shown are representative of at least 10 individual images collected from at least three independent transfections.

Phosphorylation Assay—A receptor phosphorylation assay was performed as described previously (20). In brief, CHO-K1 stable transfectants were metabolically labeled with [^{32}P]orthophosphate and then stimulated with 100 ng/ml CCL2 or 100 nM phorbol myristate acetate for 5 min. HA-D6 and HA-CCR2b were immunoprecipitated from detergent lysates via the N-terminal HA tag using the 12CA5 anti-HA antibody, and labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. The methods for determining calcium mobilization in INDO-1-loaded cells and chemotaxis in standard 48-well Boyden chamber assays were described previously (22).

Molecular Modeling—The D6 homology model was generated by using a sequence alignment with CXCR4 (23). Notably, a sequence alignment with rhodopsin places the extra residues in D6 in the middle of the eighth helix and was deemed unsatisfactory. The region of D6 that lies outside of the CXCR4 template was deleted as no structural information is available, leading to a model generated by MODELLER (24) of residues 1–374 of D6. Low density docking of β-arrestin (25) with the D6 model used GRAMM (26) with a 3-Å grid size and 10° rotation increment and generated 150 docked structures. These structures were subsequently analyzed for consistency with reported binding regions of β-arrestin (27).

RESULTS

Constitutive Internalization of D6—To determine the molecular mechanism of the D6 chemokine scavenging activity, the ability of the ligand to induce receptor internalization was analyzed. L1.2 cells stably expressing HA-tagged D6 (D6/L1.2) or CCR2b (CCR2b/L1.2) were labeled with anti-HA (Fig. 1*a*) or receptor-specific (Fig. 1*b*) monoclonal antibodies at 4 °C followed by incubation in the presence or absence of the ligand (500 ng/ml CCL2) at 37 °C. Cells were then transferred at 4 °C, labeled with the secondary antibody, and analyzed by flow cytometry. As described earlier (28), CCR2b was not internalized in the absence of the ligand, whereas CCL2 induced a significant and time-dependent decrease of cell surface CCR2b expression levels. In contrast, D6 underwent rapid and substantial internalization in the absence of the ligand, as detected by both antibodies. The presence of the ligand was unable to induce any further decrease in D6 surface expression levels (Fig. 1, *a* and *b*). Similar results were obtained in CHO-K1 cells expressing D6 (D6/CHO-K1) or CCR2b (CCR2b/CHO-K1) (Fig. 1*c*), indicating that the constitutive internalization of D6 is a cell type-independent phenomenon.

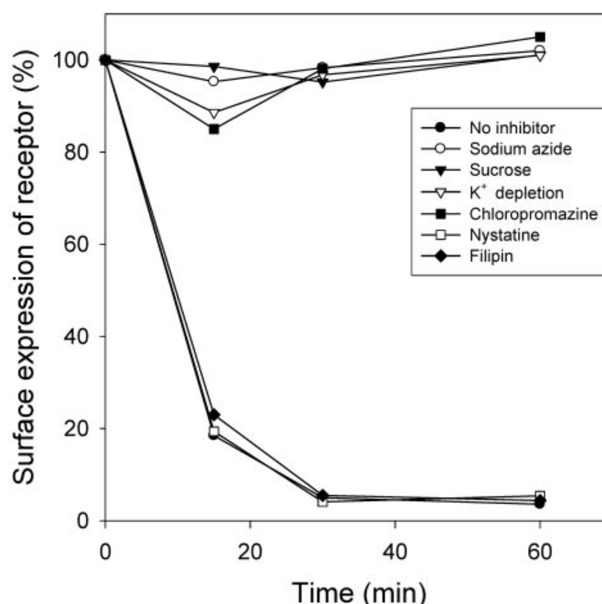

FIG. 2. **D6 internalization is dependent on clathrin-coated pit.** D6/CHO-K1 transfectants were incubated at 37 °C with the indicated inhibitors of clathrin-coated pits or caveolae-mediated pathways for 30 min, and then an internalization assay was performed as described under "Experimental Procedures." The symbols are as follows: no inhibitors (●); 0.02% sodium azide (○); 0.4 M sucrose (▼); K$^+$ depletion (▽); 25 μg/ml chloropromazine (■); 50 μg/ml nystatine (□); 5 μg/ml filipin (◆). Data from one of three independent experiments are shown.

