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Vasopressin V2 Receptor Mutants That Cause X-Linked Nephrogenic Diabetes Insipidus: Analysis of Expression, Processing, and Function

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SUMMARY

We investigated the biochemical and functional properties of five vasopressin V2 receptor mutants (L44F, L44P, W164S, S167L, and S167T) that were recently described in families with a history of X-linked nephrogenic diabetes insipidus. COS.M6 cells transfected with cDNA encoding these mutants acquired <4% specific [^{3}H]arginine vasopressin (AVP) binding sites on the cell surface in comparison with cells transfected with cDNA coding for the wild-type receptor. Membrane preparations from COS.M6 cells or human embryonic kidney 293 cells expressing these mutants did not respond with an increase in adenylyl cyclase activity in response to AVP, which is in contrast to membranes from cells expressing the wild-type. By analyzing fusion proteins of the V2 receptor and *Escherichia coli* alkaline phosphatase attached to the carboxyl terminus of the receptor moiety, we found that the mutants L44P, W164S, S167L, and S167T lacked complex glycosylation and were expressed at low levels. The data suggest that the mutants L44P, W164S, S167T, and S167L are misfolded and therefore retained within the endoplasmic reticulum and degraded. In contrast, the fusion proteins carrying the mutant L44F and the *in vitro* mutant S167A were expressed in their mature form at wild-type levels; however, only the mutant S167A was functionally active. Site-directed mutagenesis of S167 revealed that elimination of the endogenous hydroxyl group (S167A) yielded a protein with properties identical to those of the wild-type receptor, whereas both the introduction of a methyl group (S167T) and the replacement of the hydroxyl group by an isopropyl group (S167L) profoundly disturbed receptor processing. The data show that minute changes at codon 167 nearly abolish expression of a mature protein, thus defining structural requirements of this codon.

The antidiuretic action of the hormone AVP is mediated by the vasopressin V2 receptor, which is expressed in epithelial cells of renal collecting ducts. The receptor is a member of the superfamily of GPCRs (1). Its activation leads to stimulation of adenylyl cyclase via G_s. The subsequent rise in intracellular cAMP induces the insertion of the vasopressin-sensitive water channel aquaporin-2 into the luminal membrane (2).

In NDI, the kidney fails to concentrate urine despite normal or elevated levels of AVP. It is now well established that most, if not all, cases of X-linked recessive NDI are caused by inactivating mutations of the V2 receptor gene (3–6; for reviews, see Refs. 7–9), whereas mutations of the aquaporin-2 gene are responsible for autosomal recessive NDI (2, 10). Approximately 70 X-linked NDI-causing mutations of the V2 receptor gene, predominantly missense mutations, have been documented. Functional characterization of missense mutations revealed three different molecular defects that cause a loss in function: (a) defect in ligand binding, (b) defect in G protein coupling and activation, and (c) defect in transport. Missense mutations occurring in the first (R113W) and the second (R181C and R202C) extracellular loops lead to a lowered or nondetectable binding affinity, supporting the assumption that both loops contribute to the formation of the binding site for AVP (11–13). In addition, the missense mutations Y128S, within the second transmembrane domain, and P286R, within the sixth, cause a complete loss of binding, although the mutant receptors are expressed at the cell surface (12). The missense mutation R137H, in the second intracellular loop close to the third transmembrane domain, abolishes stimulation of adenylyl cyclase, underlining the importance of the second intracellular loop for G protein

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ABBREVIATIONS: AVP, 8-arginine vasopressin; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; NDI, nephrogenic diabetes insipidus; PhoA, alkaline phosphatase of *E. coli*; PCR, polymerase chain reaction; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PGE_1, prostaglandin E_1.

coupling (14). The missense mutation R143P, close to R137 within the second intracellular loop, shows, however, only a slightly reduced ability to stimulate adenylyl cyclase but is inefficiently transported to the cell surface (13). A transport defect was also found for a mutant lacking a valine at position 278 (ΔV278) of the sixth transmembrane domain (13). The causes of the transport defects have not been investigated.

We analyzed the properties of five naturally occurring V2 receptor mutants described previously (15–18). The point mutations occur within the first (L44F, L44P) or fourth (W164S, S167L, S167T) transmembrane domain. The role of S167 was studied in greater detail because the exchange of S167 by leucine (S167L) is the most frequently observed mutation in families with X-linked NDI. In addition, the conservative substitution of S167 by threonine (S167T) was found in an affected family. We also constructed and characterized the *in vitro* mutant S167A because serine or alanine is found in numerous GPCRs at the corresponding position. For immunoblot analysis, we constructed fusion proteins of wild-type and mutant V2 receptors with *Escherichia coli* alkaline phosphatase attached to their carboxyl termini.

Materials and Methods

Radiochemicals. [^{3}H]AVP (64.8 Ci/mmol) was supplied by DuPont (Bad Homburg, Germany), and [α-^{32}P]dCTP (~3000 Ci/mmol) and [α-^{32}P]ATP (~30 Ci/mmol) was supplied by Amersham (Braunschweig, Germany).

Cell culture. *Ltk*$^-$ and COS.M6 cells were cultured at 5% CO_2 in DMEM and HEK 293 cells in a mixture of DMEM/Ham's F12, both containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For selection of stably transfected *Ltk*$^-$ cells, medium was supplemented with G418 (400 μg/ml).

DNA manipulations. DNA preparations and manipulations were carried out according to standard protocols (19, 20). Nucleotide sequences were determined using the Sequenase Plasmid Sequencing kit (Amersham).

Site-directed mutagenesis of the V_2 receptor cDNA. For site-directed mutagenesis, we used the single-stranded DNA of the plasmid pRCDN2 carrying the coding region of the V2 receptor cDNA (21). Single-base substitutions were carried out with the T7-Gen *in vitro* mutagenesis kit (Amersham) with *E. coli* SDM2 as host strain. Only with the L44P mutant was an additional base changed (mutant codon 44 was changed from CCC to CCA), thereby creating a restriction site for *Fok*I. All mutations were verified by sequencing.

Construction of vectors encoding V2 receptor/PhoA fusion proteins. The gene coding for PhoA lacking the signal peptide (22) was cloned into the expression vector pRCDN2 (21). To this end, the unique *Bam*HI restriction site of pRCDN2 was eliminated by a fill-in reaction. A novel *Bam*HI restriction site was introduced by site-directed mutagenesis at position 1101 of the V2 receptor cDNA (1), generating the plasmid pEU367 (mutant primer, 5′-ccc tgg cca agg atc ctt cat cgt gag g-3′). The PhoA gene lacking the leader sequence was amplified by PCR from the plasmid pRSA468 (23); flanking primers introducing a *Bam*HI restriction site at the 5′end and a *Bgl*II restriction site at the 3′ end were used (*Bam*HI primer, 5′-gcg tcc tgg acg gat cct ttc ccg ttt tg-3′; *Bgl*II primer, 5′-gcg tat gcg ccc gag atc tgc cat taa g-3′). The *Bam*HI/*Bgl*II-cut PhoA PCR fragment was cloned in frame into the *Bam*HI site of pEU367. The resulting plasmid, pEU367.PhoA, encoded a fusion protein consisting of the entire V2 receptor (except for the last four carboxyl-terminal amino acids) and the PhoA moiety. To introduce the mutations into the V2 receptor moiety, we cloned *Sna*BI/*Nsi*I fragments from derivatives of pRCDN2 carrying the missense mutations into *Sna*BI/*Nsi*I-cut plasmid pEU367.PhoA.

