Calcium mobilization

We used a microtitre-plate-based calcium-mobilization FLIPR assay, as described previously[17], for the functional identification of ligands that activate HEK-293 cells expressing recombinant GPR14. Ligands we tested included micromolar concentrations of all known mammalian neuropeptides and ≥10 μM concentrations of all known steroid, lipid and amine transmitters.

Radioligand binding

Membranes from HEK-293 cells stably transfected with human GPR14 were pre-coupled to wheatgerm-agglutinin-coated SPA beads (Amersham). Using mono-iodinated goby U-II (^{125}I-labelled Tyr^{10}, chloramine T, 2000 Ci mmol^{-1}; 5 mM $MgCl_2$, 0.1% BSA, 20 mM Tris-HCl pH 7.4, 25 μg protein; 25 °C), equilibrium binding was measured in 96-well plates (Wallac) after 45 min[18]. Non-specific binding, determined using 1 μM unlabelled U-II, was ~10% of total binding. There was no specific binding to mock-transfected HEK-293 membranes. We determined protein using biochonic acid (Pierce). Kinetic analysis was by nonlinear least square fitting (GraphPad).

Isolated blood vessel studies

Isolated arterial rings from male Sprague–Dawley rats (400 g) and cynomolgus monkeys (*Macaca fascicularis*) (5 kg) were suspended in organ baths containing Krebs (37 °C; 95% O_2; 5% CO_2)[19]. Changes in isometric force were recorded under optimal resting tension. Cumulative agonist concentration–response curves were normalized to 60 mM KCl or 1 μM noradrenaline and fitted to a logistic equation[19]. Unless stated otherwise, responses were measured in the presence of 10 μM indomethacin using vessels denuded by rubbing (confirmed as a loss of dilator response to 10 μM carbachol).

Haemodynamics and echocardiography

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (DHSS publication NIH 85-23). Adult male cynomolgus monkeys (5–7 kg) were treated with atropine sulphate (25 μg kg^{-1} subcutaneous) and anaesthetized with ketamine (10 mg kg^{-1} intramuscular). After endotracheal intubation, anaesthesia was maintained with 1–3% isoflurane. We prepared the animals for monitoring of systemic haemodynamics. Echocardiography (Doppler, M-mode and 2-D, ATL5000) was carried out at 2–3 min intervals. Vehicle administration (0.9% NaCl) was followed by the cumulative administration of human U-II.

Statistics

Values are expressed as mean ± s.e.m. n is the number of individual observations made in a particular group. Statistical comparisons were made by one-way analysis of variance (Fisher's protected least-squares difference) and differences considered significant where $P < 0.05$.

Received 8 July; accepted 13 August 1999.

1. Bern, H. A., Pearson, D., Larson, B. A. & Nishioka, R. S. Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes. *Recent Prog. Horm. Res.* **41,** 533–552 (1985).
2. Conlon, J. M., Yano, K., Waugh, D. & Hazon, N. Distribution and molecular forms of urotensin II and its role in cardiovascular regulation in vertebrates. *J. Exp. Zool.* **275,** 226–238 (1996).
3. Coulouarn, Y. *et al.* Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl Acad. Sci. USA* **95,** 15803–15808 (1998).
4. Marchese, A. *et al.* Cloning and chromosomal mapping of three novel genes, GPR9, GPR10, and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors. *Genomics* **29,** 335–344 (1995).
5. Tal, M. *et al.* A novel putative neuropeptide receptor expressed in neural tissue, including sensory epithelia. *Biochem. Biophys. Res. Commun.* **209,** 752–759 (1995).
6. Stadel, J. M., Wilson, S. & Bergsma, D. J. Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery. *Trends Pharmacol. Sci.* **18,** 430–437 (1997).
7. Libert, F., Vassart, G. & Parmentier, M. Current developments in G-protein-coupled receptors. *Curr. Opin. Cell Biol.* **3,** 218–223 (1991).
8. O'Dowd, B. F. *et al.* The cloning and chromosomal mapping of two novel human opioid-somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. *Genomics* **28,** 84–91 (1995).
9. Ohsako, S., Ishida, I., Ichikawa, T. & Deguchi, T. Cloning and sequence analysis of cDNAs encoding precursors of urotensin II-alpha and -gamma. *J. Neurosci.* **6,** 2730–2735 (1986).
10. Itoh, H., McMaster, D. & Lederis, K. Functional receptors for fish neuropeptide urotensin II in major rat arteries. *Eur. J. Pharmacol.* **149,** 61–66 (1988).
11. Pearson, D. *et al.* Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc. Natl Acad. Sci. USA* **77,** 5021–5024 (1980).
12. Gibson, A., Wallace, P. & Bern, H. A. Cardiovascular effects of urotensin II in anesthetized and pithed rats. *Gen. Comp. Endocrinol.* **64,** 435–439 (1986).
13. Hasegawa, J., Kobayashi, Y. & Kobayashi, H. Vasodepressor effects of urotensin II in rats. *Neuroendocrinol. Lett.* **14,** 357–363 (1992).
14. Yanagisawa, M. *et al.* A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411–415 (1988).
15. Aiyar, N. *et al.* Human AT1 receptor is a single copy gene: characterization in a stable cell line. *Mol. Cell Biochem.* **131,** 75–86 (1994).
16. King, D. S., Fields, C. G. & Fields, G. B. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int. J. Pept. Protein Res.* **36,** 255–266 (1990).
17. Chambers, J. *et al.* Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* **400,** 261–265 (1999).
18. Elshourbagy, N. A. *et al.* Molecular cloning and characterization of the porcine calcitonin gene-related peptide receptor. *Endocrinology* **139,** 1678–1683 (1998).
19. Douglas, S. A. & Hiley, C. R. Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16–21) and other endothelin-related peptides in the rat. *Br. J. Pharmacol.* **104,** 311–320 (1991).