GPCR recycling may be determined by measuring surface reappearance at various times after inducing internalization by agonist. To determine D6 surface expression following internalization, D6/L1.2 cells were labeled with anti-HA or anti-D6 monoclonal antibodies either only before or before and after incubation at 37 °C for the indicated time periods, and the surface expression of D6 was analyzed. As above, cells stained only before incubation displayed a significant rate of receptor internalization. Labeling of the cells with either anti-HA or anti-D6 monoclonal antibodies after the internalization step revealed that a significant fraction of internalized receptors was replaced on the cell membrane (Fig. 1*d*). Taken together, these results indicate that D6 behaves as a constitutively active yet non-signaling receptor, undergoing rapid and ligand-independent internalization and re-expression on membrane.

Most GPCRs, including chemokine receptors, undergo ligand-induced internalization through a clathrin-coated pit mechanism (18, 29). To investigate whether a similar mechanism is involved in the ligand-independent D6 internalization process, the effect of different clathrin-coated pit or caveolae inhibitors was determined. As shown in Fig. 2, in D6/L1.2 transfectants, receptor internalization is completely inhibited by treatment with a panel of clathrin-coated pit inhibitors (chloropromazine, K$^+$ depletion, and high sucrose concentrations), whereas caveolae inhibitors (nystatin, filipin) were completely ineffective. Similar results were obtained in D6/CHO-K1 transfectants (data not shown).

Constitutive Association of D6 with β-Arrestin—The interaction of β-arrestin with clathrin and the clathrin adaptor protein AP-2 sets in motion a complex mechanism ultimately leading to GPCR sequestration from the cell surface (30). The ability of D6 to internalize in a clathrin-coated pit-dependent manner in the absence of ligand prompted us to investigate the mode of interaction of D6 with β-arrestin and its eventual role in receptor internalization. Leukotriene B_4 receptor 1 (BLT1), a