Transient and stable expression of V2 receptors or V2 receptor/PhoA fusion proteins. Transfection of COS.M6 cells for transient and of *Ltk*$^-$ cells for stable expression was performed with Lipofectin (Life Technologies, Eggenstein, Germany) as described previously (21). The protocol for the transfection of HEK 293 cells (transient expression) was identical to the former with the exception that the lipofectin/DNA complex in serum-free medium was replaced after 4 hr with DMEM/Ham's F12 supplemented with 10% heat-inactivated fetal calf serum.

Expression of V_2 receptor/PhoA fusion proteins in *E. coli*. Cells were transfected with a prokaryotic expression vector carrying the coding region for V2 receptor PhoA cDNA (see above). Plasmid construction, expression, and isolation of the fusion product were as described (23a).

[^{3}H]AVP binding assay and Scatchard analysis. Binding of [^{3}H]AVP (10 or 50 nM) to intact cells was performed as described previously (21).

Adenylyl cyclase assay. The preparation of nuclei-free crude membrane fractions from *Ltk*$^-$-derived cell lines and the adenylyl cyclase assay were performed as described previously (21). A similar protocol was used for assaying adenylyl cyclase activity in membranes of HEK 293 cells, with the exception that 2 mM EDTA and 4 mM $MgCl_2$ were used in the reaction mixture. [^{32}P]cAMP was isolated according to the two-column method (24).

Preparation of membranes from COS.M6 cells for immunoblotting. COS.M6 cells grown on 60-mm Petri dishes were washed twice with PBS and harvested with a rubber policeman in 0.5 ml of PBS containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 3.2 μg/ml trypsin inhibitor, 1.4 μg/ml aprotinin). After sonication on ice, membranes and cytosol were separated by ultracentrifugation at 150,000 × *g* for 1 hr. The membranes were washed once with 1 ml of PBS, recentrifuged at 150,000 × *g* for 30 min, resuspended in 80 μl of PBS, and stored at −80° until use.

Immunoblots. Membrane proteins of COS.M6 cells (80 μg/lane) were separated on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) using a semidry blotting apparatus as described previously (25). Protein transfer was monitored by Ponceau red staining. Nitrocellulose filters were blocked with PBS/0.5% Tween 20 (blocking solution), incubated for 1 hr with polyclonal rabbit anti-PhoA antibody diluted 1:1000 in PBS/0.05% Tween 20 (washing solution), washed three times, and then incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Dako Diagnostika, Hamburg, Germany) diluted 1:1000 in washing solution. After three washes, the filter-bound antibodies were visualized with 4-chloro-1-naphthol and H_2O_2. The properties of the polyclonal anti-PhoA antibody used in the current study have been described previously (23).

Isolation of RNA from COS.M6 cells and Northern blots. Total RNA was isolated from COS.M6 cells with the RNAzol B kit (WAK-Chemie Medical, Bad Homburg, Germany). Size separation of total RNA (7–20 μg) on 1.1% agarose gels containing 2.2 M formaldehyde was followed by capillary transfer onto nylon membranes (Qiagen, Hilden, Germany) and UV cross-linking (2 × 90 sec at 312 nm). Prehybridization and hybridization with random primed [α-^{32}P]dCTP full-length V2 receptor cDNA (2.5 × 10^6 cpm/ml) were performed in Quick-Hyb solution (Stratagene, Heidelberg, Germany) at 60° for 1 hr. Membranes were washed at increasing stringency with a final wash in 0.25× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate)/0.1% SDS at 50° for 15 min. The relative amounts of RNA loaded onto the agarose gel were assessed by detection of β-actin RNA with a probe of 617 bp derived from the human β-actin pseudogene by an *Eco*RI/*Sal*I digest (26). Labeling of the β-actin probe with [α-^{32}P]dCTP and hybridization were performed as described above. The membranes were exposed to Kodak X-Omat AR films for 12–20 hr.

