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Functional rescue of mutant V2 vasopressin receptors causing nephrogenic diabetes insipidus by a co-expressed receptor polypeptide

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Inactivating mutations in distinct G protein-coupled receptors (GPCRs) are currently being identified as the cause of a steadily growing number of human diseases. Based on previous studies showing that GPCRs are assembled from multiple independently stable folding units, we speculated that such mutant receptors might be functionally rescued by 'supplying' individual folding domains that are lacking or misfolded in the mutant receptors, by using a co-expression strategy. To test the feasibility of this approach, a series of nine mutant V2 vasopressin receptors known to be responsible for X-linked nephrogenic diabetes insipidus were used as model systems. These mutant receptors contained nonsense, frameshift, deletion or missense mutations in the third intracellular loop or the last two transmembrane helices. Studies with transfected COS-7 cells showed that none of these mutant receptors, in contrast to the wild-type V2 receptor, was able to bind detectable amounts of the radioligand, [^{3}H]arginine vasopressin, or to activate the G_s/adenylyl cyclase system. Moreover, immunological studies demonstrated that the mutant receptors were not trafficked properly to the cell surface. However, several of the nine mutant receptors regained considerable functional activity upon co-expression with a C-terminal V2 receptor peptide spanning the sequence where the various mutations occur. In many cases, the restoration of receptor activity by the co-expressed receptor peptide was accompanied by a significant increase in cell surface receptor density. These findings may lead to the design of novel strategies in the treatment of diseases caused by inactivating mutations in distinct GPCRs.

Keywords: co-expression experiments/nephrogenic diabetes insipidus/receptor mutagenesis/receptor peptide/V2 vasopressin receptor

Introduction

An extraordinarily large number of neurotransmitters, hormones and autocrine and paracrine factors exert their physiological functions via binding to plasma membrane receptors which, through coupling to different classes of heterotrimeric G proteins, can activate or inhibit specific signal transduction pathways (Watson and Arkinstall, 1994). All these G protein-coupled receptors (GPCRs) share a common molecular architecture characterized by the presence of seven α-helically arranged transmembrane domains (TM I–VII) connected by three extracellular and three intracellular loops (Dohlman *et al.*, 1991; Strosberg, 1991; Strader *et al.*, 1994). Following the cloning and sequencing of several hundred members of this receptor superfamily, mutations in distinct GPCRs that are responsible for human diseases are currently being uncovered at an ever increasing rate (Coughlin, 1994; Birnbaumer, 1995; Spiegel, 1996). At a molecular level, such mutations can lead to receptors which are constitutively active (activating mutations) or which are impaired in their ability to bind ligands and/or to couple to G proteins (inactivating mutations).

Recent *in vivo* studies suggest that GPCRs are assembled from multiple independently stable folding units (Kobilka *et al.*, 1988; Maggio *et al.*, 1993a,b; Ridge *et al.*, 1995; Schöneberg *et al.*, 1995). Studies with m2 and m3 muscarinic acetylcholine receptors, for example, have shown that co-expression of receptor fragments resulting from 'splitting' these receptors in various intracellular and extracellular loops leads to the appearance of functional receptor proteins (Maggio *et al.*, 1993a,b; Schöneberg *et al.*, 1995). These findings prompted us to speculate that the 'subunit character' of GPCRs should allow the design of strategies aimed at restoring function to clinically relevant GPCRs harboring inactivating mutations. It should be possible, for example, to functionally rescue GPCRs containing nonsense mutations by 'supplying' the receptor portion missing in the truncated receptor protein. Moreover, a similar approach might also be used to restore function to mutant receptors containing inactivating point mutations (missense mutations), assuming that such mutations specifically interrupt the structural integrity of the folding domain in which they are located.

To test the feasibility of this approach, we have chosen the V2 vasopressin receptor as a model system. Physiologically, stimulation of V2 vasopressin receptors in renal collecting tubule cells results in the activation of the G_s/adenylyl cyclase system, thus promoting, via insertion of water pores into the luminal membrane, the reabsorption of fluid (Laszlo *et al.*, 1991). Recently, many laboratories have shown (for reviews, see Raymond, 1994; Birnbaumer, 1995; Spiegel, 1996) that inactivating mutations in the V2 receptor are responsible for nephrogenic diabetes insipidus (NDI), an X-linked recessive disorder characterized by an inability of the kidney to concentrate urine despite elevated levels of the hormone, arginine vasopressin (AVP).

In this study, a series of clinically relevant mutant V2 receptors containing different mutations (nonsense, frameshift, deletion or missense mutations) in the third intracellular loop (i3) or the last two TM domains was created (Figure 1A), and transiently expressed in COS-7