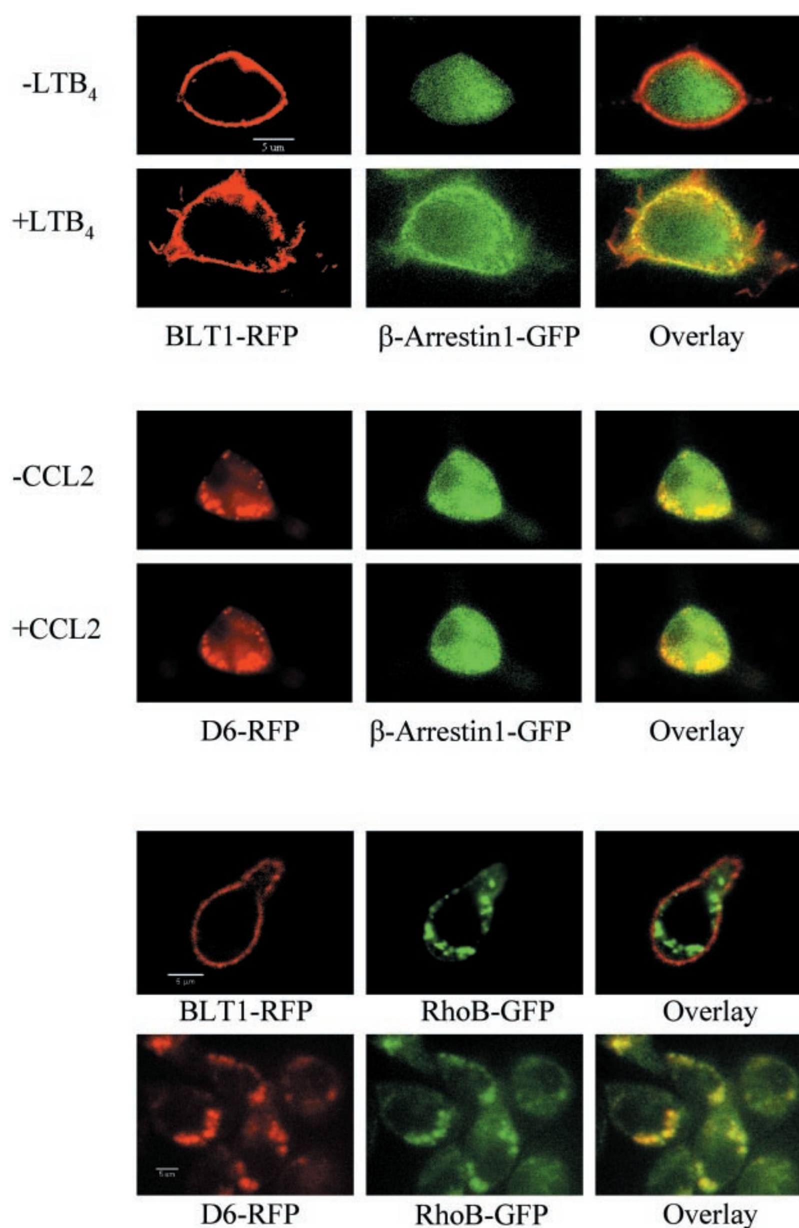

FIG. 3. **Constitutive association of D6 with β-arrestin and endosomes.** RBL-2H3 cells were transiently co-transfected with BLT1-RFP or D6-RFP along with β-arrestin 1-GFP or RhoB-GFP. 24 h after transfection, cells were stimulated with 1 μM leukotriene B_4 or 100 ng/ml CCL2 as indicated, and fluorescence images of live cells were captured as described under "Experimental Procedures." Images of fluorescence emission by RFP receptor (*left panels*), β-arrestin 1-GFP (*middle panels*), and overlay of the two (*right panels*) are shown. *Rows 1* and *2* from the *top* are images of BLT1-RFP transfectants; *rows 3* and *4* are images of D6 transfectants. *Row 5* represents the co-transfection of BLT1-RFP and RhoB-GFP, and the images were collected at ×1000 magnification. *Row 6* represents the co-transfection of D6-RFP and RhoB-GFP, and the images were collected at ×600 magnification.

chemoattractant receptor that undergoes ligand-dependent phosphorylation and internalization, was used as a reference in these experiments (22).[2] Co-expression in RBL-2H3 cells of the BLT1-RFP and β-arrestin 1-GFP chimeric molecules resulted in plasma membrane and uniform cytoplasmic localization, respectively (Fig. 3, *upper panels*). Upon exposure to the agonist (1.0 μM leukotriene B_4, 5 min), the β-arrestin 1-GFP rapidly translocates to the membrane co-localizing with BLT1-RFP and eventually redistributes inside the cell in endocytic vesicles (Fig. 3, *upper panels*). In contrast, co-expression of D6-RFP and β-arrestin 1-GFP resulted in co-localization of the receptor with β-arrestin 1-GFP in vesicles even in the absence of the ligand. The addition of D6 ligands (100 ng/ml CCL2 up to 60 min, Fig. 3, *middle panels*; 100 ng/ml CCL4 up to 60 min, data not shown) had no significant effect on receptor distribution or in coupling to β-arrestin. To determine the exact nature of these vesicles, BLT1-RFP or D6-RFP was co-expressed with RhoB-GFP, a marker for early endosomes (31). Although the BLT1-RFP displayed surface expression and RhoB-GFP labeled distinct vesicles, the D6-RFP and RhoB-GFP are clearly colocalized, presumably in early endocytic vesicles.

To investigate the role of β-arrestin in the internalization of D6, we expressed D6-RFP in the presence or absence of β-arrestin 1-GFP in β-arrestin$^{-/-}$ or in β-arrestin$^{+/+}$ mouse embryo fibroblasts (MEFs) derived from β-arrestin 1/β-arrestin 2 double-knockout mice as well their littermate wild-type animals (21). As observed in RBL-2H3 cells, D6-RFP accumulated in intracellular vacuolar compartments, co-localizing with β-arrestin 1-GFP (Fig. 4, *upper panels*) in β-arrestin$^{+/+}$ MEFs. On the contrary, in β-arrestin$^{-/-}$ MEFs, the D6-RFP displayed distinct plasma membrane localization and accumulated intracellularly only when co-expressed with β-arrestin 1-GFP (Fig. 4, *lower panels*). Again, the addition of the ligand (100 ng/ml CCL2, data not shown) did not induce internalization or alter the surface expression of D6 in these cells. Taken together, these results demonstrate a constitutive association of D6 with β-arrestin and a requirement of β-arrestin for its constitutive internalization.