Supplementary Information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We dedicate this Letter to the memory of our friend and colleague, the late J. M. Stadel. We thank D. Ashton, P. Buckley, T. Covatta, J. Culp, G. Dytko, L. Floyd, P. Hieble, M. Luttmann, G. McCafferty, D. Naselsky, P. Nuthulaganti, P. Ryan and A. Sulpizio for their assistance and comments during the preparation of this manuscript. We thank B. O'Dowd, University of Toronto for the rat GPR14 cDNA clone.

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A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β2-adrenergic receptor

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A fundamental question in cell biology is how membrane proteins are sorted in the endocytic pathway. The sorting of internalized β2-adrenergic receptors between recycling endosomes and lysosomes is responsible for opposite effects on signal transduction and is regulated by physiological stimuli[1,2]. Here we describe a mechanism that controls this sorting operation, which is mediated by a family of conserved protein-interaction modules called PDZ domains[3]. The phosphoprotein EBP50 (for ezrin–radixin–moesin(ERM)-binding phosphoprotein-50)[4] binds to the cytoplasmic tail of the β2-adrenergic receptor through a PDZ domain and to the cortical actin cytoskeleton through an ERM-binding domain. Disrupting the interaction of EBP50 with either domain or depolymerization of the actin cytoskeleton itself causes missorting of endocytosed β2-adrenergic receptors but does not affect the recycling of transferrin receptors. A serine residue at position 411 in the tail of the β2-adrenergic receptor is a substrate for phosphorylation by GRK-5 (for G-protein-coupled-receptor kinase-5) (ref. 5) and is required for interaction with EBP50 and for proper recycling of the receptor. Our results identify a new role for PDZ-domain-mediated protein interactions and for the actin cytoskeleton in endocytic sorting, and suggest a mechanism by which GRK-mediated phosphorylation could regulate membrane trafficking of G-protein-coupled receptors after endocytosis.

Surface-biotinylated β2-adrenergic receptors (β2-ARs) expressed in human embryonic kidney (HEK) 293 cells exhibited no detectable degradation when incubated in the presence of the agonist isoprenaline for 4 h (Fig. 1a, β2-AR), consistent with the rapid and efficient recycling of internalized β2-ARs to the plasma membrane in this cell type[6,7]. However, truncation of the distal portion of the cytoplasmic tail caused pronounced, ligand-induced degradation (Fig. 1a, β2-AR$_t$). Addition of various residues to the full-length tail, including a single alanine residue (β2-AR-Ala) also caused ligand-induced degradation. The $t_{1/2}$ for ligand-induced degradation of the wild-type receptor protein was >9 h. In contrast, all mutations of the distal β2-AR tail caused ligand-induced degradation that was nearly complete (≥75%) within 4 h (Fig. 1b).

To investigate the role of the distal tail in β2-AR trafficking, we visualized the membrane trafficking of antibody-labelled receptors[6] using fluorescence microscopy. Both the wild-type β2-AR and β2-AR-Ala endocytosed rapidly following activation by isoprenaline, as indicated by the translocation of antibody-labelled receptors from the plasma membrane to endocytic vesicles (Fig. 2Aa and e). After removal of the agonist, wild-type β2-AR recycled efficiently to the plasma membrane (Fig. 2Ab–d) whereas β2-AR-Ala did not (Fig. 2Af–h). Quantification by biotinylation (Fig. 2B) and flow cytometry (data not shown) confirmed that wild-type β2-ARs recycled efficiently within ≤ 30 min after agonist removal, whereas recycling of β2-AR-Ala was significantly (≥50%) inhibited.

Many vesicles containing internalized β2-AR-Ala colocalized with membrane markers of late endosomes and lysosomes, as seen by confocal microscopy (data not shown). The lysosomotropic agents chloroquine and NH_4Cl, as well as the lysosomal protease inhibitor leupeptin, significantly inhibit agonist-induced degradation of β2-AR-Ala (Fig. 2C). These results indicate that mutation of the distal cytoplasmic tail inhibits efficient recycling and causes missorting of internalized receptors to lysosomes.