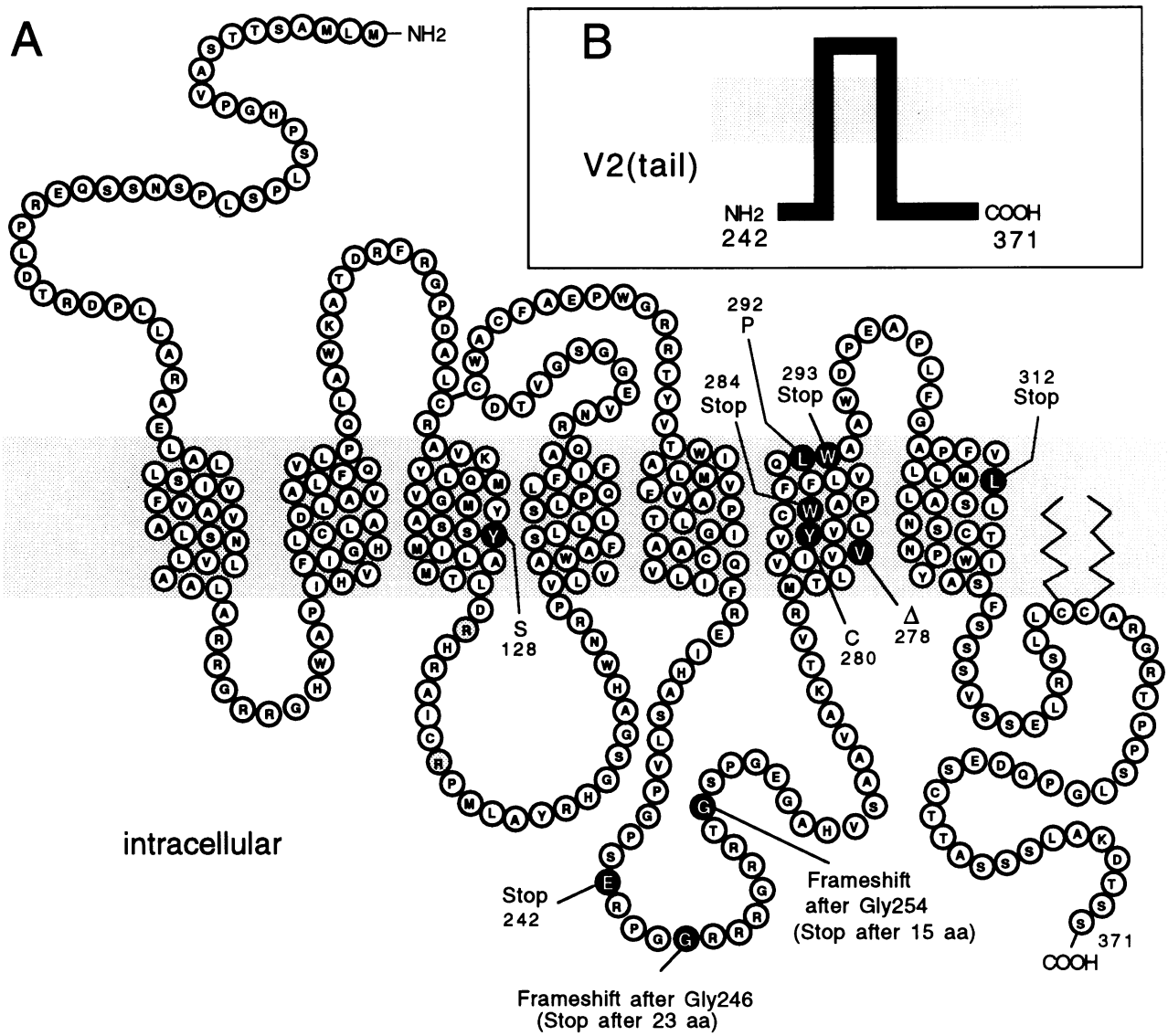

Fig. 1. Schematic representation of the human V2 vasopressin receptor (Birnbaumer *et al.*, 1992). (**A**) Summary of disease-causing mutant V2 receptors analyzed in this study. Glu242→Stop (Wildin *et al.*, 1994), Trp284→Stop (Bichet *et al.*, 1994), Trp293→Stop (Bichet *et al.*, 1994) and Leu312→Stop (Bichet *et al.*, 1993) contain nonsense mutations leading to truncated receptor proteins. In Gly246→FS (Rosenthal *et al.*, 1992) and Gly254→FS (Wildin *et al.*, 1994), the deletion of a single nucleotide after the indicated codons leads to a shift of the amino acid reading frame, resulting in a premature stop codon after 23 (AAGDAGQAAPVREPTCQQLWPRL) and 15 amino acids (APVREPTCQQLWPRL) of nonsense sequence, respectively. Tyr280→Cys (Wenkert *et al.*, 1994), Leu292→Pro (Wenkert *et al.*, 1994) and Tyr128→Ser (Pan *et al.*, 1994) contain single point mutations, whereas a single amino acid is deleted in ΔVal278 (Tsukaguchi *et al.*, 1993). (**B**) Structure of the V2(tail) polypeptide (Glu242–Ser371). To allow translation of this polypeptide, an ATG start codon was placed immediately upstream of the Glu242 codon (see Materials and methods).

cells. We demonstrated initially that all these mutant receptors were unable to bind the agonist, AVP, and to couple to G proteins. In the next step, we examined the possibility that these receptors can be functionally rescued by co-expression with a C-terminal V2 receptor fragment [V2(tail)] spanning the sequence where the various mutations occur (Figure 1B).

We could demonstrate that the presence of the V2(tail) polypeptide enabled many of the tested V2 mutant receptors to couple efficiently to the G_s/adenylyl cyclase system. These findings may open new perspectives in the treatment of the rapidly growing number of diseases caused by inactivating mutations in distinct GPCRs.

Results

Pharmacological characterization of mutant V2 receptors

Initially, we created a series of mutant V2 receptors known to occur in NDI patients (Figure 1A). These mutations (all of which are located in the C-terminal third of the receptor protein) include four nonsense (Glu242→Stop, Trp284→Stop, Trp293→Stop and Leu312→Stop) and two frameshift mutations (Gly246→FS and Gly254→FS) which result in truncated receptor proteins, a single amino acid deletion (ΔVal278), and two missense mutations (Tyr280→Cys and Leu292→Pro). A nine amino acid

Table I. Appearance of specific [^{3}H]AVP binding sites after co-expression of mutant V2 vasopressin receptors with the V2(tail) polypeptide

Construct	Amount of transfected DNA (μg)	[^{3}H]AVP binding	
		B_{max} (fmol/mg protein)	K_D (nM)
V2(wt)	4	703 ± 65	1.43 ± 0.24
V2(wt)	0.4	156 ± 1	2.06 ± 0.64
Glu242→Stop + V2(tail)	2 + 2	78 ± 4	0.90 ± 0.26
Trp284→Stop + V2(tail)	2 + 2	13 ± 3	0.30 ± 0.16
Gly246→FS + V2(tail)	2 + 2	84 ± 3	1.05 ± 0.27
Gly254→FS + V2(tail)	2 + 2	40 ± 8	0.70 ± 0.22

[^{3}H]AVP saturation binding studies were carried out with membrane homogenates prepared from transfected COS-7 cells as described in Materials and methods. Transfection of COS-7 cells with the various mutant V2 receptor constructs alone did not lead to any detectable [^{3}H]AVP binding activity. Similarly, co-expression with V2(tail) of the remaining five mutant V2 receptors not listed in the table (Trp293→Stop, Leu312→Stop, ΔVal278, Tyr280→Cys and Leu292→Pro) also did not result in the appearance of a significant number of [^{3}H]AVP binding sites. Data are presented as means ± SD of two or three independent experiments, each performed in duplicate.