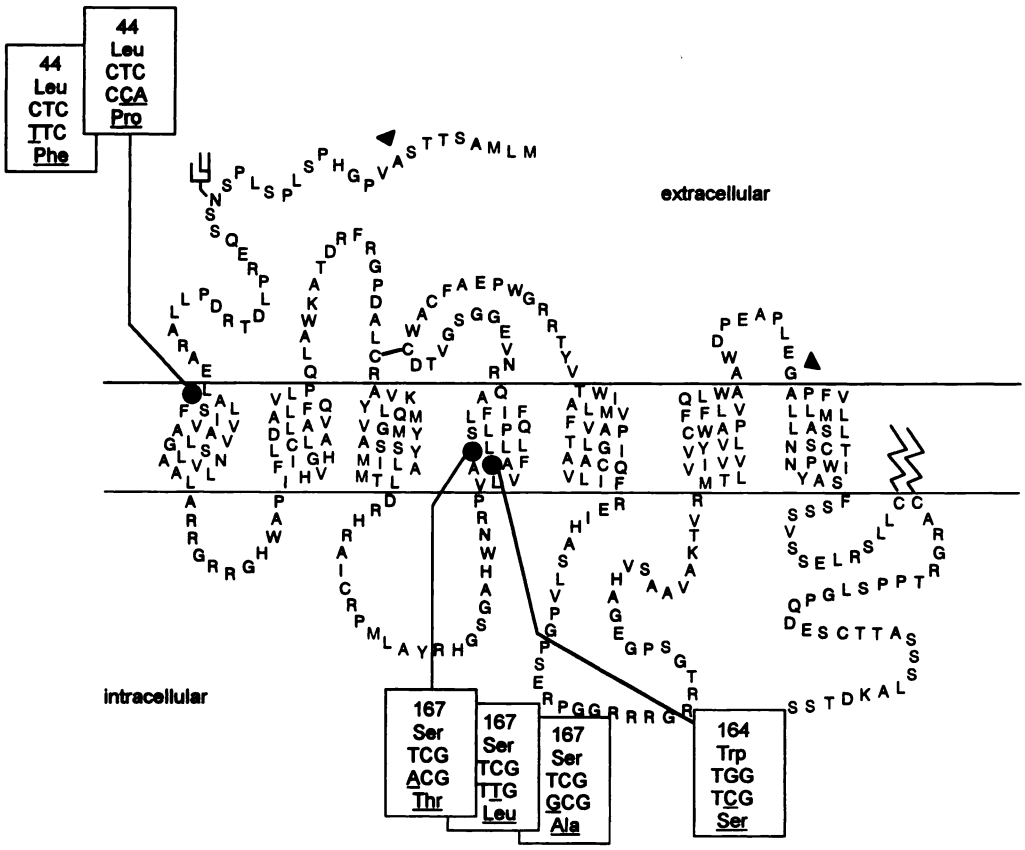

Fig. 1. Topological model of the vasopressin V2 receptor. The one-letter code for amino acids is used. Based on the presence of consensus regions for modifications in the predicted amino acid sequence, the model depicts a sugar moiety at N22 of the extracellular amino terminus; a disulfide bridge between C112 and C192 of the first and second extracellular loops, respectively; and palmitate residues at C341 and C342, attaching the intracellular carboxyl terminus to the plasma membrane. ▲, Position of introns in the genomic DNA. ●, Residues affected by the missense mutations characterized in the current report. *Boxes* (from *top* to *bottom*), number of the affected codon, the amino acid in the wild-type protein (three-letter code), the wild-type codon, the mutated codon, and the replacement amino acid. Except for the *in vitro* mutation S167A, all mutations have been found in XNDI patients. The exchange of two nucleotides (mutant L44P) is referred to in Materials and Methods.

Results

The X-linked NDI-causing V2 receptor mutations investigated here occur in the first and fourth transmembrane domains (Fig. 1). The L44F and L44P mutations were found in two unrelated families with X-linked NDI (16, 18); they involve L44 in the first transmembrane domain adjacent to the extracellular space. The W164S mutation, involving a tryptophan highly conserved in GPCRs (27), is located within the fourth transmembrane domain (15). S167, also located in the fourth transmembrane domain, appears to be a "hot spot" for mutations. The S167L mutation caused X-linked NDI in at least seven unrelated families (15–17).[1] In addition, we have described an affected family with an S167T mutation (18). The *in vitro* mutation S167A was constructed to study in greater detail the importance of S167 for receptor processing and function. Alanine was chosen as the replacement amino acid because it is found in the corresponding position in a number of GPCRs (27).

To study the functional properties of mutant receptors, intact COS.M6 cells were assayed for specific [^{3}H]AVP binding after transfection with wild-type and mutant cDNAs. In contrast to the cells transfected with wild-type cDNA, the cells transfected with the naturally occurring mutants L44F, L44P, W164S, S167L, and S167T showed no or hardly detectable (<4%) specific binding in experiments using either 10 (Fig. 2) or 50 nM [^{3}H]AVP (not shown). This finding is particularly noteworthy in the case of the S167T mutant, in which the substituted amino acid (threonine) differs from the wild-type amino acid (serine) only by an additional methyl group. Transfection with the *in vitro* mutant S167A, in which the substituted amino acid lacks the hydroxyl group of the wild-type amino acid, does, however, result in the expression of specific [^{3}H]AVP binding sites. Binding isotherms and Scatchard analyses (Fig. 3) revealed a similar number of detectable receptors at the surface of COS.M6 cells expressing the wild-type receptor and the S167A mutant (on average, 110,000 and 101,000 sites/cell, respectively). Likewise, the affinities of the wild-type receptor and S167A mutant for

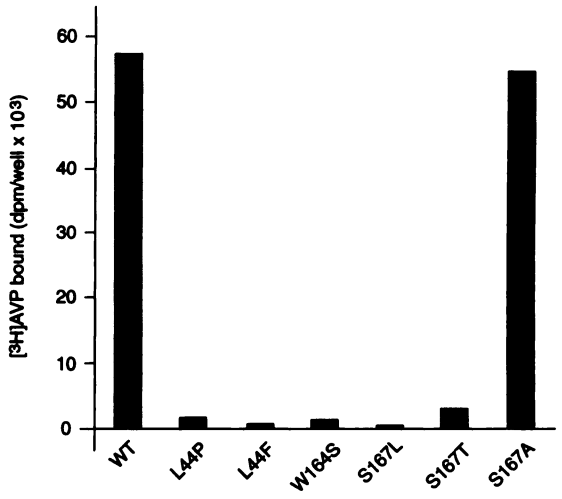

Fig. 2. Specific [^{3}H]AVP binding to intact COS.M6 cells expressing wild-type and mutant V2 receptors. [^{3}H]AVP was 10 nM. Unspecific binding (determined in the presence of a 10,000-fold excess of unlabeled AVP) was <10% of total binding. *Column height*, mean value of duplicates, which differed by <5% (wild-type and S167A mutant) or by <20% (remaining mutants). Similar data were obtained in four independent experiments with 10 or 50 nM [^{3}H]AVP.

[1] A. Oksche, A. Möller, and W. Rosenthal, unpublished observations.

