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Polarized expression of the vasopressin V2 receptor in Madin-Darby canine kidney cells

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Polarized expression of the vasopressin V2 receptor in Madin-Darby canine kidney cells.

Background. The vasopressin V2 receptor is expressed in the polarized principal cell of the renal collecting duct. Inactivating mutations of the vasopressin V2 receptor gene cause X-linked nephrogenic diabetes insipidus (NDI). Most of the mutant V2 receptors show transport defects, as analyzed in non-polarized cells, but data pertaining to polarized cells have not previously been presented.

Methods. Madin-Darby canine kidney cell (MDCK) II clones stably expressing c-myc–tagged human V2 receptors were characterized for [^{3}H]-arginine vasopressin (AVP)-binding and AVP-sensitive adenylyl cyclase activity. The V2 receptors were immunocytochemically localized using the tyramide signal amplification technique in conjunction with an anti–c-myc antibody.

Results. The introduction of the c-myc epitope at the N- or C-terminus did not affect the functional properties of the V2 receptor expressed in MDCK II clones. However, the use of standard immunofluorescence methodology for these MDCK II clones yielded only weak signals. With the tyramide signal amplification technique, strong signals were obtained, showing the V2 receptor to be mainly localized within the lateral and, to a minor extent, apical membrane. In MDCK II clones stably expressing the c-myc–tagged V2 receptor NDI mutant L44P, fluorescent signals were found exclusively within the cell.

Conclusion. The wild-type V2 receptor is expressed mainly in the lateral membrane, whereas the L44P mutant is completely retained within the cell. In conjunction with tyramide signal amplification, MDCK II cells constitute a suitable model for the analysis of transport-defective mutants of the V2 receptor.

The vasopressin V2 receptor (V2 receptor) belongs to the large family of G protein–coupled receptors characterized by a heptahelical transmembrane organization, an extracellular N-terminus, and an intracellular C-terminus, and appears to be exclusively expressed in the principal cell of the renal collecting duct [1]. Immunohistological studies of rat kidney revealed that the V2 receptor is predominantly localized in the basolateral and, to a small extent, apical membrane of principal cells [2]. Activation of the receptor by 8-arginine vasopressin (AVP) leads to stimulation of adenylyl cyclase via G_s. The increase of cAMP and the subsequent activation of protein kinase A induce the insertion of intracellularly stored water channels (aquaporin-2) into the apical membrane of the principal cells. As a consequence, the water permeability of the apical membrane is dramatically increased, facilitating the reabsorption of water (antidiuretic effect) from the hypotonic urine in the collecting duct.

In nephrogenic diabetes insipidus (NDI), the kidney fails to concentrate urine despite normal or even elevated levels of AVP. Inactivating mutations of the V2 receptor gene have been identified as the cause of X-linked NDI, whereas inactivating mutations of the aquaporin-2 gene lead to autosomal-recessive NDI [reviewed in 3–5]. The functional characterization of V2 receptor mutants has revealed three different types of molecular defects that lead to loss of function: (*a*) defects in the transport of the receptor to the plasma membrane, (*b*) defects in ligand binding, and (*c*) defects in G-protein coupling and activation [6]. Most of the mutants analyzed exhibit a transport defect. Functional analysis has hitherto been performed in only non-polarized cell systems. Because kidney tissue from patients suffering from X-linked NDI is not available, the consequences of intracellular transport defects in the polarized principal cell are not amenable to direct investigation. Therefore, we established an expression system for the V2 receptor in Madin-Darby canine kidney (MDCK) II cells, a polarized cell line derived from the canine renal collecting duct [7]. A c-myc epitope was engineered at the N- or C-terminus of the human V2 receptor to facilitate detection of the receptor by the recently established tyramide signal amplification technique [8]. The epitope-tagged

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wild-type V2 receptor was detected predominantly in the lateral membranes of polarized MDCK II cells. The L44P mutant was exclusively found intracellularly, in agreement with recent data showing the mutant to be retained within the endoplasmic reticulum [9]. MDCK II cells therefore represent a suitable expression system to analyze the transport defects of mutant V2 receptors occurring in families with X-linked NDI.