Table II. AVP-induced stimulation of cAMP accumulation mediated by mutant V2 vasopressin receptors upon co-expression with the V2(tail) polypeptide

Construct	AVP EC_{50} (nM)	Maximum increase in cAMP levels (-fold above basal)
V2(wt) (4 μg)	0.16 ± 0.09	13.0 ± 3.0
V2(wt) (0.4 μg)	0.10 ± 0.01	9.2 ± 1.2
Glu242→Stop + V2(tail)	0.13 ± 0.06	7.8 ± 2.1
Trp284→Stop + V2(tail)	0.22 ± 0.01	6.3 ± 2.3
Trp293→Stop + V2(tail)	1.27 ± 0.34	2.4 ± 0.3
Leu312→Stop + V2(tail)	0.36 ± 0.02	3.0 ± 0.5
Gly246→FS + V2(tail)	0.17 ± 0.01	6.1 ± 2.4
Gly254→FS + V2(tail)	0.14 ± 0.01	13.0 ± 1.2
ΔVal278 + V2(tail)	0.39 ± 0.28	2.7 ± 0.3
Tyr280→Cys + V2(tail)	0.51 ± 0.26	4.1 ± 1.3
Leu292→Pro + V2(tail)	1.18 ± 0.42	2.3 ± 0.4

cAMP assays were carried out with transfected COS-7 cells as described in Materials and methods. Cells transfected with the nine mutant V2 receptor constructs alone did not mediate a significant increase in cAMP production. In the co-expression experiments, 2 μg of each plasmid (per plate) were used for transfections. To lower wild-type V2 receptor levels, the amount of transfected V2(wt) DNA was reduced to 0.4 μg (supplemented with 3.6 μg of vector DNA). Basal cAMP levels for the V2(wt) receptor amounted to 877 ± 180 c.p.m./well. The basal cAMP levels observed in the co-expression experiments were not significantly different from this value. Data are given as means ± SEM of two or three independent experiments, each performed in triplicate.

hemagglutinin epitope tag was added to the N-terminus of the wild-type V2 receptor and all mutant constructs in order to be able to study the cellular distribution of the various receptor proteins by immunological techniques. The presence of the epitope tag had no significant effect on the ligand binding and functional properties of the wild-type V2 receptor (data not shown). For clarity, the epitope-tagged version of the wild-type V2 receptor is simply referred to as V2(wt) in the following.

Initially, the V2(wt) receptor and the various mutant constructs (all epitope-tagged) were transiently expressed in COS-7 cells and studied for their ability to bind the radioligand, [^{3}H]AVP, and to mediate AVP-induced increases in adenylyl cyclase activity. Whereas non-transfected cells did not display any specific [^{3}H]AVP binding activity, COS-7 cells transfected with V2(wt) yielded a significant number of high-affinity [^{3}H]AVP binding sites (Table I). Moreover, V2(wt)-transfected cells were able to mediate a pronounced increase in intracellular cAMP levels (9- to 13-fold above basal; Table II) upon stimulation with the agonist, AVP.

In contrast to V2(wt), none of the nine mutant V2 receptors examined was able to bind detectable amounts of [^{3}H]AVP. Moreover, AVP stimulation of cells expressing the various mutant constructs did not result in an appreciable increase in cAMP production, indicating that the nine mutant receptors are devoid of any pharmacological/functional activity. These findings are in agreement with previous studies showing that the Tyr280→Cys, Leu292→Pro and ΔVal278 mutant receptors are functionally inactive (Tsukaguchi *et al.*, 1995; D.Wenkert and A.Spiegel, submitted).

Cellular localization of wild-type and mutant V2 receptors

To study whether the various mutant V2 receptors were properly transported to the cell surface, their cellular localization was studied by confocal fluorescence microscopy, using a monoclonal antibody (12CA5) directed against the N-terminal epitope tag present in all V2 receptor constructs. COS-7 cells (non-permeabilized) expressing the V2(wt) receptor showed an intense staining of the plasma membrane (Figure 2A), whereas cells (non-permeabilized) expressing the various mutant constructs displayed only a faint surface staining (shown for Glu242→Stop in Figure 2C). In contrast, studies with permeabilized cells showed that all mutant receptors, similarly to V2(wt) (Figure 2B), were present at high levels in the cell interior [endoplasmatic reticulum (ER)/Golgi complex; shown for Glu242→Stop in Figure 2D].

To quantify the receptor protein present on the cell surface, an indirect cellular enzyme-linked immunosorbent assay (ELISA) system was employed. COS-7 cells (non-permeabilized) expressing V2(wt) or the various V2 mutant receptors were first incubated with the monoclonal antibody, 12CA5, followed by the addition of a peroxidase-conjugated secondary antibody and the photometric determination of peroxidase activity. Consistent with the microscopic studies, the ELISA experiments showed that the nine mutant receptors, in contrast to V2(wt), were expressed only at rather low levels on the cell surface (Table III).