To identify candidate proteins involved in this sorting operation, we searched for proteins that bind to the cytoplasmic tail of the wild-type β2-AR but not to β2-AR-Ala. A predominant immunoreactive protein present in HEK293 cell lysates that bound specifically to the wild-type β2-AR tail was EBP50, as identified using an antibody recognizing members of the EBP50/NHERF/E3KARP protein family[4] (Fig. 3A). EBP50 is a human homologue of rabbit NHERF[8] and a relative of E3KARP[9]. NHERF is a N^+/H^+-exchanger regulator factor[8,9] that functions in β2-AR-mediated signal transduction at the plasma membrane[10]. NHERF and E3KARP bind to the cytoplasmic tail of the β2-AR by an amino-terminal PDZ domain[11]. We confirmed, by affinity chromatography, that the homologous PDZ domain from EBP50 bound to the cytoplasmic tail of the β2-AR but not β2-AR-Ala (data not shown). Endogenous EBP50 co-immunoprecipitated with full-length but not mutant β2-ARs, indicating that this protein interaction may occur *in vivo* (Fig. 3B).

EBP50 can link membrane proteins with the cortical actin cytoskeleton through association with ERM proteins[4]. If such a linker function is required for endocytic sorting of the β2-AR, disruption of the EBP50–ERM interaction may have a similar effect on sorting of the wild-type β2-AR to that of disruption of the β2-AR–EBP50 interaction caused by mutation of the receptor tail. We constructed a truncated mutant form of EBP50 (EBP50Δ) that lacks the ERM-binding domain[12] but retains the PDZ domain that is required for specific interaction with the β2-AR. This was confirmed by affinity chromatography, showing binding of EBP50Δ to the cytoplasmic tail of the wild-type β2-AR but not to the β2-AR-Ala (data not shown). Overexpression of EBP50Δ in stably transfected cells caused missorting and enhanced lysosomal degradation of the full-length wild-type β2-AR after agonist stimulation (Fig. 3C). Overexpression of the wild-type EBP50 protein at similar levels (determined by immunoblotting) had a much smaller effect on β2-AR degradation (Fig. 3C). In contrast, neither EBP50Δ nor overexpressed wild-type EBP50 affected the recycling of transferrin receptors analysed in the same cell extracts (not shown). These results indicate that the proper sorting of internalized β2-ARs requires EBP50 to interact, through distinct domains, with both the β2-AR cytoplasmic tail and ERM proteins.

Interaction with ERM-family proteins links EBP50 to the cortical actin cytoskeleton[4], indicating that the actin cytoskeleton may be

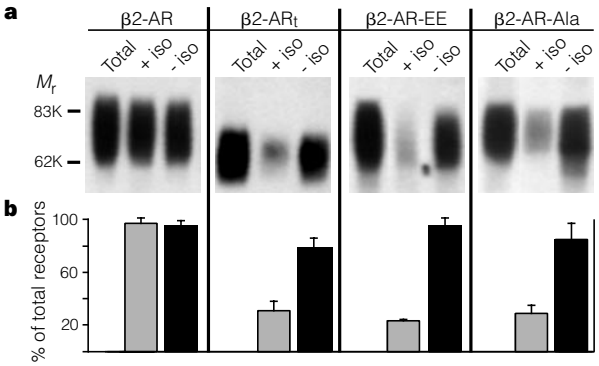

Figure 1 β2-AR mutants are degraded. **a**, Mutation of the β2-AR cytoplasmic tail results in receptor degradation. The biotinylation assay of receptor degradation was used to compare agonist-induced degradation of wild-type β2-AR (β2-AR) and C-terminal truncation (β2-AR$_t$) or addition (β2-AR-EE and β2-AR-Ala) mutations. M_r, relative molecular mass **b**, Quantification of receptor degradation by densitometric scanning of streptavidin overlays. Bars indicate mean biotinylated receptor protein recovered 4 h after biotinylation. Error bars represent s.d.

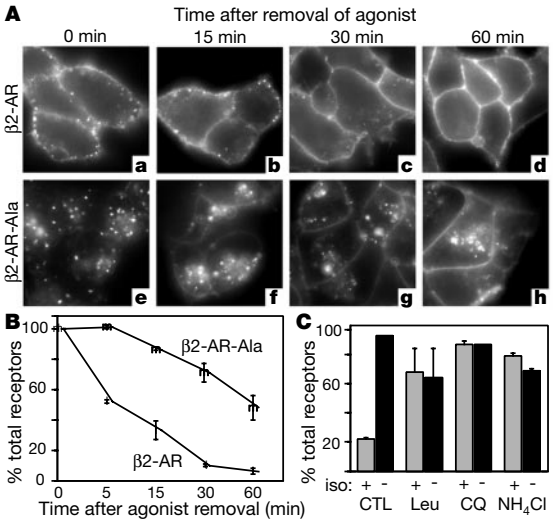

Figure 2 Missorting of mutant β2-ARs to lysosomes. **A**, Recycling of β2-AR-Ala is inhibited. Agonist-internalized β2-ARs present in endocytic vesicles (**a**) returned to the plasma membrane following agonist removal (**b**–**d**), whereas internalized β2-AR-Ala (**e**) remained in endocytic vesicles (**f**–**h**). **B**, Inefficient recycling of β2-AR-Ala measured using a biotinylation recycling assay. Efficient recycling of wild-type β2-AR but not β2-AR-Ala was observed by glutathione-resistance. **C**, Lysosomal degradation of β2-AR-Ala. Degradation of biotinylation β2-AR-Ala was significantly inhibited in cells treated with leupeptin (Leu), chloroquine (CQ) or NH_4Cl. Ctl, control. Error bars represent s.d.