METHODS

DNA manipulation

Standard DNA manipulations were carried out according to the handbook of Sambrook, Fritsch, and Maniatis [10]. Automated sequencing of DNA fragments was performed using the ABI PRISM Dye Terminator Cycle Sequencing kit (Amplitaq DNA Polymerase FS; Perkin Elmer, Langen, Germany) on the Applied Biosystems 373A DNA Sequencer (Weiterstadt, Germany).

Construction of c-myc epitope-tagged receptors

The V2 receptor cDNA was cloned from plasmid pRCD N2 [11] into M13 mp19 (Pharmacia, Freiburg, Germany) as a *BamHI*/*XbaI* fragment. A c-myc epitope (EQKLISEEDL, residues 408 to 439 of c-myc) was inserted by site-directed mutagenesis either between methionine 3 and alanine 4 in the N-terminus or between serine 371 and the stop-codon at the C-terminus of the V2 receptor cDNA. Site-directed mutagenesis was carried out with the Sculptor *in vitro* Mutagenesis System (Amersham, Braunschweig, Germany). The oligonucleotides were 5′CCCCACC ATG CTC ATG **GAA CAA AAG CTT ATT TCT GAA GAA GAC TTG** GCC TCC ACC ACT TCC GCT GTG 3′ (N-tag) and 5′CTG GCC AAG GAC ACT TCA TCG **GAA CAA AAG CTT ATT TCT GAA GAA GAC TTG** TGAGGATCTTCTAGAGGGCCC 3′ (C-tag). The mutant cDNAs were cloned as *BamHI*/*XbaI* fragments into the eukaryotic expression vector pCDNAI.Neo (Invitrogen, Leek, The Netherlands), yielding plasmids encoding the N-tagged (pNV2Rmyc) and C-tagged (pCV2Rmyc) V2 receptor, respectively. The L44P mutant [9] was cloned as an *Nde*I/*Pml*I fragment into the *Nde*I/*Pml*I cut plasmid pCV2Rmyc. Mutagenesis was verified by sequencing.

Cell culture

Madin-Darby canine kidney II cells and COS.M6 cells were generously donated by K. Simons (EMBL, Heidelberg, Germany) and F. Fahrenholz (Frankfurt, Germany), respectively. The cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For cultivation of stably transfected MDCK II cells, the medium was supplemented with geniticin 418 (400 μg/ml; GIBCO BRL, Eggenstein, Germany).

Stable expression of wild-type and mutant V2 receptors in Madin-Darby canine kidney II cells

Stable transfection of MDCK II cells was performed as recently described [11]. In brief, 200,000 cells per 60 mm Petri dish were transfected overnight with a mixture of Lipofectin (GIBCO BRL, Eggenstein, Germany) and plasmid DNA in serum-free DMEM according to the manufacturer's protocol. Cells were incubated in complete medium (3 days), and geniticin was then added for selection. Seven days after transfection, cells were harvested, and serial dilutions were made. After four to seven days, individual clones were selected, cultivated, and tested for ligand binding.

Transient expression of V2 receptors in COS.M6 cells

Transfection of COS.M6 cells was carried out as described previously [9]. Control cells were mock transfected with pcDNAI/Neo (Invitrogen).

[^{3}H]AVP binding assay to intact cells and membranes of Madin-Darby canine kidney II clones and Scatchard analysis

Binding of [^{3}H]AVP to intact MDCK cells was performed as described [11]. Unspecific binding, determined in the presence of a 100- to 1000-fold excess of unlabeled AVP, was less than 10% of total binding. Binding of [^{3}H]AVP to membranes of MDCK cells was performed as described [12]. 8-Arginine vasopressin (AVP) and the V2-selective agonist 1-deamino-cys-8-D-arginine vasopressin (dDAVP, desmopressin) were purchased from Sigma-Aldrich (Deisenhofen, Germany). The V2-selective antagonist SR 121463A [13] was kindly provided by Claudine Serradeil-Le Gal, Sanofi Recherche, Toulouse, France.

