**GTP loading.** Cells were transfected with 15 μg His-Rap or His-Ras and the indicated plasmids as described. Before treatment, cells were serum-starved in DMEM for 4 hours, washed three times and incubated in phosphate-free DMEM at 37 °C. After one hour, 0.5 mCi $ml^{-1}$ [$^{32}$P]orthophosphate in DMEM was added and cells were incubated for an additional 1–2 h. Rap1 was precipitated with Ni-NTA agarose and GTP loading was calculated as described[9].

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# The $\beta_2$-adrenergic receptor interacts with the $Na^+/H^+$-exchanger regulatory factor to control $Na^+/H^+$ exchange

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**Stimulation of $\beta_2$-adrenergic receptors on the cell surface by adrenaline or noradrenaline leads to alterations in the metabolism, excitability, differentiation and growth of many cell types. These effects have traditionally been thought to be mediated exclusively by receptor activation of intracellular G proteins[1]. However, certain physiological effects of $\beta_2$-adrenergic receptor stimulation, notably the regulation of cellular pH by modulation of $Na^+/H^+$ exchanger (NHE) function, do not seem to be entirely dependent on G-protein activation[2–7]. We report here a direct agonist-promoted association of the $\beta_2$-adrenergic receptor with the $Na^+/H^+$ exchanger regulatory factor (NHERF), a protein that regulates the activity of the $Na^+/H^+$ exchanger type 3 (NHE3)[8]. NHERF binds to the $\beta_2$-adrenergic receptor by means of a PDZ-domain-mediated interaction with the last few residues of the carboxy-terminal cytoplasmic domain of the receptor. Mutation of the final residue of the $\beta_2$-adrenergic receptor from leucine to alanine abolishes the receptor's interaction with NHERF and also markedly alters $\beta_2$-adrenergic receptor regulation of NHE3 in cells without altering receptor-mediated activation of adenylyl cyclase. Our findings indicate that agonist-dependent $\beta_2$-adrenergic receptor binding of NHERF plays a role in $\beta_2$-adrenergic receptor-mediated regulation of $Na^+/H^+$ exchange.**

$\beta_2$-Adrenergic receptors belong to the class of seven-transmembrane-domain receptors for hormones and neurotransmitters that possess extracellular amino termini and intracellular carboxy termini[1]. To identify proteins that might interact with the intracellular C-terminal tail of $\beta_2$-adrenergic receptors, we probed tissue extracts from various organs in blot overlay experiments using the $\beta_2$-receptor tail expressed as a fusion protein with glutathione-*S*-transferase (GST). These overlay assays revealed a single prominent band corresponding to a relative molecular mass of ~50K which was greatly enriched in kidney relative to other tissues. This protein was purified in one step from CHAPS-solubilized bovine kidney extract using a matrix consisting of the C-terminal tail of the $\beta_2$ receptor expressed as a GST-fusion protein and adsorbed to glutathione–agarose beads (Fig. 1a). Tryptic digestion and sequencing of the purified peptides yielded two fragments (VGQYIRLVEPGSPAEK

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and LLVVDRETDEFFK); a database search revealed that both fragments matched internal sequence stretches of NHERF, a 50K protein that is expressed in many tissues and at especially high levels in the kidney[8,9].

The identity of NHERF as the $\beta_2$-adrenergic receptor-interacting species was confirmed in two ways. First, samples that had been purified from kidney with the $\beta_2$-receptor tail fusion protein bead matrix were specifically recognized by anti-NHERF antibodies (Fig. 1b). Second, purified recombinant NHERF bound to the $\beta_2$-receptor tail in blot overlay experiments (Fig. 1c). The NHERF protein has a bipartite structure, with the two halves of the molecule (domains 1 and 2) containing one PDZ domain each. Full-length NHERF and NHERF domain 1 (the N-terminal 151 amino acids) were each recognized by the $\beta_2$-receptor tail probe whereas in overlay assays NHERF domain 2 (the C-terminal 207 amino acids) was not. Because phosphorylation by cyclic AMP-dependent protein kinase (PKA) is required for NHERF inhibition of NHE3 (ref. 10), we also examined whether PKA phosphorylation of NHERF would affect its binding to the tail of the $\beta_2$ receptor in the overlay experiments. No effect of NHERF phosphorylation by PKA was observed on $\beta_2$-receptor tail binding (Fig. 1c), consistent with the observation that the only consensus PKA phosphorylation sites on NHERF are located in domain 2 (ref. 8).