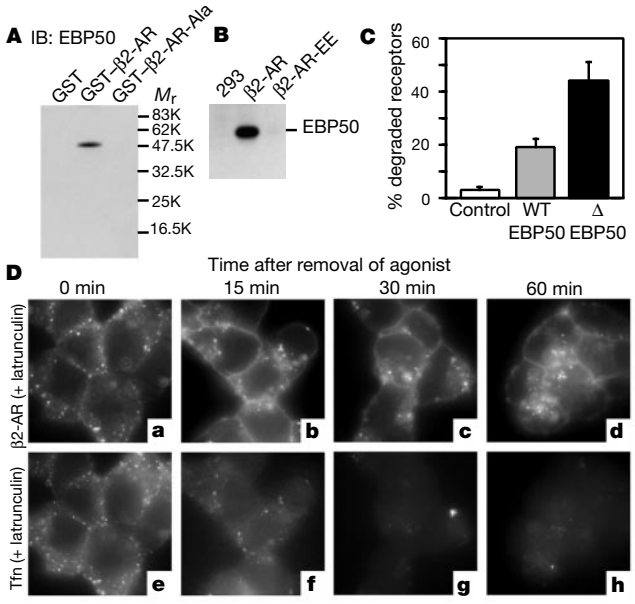

Figure 3 Role of EBP50 and actin in β2-AR sorting. **A**, EBP50 binds to the wild-type β2-AR cytoplasmic tail. Binding of EBP50 from HEK293 extracts to wild-type but not β2-AR-Ala tail fusion proteins. **B**, EBP50 associates with β2-AR *in vivo*. Anti-FLAG immunoprecipitates from untransfected 293 cells (293) and cells expressing FLAG-tagged β2-ARs (β2-AR) or mutant β2-AR (β2-AR-EE) were immunoblotted with anti-EBP50. **C**, Disrupting EBP50–ERM interaction increases β2-AR degradation. Biotin degradation assay of wild-type β2-ARs in cells expressing native EBP50 (control) or overexpressing truncated EBP50 (EBP50Δ) or wild-type protein (WT EBP50). Error bars represent s.e. ($n \geq 4$) **D**, The actin cytoskeleton is required for β2-AR recycling. Latrunculin B treatment inhibited recycling of β2-ARs (**a**–**d**) but not of transferrin receptors (Tfn; **e**–**h**).

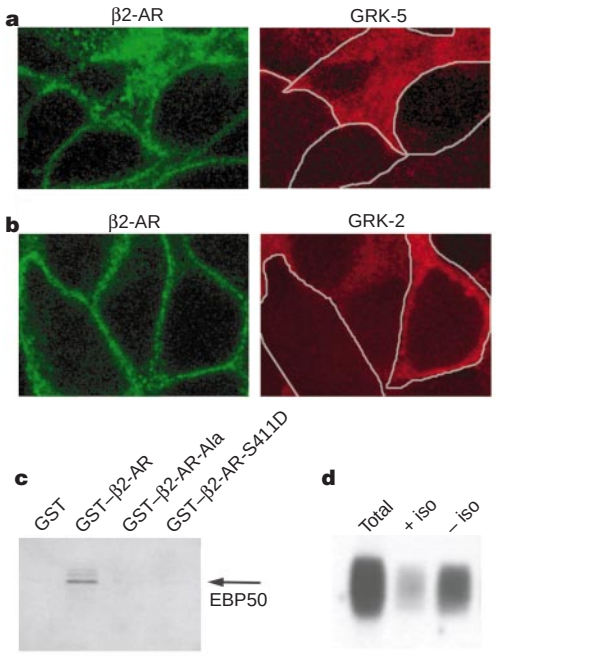

Figure 4 Regulation of β2-AR sorting by GRK-5. **a**, GRK-5 inhibits recycling of β2-ARs. Residual antibody-labelled β2-ARs (green) were observed following agonist removal in endocytic vesicles of transiently transfected cells overexpressing GRK-5 (red) but not adjacent cells not expressing GRK-5. **b**, Efficient recycling of antibody-labelled β2-ARs (green) was observed in cells overexpressing GRK-2 (red). Lines superimposed on red images indicate cell borders. **c**, S411D mutation disrupts β2-AR–EBP50 interaction. EBP50 bound specifically to GST fusion proteins containing cytoplasmic tails from wild-type β2-AR but not β2-AR-Ala or -S411D. **d**, Agonist-induced degradation of biotinylated β2-AR-S411D.

essential in endocytic sorting of the β2-AR. To test this idea, we examined endocytic sorting of wild-type β2-AR in cells pretreated with latrunculin B, which depolymerizes actin[13]. Latrunculin B markedly inhibited recycling of wild-type β2-ARs and caused internalized receptors to redistribute to perinuclear compartments (Fig. 3Da–d). This manipulation specifically disrupted efficient recycling of β2-ARs without causing detectable effects on recycling of transferrin receptors in the same cells (indicated by the complete disappearance of labelled transferrin from endocytic structures and dissociation of apo-transferrin)[14] (Fig. 3De–h), even though both receptors recycle by a similar membrane pathway[7]. Efficient recycling of transferrin receptors in the absence of an intact actin cytoskeleton has been shown in previous studies[13,15]. These results indicate that EBP50, ERM proteins and the actin cytoskeleton have a specialized function in the endocytic sorting of a subset of membrane proteins.