Isolation of RNA from Madin-Darby canine kidney cell II clones and Northern blots

Total RNA was isolated from MDCK II clones with the RNAzol™ B kit (WAK-Chemie Medical GmbH, Bad Homburg, Germany). Size separation of total RNA (20 μg) on 1.1% agarose gels containing 2.2 M formaldehyde was followed by capillary transfer onto nylon membranes (Qiagen, Hilden, Germany) and ultraviolet cross-linking (2×90 seconds at 312 nm). Prehybridization and hybridization with random primed full-length V2 receptor cDNA (2.5×10^6 cpm/ml; Prime It Kit; Stratagene, Heidelberg, Germany) was performed in Quick-Hyb® solution (Stratagene) at 60°C for one hour. Membranes were washed at increasing stringency with a final wash in 0.25 × sodium salt citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 50°C for 15 minutes. The membranes were exposed to Kodak X-Omat AR films for 12 hours.

Adenylyl cyclase assay

The preparation of nuclei-free crude membrane fractions from MDCK II clones and the adenylyl cyclase assay were performed as described previously [14], with the exception that 1.5 mM $MgCl_2$ and 1 mM ethylenediaminetetraacetic acid (EDTA) were present in the reaction mixture. [^{32}P]cAMP was isolated by the two-column method [15].

Antibodies

Monoclonal anti–c-myc antibody (clone 9E10) was purchased from Dianova (Hamburg, Germany). Polyclonal anti–c-myc antibody was raised against a synthetic peptide corresponding to the c-myc fragment EQKLISEEDL coupled through an extra C-terminal tyrosine to the carrier protein keyhole limpet hemocyanin (KLH; Calbiochem, Bad Soden, Germany) [16]. The titer of the antiserum was determined by enzyme-linked immunosorbent assay, and its specificity was verified by immunoblotting. Polyclonal anti–c-myc antibody was purified with protein G-Sepharose 4 Fast Flow (Pharmacia, Freiburg, Germany) according to the manufacturer's protocol. The eluted IgG fraction was dialyzed against phosphate-buffered saline (PBS) and stored at −20°C.

Immunoblots

The preparation of membranes from COS.M6 clones and immunoblotting was performed as described previously [9]. Nitrocellulose membranes were incubated with either monoclonal or polyclonal anti–c-myc antibody. As secondary antibody, either horseradish peroxidase-conjugated antirabbit IgG or ^{125}I-labeled antirabbit and antimouse IgG were used. The washed filters were visualized with 4-chloro-1-naphthol and H_2O_2 or were exposed to Kodak X-Omat AR films for two to five days.

Immunocytochemistry with anti–c-myc antibody

Cells grown on glass cover slips. Five × 10^5 cells were seeded in a 35-mm diameter dish containing four cover slips and cultured for four days to confluence. Cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.4), fixed with freshly made 3% paraformaldehyde in PBS for 10 minutes at room temperature, and washed three times with PBS. The cover slips were transferred to a humidifying chamber. For permeabilization, cells were incubated in PBS containing 0.1% Triton X-100 for five minutes and washed three times with PBS prior to transfer. Unspecific binding was blocked with 5% normal goat serum (Dianova) for 20 minutes at 37°C, followed by three rinses for 5 minutes with PBS. Cover slips were incubated for one hour at 37°C or overnight at 4°C with 25 μl protein G-Sepharose–purified polyclonal anti–c-myc antibody (1:160 in PBS) and washed three times for 10 minutes with PBS. The cover slips were incubated with 30 μl biotin-SP–conjugated AffiniPure F(ab′)$_2$ fragment goat antirabbit IgG (H + L; 1:500 in PBS; Dianova) for one hour at 37°C. A modified protocol for tyramide signal amplification (TSA-Direct Green; NEN, Köln, Germany) was subsequently followed. The cover slips were incubated with 30 μl of streptavidin-horseradish peroxidase conjugate (1:2000) and fluorescein tyramide (1:200) and mounted with 50% glycerol in PBS containing 100 mg/ml 1,4-diazabicyclo [2.2.2]octane (DABCO; Sigma, München, Germany). The cover slips were sealed with nail polish. At least three independent preparations were analyzed with a Zeiss LSM410 inverted laser scanning microscope or with a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Jena, Germany). Representative pictures were taken with the camera system Sony 3CCD on Kodak T-MAX400. The controls consisted of (*a*) clones expressing V2 receptor without the c-myc tag, (*b*) incubation without a primary antibody, or (*c*) no amplification reagent (fluorescein tyramide).