Structural Determinants of D6 Supporting Interaction with β-Arrestin—Agonist-dependent phosphorylation of serine and threonine residues in the C-terminal tail of the receptor plays a major role in GPCR interaction with β-arrestin (14). Both naturally occurring and mutation-induced constitu-

tively active chemokine receptors (32–34) undergo constitutive internalization, which was shown to correlate with constitutive receptor phosphorylation. To investigate the role of receptor phosphorylation in D6 internalization, we analyzed its phosphorylation status in D6/CHO-K1 cells in the presence and absence of ligand (100 ng/ml CCL2, 5 min), with CCR2b/CHO-K1 cells as controls. As shown in Fig. 5*b*, CCR2b phosphorylation was induced by CCL2. In contrast, D6 was not phosphorylated in the presence or absence of the ligand (Fig. 5*b*). Similarly, CCR2b was phosphorylated in response to phorbol myristate acetate treatment (100 ng/ml, 5 min), whereas D6 was not (data not shown). Western blotting of total cellular lysates as well as anti-HA antibody immunoprecipitates from HA-tagged D6-expressing cells showed that anti-HA antibody quantitatively immunoprecipitated HA-tagged D6 (data not shown). Thus, β-arrestin association with D6, which is required for D6 internalization, occurs in the absence of receptor phosphorylation.

To identify structural determinants of D6 involved in β-arrestin association, a series of C-terminal tail deletion mutants of D6 was generated (Fig. 5*a*). These included the deletion of an acidic region at the C terminus (D6-Δ1; Gln356-stop), an isoleucine-leucine motif (D6-Δ2; Ser351-stop), most cytoplasmic serine/threonine residues (D6-Δ3; Ala342-stop), and the entire cytoplasmic tail (D6-Δ4; Arg318-stop). All mutants have been expressed, at comparable levels, in distinct stable CHO-K1 clones (data not shown). None of these deletion mutants, including D6-Δ1, which retained most of the phosphorylation sites, were phosphorylated as seen with the native D6 (Fig. 5*b*). Interestingly, all mutants, however, lost their capacity to undergo internalization, either in the absence (Fig. 5*c*) or in the presence (Fig. 5*d*) of the ligand. These results highlight the existence of structural determinants located in the most distal acidic region of the D6 C-terminal domain responsible for constitutive receptor internalization.

To define whether the inability to internalize was due to impaired association with β-arrestin, all D6 deletion mutants were tagged with RFP at the C terminus and expressed in wild-type or β-arrestin double-knockout MEFs either with or without β-arrestin 1-GFP (Fig. 6). The data with native D6 are consistent, where D6-RFP constitutively colocalizes with β-arrestins (Fig. 6, *left bottom three panels*) but remains on the membrane in β-arrestin$^{-/-}$ cells. In contrast, none of the mutants of D6 were able to colocalize with β-arrestins in either the β-arrestin$^{+/+}$ or β-arrestin$^{-/-}$ cell line (Fig. 6, *right four columns*). The addition of ligand (100 ng/ml CCL2 up to 60 min) did not alter the surface expression nor induce internalization of any of the mutant D6 receptors in either cell type (data not shown). Taken together, these results demonstrate that structural determinants in the C-terminal tail of D6 are required for the constitutive association with β-arrestin and consequent receptor constitutive internalization.

In addition to its role in mediating the internalization of GPCRs, β-arrestin also enhances the uncoupling of phosphorylated receptors from G-proteins (35). Thus, the lack of G-protein-mediated signaling by D6 may be attributed to its constitutive association with β-arrestin. To examine whether this indeed was the case, CCL2-induced calcium mobilization and chemotaxis responses were determined in CHO-K1 cells stably expressing either native D6 or each of the mutant D6 receptors. None of the cells expressing individual mutant D6 receptors showed chemotaxis or calcium mobilization response to CCL2, whereas CCR2b/CHO-K1 cells showed robust responses to the same ligand (data not shown). In addition, transient expression of D6 in arrestin-deficient cells also did not result in any detectable CCL2-induced calcium mobilization (data not shown).