To correlate the OD readings obtained in the ELISA studies with the amount of surface receptor protein, the V2(wt) receptor was expressed at different densities (B_{max}, determined in [^{3}H]AVP saturation binding studies; 40–500 fmol/mg) by stepwise reduction of the amount of transfected plasmid DNA (0.0625–4.0 μg, supplemented with vector DNA to keep the amount of transfected plasmid DNA constant at 4 μg). In parallel, ELISA experiments were carried out with cells derived from the same batch of cells used for the B_{max} determinations. These experiments showed that the OD readings (0.03–0.70) were directly proportional to the number of receptors detected in the radioligand binding studies (data not

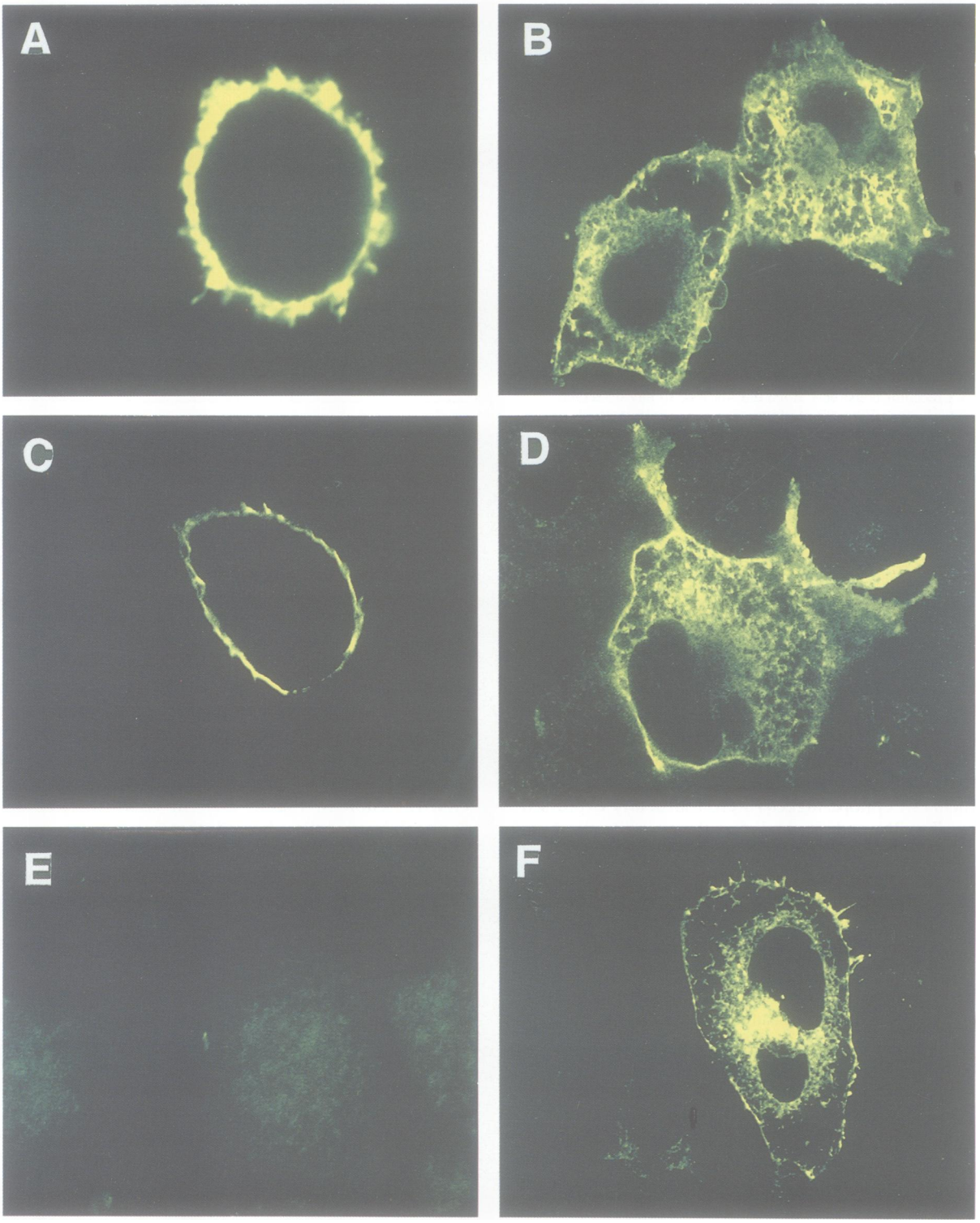

Fig. 2. Immunocytochemical localization of V2(wt), Glu242→Stop and the V2(tail) polypeptide. Immunofluorescence studies were carried out with transfected COS-7 cells grown on glass coverslips as described in Materials and methods. Cells expressing V2(wt) (**A** and **B**) and Glu242→Stop (**C** and **D**) were probed with the monoclonal antibody, 12CA5, directed against the N-terminal epitope tag present in these constructs. For the detection of the V2(tail) polypeptide (**E** and **F**), an affinity-purified polyclonal antibody directed against the C-terminal 29 amino acids of the human V2 vasopressin receptor was employed. Experiments were carried out with non-permeabilized (A, C and E) and permeabilized cells (B, D and F), respectively. Fluorescence images were obtained with a confocal laser scanning microscope (MRC-600, Bio-Rad), using a FITC-linked species-specific IgG secondary antibody. Each picture is representative of three independent experiments.

shown). It can be estimated, therefore, that the density of mutant receptors expressed on the cell surface is reduced by 9- to 25-fold [as compared with V2(wt)].

Functional rescue of mutant V2 receptors

All of the mutant V2 receptors investigated in this study either lack a C-terminal portion of the V2 receptor protein

Table III. Cell surface expression of mutant V2 receptors studied by ELISA

Construct	OD_{490nm}	
	transfection of receptor DNA alone	co-transfection with V2(tail)
V2(wt)	0.774 ± 0.022	0.580 ± 0.016
Glu242→Stop	0.060 ± 0.006	0.176 ± 0.007
Trp284→Stop	0.031 ± 0.005	0.047 ± 0.007
Trp293→Stop	0.045 ± 0.005	0.033 ± 0.005
Leu312→Stop	0.048 ± 0.006	0.035 ± 0.003
Gly246→FS	0.066 ± 0.005	0.190 ± 0.006
Gly254→FS	0.035 ± 0.006	0.109 ± 0.008
ΔVal278	0.048 ± 0.004	0.035 ± 0.005
Tyr280→Cys	0.088 ± 0.007	0.050 ± 0.004
Leu292→Pro	0.067 ± 0.004	0.038 ± 0.004