Serine 411 in the −2 position of the PDZ-binding domain (D SLL) of the β2-AR tail is a potential site for regulatory phosphorylation by G-protein-coupled receptor kinases (GRKs)[5]. GRKs are a family of protein kinases that phosphorylate ligand-activated β2-ARs and play a key role in rapid desensitization of receptors by promoting receptor–G-protein uncoupling[16] and endocytosis of receptors by clathrin-coated pits[17]. Ser 411 is phosphorylated *in vitro* by GRK-5 but not GRK-2, even though other residues in the cytoplasmic tail are phosphorylated by both kinases[5] and both kinases can promote uncoupling and endocytosis of the β2-AR[18]. Although the *in vivo* substrate specificities of GRK-2 and GRK-5 have not been established, precedent from studies of GRK-1-mediated phosphorylation of rhodopsin indicate that kinase specificity may be similar *in vitro* and *in vivo*[5,19]. As HEK293 cells express relatively high levels of GRK-2 (ref. 18) but not detectable GRK-5 (ref. 20), we examined the effects of overexpressing GRK-5 on β2-AR trafficking. Recycling of wild-type β2-AR was inhibited in transfected cells overexpressing GRK-5 but not in adjacent nontransfected cells (Fig. 4a). In contrast, overexpression of GRK-2 had no detectable effect on β2-AR recycling (Fig. 4b).

Replacing Ser 411 with an acidic residue may mimic the effects of protein phosphorylation and disrupts PDZ-mediated protein interaction with NHERF and E3KARP (NHERF-2)[11]. Replacement of Ser 411 with aspartic acid (S411D) blocked interaction of the β2-AR tail with EBP50 (Fig. 4c) and caused inefficient recycling and ligand-induced degradation of receptors (Fig. 4d). Although acidic substitution of Ser 411 does not necessarily mimic the effects of protein phosphorylation, crystallographic and mutational studies indicate that PDZ-mediated protein interactions involve hydrogen-bonding with the free hydroxyl group at the −2 position[21,22]. Consistent with this, alanine mutation of Ser 411 also blocked the interaction of EBP50 with the β2-AR tail and caused missorting of mutant receptors to lysosomes (not shown). These results indicate that Ser 411 is critical for β2-AR interaction with EBP50 and that GRK-mediated phosphorylation may disrupt this interaction and thereby regulate the sorting of internalized β2-ARs.

We conclude that sorting of internalized β2-ARs between recycling and degradative endocytic pathways is controlled by a protein interaction involving the distal carboxyl-terminal cytoplasmic domain of the β2-AR. We propose that this sorting operation is mediated by PDZ-mediated interaction of the β2-AR with EBP50/NHERF family proteins. This sorting function also involves inter-

action of EBP50 with ERM proteins and an intact actin cytoskeleton, indicating that EBP50 may link β2-ARs to the actin cytoskeleton. The efficient recycling of transferrin receptors observed under conditions that inhibit recycling of β2-ARs indicates that the PDZ-mediated sorting mechanism may specifically control the membrane trafficking of certain signalling receptors, in contrast to the constitutive recycling of many other membrane proteins (including the transferrin receptor) that occurs by 'default' membrane flow[23]. Our data provide evidence for a role for PDZ domains and the cortical cytoskeleton in regulating the sorting of receptors after endocytosis. In addition, they suggest a new function for specific GRK isoform(s) in regulating the membrane trafficking of G-protein-coupled receptors after endocytosis. Recycling of internalized β2-ARs back to the plasma membrane promotes dephosphorylation and functional resensitization of receptors, whereas sorting of internalized receptors to lysosomes promotes downregulation of receptors and long-term desensitization of receptor-mediated signal transduction[2]. Thus, the sorting mechanism identified here may be fundamental in the physiological regulation of signal transduction. □

Methods

Complementary cDNA constructs

N-terminally tagged (haemagglutinin (HA) and FLAG) human β2-ARs were constructed as described[7]. β2-AR_t was constructed by removing the coding sequence for the C-terminal 48 residues. β2-AR-EE was constructed by appending cDNA encoding the Glu-Glu epitope to the 3′ end of the full-length β2-AR coding sequence. β2-AR-Ala and S411D were constructed using the Quick-Change mutagenesis kit (Stratagene). Wild-type EBP50 (accession no. AF015926) was C-terminally HA-tagged, and EBP50Δ was generated by replacing the terminal 61 residues with an HA epitope. All cDNAs were cloned into pcDNA 3.0 (Invitrogen) and verified by dideoxynucleotide sequencing.