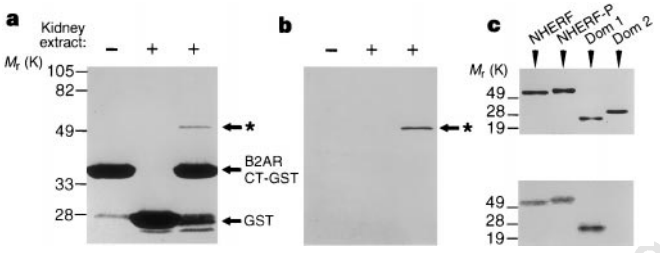


**Figure 1 a**, Purification of a $\beta_2$-receptor tail interacting protein. A Coomassie-blue stained SDS–PAGE gel of proteins eluted from glutathione–agarose resin is shown: from left to right, the resin was bound with $\beta_2$-receptor tail–GST fusion protein alone (B2ARCT-GST), bound with control GST that had been incubated with CHAPS-solubilized bovine kidney extract, or bound with $\beta_2$-receptor tail–GST fusion protein that had been incubated with CHAPS-solubilized bovine kidney extract. A 50K protein (asterisk) from the kidney extract that bound to the $\beta_2$-receptor tail–GST but not to control GST is indicated. **b**, The $\beta_2$-receptor tail interacting protein is recognized by anti-NHERF antibodies. A western blot of the three samples from **a** with an anti-NHERF antibody revealed specific labelling of the purified 50K species (asterisk). **c**, The $\beta_2$-receptor tail binds to recombinant NHERF fusion proteins on a blot overlay. $^{32}$P-phosphorylated $\beta_2$-receptor tail was overlaid onto four blotted samples: NHERF, PKA-phosphorylated NHERF ('NHERF-P'), NHERF domain 1 ('Dom 1') and NHERF domain 2 ('Dom 2'): top, Coomassie-blue stained gel. Results were similar from three experiments.

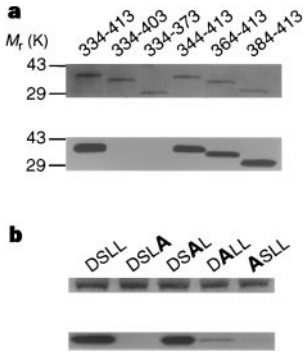


**Figure 2 a**, NHERF binds to the C-terminal 10 amino acids of the $\beta_2$-receptor tail. Five truncated $\beta_2$-receptor tail–GST fusion proteins were expressed and purified, and 5 μg of each was run on SDS–PAGE (top). A blot of these samples was overlaid with 50 nM NHERF domain-1 fusion protein (bottom). The numbers at the top of the gel represent the amino acids of the wild-type human $\beta_2$ receptor to which each truncated mutant fusion protein corresponds. **b**, NHERF binding to the $\beta_2$-receptor tail is inhibited by point mutations at or near the $\beta_2$-receptor C terminus. GST fusion proteins of the $\beta_2$-receptor tail with one of the final four amino acids (DSLL, as labelled at the top of the gel) mutated to alanine were expressed and purified, and 25 μg of each was run on SDS–PAGE (top). Samples were blotted and overlaid with 50 nM NHERF domain-1 fusion protein (bottom).