COS-7 cells were transfected with V2(wt) and the various mutant V2 vasopressin receptor constructs (containing an HA tag at their N-terminus), either alone (4 μg of receptor cDNA/plate) or together with the V2(tail) construct (2 μg of each plasmid/plate). ELISA measurements were carried out with non-permeabilized COS-7 cells in 96-well plates as described in Materials and methods. The OD readings were corrected for background extinction (0.150 ± 0.002), as determined with cells expressing a non-tagged version of the wild-type V2 receptor. Data are presented as means ± SD of two independent experiments, each performed in triplicate.

or contain specific mutations in this domain. Since previous studies have shown that GPCRs consist of multiple independently stable folding units (Kobilka *et al.*, 1988; Maggio *et al.*, 1993a,b; Ridge *et al.*, 1995; Schöneberg *et al.*, 1995), we have tested the hypothesis that the functionally inactive mutant V2 receptors can be pharmacologically rescued by co-expression with a C-terminal V2 receptor fragment [V2(tail)] spanning the sequence where the various mutations occur (Figure 1B).

To study the cellular localization of V2(tail) when expressed alone, an affinity-purified polyclonal antibody raised against a peptide corresponding to the C-terminal 29 amino acids of the human V2 receptor was used. Immunocytochemical studies with permeabilized COS-7 cells showed that the V2(tail) fragment was localized intracellularly (ER/Golgi complex) as well as in the plasma membrane (Figure 2F). In contrast, no signal was obtained with non-permeabilized cells (Figure 2E), indicating that the V2(tail) polypeptide is properly inserted (C-terminus intracellular) into the plasma membrane.

In the next step, COS-7 cells co-transfected with the individual mutant V2 receptors and V2(tail) were studied for their ability to gain [^{3}H]AVP binding and AVP-induced G protein coupling. Co-expression of four of the nine mutant receptors examined (Glu242→Stop, Trp284→Stop, Gly246→FS and Gly254→FS) with V2(tail) resulted in the appearance of a significant number of high affinity [^{3}H]AVP binding sites (up to 84 fmol/mg; Table I). Moreover, cells co-transfected with these four mutant receptors and V2(tail) also gained the ability to mediate pronounced increases in intracellular cAMP levels (6- to 13-fold) following AVP stimulation (Table II; Figure 3). Co-expression of the remaining five mutant receptors (Trp293→Stop, Leu312→Stop, Tyr280→Cys, Leu292→Pro and ΔVal278) with V2(tail) did not result in any detectable ligand binding activity. Interestingly, however, these mutant receptors [when co-expressed with V2(tail)]

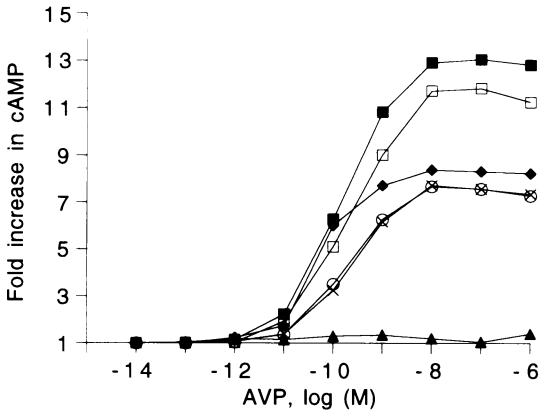

Fig. 3. AVP-induced cAMP accumulation mediated by the wild-type V2 vasopressin receptor [V2(wt)] and mutant V2 receptors co-expressed with the V2(tail) polypeptide. COS-7 cells were transfected with V2(wt) DNA (0.4 μg, supplemented with 3.6 μg of vector DNA) or mixtures of mutant V2 receptor constructs and the V2(tail) gene fragment (2 μg of each plasmid). The following symbols are used: V2(wt) (□), Glu242→Stop + V2(tail) (◆), Trp284→Stop + V2(tail) (×), Gly246→FS + V2(tail) (○) and Gly254→FS + V2(tail) (■). cAMP assays were carried out in six-well plates as described in Materials and methods. Cells transfected with the four mutant receptor constructs alone did not mediate a significant increase in cAMP production [shown for Gly254→FS (▲)]. Data are presented as fold increase in cAMP above basal levels in the absence of AVP. Each curve is representative of two or three independent experiments, each carried out in triplicate.

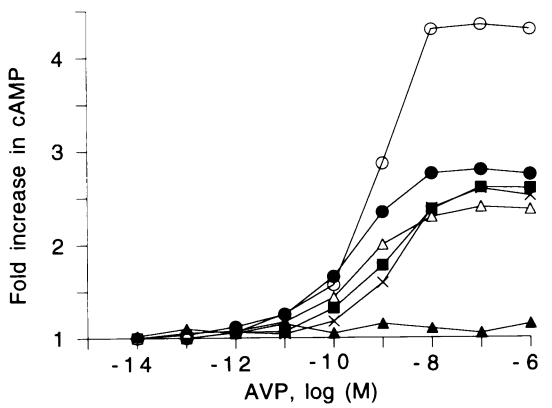

Fig. 4. AVP-induced cAMP production mediated by mutant V2 vasopressin receptors co-expressed with the V2(tail) polypeptide. COS-7 cells were co-transfected with the indicated mutant V2 vasopressin receptor constructs and the V2(tail) gene fragment (2 μg of each plasmid). The following symbols are used: Trp293→Stop + V2(tail) (×), Leu312→Stop + V2(tail) (△), Tyr280→Cys + V2(tail) (○), Leu292→Pro + V2(tail) (■) and ΔVal278 + V2(tail) (●). cAMP assays were carried out in six-well plates as described in Materials and methods. COS-7 cells transfected with the five mutant receptor constructs alone did not mediate a significant increase in cAMP production [shown for ΔVal278 (▲)]. Data are presented as fold increase in cAMP above basal levels in the absence of AVP. Each curve is representative of two or three independent experiments, each carried out in triplicate.

still gained the ability to mediate 2- to 4-fold increases in cAMP production in the presence of AVP (Table II; Figure 4). The cAMP responses to AVP stimulation of all nine mutant receptors in the co-transfection experiments were characterized by high AVP potencies (EC_{50}: 0.1–1.2 nM; EC_{50} for the wild-type V2 receptor: 0.1–0.2 nM; Table II).