Cell culture

HEK293 cells were passaged and stably transfected as described[7]. Cells transiently expressing GRK-2 or GRK-5 (gifts from J. Benovic) were analysed 48 h after transfection.

Degradation of biotinylated receptors

Cells were surface biotinylated[24] with 300 μg ml^{-1} sulfo-NHS-biotin (Pierce), left at 4 °C to determine total biotinylated proteins, or incubated with or without 10 μM isoprenaline (RBI) for 4 h. Immunoprecipitation and detection of biotinylated receptors were done as described[24]. Studies using lysosomal inhibitors (200 μg ml^{-1} leupeptin (Sigma), 200 μM chloroquine (Sigma) or 50 mM NH_4Cl (Fisher Scientific)) were performed by 90 min preincubation before biotinylation, and inhibitors were present during all subsequent manipulations.

Immunofluorescence recycling assay

HA-tagged β2-ARs were surface labelled with 30 μg ml^{-1} HA.11 antibody and cells were incubated with 10 μM isoprenaline for 15 min to drive agonist-induced internalization. Residual agonist and antibody were removed, and coverslips were immediately fixed (to determine total internalized receptors) or incubated with the antagonist alprenolol (100 μM; Sigma) for 15, 30 or 60 min at 37 °C before fixation and processing for fluorescence microscopy[24]. GRK-2 and -5 were detected in permeabilized specimens using 0.8 mg ml^{-1} rabbit anti-GRK serum (Santa Cruz Biotechnology).

For receptor recycling experiments in the presence of latrunculin B (Alexis Corporation), cells were labelled with HA.11 and 10 μg ml^{-1} transferrin Texas Red (to label transferrin receptors). Following endocytosis (induced as above), cells were incubated at 4 °C with 10 μg ml^{-1} latrunculin B for 1 h, washed with PBS, warmed to 37 °C and subjected to recycling conditions (as described above) in the presence of 200 μg ml^{-1} unlabelled holotransferrin (Sigma).

Recycling of biotinylated receptors

Cells were preincubated with 100 μg ml^{-1} leupeptin for 90 min before biotinylation with sulpho-NHS-S-S-biotin[24]. Leupeptin was included in all subsequent steps to inhibit lysosomal proteolysis of internalized receptors. Cells were incubated with 10 μM isoprenaline for 15 min to drive endocytosis of biotinylated receptors, then agonist was washed away and cells were incubated with 100 μM alprenolol for 0, 5, 15, 30 or 60 min to block additional endocytosis and allow internalized receptors to recycle. Surface-associated biotin was cleaved quantitatively by treatment of intact cells with glutathione and residual biotinylated (internalized) receptors were detected as described[24].

Affinity chromatography and western blotting

HEK293 cells were lysed (50 mM Tris, pH 7.4, 1 mM EDTA and protease inhibitors), homeogenized and spun at 30,000*g* for 15 min. The supernatant was solubilized with 1% CHAPS and 150 mM NaCl (final concentration) and re-spun. Extracts were incubated with glutathione *S*-transferase fusion proteins containing wild-type (last 84 residues) or mutant β2-AR cytoplasmic tails as described[25]. Beads were washed with the lysis buffer including 1% CHAPS and 150 mM NaCl. Membranes were probed with a rabbit polyclonal antibody (B61) recognizing EBP50/NHERF/E3KARP[4] family members. Binding of ^{35}S-methionine-labelled full-length EBP50 to receptor tails was performed as described[25].

Co-immunoprecipitation

Cells expressing comparable levels of β2-ARs were treated with dithiobis(succinimidyl-propionate) (DSP) (Pierce), essentially as described[26], and FLAG-tagged β2-ARs were immunoprecipitated with M2-agarose (Sigma). The immunoprecipitates were washed[12] without reducing agents, and bound proteins were extracted in SDS sample buffer with 100 mM DTT and 200 mM β-ME.

Received 10 May; accepted 15 July 1999.