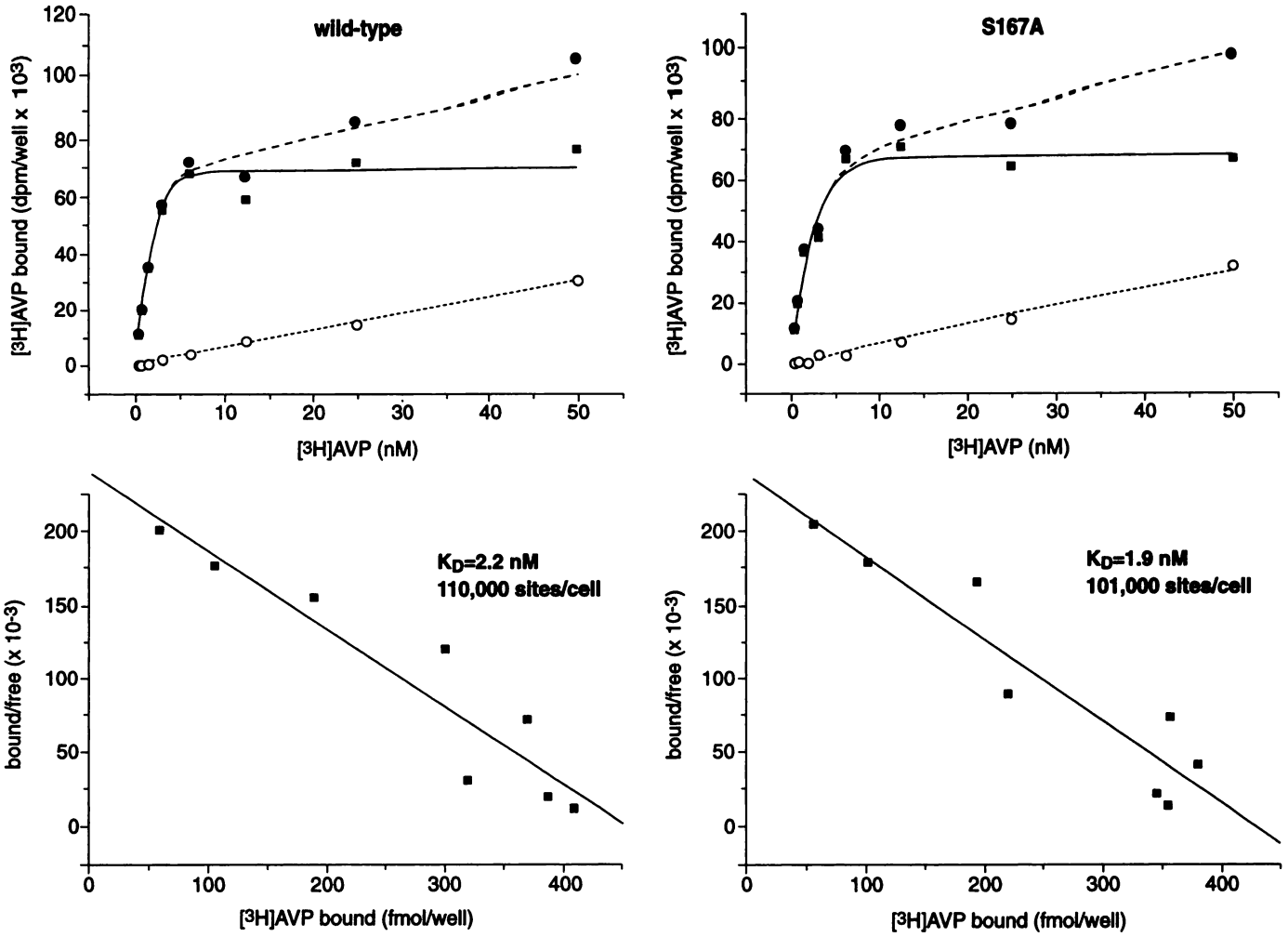

Fig. 3. [^{3}H]AVP binding profiles of COS.M6 cells transfected with wild-type V2 receptor (*left*) and the mutant S167A (*right*). *Top*, binding isotherms. Data for total binding (●) and unspecific binding (○) of [^{3}H]AVP to intact cells represent mean values of duplicates, which differed by <5%. ■, Specific binding. *Bottom*, Scatchard transformations. Shown are calculated K_D values for binding of [^{3}H]AVP to intact cells and the number of receptors per cell. Similar data were obtained in three independent experiments.

[^{3}H]AVP were essentially the same (2.2 and 1.9 nM, respectively). The data were confirmed with clonal cell lines derived from L*tk*$^-$ cells stably expressing the wild-type receptor and the S167A mutant (data not shown); for further functional analysis of the S167A mutant, see below.

The fact that the naturally occurring mutations induce no or barely detectable expression of specific [^{3}H]AVP binding sites could be a result of a lowered transcription rate or RNA instability. To exclude these possibilities, we performed Northern blot analyses of RNA isolated from COS.M6 cells transfected with the various constructs. Using radiolabeled V2 receptor cDNA as a probe, transcripts of the expected size (~1.8 kb) were detected in transfected but not in control COS.M6 cells (Fig. 4A). Only minor quantitative differences between wild-type and mutant mRNAs were detected. These derived, at least in part, from the different amounts of mRNA transferred to the filter as indicated by the signal obtained with an actin probe. Consistent with the Northern blot experiments, a PCR product (1150 bp) comprising the entire coding region of the V2 receptor cDNA was obtained by reverse transcription-PCR with RNA from transfected but not from control cells (not shown). Thus, the functional defect of transfected cells to bind [^{3}H]AVP seems to be due either to the expression of mature, albeit nonfunctional, receptor at the cell surface or to an abnormal receptor processing, resulting in a decrease or abolition of cell surface expression.

To differentiate between the two possibilities, we attempted to analyze the wild-type and mutant V2 receptors on the protein level. The generation of receptor specific antibodies suitable for immunoblots, however, proved to be unsuccessful. Therefore, wild-type and mutant V2 receptors were expressed as fusion proteins with PhoA (47.5 kDa) attached to their carboxyl termini; the fusion proteins were detected by antibodies against the PhoA moiety. Previous studies have shown that PhoA fusions, particularly when directed to the hydrophilic carboxyl termini of membrane proteins (28), carry minimal risk of disrupting topogenic signals. Before protein analysis, fusion transcripts were detected by Northern blot analysis. In all transfected cells, transcripts of the expected size (~3.8 kb) were detected (Fig. 4B). Levels of mRNA encoding wild-type and mutant fusion proteins did not vary significantly, as observed for mRNA encoding wild-type and mutant V2 receptors without the PhoA moiety (Fig. 4A). A comparison of the amounts of transcripts obtained with wild-type cDNAs with and without PhoA, however, revealed that the former were considerably lower than the