With the exception of Glu242→Stop, all mutant receptors contain sequences that partly overlap with the V2(tail)

sequence. To exclude the possibility that the appearance of functional receptors in the co-expression experiments is caused by homologous recombination events, all mutant receptors were co-expressed with a V2(tail) fragment lacking the initiating ATG translation start codon. In this case, co-transfected COS-7 cells did not display any detectable [^{3}H]AVP binding activity or any agonist-induced increases in intracellular cAMP levels. Similar results were obtained when the various mutant receptors were co-expressed with a V2(tail) fragment in which one extra nucleotide (G) was inserted after the initiating ATG codon (data not shown). Taken together, these results eliminate the possibility that the functional rescue of the various mutant receptors upon co-expression with V2(tail) is due to homologous recombination events.

Effect of V2(tail) on cell surface receptor density

To study the effect of the V2(tail) polypeptide on the cell surface expression of the different V2 mutant receptors, ELISA measurements were carried out with non-permeabilized, co-transfected COS-7 cells (Table III). Interestingly, up to 3-fold higher levels of cell surface receptors were found when the Glu242→Stop, Trp284→Stop, Gly246→FS and Gly254→FS mutant receptors were co-expressed with V2(tail) (Table III). These increases in receptor density correlated well with the ability of these four mutant receptors to gain high affinity [^{3}H]AVP binding and efficient coupling to G_s upon co-expression with V2(tail) (see previous paragraph). In contrast, co-expression of V2(tail) with V2(wt) and the remaining five V2 mutant receptors resulted in a 25–43% reduction in the number of cell surface receptors, as determined by ELISA (Table III). A possible explanation for this phenomenon is that the two co-transfected plasmids [coding for V2(tail) and the individual mutant receptors or V2(wt), respectively] compete with each other for amplification in COS-7 cells, thus leading to reduced copy numbers for each individual plasmid.

Specificity of rescue by the V2(tail) polypeptide

In a separate set of experiments, the Glu242→Stop and Gly254→FS mutant receptors were co-expressed with a C-terminal m3 muscarinic receptor fragment (Schöneberg *et al.*, 1995) structurally homologous to the V2(tail) polypeptide. In these experiments, no specific [^{3}H]AVP binding, stimulation of adenylyl cyclase activity or increase in cell surface expression was observed for the two studied mutant receptors. Similar results were obtained when these two mutant receptors were co-expressed with V2(tail) fragments containing the Tyr280→Cys or Leu292→Pro point mutation, respectively (data not shown). In contrast to V2(tail), the mutated V2(tail) fragments also failed to (partially) rescue the 'full-length' mutant V2 receptors containing these two point mutations.

In another control experiment, a mutant V2 vasopressin receptor was created that contained an inactivating point mutation in the N-terminal portion (TM III) of the receptor protein (Tyr128→Ser; Figure 1A). As previously reported by Pan *et al.* (1994), this mutant receptor was unable to bind significant amounts of the radioligand, [^{3}H]AVP, and was severely impaired in its ability to stimulate adenylyl cyclase activity (E_{max} <30% of the wild-type response). Moreover, ELISA experiments showed that this mutant receptor was only poorly expressed on the cell surface (15% of wild-type receptor density). Co-expression of the Tyr128→Ser mutant receptor with the V2(tail) fragment did not result in detectable [^{3}H]AVP binding activity, increased cell surface expression or improved coupling to the G_s/adenylyl cyclase system.

Discussion

Inactivating mutations in distinct GPCRs are known to be responsible for a steadily growing number of human diseases (Coughlin, 1994; Raymond, 1994; Spiegel, 1996). In most cases, nonsense, frameshift, deletion or missense mutations result in truncated or misfolded receptor proteins which are unable to bind ligands and/or to couple to G proteins. From a therapeutic point of view, it would be highly desirable to devise pharmacological or molecular genetic strategies aimed at restoring function to such inactive mutant receptors.

Previous studies have shown that GPCRs, similar to the structurally closely related bacterial membrane protein, bacteriorhodopsin (Popot and Engelman, 1990; Kahn and Engelman, 1992), are assembled from several autonomous folding units (Kobilka *et al.*, 1988; Maggio *et al.*, 1993a,b; Ridge *et al.*, 1995; Schöneberg *et al.*, 1995). *In vivo* studies with 'split' m3 muscarinic acetylcholine receptors, for example, have shown that receptor fragments containing as few as two TM domains can be stably integrated into lipid membranes, apparently in the proper orientation and secondary structure (Maggio *et al.*, 1993a; Schöneberg *et al.*, 1995). We speculated, therefore, that clinically relevant mutant GPCRs that contain truncations or mutations which interfere with the structural integrity of individual folding units might be functionally rescued by 'supplying' the correctly folded receptor domain (which is misfolded or lacking in the mutant receptors) by a co-expression strategy.

To test the feasibility of this approach, we initially created a series of mutant V2 vasopressin receptors known to be the cause of X-linked NDI (Figure 1A). The mutant V2 receptors chosen for this study all contained mutations in the C-terminal third of the receptor protein. We found that none of the nine mutant receptors, when transiently expressed in COS-7 cells, was able to bind detectable amounts of the radioligand, [^{3}H]AVP, or to couple to the G_s/adenylyl cyclase system.