1. Ferguson, S. S., Zhang, J., Barak, L. S. & Caron, M. G. Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci.* **62,** 1561–1565 (1998).
2. Lefkowitz, R. J., Pitcher, J., Krueger, K. & Daaka, Y. Mechanisms of β-adrenergic receptor desensitization and resensitization. *Adv. Pharmacol.* **42,** 416–420 (1998).
3. Craven, S. E. & Bredt, D. S. PDZ proteins organize synaptic signaling pathways. *Cell* **93,** 495–498 (1998).
4. Reczek, D., Berryman, M. & Bretscher, A. Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. *J. Cell Biol.* **139,** 169–179 (1997).
5. Fredericks, Z. L., Pitcher, J. A. & Lefkowitz, R. J. Identification of the G protein-coupled receptor kinase phosphorylation sites in the human β2-adrenergic receptor. *J. Biol. Chem.* **271,** 13796–13803 (1996).
6. von Zastrow, M. & Kobilka, B. K. Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J. Biol. Chem.* **269,** 18448–18452 (1994).
7. von Zastrow, M. & Kobilka, B. K. Ligand-regulated internalization and recycling of human β2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267,** 3530–3538 (1992).
8. Weinman, E. J., Steplock, D., Wang, Y. & Shenolikar, S. Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane $Na^{(+)}$-H^+ exchanger. *J. Clin. Invest.* **95,** 2143–2149 (1995).
9. Yun, C. H. *et al.* cAMP-mediated inhibition of the epithelial brush border Na^+/H^+ exchanger, NHE3, requires an associated regulatory protein. *Proc. Natl Acad. Sci. USA* **94,** 3010–3015 (1997).
10. Hall, R. A. *et al.* The β2-adrenergic receptor interacts with the Na^+-H^+-exchanger regulatory factor to control Na^+-H^+ exchange. *Nature* **392,** 626–630 (1998).
11. Hall, R. A. *et al.* A C-terminal motif found in the β2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na^+-H^+ exchanger regulator factor family of PDZ proteins. *Proc. Natl Acad. Sci. USA* **95,** 8496–8501 (1998).
12. Reczek, D. & Bretscher, A. The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. *J. Biol. Chem.* **273,** 18452–18458 (1998).
13. Lamaze, C., Fujimoto, L. M., Yin, H. L. & Schmid, S. L. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* **272,** 20332–20335 (1997).
14. Klausner, R. D., Ashwell, G., van Renswoude, J., Harford, J. B. & Bridges, K. R. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl Acad. Sci. USA* **80,** 2263–2266 (1983).
15. Durrbach, A., Louvard, D. & Coudrier, E. Actin filaments facilitate two steps of endocytosis. *J. Cell Sci.* **109,** 457–465 (1996).
16. Pitcher, J. A., Freedman, N. J. & Lefkowitz, R. J. G protein-coupled receptor kinases. *Annu. Rev. Biochem.* **67,** 653–692 (1998).
17. Goodman, O. J. *et al.* β-arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. *Nature* **383,** 447–450 (1996).
18. Ménard, L. *et al.* Synergistic regulation of β2-adrenergic receptor sequestration: intracellular complement of β-adrenergic receptor kinase and β-arrestin determine kinetics of internalization. *MOl. Pharmacol.* **51,** 800–808 (1997).
19. Ohguro, H., Van Hooser, J. P., Milam, A. H. & Palczewski, K. Rodopsin phosphorylation and dephosphorylation *in vivo*. *J. Biol. Chem.* **270,** 14259–14262 (1995).
20. Seibold, A., January, B. G., Friedman, J., Hipkin, R. W. & Clark, R. B. Desensitization of β2-adrenergic receptors with mutations of the proposed G protein-coupled receptor kinase phosphorylation sites. *J. Biol. Chem.* **273,** 7637–7642 (1998).
21. Doyle, D. A. *et al.* Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell* **85,** 1067–1076 (1996).
22. Songyang, Z. *et al.* Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275,** 73–77 (1997).
23. Mayor, S., Presley, J. F. & Maxfield, F. R. Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J. Cell Biol.* **121,** 1257–1269 (1993).
24. Cao, T. T., Mays, R. W. & von Zastrow, M. Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J. Biol. Chem.* **273,** 24592–24602 (1998).
25. Klein, U., Ramirez, M. T., Kobilka, B. K. & von Zastrow, M. A novel interaction between adrenergic receptors and the α-subunit of eukaryotic initiation factor 2B. *J. Biol. Chem.* **272,** 19099–19102 (1997).
26. Hinck, L., Nathke, I. S., Papkoff, J. & Nelson, W. J. Dynamics of cadherin/catenin complex formation:

novel protein interactions and pathways of complex assembly. *J. Cell Biol.* **125,** 1327–1340 (1994).

Acknowledgements

We thank P. Tsao for generating the β2-AR$_t$ cell line and for advice and assistance with flow cytometric experiments; U. Klein for the β2-AR tail GST fusion protein; J. Benovic for GRK-2 and -5 cDNAs; and R. Kelly, P. Walter, F. Brodsky, P. Peluso and J. Wilhelm for valuable discussion. T.T.C. is supported by an NIH Institutional Training Grant. These studies were supported by the NIH.

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EPS8 and E3B1 transduce signals from Ras to Rac

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The small guanine nucleotide (GTP)-binding protein Rac regulates mitogen-induced cytoskeletal changes and c-Jun amino-terminal kinase (JNK), and its activity is required for Ras-mediated cell transformation[1]. Epistatic analysis placed Rac as a key downstream target in Ras signalling[2]; however, the biochemical mechanism regulating the cross-talk among these small GTP-binding proteins remains to be elucidated. Eps8 (relative molecular mass 97,000) is a substrate of receptors with tyrosine kinase activity[3] which binds, through its SH3 domain, to a protein designated E3b1/Abi-1 (refs 4, 5). Here we show that Eps8 and E3b1/Abi-1 participate in the transduction of signals from Ras to Rac, by regulating Rac-specific guanine nucleotide exchange factor (GEF) activities. We also show that Eps8, E3b1 and Sos-1 form a tri-complex *in vivo* that exhibits Rac-specific GEF activity *in vitro*. We propose a model in which Eps8 mediates the transfer of signals between Ras and Rac, by forming a complex with E3b1 and Sos-1.