To examine the NHERF-binding determinants on the $\beta_2$ receptor, five truncated versions of the $\beta_2$ receptor C-terminal tail were expressed as GST fusion proteins and examined for their ability to bind NHERF in the blot overlay assay (Fig. 2a). The three tails that were truncated from the N-terminal side all interacted with NHERF with an affinity equal to that of the wild-type tail, whereas the two tails that were truncated from the C-terminal side did not detectably bind NHERF. As one of the C-terminal truncations was only missing ten amino acids, these results demonstrate that NHERF binds to the extreme C terminus of the $\beta_2$-receptor tail and suggest that this interaction is mediated by the first PDZ domain of NHERF. PDZ domains bind to three- or four-amino-acid stretches of C-terminal sequence on target proteins[11]. The first PDZ-domain-containing protein for which a preferred target protein C-terminal sequence was characterized was PSD-95, which was found to prefer Val at the C-terminal position and Thr or Ser at the −2 position of binding partners[12]. Subsequent analysis of other PDZ-domain proteins has revealed preferences for different C-terminal sequences[13]. To identify the C-terminal residues on the $\beta_2$-receptor tail required for NHERF binding, we sequentially replaced the C-terminal four amino acids of the $\beta_2$-receptor tail with alanine (Fig. 2b). Mutation of Leu to Ala at the terminal position resulted in a complete loss of NHERF binding to the tail. Mutation of Ser to Ala at the −2 position and Asp to Ala at the −3 position resulted in markedly reduced NHERF binding, whereas mutation of Leu to Ala at the −1 position had no effect.

To examine whether NHERF might interact with full-length $\beta_2$ receptors *in vivo*, we used immunocytochemistry to analyse HEK-293 cells expressing epitope-tagged versions of NHERF and either the wild-type $\beta_2$ receptor or a mutant $\beta_2$ receptor (L413A) in which the final residue was changed from leucine to alanine: according to our *in vitro* binding results, this point mutation should abolish the binding of NHERF to the $\beta_2$ receptor. Living cells were incubated with antibodies to detect the tagged $\beta_2$ receptors and to promote cell-surface receptor crosslinking into large aggregates that would be easily visible. The cells were then fixed, permeabilized and labelled with antibodies to detect NHERF that had been tagged with a different epitope.

As expected, the $\beta_2$ receptors aggregated into large patches in all cells and under all conditions as a result of antibody crosslinking (Fig. 3 a, c, e, g). Under control conditions, staining for NHERF was uniform throughout the cytoplasm and cell membrane (Fig. 3, b, f). Following stimulation of wild-type cells transfected with $\beta_2$ receptor by the specific β-adrenergic-receptor agonist isoprenaline, however, NHERF staining was notably more patchy (Fig. 3d), with most of these patches co-localizing with $\beta_2$ receptor-staining patches. This effect of isoprenaline was blocked by the $\beta_2$-receptor antagonist propranolol (100 μM) (data not shown). Treatment of the cells with forskolin (10 μM) had no effect on the subcellular distribution of NHERF (data not shown), indicating that the changes seen upon agonist stimulation were due to receptor activation and not simply to $\beta_2$-receptor-mediated adenylyl cyclase activation. In contrast to the effect of wild-type $\beta_2$-receptor stimulation on NHERF localization, cells transfected with the L413A mutant receptor showed no agonist-induced change in NHERF subcellular distribution after stimulation with isoprenaline (Fig. 3h). The targeting of NHERF to receptor-rich cellular domains following isoprenaline stimulation of wild-type but not L413A mutant $\beta_2$ receptors strongly suggests that full-length wild-type $\beta_2$ receptors associate with NHERF in cells, that this association depends on agonist activation of the

receptors, and that it is mediated by the receptors' most C-terminal amino acids.

As NHERF is known to be a regulator of NHE3 (refs 8, 10, 14), we did co-reconstitution experiments to address whether binding of NHERF to the $\beta_2$-receptor tail might affect NHERF regulation of the NHE3. Reconstituted kidney brush-border membranes were prepared[10] in such a way that native NHE3 was fully functional but native NHERF was removed. Recombinant NHERF was then added back to the preparation in the presence of active PKA and ATP, resulting in more than 30% inhibition of NHE3 (Fig. 4). The effect of NHERF on NHE3 function was completely blocked in the presence of the $\beta_2$-receptor tail expressed as a soluble GST fusion protein. The right-hand bar in Fig. 4 indicates that the $\beta_2$-receptor tail fusion protein in the absence of NHERF has no effect on NHE3 function. Further control studies revealed that a GST protein without the $\beta_2$-receptor tail fused to it had no effect on NHERF-mediated inhibition of NHE3 (data not shown). These results indicate that the binding of NHERF to the $\beta_2$-receptor tail prevents NHERF from regulating NHE3, either by preventing the NHERF/NHE3 interaction or by altering the conformation of NHERF so that it can no longer inhibit NHE3.