Immunocytochemical studies with permeabilized COS-7 cells demonstrated that all mutant V2 receptors, similarly to the V2(wt) receptor, were expressed at high levels in the cell interior (ER/Golgi network). However, ELISA measurements showed that the number of cell surface receptors found with the nine mutant constructs was 9- to 25-fold lower than that observed with the V2(wt) receptor, indicating that the mutant receptors are not properly trafficked to the cell surface. Consistent with this finding, many mutant forms of the photoreceptor rhodopsin, which cause autosomal dominant retinitis pigmentosa, are also retained intracellularly when stably expressed in cultured cells (Sung *et al.*, 1991). These findings suggest the existence of a mechanism in the ER that recognizes, retains and possibly degrades improperly folded receptor proteins (Klausner and Sitia, 1990). It should be noted, however, that the reduction in cell surface

receptor density observed in the present study is not sufficient to account for the lack of functional activity found with all nine mutant receptors, since expression of the V2(wt) receptor at similarly low levels (as determined by ELISA) still allowed detectable [^{3}H]AVP binding and efficient G_s activation (Tables I and II).

We next tested the possibility that a C-terminal V2 receptor peptide [V2(tail)] spanning the region in which the various mutations occur can functionally rescue the various V2 mutant receptors in co-expression experiments. Expression in COS-7 cells of V2(tail) alone showed that this polypeptide is stably inserted, in the proper orientation, into lipid membranes, including the plasma membrane. Remarkably, co-expression of V2(tail) with four of the nine mutant V2 receptors examined (Glu242→Stop, Trp284→Stop, Gly246→FS and Gly254→FS) resulted in the appearance of a significant number of specific, high affinity [^{3}H]AVP binding sites. In addition, all four mutant receptors gained the ability to mediate pronounced increases (6- to 13-fold) in intracellular cAMP levels and showed significantly increased (up to 3-fold) levels of cell surface expression (determined by ELISA).

In a set of control experiments, co-expression studies were carried out with a C-terminal m3 muscarinic receptor fragment (Schöneberg *et al.*, 1995) structurally homologous to the V2(tail) polypeptide as well as with V2(tail) polypeptides containing single point mutations in TM VI (Tyr280→Cys or Leu292→Pro) that functionally inactivate the wild-type V2 receptor. We found that these receptor fragments, in contrast to V2(tail), were unable to restore function to a series of selected V2 mutant receptors (Glu242→Stop, Gly254→FS, Tyr280→Cys and Leu292→Pro). Similarly, the V2(tail) fragment failed to pharmacologically rescue a mutant V2 receptor (Tyr128→Ser; Pan *et al.*, 1994) containing a point mutation in the N-terminal portion of the receptor protein (TM III). Taken together, these findings strongly suggest that the ability of the V2(tail) polypeptide to restore function to mutant V2 receptors containing mutations in their C-terminal domain is due to the formation of specific complexes between V2(tail) and the N-terminal portion (including TM I–V) of the receptor proteins, resulting in the 'reconstitution' of the ligand binding pocket (Chini *et al.*, 1995). It is likely that such complexes, after their assembly in ER/Golgi membranes, can be delivered to the cell surface with greater efficiency than the mutant receptors alone. Such a mechanism may explain the increase in cell surface receptor density observed after co-expression of some of the mutant receptors (see above) with the V2(tail) polypeptide. However, the possibility cannot be excluded that the V2(tail) fragment can also interact with and stabilize mutant receptor molecules already present in the plasma membrane. In any case, it is likely that the increase in cell surface receptor density observed with some of the mutant receptors [after co-expression with V2(tail)] is secondary to complexation with the V2(tail) fragment and the 'reconstitution' of the AVP binding site. However, the increase in the number of cell surface receptors [complexed with V2(tail)] may contribute to the efficiency of the observed functional rescue.

Whereas co-expression of the Trp293→Stop, Leu312→Stop, Tyr280→Cys, Leu292→Pro and ΔVal278 mutant receptors with the V2(tail) polypeptide did not result in detectable [^{3}H]AVP binding activity, all five mutant receptors gained the ability to stimulate the G_s/adenylyl cyclase system with weak to moderate efficacy (2- to 4-fold increases in AVP-stimulated cAMP levels) but high AVP potency. It is likely that these effects are caused by small numbers of functional mutant receptor–polypeptide complexes which can no longer be detected in our [^{3}H]AVP binding assay (detection limit <10 fmol/mg). This notion is consistent with the observation that the number of cell surface receptors detected by ELISA was not increased (but rather decreased) upon co-expression of these five mutant receptors with the V2(tail) polypeptide. These results suggest that the V2 receptor is coupled to the G_s/adenylyl cyclase system in a very efficient manner and that the functional rescue of a rather small number of mutant V2 receptors is sufficient to allow at least a certain degree of G_s activation.

The observation that the V2(tail) polypeptide cannot only functionally rescue truncated V2 receptors (such as Glu242→Stop or Gly254→FS) but also (at least partially) mutant receptors containing single point mutations (such as Tyr280→Cys) is of particular interest since the majority of disease-causing mutations in GPCRs represent single amino acid substitutions. The molecular mechanism by which the V2(tail) polypeptide is able to (partially) rescue the function of mutant receptors such as Tyr280→Cys is unclear at present. One possibility is that the V2(tail) fragment can compete, during the assembly of the receptor in the ER, with the C-terminal (mutated) receptor domain for binding to the 'bulk' of the receptor protein (containing TM I–V).

In conclusion, this is the first report demonstrating that GPCRs containing inactivating mutations responsible for a human disease can be functionally rescued by co-expression with a short receptor peptide. Using a similar approach, it should also be possible, based on the 'subunit character' of GPCRs, to restore function to GPCRs containing mutations in regions other than those described here. During the past decade, advances in gene transfer technology have enabled the introduction of exogenous genes or gene fragments into distinct mammalian organs, including the kidney (for a review, see, for example, Kitamura, 1994). We speculate, therefore, that the targeted expression of specific receptor polypeptides in physiological tissues may lead to novel strategies in the treatment of diseases caused by inactivating GPCR mutations. Since these peptides are expected to interact only with those (mutant) receptors from which they are derived, this approach would offer the great advantage that the polypeptides exert their pharmacological effects only in those tissues or cells in which the (mutant) receptors are physiologically expressed.