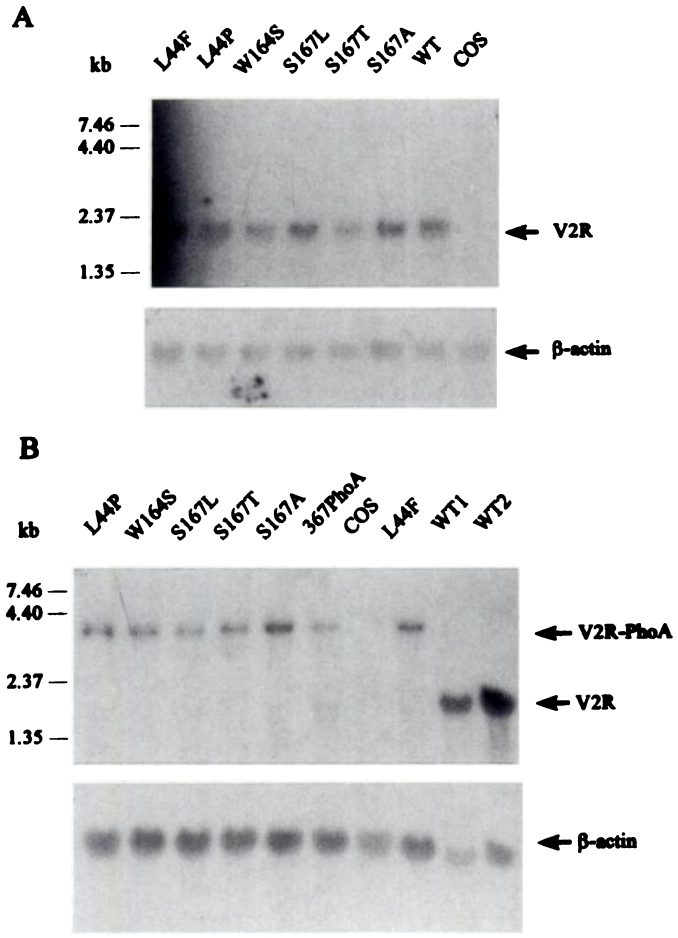

Fig. 4. Northern blot analysis of V2 receptor transcripts isolated from COS.M6 cells transfected with wild-type and mutant V2 receptor cDNA without (A) and with (B) PhoA moiety. The blots were hybridized with radiolabeled V2 receptor cDNA (*top* in A and B). To assess relative amounts of RNA, the blots were also hybridized with a human β-actin cDNA probe (*bottom* in A and B). *Right*, specific messages. *Left*, size standards. A, Seven micrograms of total RNA of the indicated mutants was analyzed. *COS*, untransfected cells; *WT*, wild-type. B: *L44P*, *W164S*, *S167L*, *S167T*, and *S167A*, respective mutant V2 receptor fusions with PhoA. *367PhoA*, wild-type V2 receptor fusion with PhoA. In the case of the fusions, 20 μg of RNA was used. *WT1* (7 μg of RNA) and *WT2* (20 μg of RNA), wild-type V2 receptor without PhoA. *COS*, untransfected cells.

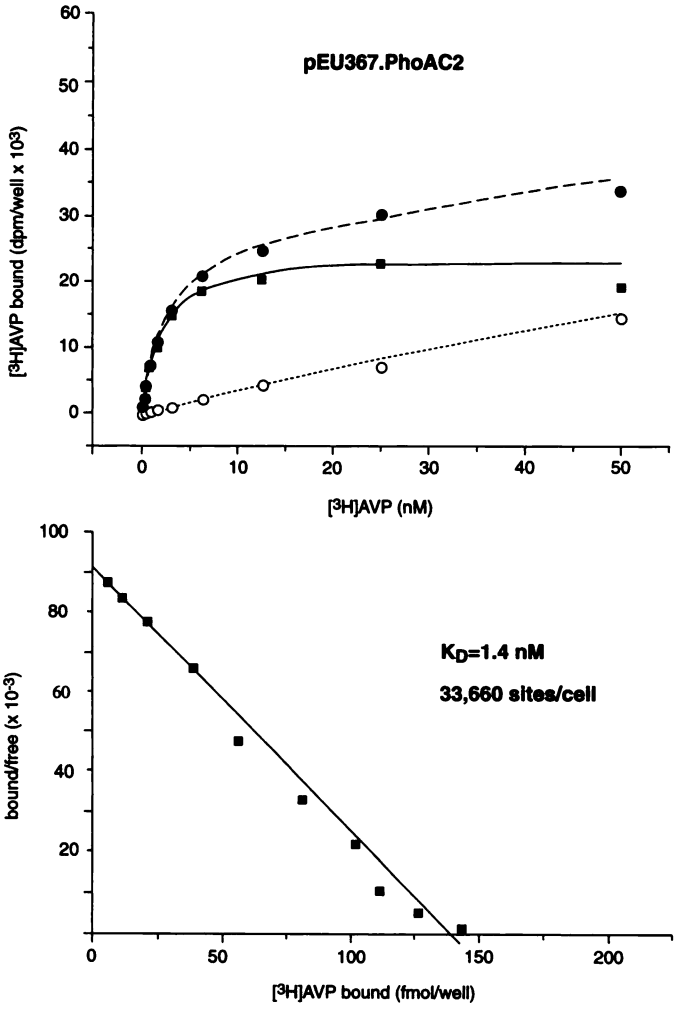

Fig. 5. [^{3}H]AVP binding profiles of a cell clone (derived from *Ltk*$^-$ cells) expressing the V2 receptor/PhoA fusion protein (pEU367.PhoAC2). *Top*, binding isotherms. Data for total binding (●) and unspecific binding (○) of [^{3}H]AVP to intact cells represent mean values of duplicates, which differed by <5%. ■, Specific binding. *Bottom*, Scatchard transformations. Shown are calculated K_D values for binding of [^{3}H]AVP to intact cells and the number of receptors per cell (average). Similar data were obtained in three independent experiments.

latter (compare *WT2* with fusions, Fig. 4B). This may be due either to a less efficient transcription of the cDNAs encoding the fusion proteins or to a decreased stability of the mRNAs. It is also plausible that the increased size of the plasmid encoding the fusion protein results in a reduced transfection efficiency. Binding of [^{3}H]AVP (10 nM) to intact COS.M6 cells expressing the wild-type fusion protein was 47–68% of that of cells expressing the wild-type receptor without PhoA moiety (three independent experiments). Based on the lower transcript levels for the fusion protein, these values suggest that transport of the fusion protein to the plasma membrane was comparable to that of the receptor without the PhoA moiety. The affinity of the receptor for [^{3}H]AVP was not affected by the PhoA moiety as demonstrated in binding studies and Scatchard analyses with cells derived from *Ltk*$^-$ cells stably expressing the wild-type fusion protein (Fig. 5): the K_D value obtained with cells expressing the receptor without PhoA (V2IIB1 cells; 180,000 sites/cell, see Fig. 6) and with PhoA (pEU367.PhoAC2; 33,600 sites/cell, see Fig. 5) was 2.2 and 1.4 nM, respectively. The results for the fusion protein were confirmed with a second, independently derived cell clone. In membranes from the parental cell line (Ltk$^-$), adenylyl cyclase activity is increased 5–6-fold by PGE_1, acting via endogenous prostaglandin receptors (11, 14). A similar increase is observed in response to AVP in cell lines derived from *Ltk*$^-$ cells, stably expressing the human V2 receptor (14). Adenylyl cyclase assays with membrane preparations of the cell clones expressing the fusion proved that the V2 receptor with the PhoA moiety retained fully the ability to stimulate adenylyl cyclase but with a marked rightward shift (EC_{50} = 0.36 and 46.8 nM for the receptor without and with PhoA, respectively; Fig. 6). Thus, the V2 receptor fusion protein seems to be normally processed as indicated by an efficient expression at the cell surface, has normal ligand binding properties, and retains its ability to stimulate adenylyl cyclase. The rightward shift of the concentration-response curve with regard to stimulation of adenylyl cyclase may