To test whether binding of NHERF to the $\beta_2$ receptor affects adrenergic regulation of NHE3, we examined the effect of $\beta_2$ receptor stimulation on NHE3 function in intact cells in a mutant Chinese hamster ovary (CHO) cell line[15,16] which stably expresses NHE3 but lacks all other NHE isoforms. CHO cells express high levels of NHERF[14], a finding we verified by western blot analysis of our mutant CHO cells (data not shown). CHO cells expressing NHE3 were stably transfected with either wild-type $\beta_2$ receptor or the L413A mutant receptor. The two cell lines were similar with respect to ligand binding ($B_{max}$ for both cell lines was 1.4 pmol receptor per mg total protein) and agonist-induced cAMP accumulation (wild-type line showed 1.28 ± 0.29% conversion; the L413A mutant line, 1.09 ± 0.25%; non-transfected CHO cells, 0.05 ± 0.02%). Stimulation with forskolin inhibits $Na^+/H^+$ exchange in CHO cells expressing NHE3 (refs 15, 16), and we found an equivalent amount of forskolin-induced inhibition of $Na^+/H^+$ exchange in the two stable $\beta_2$ receptor cell lines (Fig. 5). The two cell lines differed markedly, however, with regard to adrenergic regulation of $Na^+/H^+$ exchange: stimulation with isoprenaline of the wild-type $\beta_2$ receptor cell line had little effect on NHE3 activity (Fig. 5, left), whereas isoprenaline stimulation of the L413A mutant $\beta_2$ receptor cell line inhibited NHE3 activity by ~50% (Fig. 5, right).

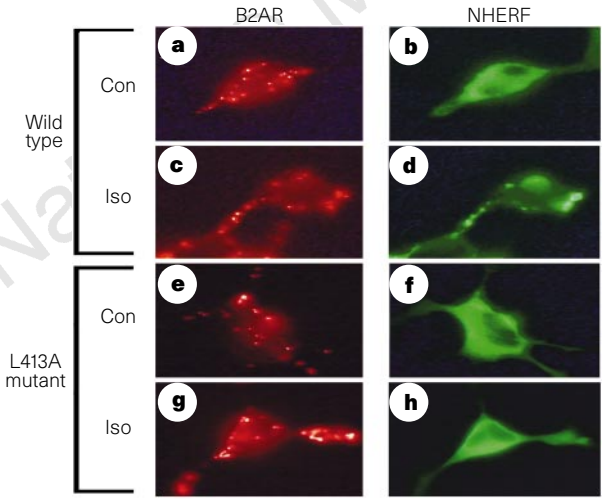


**Figure 3** NHERF co-localizes in cells with full-length wild-type $\beta_2$ receptor, but not with full-length L413A mutant $\beta_2$ receptor, after receptor stimulation with agonist. Left panels show immunostaining of Flag-tagged $\beta_2$ receptor (B2AR) over-expressed in HEK-293 cells (red); right panels show HA-tagged NHERF staining in the same cells (green). Cells are shown under control conditions (Con), when $\beta_2$ receptor distribution was extremely patchy (**a**, **e**) because receptors were crosslinked with antibody (see Methods) and NHERF distribution was quite uniform (**b**, **f**). Following stimulation with 50 μM isoprenaline (Iso) the pattern of $\beta_2$ receptor distribution was unchanged (**c**), but NHERF staining was more patchy (**d**), with most of the patches corresponding to regions rich in $\beta_2$ receptor staining. This agonist induced co-localization of $\beta_2$ receptor and NHERF was not observed in the cells expressing the L413A mutant $\beta_2$ receptor (**g**, **h**). Cells are representative of 4–6 independent experiments.