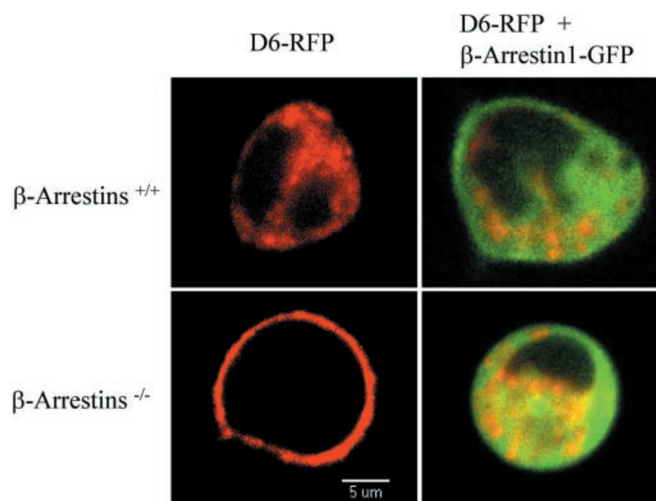

FIG. 4. **β-Arrestin-dependent constitutive internalization of D6.** MEFs derived from β-arrestin 1/β-arrestin 2 double-knockout mice (β-arrestin$^{-/-}$) or wild-type littermates (β-arrestin$^{+/+}$) were transiently transfected with D6-RFP alone or co-transfected along with β-arrestin 1-GFP as described under "Experimental Procedures." 24 h after transfection, the fluorescence images of live cells were captured using a spinning disc confocal microscope as described under "Experimental Procedures." The *left panels* are images of D6-RFP alone in wild-type (*top*) or β-arrestin-deficient (*bottom*) cells, and the *right panels* are overlay images of D6-RFP and β-arrestin 1-GFP.

Modeling the D6-β-Arrestin Interaction—To investigate the possible binding sites of β-arrestin to D6, molecular modeling studies were undertaken. A homology model of D6 was built based on recent models of CXCR4 (36) and docked with the crystal structure of β-arrestin (25). The majority of the docked solutions interacted with the exposed cytoplasmic loops and the C terminus. One of the low energy docked structures (Fig. 7) is consistent with some previously identified regions of interactions of β-arrestin (27). The reported C-domain β-strands XV and XVI of β-arrestin are in contact with the C terminus of D6, whereas the residues in the N-domain, which are reportedly important for phosphate binding, do not directly interact with D6. As D6 does not require phosphorylation for β-arrestin binding, it is possible for β-arrestin to bind to D6 by a different mechanism.

DISCUSSION

The results presented here demonstrate that the chemokine receptor D6 has unique functional and structural features, which make it ideally adapted for the chemokine scavenging function. Ligand binding of a typical GPCR results in a series of events including G-protein activation, receptor phosphorylation, desensitization, β-arrestin association, sequestration, and/or downregulation. This sequence of events leads to distinct biological responses as well as other mechanisms for regulating such responses. An alteration in one or more of these events offers control of additional biological functions such as ligand scavenging. In the case of D6, a number of changes in typical GPCR mechanisms appear to have been incorporated, allowing it to function as a chemokine decoy receptor.

Chemokine receptors in general have broad specificity, with several receptors binding to multiple chemokines and transducing signals (4). Recent studies from us and others have shown that D6 has an unusually broad chemokine binding specificity, interacting and scavenging 12 of the 20 CC-chemokines tested to date (6, 7, 42). Interestingly, all of these are chemokines involved in inflammatory responses, whereas homeostatic chemokines do not bind to D6. It is of interest to note that the other chemokine scavenger, Duffy antigen, also dis-