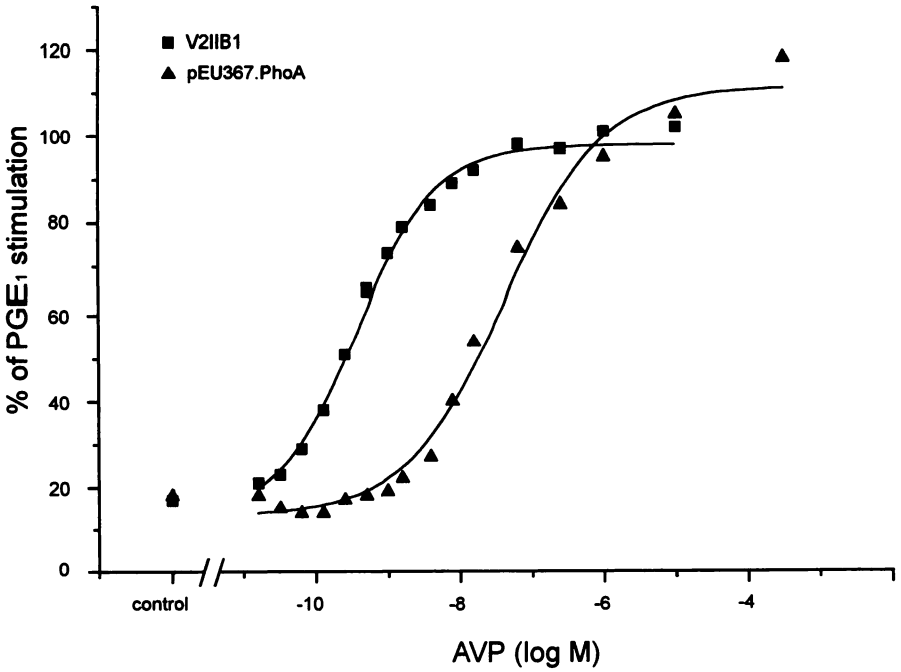

Fig. 6. Adenylyl cyclase activity (shown as AVP concentration-response curves) in membranes from a cell line stably expressing the wild-type V2 receptor and the V2 receptor/PhoA fusion protein. V2 receptor-expressing clonal cell lines were established by transfection of Ltk^- cells with the respective cDNAs. A cell line that expressed the wild-type V2 receptor (V211B1, 180,000 sites/cell) and one that expressed the V2 receptor/PhoA fusion protein (pEU367.PhoAC2, 33,600 sites/cell) were analyzed. Data are expressed as percentage of adenylyl cyclase activity after maximal PGE_1 stimulation (100 μM PGE_1). Data represent mean values of duplicates, which differed by <5%. Basal and PGE_1-stimulated adenylyl cyclase activities in pmol of cAMP/mg of protein/min were V2IIB1, 8.8 and 53.6; and pEU367.PhoAC2, 3.1 and 20.6, respectively. EC_{50} values were 0.35 nM for the wild-type receptor and 46.8 nM for the pEU367/PhoA fusion protein.

derive from the relatively low receptor density on cells expressing the fusion protein. In addition, the PhoA moiety may interfere with the interaction of the activated receptor and G_s.

Immunoblots of crude membrane fractions from control COS.M6 cells and those expressing the wild-type V2 receptor fusion protein were probed with a polyclonal anti-PhoA antibody (Fig. 7A). Multiple bands migrating faster than a 50-kDa standard protein were detected by the anti-PhoA antibody in both cases, indicating that these bands do not represent proteolytic products of the fusion protein. In contrast, bands of >70 kDa were detected only in cells expressing the V2 receptor fusion protein. In particular, a prominent band migrating at ~76 kDa and a broad band migrating at ~97 kDa were detected by the antibody. In four of six membrane preparations, a faint band was detectable at ~78 kDa (Fig. 7A), forming a doublet with the prominent ~76-kDa band. The reason for the variability is not known.

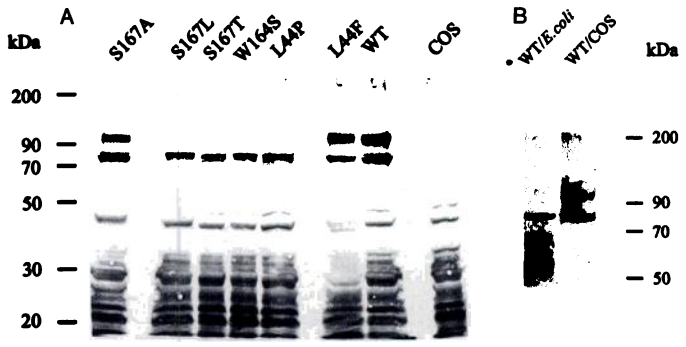

Fig. 7. Immunoblot analysis of wild-type and mutant V2 receptor/PhoA fusion proteins. Fusion proteins were separated on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose filters, and detected with rabbit polyclonal anti-PhoA antibody as the first antibody and horseradish peroxidase-conjugated anti-rabbit IgG as the second antibody. A, Membrane proteins (80 μg) from COS.M6 cells transfected with cDNA encoding wild-type or mutant V2 receptor/PhoA fusion proteins were analyzed. *COS*, untransfected cells. *WT*, cells transfected with wild-type cDNA. The position of marker proteins is indicated. B, Comparison of the wild-type V2 receptor/PhoA fusion protein expressed in *E. coli* (*WT/E. coli*) and COS.M6 cells (*WT/COS*). Eighty micrograms of membrane proteins (COS.M6 cells) and 60 μg of cytosolic protein (*E. coli*) were analyzed. Bands migrating faster than the 70-kDa standard protein in *E. coli* cytosol preparations most likely represent proteolytic products of the fusion protein.

In membranes from COS.M6 cells expressing the mutant fusion proteins L44P, W164S, S167L, or S167T, the ~76/78-kDa doublet was invariably found in six experiments (Fig. 7A). In contrast to the wild-type V2 receptor/PhoA fusion protein, the ~78-kDa band was always prominent, and the ~97-kDa protein was only barely visible after prolonged staining of filters (data not shown). The low intensity of the bands rules out an intracellular accumulation of these mutant fusion proteins and is consistent with an increased degradation. The fusion proteins carrying the naturally occurring mutant L44F and the *in vitro* mutant S167A yielded patterns resembling that of the wild-type fusion protein, indicating that they are expressed as mature proteins at normal levels.

The calculated molecular mass of the unmodified fusion proteins was 87.3 kDa (V2 receptor moiety, 39.8 kDa; PhoA moiety, 47.5 kDa). Thus, the band at ~97 kDa may represent the post-translationally modified, glycosylated protein. This possibility is supported by its fuzzy appearance and the finding that treatment with *N*-glycosidase F, which removes the asparagine-linked (i.e., *N*-linked) sugar moiety, caused a shift to ~85 kDa (data not shown). The discrepancy between the apparent molecular mass of the deglycosylated protein (~85 kDa) and its calculated molecular mass (~87.3 kDa) is probably caused by incomplete unfolding of the fusion protein in the presence of SDS. The fusion protein expressed in *E. coli* comigrated with the ~76/78-kDa doublet; larger species were not detected (Fig. 7B). The data indicate that the fusion proteins show abnormal behavior during SDS-PAGE, as is frequently observed for membrane proteins. The ~97-kDa and ~85-kDa forms seem to correspond to the mature and the non-*N*-glycosylated protein (see Fig. 1), respectively. The ~76/78-kDa doublet may represent the protein without mod-

ifications or with modifications that do not influence mobility on SDS-PAGE to a major degree (e.g., phosphorylation).