Rac proteins belong to the sub-family of Rho-GTPases that become activated when bound GDP is exchanged for GTP. Within this family, Rho regulates the assembly of stress fibres, Rac controls membrane ruffling and lamellipodia and Cdc42 mediates the formation of filopodia. Rho-GTPases are organized in hierarchical cascades, in which activated Ras or Cdc42 can independently activate Rac[1,2]. Phosphatidylinositol 3-phosphate kinase (PI(3)K) is the immediate downstream effector of Ras[6], and might activate Rac through the products of its catalytic activity, phosphoinositide phosphates (PtdInsPs), which can bind and modulate the activity of GEFs for Rac, like Vav and h-Sos-1 (refs 7, 8). Initial results indicated that overexpression of Eps8 increased JNK activity, leaving mitogen-activated protein kinase (MAPK) unperturbed, leading to the testable hypothesis that Eps8 might be involved in the regulation of Rac (G.S. and P.P.D.F., unpublished observations).

We engineered an *eps*8 targeting vector (Fig. 1a) and used a targeted embryonic stem-cell clone (Fig. 1b) to derive mice heterozygous and homozygous for the *eps*8 mutated allele. Eps8-null mice displayed no obvious phenotype. We established *Eps*8 +/+, +/– and –/– immortalized fibroblast-like cell lines that displayed the predicted expression of Eps8 (Fig. 1c) and comparable levels of the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (see Supplementary Information). PDGF induced actin reorganization and membrane ruffles in +/+ but not in –/– (Fig. 1d). Re-expression of Eps8 in –/– cells restored ruffle formation (Fig. 1d). Similar results were obtained with EGF (data not shown). 12-*O*-tetradecanoylphorbal-13-acetate (TPA), which induces actin reorganization through mechanisms that are different from those of growth factors[9], induced ruffling in both –/– and +/+ fibroblasts. Thus Eps8 acts specifically on growth-factor-dependent actin remodelling.

Expression vectors encoding the activated versions of Ras, Rac, Cdc42 and PI(3)K (RasV12, RacQL, Cdc42QL and rCD2p110, respectively) induced ruffling in +/+ cells (Fig. 2). In –/– cells, RasV12 and CD2p110 did not induce ruffles (Fig. 2), despite readily detectable synthesis and intrinsic activity of both proteins (see Supplementary Information). Microinjection of vectors for

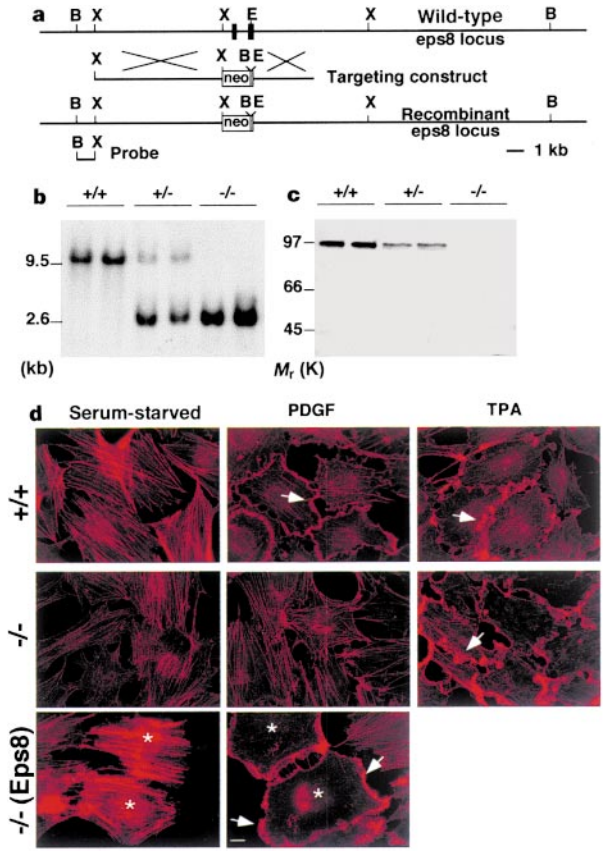

Figure 1 Disruption of *eps8* locus abrogates PDGF-induced membrane ruffling. **a**, Restriction map of the mouse *eps*8 locus and of the targeting construct. B, *Bam*HI; X, *Xho*I; E, *Eco*RV; solid bars indicate the two exons encoding the SH3 domain. **b**, Southern blot of *Bam*HI-digested mouse tail DNAs from 2+/+, 2+/– and 2–/– mice, probed with the 1-kb fragment indicated in **a**. **c**, Immunoblot, with the anti-Eps8 peptide serum (amino acids 32–55) Eps8 antibody, of cellular lysates from *eps*8+/+, +/– and –/– fibroblasts (four independently derived clones in each case, of which two are shown). **d**, *Eps*8+/+ (top) and –/– (middle and bottom) fibroblasts were treated as indicated and stained for actin. In the bottom panels–/– cells were also microinjected with pCR3–eps8 (–/–(Eps8)). Results in this and all subsequent experiments are representative of independently derived clones of +/+ and –/– fibroblasts (4 of each). Asterisks identify microinjected cells; arrowheads point to ruffles. Bar represents 10 μm.