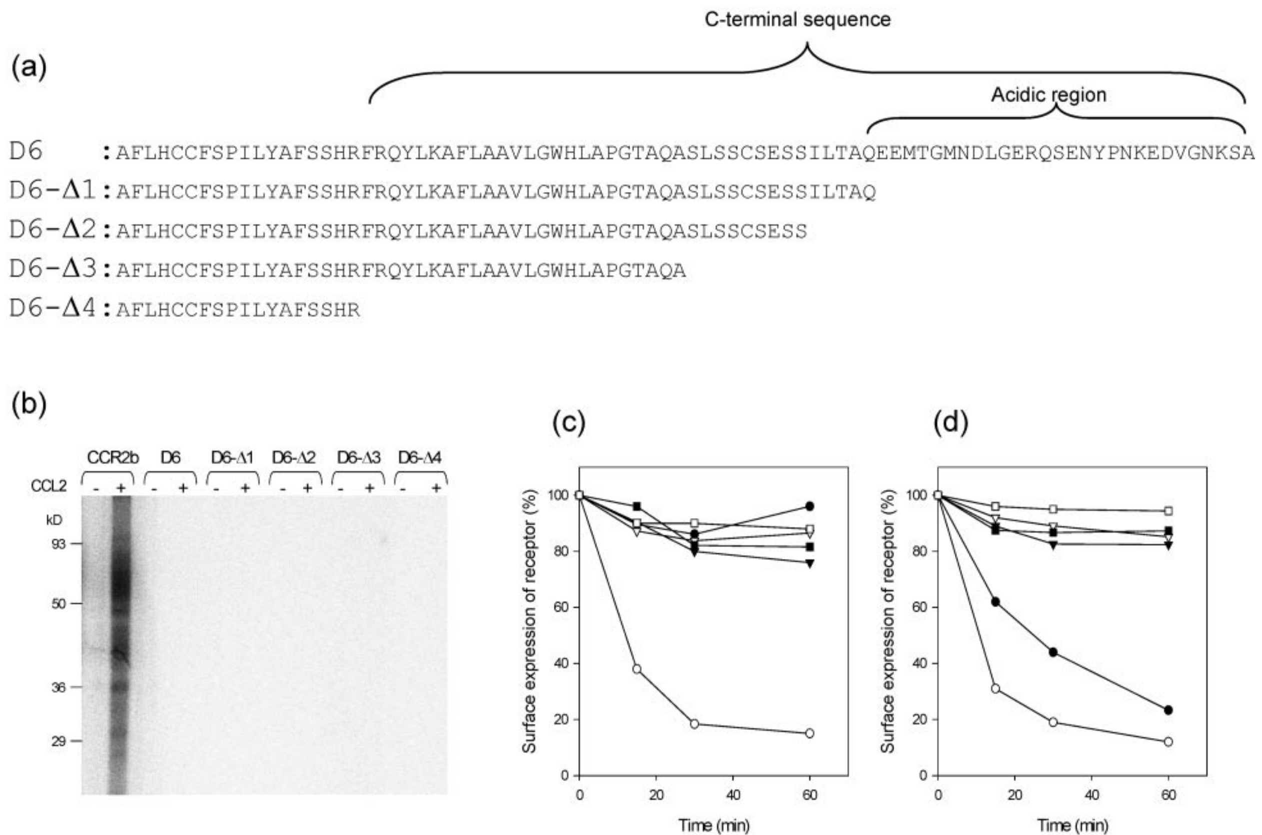

FIG. 5. **The acidic region on the cytoplasmic tail of D6 is essential for receptor internalization.** *a*, the sequence of the cytoplasmic tail and C-tail truncation mutants of D6. *b*, phosphorylation of CCR2b and D6 wild-type and C-tail truncation mutants. CHO-K1 cells stably transfected with the indicated HA-tagged receptor were labeled with [^{32}P]orthophosphate and stimulated for 5 min with 100 ng/ml CCL2 as indicated. Cell lysates immunoprecipitated with anti-HA antibody were separated on SDS-PAGE and visualized by autoradiography as described under "Experimental Procedures." One experiment representative of three performed is shown. *c* and *d*, lack of internalization of D6 C-tail truncation mutants. CHO-K1 cells stably transfected with the indicated HA-tagged receptor were treated with phosphate-buffered saline (*c*) or with 500 ng/ml CCL2 (*d*), and an internalization assay was performed as described under "Experimental Procedures" with anti-HA-antibody, D6 (○), CCR2b (●); D6-Δ1 (▼); D6-Δ2 (▽); D6-Δ3 (■); D6-Δ4 (□). Data from one of three independent experiments are shown.