Materials and methods

DNA constructs

A 1.7 kb *Eco*RI–*Xba*I fragment was cut out from hV2-pcDNA I/Amp, a plasmid containing the genomic human V2 vasopressin receptor sequence, and subcloned into the pcD-PS mammalian expression vector (Bonner *et al.*, 1988) using the *Eco*RI and *Spe*I sites present in the pcD-PS polylinker sequence. To be able to study the cellular localization of the V2 receptor protein by immunological techniques, a stretch of nucleotides coding for a nine amino acid epitope (YPYDVPDYA; Kolodziej and Young, 1991) derived from the influenza virus hemag-

glutinin protein (HA-tag) was inserted after the initiating Met codon. In addition, the two introns interrupting the V2 receptor coding sequence were removed by using standard PCR mutagenesis techniques (Higuchi, 1989). All mutations (Figure 1A) were introduced into this intronless, HA-tagged vasopressin V2 receptor construct by standard PCR mutagenesis approaches. For the construction of the V2(tail) gene fragment (coding for amino acids Glu242–Ser371; Figure 1B), the 5′ PCR primer was designed to contain an *Eco*RI site at its 5′ end (to link the PCR fragment to the *Eco*RI site in the pcD-PS polylinker sequence), followed by six bases of 5′-untranslated sequence adjacent to the ATG translation initiation codon in the V2 vasopressin receptor gene (CCCACC) and an in-frame ATG start codon. The correctness of all PCR-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids (Sanger *et al.*, 1977).

Transient expression of mutant V2 vasopressin receptors

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified 5% CO_2 incubator. For transfections, 2×10^6 cells were seeded into 100 mm dishes. About 24 h later, cells were transfected with the various V2 receptor constructs (4 μg of plasmid DNA per dish) by a DEAE–dextran method (Cullen, 1987). In co-expression experiments, COS-7 cells were co-transfected with the individual mutant V2 receptor constructs and the V2(tail) gene fragment (2 μg of each plasmid).

Radioligand binding assays

For radioligand binding studies, COS-7 cells were harvested ~72 h after transfections, and membrane homogenates were prepared as described previously by Dörje *et al.* (1991). Binding buffer consisted of 50 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin (BSA) and 0.1 mg/ml bacitracin. Incubations were carried out for 1 h at 22°C, in a 0.5 ml volume, with increasing concentrations of the radioligand [^{3}H]AVP (81 Ci/mmol; NEN). Six different concentrations of the radioligand (0.2–8 nM) were used. Non-specific binding was defined as binding in the presence of 5 μM AVP. The protein content of samples was determined by the method of Bradford (1976).

Binding data were analyzed by a non-linear least squares curve-fitting procedure using the computer program LIGAND (Munson and Rodbard, 1980).

cAMP assays

Approximately 20–24 h after transfections, cells were transferred into six-well plates (~0.3×10^6 cells/well), and 2 μCi/ml of [^{3}H]adenine (15 Ci/mmol; American Radiolabeled Chemicals Inc.) was added to the growth medium. After a 20–24 h labeling period, cells were pre-incubated in Hank's balanced salt solution containing 20 mM HEPES and 1 mM 3-isobutyl-1-methylxanthine for 15 min (37°C), and then stimulated for 45 min at 37°C with increasing concentrations of AVP. The reaction was terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM ATP and 1 mM cAMP. Increases in intracellular [^{3}H]cAMP levels were then determined by anion-exchange chromatography as described (Salomon *et al.*, 1974).

ELISA

ELISA measurements were carried out with non-permeabilized cells essentially as described previously by Schöneberg *et al.* (1995). COS-7 cells expressing HA-tagged versions of wild-type and mutant V2 vasopressin receptors were transferred into 96-well plates ($4–5\times10^4$ cells/well) ~20–24 h after transfections. About 48 h later, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min at 37°C. After washing with PBS and blocking with DMEM containing 10% FCS, cells were incubated for 3 h at 37°C with a monoclonal antibody directed against the HA-epitope tag (12CA5, 20 μg/ml; Boehringer Mannheim). Plates were then washed with PBS and incubated with a 1:2500 dilution of a peroxidase-conjugated species-specific IgG antibody (Sigma) for 1 h at 37°C. H_2O_2 and *o*-phenylenediamine (2.5 mM each in 0.1 M phosphate–citrate buffer, pH 5.0) were then added to serve as substrate and chromogen, respectively. The enzymatic reaction (carried out at room temperature) was stopped after 30 min with 1 M H_2SO_4 containing 0.05 M Na_2SO_3, and the color development was measured bichromatically at 490 and 630 nm (background), using the BioKinetics reader (EL 312, Bio Tek Instruments, Inc.).

Immunofluorescence microscopy

Approximately 20–24 h after transfections, COS-7 cells were transferred into six-well plates ($1–2\times10^5$ cells/well) containing sterilized glass coverslips. About 48 h later, cells were fixed with 4% formaldehyde (in PBS) for 15 min at room temperature. After rinsing with PBS, unspecific binding was blocked with DMEM containing 10% FCS. Cells (non-permeabilized or permeabilized) expressing epitope-tagged versions of the wild-type V2 and the various V2 mutant receptors were then incubated for 3 h at 37°C with monoclonal antibody 12CA5 (20 μg/ml in DMEM/10% FCS). In a similar fashion, cells expressing the V2(tail) polypeptide were incubated with an affinity-purified polyclonal antibody (10 μg/ml) raised (in rabbits) against a peptide corresponding to the C-terminal 29 amino acids of the human V2 vasopressin receptor (kindly provided by Dr Paul Goldsmith, NIH). To permeabilize cell membranes, cells were treated with 0.1% saponin (Sigma) in PBS for 10 min at room temperature before the addition of the primary antibody. After washing off the excess of unbound primary antibody with PBS, cells were incubated for 1 h at 37°C with a 1:100 dilution of a FITC-conjugated species-specific IgG antibody (Sigma). Unbound secondary antibody was removed by washing with PBS, and coverslips were mounted on microscope slides using a glycerol–PBS mixture (1:1, v/v). Images were obtained using a confocal laser-scanning fluorescence microscope (MRC-600, Bio-Rad).

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