Cells grown on filters. Five × 10^5 cells were grown on permeable polycarbonate filter supports (24 mm diameter Transwell permeable supports, 0.4 μm pore polycarbonate membrane; Costar, Bodenheim, Germany) for four days. After fixation and permeabilization (discussed earlier in this article) the filters were cut into two to three pieces (approximately 5 mm square and marked at one edge) and transferred onto Parafilm in a humidifying chamber. Each filter piece was incubated with 50 μl 5% unspecific goat serum on both sides for 20 minutes at 37°C, washed with PBS, and incubated with purified polyclonal anti–c-myc antibody (1:130) overnight at 4°C. Filters were washed in the wells of the cell culture plates. Subsequent incubation steps were as described earlier in this article. The filters were mounted on two plastic spacers on a slide with the cells facing up. A cover slip was placed above the filter and sealed with nail polish [17].

Immunocytochemistry with anti–ZO-1 antibody

For ZO-1 localization, the conventional immunostaining procedure with rat polyclonal anti–ZO-1 antibody (1:200 in PBS; DPC Biermann, Bad Nauheim, Germany) and TRITC-conjugated sheep antirat IgG (1:200 in PBS; Dianova) was followed. Permeabilization and processing for laser scanning microscopy was as described earlier in this article.

RESULTS

Immunoblot analysis of COS.M6 cells expressing c-myc–tagged wild-type V2 receptor

C- or N-terminally c-myc–tagged V2 receptors were expressed transiently in COS.M6 cells, and membrane preparations of these cells were analyzed by Western blotting. Incubation with polyclonal anti–c-myc antibody