The effect of forskolin on NHE3 function is believed to be mediated by PKA phosphorylation of both the NHE3 and NHERF, which leads to NHERF binding to and inhibition of NHE3 (refs 15, 16). According to this model, activation of $G_s$-protein-coupled receptors such as the $\beta_2$ receptor should also inhibit NHE3 activity. Consistent with this, agonist activation of the L413A mutant $\beta_2$ receptor does inhibit NHE3, but agonist activation of the wild-type receptor does not. As each receptor activates adenylyl cyclase to the same extent and the only apparent difference between the two receptors is that the wild-type receptor binds NHERF and the mutant does not, the wild-type receptor probably mediates two distinct effects following agonist activation: it binds $G_s$, activating adenylyl cyclase, but it also binds NHERF, thereby preventing the NHE3 inhibition that would be expected to follow adenylyl cyclase activation.

The effect of hormone stimulation on $Na^+/H^+$ exchanger activity is not completely understood[17–19]: for example, $\beta_2$ receptor stimulation regulates both NHE1 (refs 2–5) and NHE3 (refs 6, 7) in a manner that cannot be explained by the ability of $\beta_2$ receptors to activate adenylyl cyclase through G-protein signalling. These apparently anomalous findings can be explained, at least in part, by the discovery that $\beta_2$ receptors bind in an agonist-dependent manner to a protein, NHERF, which is known to inhibit NHE activity. In the case of NHE3, activation of PKA by stimulation with parathyroid hormone or by incubation with cAMP analogues inhibits NHE3 activity in membrane-reconstitution studies[20–22] and decreases sodium reabsorption in the kidney proximal tubule[23]. However, stimulation of $\beta_2$ receptors paradoxically increases sodium reabsorption in the kidney proximal tubule by apparently stimulating NHE3 activity[6,7]. This paradox could be explained if there is a basal level of PKA activation and thus of NHERF-mediated NHE3 inhibition in proximal tubules: the binding of activated $\beta_2$ receptors to NHERF might relieve this basal inhibition. Receptor activation may thus stimulate NHE3 activity if the receptor density is high enough to bind a significant fraction of activated cellular NHERF.

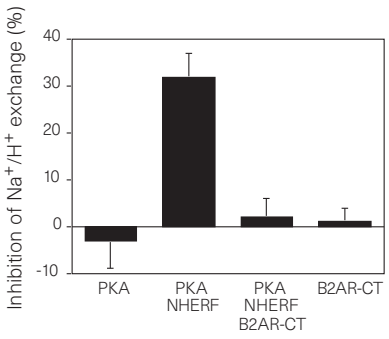


**Figure 4** $\beta_2$-receptor tail–GST fusion protein blocks the ability of NHERF to regulate NHE3. The effects of various recombinant proteins on $Na^+/H^+$ exchange mediated through NHE3 in a partially purified reconstituted brush-border membrane preparation were examined. Addition of PKA (50 U ml$^{-1}$) had no effect, but addition of NHERF (1 μg ml$^{-1}$) along with PKA gave >30% inhibition of sodium flux. This effect was blocked by co-application of $\beta_2$-receptor tail–GST fusion protein (1 μg ml$^{-1}$; B2AR-CT); the receptor tail–GST by itself (1 μg ml$^{-1}$) had no effect on sodium flux. Bars and error bars represent the mean ± s.e.m. for 3 experiments. The average basal value for $^{22}Na^+$ uptake in these experiments was 15.8 pmol per mg protein.