We attempted to exclude more rigorously the possibility that naturally occurring mutations lead to the expression of a receptor protein with residual function (e.g., low affinity receptor). We therefore used the adenylyl cyclase assay, which is more sensitive than the [^{3}H]AVP binding assay. Because the assay is performed with a broken cell preparation, it should also be possible to detect functional receptors retained within the cell. Except for the wild-type and the *in vitro* mutant S167A (see below), none of the NDI mutants (L44P, L44F, W164S, S167L, S167T) rendered the adenylyl cyclase system of transfected COS.M6 or HEK 293 cells sensitive to AVP (≤1 μM was used).

The *in vitro* mutant S167A was studied in greater detail. To this end, cell clones derived from L*tk*$^-$ cells expressing the S167A mutant were developed. Fig. 8A shows that the concentration-response curves for stimulation of adenylyl cyclase activity by AVP are almost superimposable for membranes from cells expressing either the wild-type receptor or the S167A mutant. The EC_{50} values were 0.35 nM in membranes from a representative cell clone (V2IIB1) expressing the wild-type receptor protein and 0.43 and 0.63 nM in membranes from two independently developed cell lines expressing the S167A mutant (S167AB1, 110,000 sites/cell; S167AB2, 96,000 sites/cell). Thus, all tested properties of the S167A mutant are indistinguishable from those of the wild-type receptor. In contrast, cell lines stably expressing the mutant L44F (as demonstrated by the detection of V2 receptor transcripts by reverse transcription-PCR; data not shown) failed to respond to 1 μM AVP; the responses to PGE_1 and to forskolin, an activator of adenylyl cyclase, were retained (Fig. 8B). The data provide further evidence for the assumption that the normally processed L44F mutant does not possess any residual function.

Discussion

In the current report, we show on a biochemical level that the five naturally occurring point mutations in the V2 receptor found in families with X-linked NDI (L44F, L44P, W164S, S167L, and S167T) are responsible for the disease. When expressed in COS.M6 cells, all of these mutants failed to bind or bound very little (<4%) [^{3}H]AVP. They also failed to confer AVP sensitivity to adenylyl cyclase when transiently expressed in COS.M6 or HEK 293 cells. The functional impairment does not seem to be a consequence of reduced mRNA stability or of inefficient transcription because Northern blots revealed identical sizes and essentially similar amounts of wild-type and mutant V2 receptor mRNAs. It is thus more likely that functional defect results from cell surface expression of mature but binding-deficient proteins or from a decreased or absent cell surface expression due to incomplete processing and degradation of the mutant receptor proteins.

To investigate these possibilities, we studied fusion proteins with PhoA attached to the carboxyl-terminal hydro-

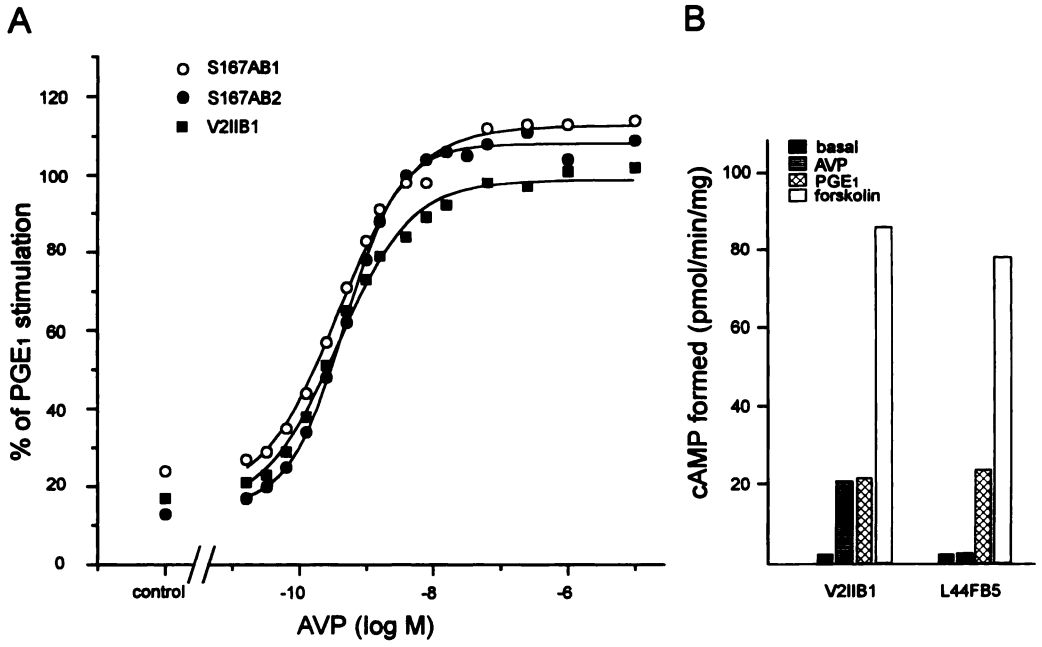

Fig. 8. Adenylyl cyclase activity in membranes from cell lines stably expressing the wild-type V2 receptor and the mutants S167A and L44F. V2 receptor-expressing clonal cell lines were established by transfection of L*tk*$^-$ cells with the respective cDNAs. A, AVP concentration-response curves. One representative cell line expressing the wild-type V2 receptor (V2IIB1, 180,000 sites/cell) and two cell lines expressing the S167A *in vitro* mutation (S167AB1, 96,000 sites/cell; S167AB2, 110,000 sites/cell) were analyzed. Data are expressed as percentage of adenylyl cyclase activity after maximal PGE_1 stimulation (100 μM PGE_1). The concentration-response curve for the wild-type receptor (V2IIB1) is the same as in Fig. 6. Data represent mean values of duplicates, which differed by <5%. Basal and PGE_1-stimulated adenylyl cyclase activities in pmol of cAMP/mg of protein/min were V2IIB1, 8.8 and 53.6; S167AB1, 12 and 52.7; and S167AB2, 4.0 and 31.8, respectively. EC_{50} values were 0.35 nM for the wild-type receptor, 0.43 nM for the S167AB1, and 0.63 nM for the S167AB2 mutant. B, Adenylyl cyclase activity was determined in membranes from cells expressing the wild-type V2 receptor (V2IIB1 cells; see A) and in membranes from one representative cell line expressing the L44F mutant (L44FB5). *Filled bars*, adenylyl cyclase activity under basal conditions. *Hatched bars*, adenylyl cyclase activity in the presence of 1 μM AVP. *Cross-hatched bars*, adenylyl cyclase activity in the presence of 100 μM PGE_1. *Open bars*, adenylyl cyclase activity in the presence of 100 μM forskolin, respectively. *Column height*, mean value of triplicates, which differed by <5% from each other.