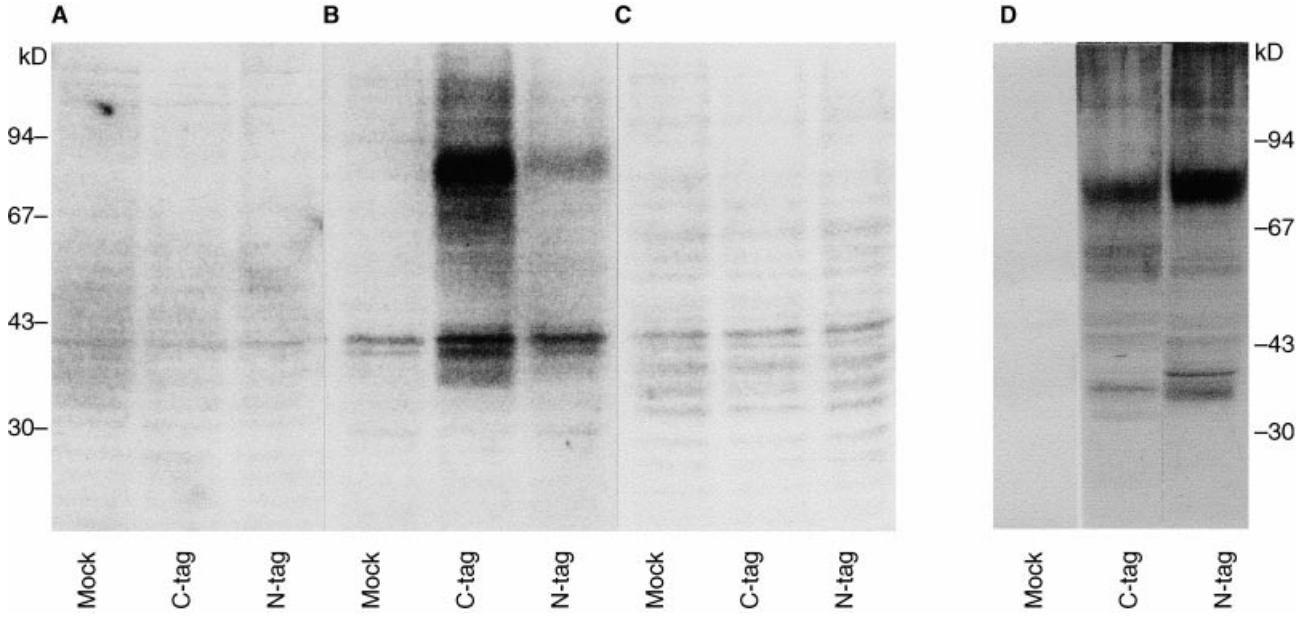

Fig. 1. Immunoblot analysis of c-myc–tagged V2 receptor with anti–c-myc antibody. Membrane preparations (60 μg protein) from COS.M6 cells transfected with cDNA encoding c-myc–tagged V2 receptor were separated on 12% sodium dodecyl sulfate-polyacrylamide gels, transferred onto nitrocellulose filters and incubated with polyclonal rabbit anti–c-myc antibody or monoclonal mouse anti–c-myc antibody. Specific binding was detected with peroxidase-conjugated antirabbit IgG (*A–C*) or with [^{125}I]-labeled antimouse IgG (*D*). (A) Incubation with rabbit preimmune serum (1:500). (B) Incubation with polyclonal anti–c-myc antibody (1:500). (C) Incubation with polyclonal anti–c-myc antibody as in (B) but serum preincubated with c-myc peptide (3.5 mM). (D) Incubation with monoclonal anti–c-myc antibody (2.5 μg/ml). Mock, vector without V2 receptor cDNA. C-tag, vector with C-terminal–tagged V2 receptor cDNA. N-tag, vector with N-terminal–tagged V2 receptor cDNA. Similar results were obtained in at least two independent experiments.

resulted in immunoreactive signals of apparent molecular masses of 37, 40, and 75 to 85 kDa and multiple bands in the region of 45 to 60 kDa (Fig. 1B). The immunoreactive band migrating at 40 kDa was presumed to be unspecific, as it was also found in mock-transfected cells (Fig. 1A). The specificity of the polyclonal anti–c-myc antibody was verified by peptide competition. Preincubation of the polyclonal anti–c-myc antibody with c-myc peptide abolished the immunoreactivity with the exception of the band migrating at 40 kDa (Fig. 1C). The results obtained with the polyclonal anti–c-myc antibody were confirmed with the monoclonal anti–c-myc antibody (Fig. 1D). The intensities observed, especially in the case of the 75 to 85 kDa band, were variable in each experiment. These differences did not correlate with the position of the c-myc epitope in the V2 receptor but varied between the transfection experiments. The 37 kDa band most likely represents the immature V2 receptor, and the broad band migrating at 75 to 85 kDa dimers of the same, as suggested by the work of Sadehgi, Innamorati, and Birnbaumer [18]. Interestingly, the bands in the region of 45 to 60 kDa were easily visualized in the case of the C-terminally tagged receptor but were hardly detectable for the N-terminally tagged receptor. Thus, the c-myc epitope might be thought to impair glycosylation of the V2 receptor, or alternatively, the glycosylation (which takes place 19 amino acids C-terminal of the c-myc epitope) might mask the c-myc epitope from the antibody. In membrane preparations of MDCK II clones expressing the c-myc–tagged V2 receptor, we failed to detect any specific signal in immunoblot experiments.