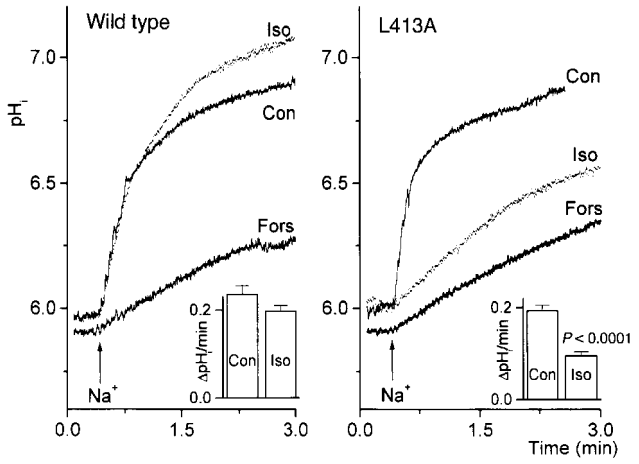


**Figure 5** Agonist stimulation of L413A mutant $\beta_2$ receptors but not wild-type $\beta_2$ receptors inhibits NHE3 in whole cells. $pH_i$ was measured fluorometrically in stable cell lines, expressing either NHE3/wild type $\beta_2$ receptor (left) or NHE3/L413A mutant $\beta_2$ receptor (right), which were acid-loaded (see Methods) and bathed in $Na^+$-free solution. Where indicated, NHE was activated by addition of $Na^+$-rich medium (arrow). In the presence of forskolin ('Fors'), NHE3 activity was inhibited in both cell lines. This effect was mimicked by stimulation of the L413A mutant receptor cell line with isoprenaline ('Iso') but not by stimulating the wild-type $\beta_2$-receptor cell line. Traces are representative of at least 4 independent experiments for each condition. Inset, quantification of the initial rate of $pH_i$ recovery after an acute acid load. Values were calculated by deriving the change of $pH_i$ with time every 2.5 s for 60 s after $Na^+$ addition. Values are means ± s.e. for 6 and 8 determinations for untreated ('Con') and isoprenaline-treated wild-type $\beta_2$-receptor-expressing cells, respectively, and for 12 and 10 determinations for untreated and isoprenaline-treated L413A-mutant-receptor-expressing cells, respectively. Statistics were done using the Student's *t*-test.

The agonist-dependence of the association between $\beta_2$ receptors and NHERF is analogous with the association of other proteins with $\beta_2$ receptors: for example, both G proteins and β-arrestins associate with $\beta_2$ receptors in an agonist-promoted fashion[1,24]. However, the primary determinants of G-protein[1] and β-arrestin[24] interaction with the $\beta_2$ receptor are located in the third intracellular loop of the receptor, whereas NHERF interacts with the receptor C-terminal tail. As mutant $\beta_2$ receptors with truncated C-terminal tails signal normally through adenylyl cyclase[25–27], it has been assumed that the C-terminal tail is not essential for $\beta_2$ receptor signalling. Our results, however, indicate that the $\beta_2$ receptor tail is important for signal generation, in particular for the regulation of NHE activity; they also reveal a mechanism distinct from G-protein activation by which seven-transmembrane-domain receptors like the $\beta_2$ receptor can influence intracellular events. □

## Methods

**SDS–PAGE and tissue overlays.** A probe was made using $\beta_2$-receptor tail GST fusion protein (the C-terminal 80 amino acids of the human $\beta_2$-adrenergic receptor) adsorbed to glutathione–agarose beads and phosphorylated with 2 μg ml$^{-1}$ PKA and 0.1 mCi ml$^{-1}$ [$^{32}$P]-ATP for 1 h at 30 °C. The beads were then washed extensively with PBS and the radiolabelled $\beta_2$-receptor tail was cleaved away from the GST-bead mix using recombinant thrombin in a buffer supplied by the manufacturer (Boehringer Mannheim). Overlay experiments using this probe were done by running samples of bovine tissues (50 μg per lane) on 4–20% SDS–PAGE (Novex) for 1 h at 150 V and then blotting onto nitrocellulose for 40 min at 12 V. Blots were blocked in 2% milk/0.1% Tween-20 in PBS ('blot buffer') and incubated with the phosphorylated $\beta_2$-receptor tail probe in blot buffer for 1 h. Blots were washed five times with blot buffer, once in PBS, and then exposed to Kodak X-OMAT film.