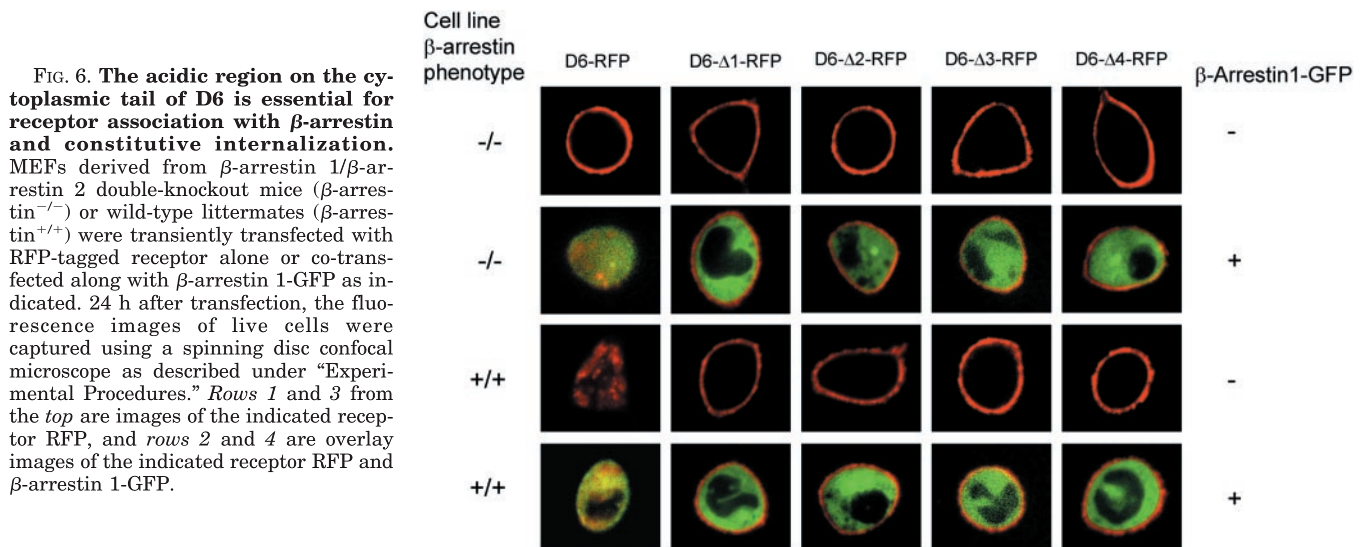

FIG. 6. **The acidic region on the cytoplasmic tail of D6 is essential for receptor association with β-arrestin and constitutive internalization.** MEFs derived from β-arrestin 1/β-arrestin 2 double-knockout mice (β-arrestin$^{-/-}$) or wild-type littermates (β-arrestin$^{+/+}$) were transiently transfected with RFP-tagged receptor alone or co-transfected along with β-arrestin 1-GFP as indicated. 24 h after transfection, the fluorescence images of live cells were captured using a spinning disc confocal microscope as described under "Experimental Procedures." *Rows 1* and *3* from the *top* are images of the indicated receptor RFP, and *rows 2* and *4* are overlay images of the indicated receptor RFP and β-arrestin 1-GFP.

plays an unusually broad ligand profile (37). Thus, acquiring a broad ligand binding specificity might be an important step for effective scavenging function.

The loss of signaling capacity is the essential property of a decoy receptor, as was demonstrated for the interleukin-1 type II receptor (38). D6 clearly does not activate any of the known signaling pathways of chemokine receptors including chemotaxis, calcium mobilization, or ligand-induced receptor phosphorylation. Although the mechanisms are still unknown, the primary sequence of D6 contains several differences in highly conserved sequence motifs found in signaling chemokine receptors. These include an asparagine in place of an aspartic acid in the second transmembrane domain and a change in the canonical DRYLAIV motif in the second cytoplasmic loop to DKYLEIV in D6. Both regions were shown to be important in G protein-dependent signaling in chemokine receptors (8). However, reverse