[^{3}H]AVP binding and adenylyl cyclase activity

To study the functional properties of MDCK II clones stably expressing c-myc–tagged V2 receptor, intact cells were assayed for specific [^{3}H]AVP binding, and membrane preparations were analyzed for adenylyl cyclase activity. Saturation binding and Scatchard analyses revealed similar K_D values for the cells expressing moderate (up to 200,000 sites/cell) or high levels (>300,000 sites/cell) of the wild-type or c-myc–tagged V2 receptors (Table 1). The EC_{50} values ranged between 2 and 10 nM for the different cell clones and did not reveal any influence of the tag at either the N- or C-terminus. Membrane preparations of untransfected MDCK II cells showed neither significant [^{3}H]AVP binding nor AVP-induced stimulation of adenylyl cyclase activity, suggesting the absence of endogenous V2 receptors, in contrast to the data of Schöneberg et al [19] and Deen et al [20]. This discrepancy may arise from the different MDCK clones used. We also established MDCK II clones expressing the L44P mutant V2 receptor. Because

Table 1. [^{3}H]AVP binding and adenylyl cyclase activity of MDCK II clones stably expressing the wild-type or c-myc V2 receptor

MDCK II clone	[^{3}H]AVP binding		Adenylyl cyclase activity			
			Basal	PGE_1 100 μM	AVP 10 μM	
	K_D nM	Sites/cell	*pmol cAMP/mg protein/min*			EC_{50} for AVP nM
Clones with moderate levels of V2 receptor						
Wild-type (V2RWT A4)	3.4	189,000	9.4	27.6	27.3	3.2
N-terminal tagged (NV2R B5)	2.5	177,000	3.6	25.9	25.3	2.3
C-terminal tagged (CV2R A5)	4.1	156,000	3.4	27.5	25.8	3.5
Clones with high levels of V2 receptor						
Wild-type (V2RWT A2)	3.2	302,000	6.5	16.0	21.1	2.9
N-terminal tagged (NV2R B4)	3.9	940,000	3.9	9.8	8.7	9.5
C-terminal tagged (CV2R A1)	3.6	431,000	6.4	19.6	19.0	6.4

Stable MDCK II clones expressing wild-type or c-myc tagged V2 receptor were tested for [^{3}H]AVP binding to intact cells and adenylyl cyclase assays as described in the **Methods** section. These clones were used for immunocytochemistry. K_D values were calculated from saturation experiments (specific binding of [^{3}H]AVP to intact cells). Data represent mean values of duplicates, which differed by less than 10%. Similar results were obtained in at least two independent experiments. EC_{50} values are the AVP concentrations required for half-maximal activation of adenylyl cyclase in crude membrane fractions. AVP was assayed at concentrations from 30 pM to 10 μM. Data represent mean values of duplicates, which differed by less than 10%. Similar results were obtained in at least three independent experiments.

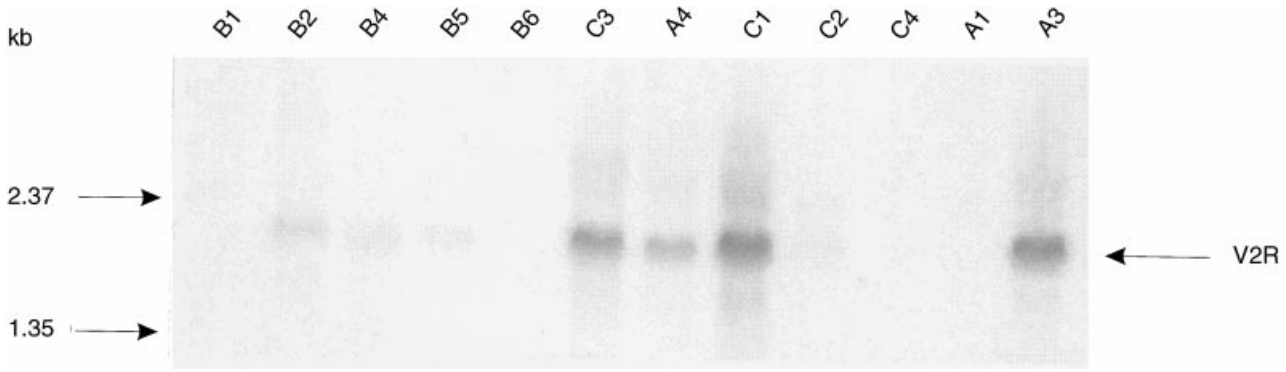

Fig. 2. Northern blot analysis of Madin-Darby canine kidney (MDCK) CL44P.myc clones. Total RNA isolated from MDCK II clones stably transfected with the L44P mutant V2 receptor cDNA were screened in Northern blot analysis (20 μg/lane) for specific V2 receptor transcripts. The blots were hybridized with radiolabeled V2 receptor cDNA as described in the **Methods** section. At the top, the code of the individual MDCK CL44P.myc clones is indicated. Left, size standards. Right, specific message. V2R is vasopressin V2 receptor mRNA.