**Purification and sequencing.** To purify $\beta_2$-receptor-interacting proteins, salt-washed bovine kidney membranes (500 mg total protein) were solubilized for 1 h in a buffer containing 10 mM HEPES, pH 7.5, 50 mM NaCl, 1% CHAPS, 1 mM PMSF, 1 mM benzamidine and 1 mM EDTA ('solubilization buffer'). After a 20 min centrifugation at 48,000*g*, to remove insoluble material, the soluble supernatant was incubated with $\beta_2$-receptor tail–GST fusion protein adsorbed to glutathione–agarose for 1 h at 4 °C. The beads were pelleted by brief centrifugation and washed five times with 40 ml fresh solubilization buffer. Finally, the beads were incubated with SDS–PAGE sample buffer for 30 min at 37 °C to elute adsorbed proteins from the bead matrix. Samples were then run on 4–20% SDS–PAGE and visualized by staining with Coomassie blue. Peptides for sequencing were generated by cutting the bands of interest out of the gel, followed by trypsin digestion and HPLC purification.

**Fusion protein overlays and western blotting.** Hexahistidine-tagged and S-tagged NHERF, NHERF domain 1 (1–151) and NHERF domain 2 (152–358) fusion proteins were created by insertion of rabbit NHERF cDNA[8] into pET-30A (Novagen). Fusion proteins were expressed according to manufacturer's instructions. Purified NHERF fusion proteins were run on SDS–PAGE and overlay assays done as described. In other experiments, wild-type or mutant $\beta_2$-receptor tails expressed as GST fusion proteins were examined for NHERF binding by a far-western blot overlay technique. Mutant $\beta_2$-receptor tail–GST constructs were created by PCR using mutant sequence oligonucleotides and inserting the PCR products in a pGEX-2T vector (Pharmacia); all mutations were confirmed by sequencing with an ABI377 automated sequencer. Fusion proteins were run on SDS–PAGE gel, blotted and overlaid with 50 nM NHERF domain-1 fusion protein in blot buffer for 1 h at room temperature. Blots were washed three times, incubated for 1 h at room temperature with an HRP-conjugated anti-S-tag antibody (Novagen), washed three more times and visualized with an enhanced chemiluminescence kit (Boehringer Mannheim).

An anti-NHERF antibody was created by injecting full-length NHERF fusion protein into rabbits, boosting at four weeks and then bleeding at 10 weeks. The blood serum was passed over a DEAE Affi-gel blue column (BioRad). The resulting purified IgG fraction demonstrated specific binding to recombinant NHERF and NHERF domain 1 but not to other fusion proteins. This antibody also binds to a single major 50K species in bovine kidney (presumably bovine NHERF). Western blots were performed via standard procedures.

**Immunocytochemistry.** A haemagglutinin (HA)-tagged NHERF construct was prepared by subcloning rabbit full-length NHERF into a modified version of the pBK-CMV vector (Stratagene) which places an HA tag at the N terminus of expressed proteins. HEK-293 cells on coverslips in 6-well plates were co-transfected with 0.5 μg of this construct and 0.5 μg of a Flag-tagged $\beta_2$-adrenergic receptor construct in the vector pCDNA1 (ref. 28) by calcium phosphate co-precipitation[28]. Two days later, cells were washed with MEM (Gibco-BRL) and incubated for 30 min at room temperature with M2 monoclonal Flag antibody (Kodak) at a dilution of 1:400. Following three washes with MEM, cells were incubated with a goat anti-mouse Texas red-conjugated whole secondary antibody (Molecular Probes) at a dilution of 1:250 for 30 min. This allows visualization of the transfected $\beta_2$ receptors and artificially induces clustering of the receptors into large patches so that the co-localization of proteins with $\beta_2$ receptors may be studied[29]. After three washes with MEM, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 1% BSA/0.04% saponin in PBS ('saponin buffer'), then incubated successively with rabbit anti-HA (Berkeley Antibody) at 1:100 in saponin buffer and a fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes) at 1:250; each step was followed by three washes with 1 ml saponin buffer. Coverslips were examined using a Leica DM model 50 microscope at 100× magnification. Control experiments used untransfected or singly transfected cells to verify that each antibody was specifically detecting the appropriate transfected protein.