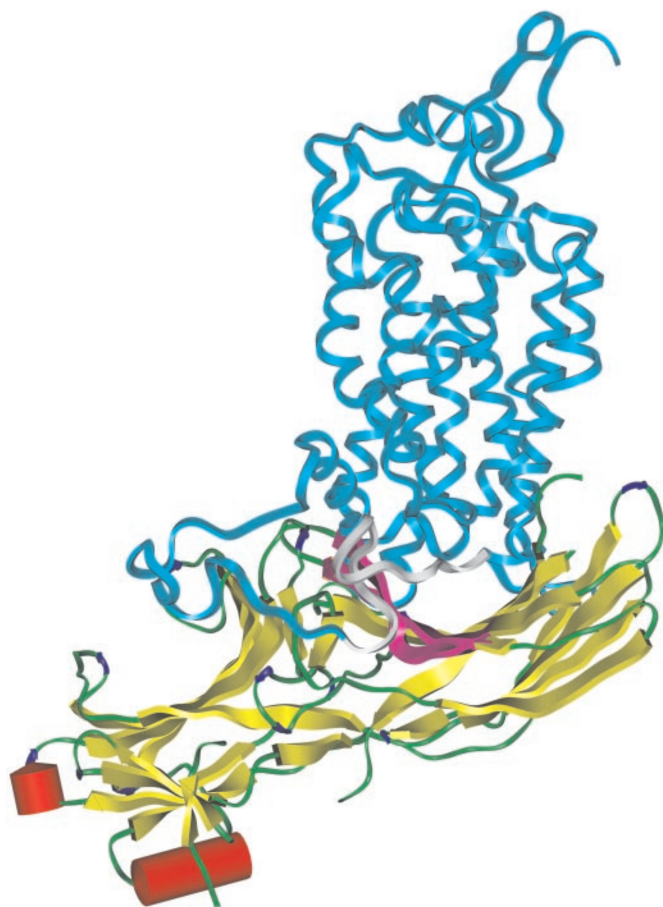

FIG. 7. **Potential sites of D6 interaction with β-arrestin.** The homology model of D6 is shown in *cyan ribbon representation* with β-arrestin colored by secondary structure, β sheets colored *yellow*, helices colored *red*, the random coil colored *green*, and turns colored *blue*. The β sheet region (β-strands XV and XVI) previously identified as being important for binding is shown in *purple* (27). The C-terminal region of D6, residues 357–374, corresponding to the D6-Δ1 deletion, is colored *gray*.

mutations of these regions by mutagenesis did not result in a signaling D6 receptor.[3] Therefore, multiple mutations may have occurred to generate D6 as a non-signaling receptor.

Among the typical GPCR functions, D6 not only retains the ability to associate with β-arrestin and internalize in a clathrin-coated pits-dependent mechanism, but it does so even in the absence of ligand. The data with arrestin knockout MEFs clearly showed that constitutive internalization of D6 is dependent on β-arrestin expression. Two potential mechanisms might explain this observation. In the first case, D6 is in a constitutively active conformation but is unable to transduce G-protein-mediated signals due to specific mutations in regions of the receptor essential for G-protein activation. Indeed, two other chemokine receptors, US28, a cytomegalovirus-encoded chemokine receptor with broad ligand specificity, and a constitutively active mutant of CXCR4 (CXCR4-CAM) are known to undergo constitutive internalization (32, 34). However, unlike D6, these receptors transduce G-protein-mediated signals and showed constitutive phosphorylation. Although β-arrestin has been reported as being dispensable for the constitutive internalization of US28 (39), more recent studies demonstrated a direct association of β-arrestin with US28 (33). A naturally occurring mutation in vasopressin V2 receptor (R137H), associated with familial nephrogenic diabetes insipidus, is constitutively phosphorylated and localizes to endosomal vesicles together with β-arrestin (40). Mutation of the R137H receptor that eliminates β-arrestin binding restores surface expression and signaling to this receptor. In the case of D6, elimination of β-arrestin binding restored receptor surface expression, but it did not convert it to a signaling receptor. Thus, constitutive interaction with β-arrestin is not the sole determinant for the lack of signaling function in D6. Although D6 does not signal through G proteins, signaling through β-arrestins remains a possibility. The complete absence of either constitutive or ligand-induced phosphorylation indicates that the mechanisms of interaction between β-arrestin and D6 may be different from other GPCRs.

A second possible mechanism is that the cytoplasmic tail of D6 contained a structural element allowing it to constitutively associate with β-arrestin and internalize. The demonstration that arrestin association to D6 does not require receptor phosphorylation may indicate that these interactions are different from other typical GPCRs. This hypothesis is consistent with a model of the possible complex of D6 and β-arrestin shown in Fig. 7. However, the regions that have been reported as important for the binding of arrestins to GPCRs contain the two concave β-sheet regions, and the region identified as being most important for receptor specificity is in contact with D6 in the model. Upon examination of the β-arrestin regions, it is unlikely that all of these regions are making direct contact with the GPCR due to the physical size of both proteins, although the C terminus of D6 will be dynamic. It is likely that the conformation and topology of the domains of β-arrestin rely on each other through interdomain contacts, thus making both domains crucial. Further work is required to map the precise β-arrestin binding sites in the case of D6, but data from the deletion mutants suggest that the acidic region in the C-tail might contain such a binding site. Thus, by virtue of altered structural determinants, D6 receptor interaction with β-arrestin has been shifted from a functional to a structural property, leading to the acquisition of ligand-independent internalization and recycling capacities, ideal for the receptor-mediated ligand scavenging function.

D6 has restricted tissue distribution with high expression in placenta and afferent lymphatic endothelial cells (13). Inflammatory chemokines are transferred via lymphatic vessels to lymph nodes, where they act as a “remote control” mechanism for leukocyte recruitment (41). On afferent lymphatic endothelium, D6 may act as a gatekeeper, preventing excessive accumulation of inflammatory chemokines in lymph nodes and uncontrolled, disruptive leukocyte recruitment (8, 13). The results presented here show that D6 is constitutively internalized via a ligand-independent, phosphorylation-independent association with β-arrestin. These unique properties underlie inflammatory chemokine scavenging by the D6 decoy receptor.

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