philic tail of the V2 receptor. Fusion proteins with a PhoA moiety expressed in *E. coli* have proved to be useful for studying the membrane topology of bacterial proteins (28) and of various eukaryotic proteins, including β_2-adrenergic receptor (29), a cyclic nucleotide-gated cation channel, (30) and the multidrug resistance protein (31). These studies show that PhoA fusions, particularly when directed to the hydrophilic carboxyl termini of membrane proteins (28), carry minimal risk of disrupting topogenic signals. We have extended this observation to eukaryotic cells. Here, we show that wild-type fusion protein is efficiently expressed at the cell surface, does not differ from the wild-type receptor without PhoA moiety in its affinity for [^{3}H]AVP, and has retained its ability to activate fully the G_s/adenylyl cyclase system (Figs. 5 and 6), although a marked rightward shift of the dose-response curve for AVP was observed. It is likely that this rightward shift is at least in part due to the difference in the receptor densities of the cell clones expressing the wild-type V2 receptor with (33,600 sites/cell) and without (180,000 sites/cell) the PhoA moiety. This assumption is based on the finding that a cell line expressing 39,000 receptors without PhoA moiety shows an increased EC_{50} value for AVP (5 nM; data not shown) compared with a cell line expressing the wild-type receptor at a higher density (see above). In addition, the higher EC_{50} value may be attributed to the intracellularly located PhoA moiety, which possibly interferes with G protein coupling.

In immunoblots with membrane preparations of COS.M6 cells transfected with wild-type V2 receptor PhoA cDNA, two prominent bands of ~97 kDa and ~76 kDa were specifically stained with polyclonal anti-PhoA antibody; in the majority of experiments, a faint ~78-kDa band was also visible. We propose that the broad band at ~97 kDa, which is *N*-glycosidase F sensitive, represents the mature fusion protein bearing complex oligosaccharides at an extracellular asparagine (see Fig. 1). The ~76/78-kDa doublet apparently represents immature proteins that lack post-translational modifications rather than a proteolytic product of the ~97-kDa species. This view is strongly supported by the finding that the doublet comigrates closely with the wild-type V2 receptor/PhoA fusion protein expressed in *E. coli*. The ~76- and ~78-kDa polypeptides expressed in the eukaryotic system may differ from each other by post-translational modifications that do not significantly influence migration during SDS-PAGE (e.g., palmitoylation or phosphorylation).

The fusion proteins carrying the mutations L44P, W164S, S167L, and S167T are expressed at low levels as immature proteins. Notably, the ~97-kDa species was barely detectable, and the ~78-kDa species, which was invariably detectable, was more prominent than the ~76-kDa species. The data strongly indicate that the mutations severely impair glycosylation and possibly other post-translational modifications. Because the addition of complex sugars takes place in the Golgi apparatus, it is highly likely that this group of mutant receptors is retained in a pre-Golgi compartment, is degraded, and fails to reach the cell surface. This applies to the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator, which is only core glycosylated and becomes rapidly degraded within a pre-Golgi compartment (32). A severe disturbance of protein processing by the V2 receptor mutations is plausible because they cause either the exchange of amino acids (W164 and S167) that are highly conserved among GPCRs (27) or the introduction of a helix-breaking proline residue.

There are ~20 invariant amino acids among the GPCRs, most of which are located within the transmembrane domains (33). A tryptophan corresponding to W164 of the V2 receptor is found in 99 of 105 GPCRs, and a serine or an alanine corresponding to S167 is found in 88 of 105 GPCRs (27). Substitution of the conserved tryptophan in the rat muscarinic m3 receptor by phenylalanine (W192F) reduces the expression level but only marginally affects receptor function (34). The reduced expression levels are possibly due to retention within the cell or to increased degradation because mRNA levels were not substantially altered. An exchange of the conserved tryptophan for serine (V2 receptor) is much more profound than its substitution by phenylalanine (m3 muscarinic receptor). This could account for the differences between the data obtained with the m3 muscarinic receptor and the V2 receptor.

The interchangeability of alanine and serine corresponding to S167 in the V2 receptor is reflected within the family of primate opsins. Those with a serine or an alanine residue differ only by a 6-nm shift in the peak spectral sensitivity (35). In the human α-adrenergic receptor, the corresponding serine can be replaced by alanine without an effect on either receptor expression or ligand affinity (36). We show here that substitution of S167 by alanine in the V2 receptor does not interfere with its affinity for AVP, ability to stimulate adenylyl cyclase, expression level, or processing. Thus, the hydroxyl group of S167 is not obligatory for receptor synthesis, transport, or function. Substitution of S167 by larger amino acids, however (e.g., the S167T or S167L mutant), abolishes receptor function. These mutants are almost exclusively expressed as immature proteins. The data allow the conclusion that a subtle change, such as the introduction of a methyl group at this position (S167T mutant), causes X-linked NDI as a result of incomplete processing. Because detailed structural knowledge of GPCRs is lacking, the precise cause of the severe disturbance of protein processing is speculative. Although substitution of the conserved, membrane-embedded serine by an alanine may be tolerated sterically, an additional methyl group at this position might give rise to a misfolded receptor and result in its retention in the endoplasmic reticulum and subsequent degradation.

In contrast to the substitution of L44 by proline, the substitution at this position by phenylalanine (L44F), although a nonconservative exchange, does not seem to interfere with maturation of the V2 receptor protein. This mutant seems to be fully glycosylated, indicating that it is correctly inserted into the membrane of the endoplasmic reticulum and transported into the Golgi apparatus. The findings are consistent with synthesis of normal amounts of a mature protein, which should be expressed on the cell surface. Transfection of COS.M6 cells with L44F cDNA, however, does not result in the appearance of [^{3}H]AVP binding sites on the cell surface, nor does the mutant receptor stably expressed in cell lines derived from L*tk*$^-$ cells render the adenylyl cyclase system AVP sensitive, as determined in a broken cell preparation. The latter assay should have detected any residual function of receptors not expressed at the cell surface. We propose that the L44F mutant, although possibly expressed at the cell surface, is nonfunctional.

The mutations of the V2 receptor characterized to date

were reported to cause X-linked NDI by a decreased or absent affinity for AVP (11–13), a defect in the transport to the cell surface (11, 13, 14), or a decreased or absent ability to stimulate adenylyl cyclase (11, 14). Here, we show that four of five X-linked NDI mutations analyzed severely disturb protein processing. As a consequence, immature forms of receptor proteins are prevalent, whereas mature forms are almost undetectable. As discussed above, mutant proteins may fold incorrectly and therefore be incompletely processed and rapidly degraded within a pre-Golgi compartment. At present, the structural requirements for correct folding, processing, and sorting of membrane proteins, including GPCRs, are poorly understood. Naturally occurring mutations may help elucidate these structural requirements.

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