**Creation of wild-type or L413A mutant $\beta_2$ receptor stable cell lines.** A Flag-tagged L413A mutant $\beta_2$ receptor was prepared by PCR amplification using a primer bearing the desired mutation followed by replacement of a cassette in the Flag-tagged wild-type $\beta_2$ receptor construct. NHE3-AP1 cells[15,16] were transfected by the calcium phosphate method[28] with the plasmid pSV2-neo and either the Flag-tagged wild-type $\beta_2$ receptor or the Flag-tagged L413A mutant receptor. Stable transfectants were selected for two weeks by adding 1 mg ml$^{-1}$ G418 to the medium. Transfection was confirmed by immunocytochemistry, radioligand binding and adenylyl cyclase studies[30].

**Reconstitution assay.** The preparation of kidney brush-border membrane

vesicles for reconstitution assays has been described[10,20–22]. Briefly, the internal pH of the vesicles was set at 6.0 by dialysis. The final uptake solution contained 1 mM $^{22}Na^+$, 250 mM mannitol, 30 mM potassium gluconate, 1 μg ml$^{-1}$ valinomycin and 50 mM Tris/MES at either pH 6.0 or 8.0. Sodium uptake was determined by application of the reaction mix to 1 ml Dowex 50X8 (Tris), 100-mesh columns and rapid elution with vacuum suction with 1 ml 300 mM mannitol (pH 8.0) at 0 °C. Under these voltage-clamped conditions, the proton-stimulated component of sodium uptake was taken as a measure of $Na^+/H^+$ exchange rate.

**Quantification of $Na^+/H^+$ exchanger activity by cytosolic pH measurement.** The intracellular pH ($pH_i$) of small groups of cells was determined by microphotometry of the fluorescence emission of the pH-sensitive dye, 2′7′bis-(2-carboxyethyl)-5(and 6)carboxyfluorescein, using dual wavelength excitation[15,16]. Cells grown to 70–80% confluence on glass coverslips were concurrently loaded with 25 mM $NH_4Cl$ and 2 mg ml$^{-1}$ of the acetoxymethyl ester precursor of 2′7′bis-(2-carboxyethyl)-5(and 6)carboxyfluorescein in PBS for 10 min at 37 °C. Where indicated, 10 μM forskolin or 50 μM isoprenaline was also added. Acid loading was accomplished by washing cells with a $Na^+$-free medium containing (in mM): 117 *N*-methyl-D-glucammonium chloride, 1.66 $MgSO_4$, 1.36 $CaCl_2$, 5.36 KCl, 25 HEPES, 5.55 glucose, pH 7.5, 290 ± 10 mosM. Sodium-dependent $pH_i$ recovery was then initiated by perfusing the cells with a $Na^+$-rich solution composed of (in mM): 117 NaCl, 1.66 $MgSO_4$, 1.36 $CaCl_2$, 5.36 KCl, 25 HEPES, 5.55 glucose, pH 7.5. To measure fluorescence, the coverslip was placed into a holding chamber attached to the stage of a Nikon Diaphot TMD inverted microscope equipped with a Nikon Fluor 40x/1.3 N.A. oil-immersion objective. Clusters of 6–12 cells were selected for analysis with an adjustable diaphragm. Excitation light provided by a Xenon lamp was alternately selected using 495 ± 10 nm and 445 ± 10 nm filters at a rate of 50 Hz and then reflected onto the cells by a 510-nm dichroic mirror. Emitted light was directed to the photometer through a 530 ± 30 nm band-pass filter. Photometric data were acquired at 10 Hz using a 12-bit A/D board (Labmaster, National Instruments) interfaced to a Dell 486 computer and analysed with Felix software (Photon Technologies). Calibration of fluorescence intensity with $pH_i$ was done in the presence of 5 mM nigericin in high-$K^+$ medium (140 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose) as described[15,16]

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## corrections

### Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein

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*Nature* **379,** 343–346 (1996)

In this Letter, "96P" indicates a proline at residue 96, and not phenylalanine as published. □

### A new pattern for helix–turn–helix recognition revealed by the PU.1 ETS-domain–DNA complex

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In the legend to Fig. 3, lines 11 and 12 should read: "Arg 232 (NH1) makes two hydrogen bonds with G9(06) and G9(N7)." In panel b, a dashed line indicating a hydrogen bond to base A10 should be deleted. The coordinates for the complex remain unchanged as originally submitted to the Brookhaven Protein Data Bank under accession number 1 pue. □