this mutation was recently shown to display no significant binding [9], the individual clones were screened for specific transcripts by Northern blot analysis (Fig. 2). MDCK clones displaying abundant specific transcripts of the expected size of approximately 1.8 kb were investigated using the tyramide signal amplification technique (clones A3, A4, C1, and C3). In membrane preparations of these clones, we failed to detect significant [^{3}H]AVP binding (Fig. 3), thereby confirming the data previously found for the L44P mutant expressed in COS.M6 cells [9].

Immunocytochemical localization

Conventional immunostaining of MDCK II clones expressing the c-myc–tagged V2 receptor with anti–c-myc primary antibody and Cy3-coupled secondary antibody failed to give signals of sufficient intensity for analysis by either epifluorescence or laser scanning microscopy, in sharp contrast to COS.M6 cells transiently expressing the N- or C-terminally tagged V2 receptor [12]. By applying the direct tyramide signal amplification (TSA) technique [8], we were able, however, to detect V2 receptors in MDCK II clones. The cells were incubated with protein G-Sepharose–purified polyclonal anti–c-myc antibody, followed by biotinylated secondary antibody. After binding of streptavidin/horseradish peroxidase complex to biotin, the peroxidase catalyzes the formation of fluorescein tyramide radicals in the presence of hydrogen peroxide. The radicals react with proteins in the immediate vicinity of the enzyme, hence immobilizing fluorescein tyramide at the site of immunoreactivity. The polyclonal antibody yielded more intense signals than the monoclonal antibody 9E10 (not shown). Staining of permeabilized, confluent MDCK II clones expressing the C- or N-terminally tagged V2 receptors resulted in a honeycomb pattern, which is typical for proteins expressed in the lateral membranes of polarized cells (Fig. 4 B, C). The C-terminally tagged V2 receptor was detectable only in permeabilized cells (Fig. 4C), whereas the

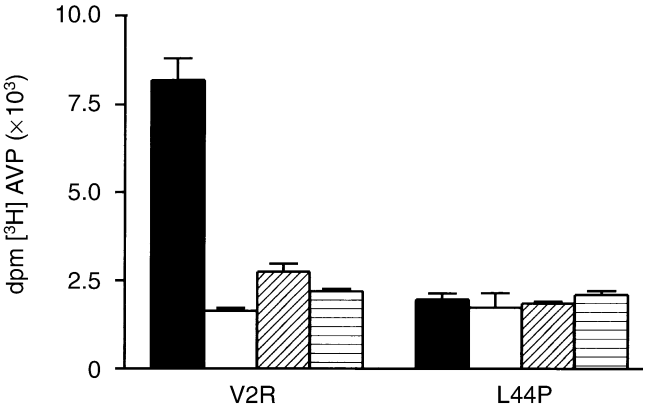

Fig. 3. [^{3}H]AVP-binding to membrane preparations from Madin-Darby canine kidney (MDCK) II clones. Stable MDCK II clones expressing either c-myc epitope-tagged V2 receptor (N**V2R** B5) or the L44P mutant V2 receptor (C**L44 P**V2R C3) were tested for [^{3}H]AVP binding to membrane preparations as described in the **Methods** section. Binding was performed with 10 nM [^{3}H]AVP in the presence or absence of 1 μM unlabeled agonist AVP, 1 μM V2-selective agonist dDAVP (desmopressin), and 10 μM V2-selective antagonist SR 121463A, respectively. The data represent triplicates ± SD. Similar results were obtained in two independent experiments. Symbols are: (■) [^{3}H]AVP; (□) [^{3}H]AVP/1 μM AVP; (▨) [^{3}H]AVP/1 μM dDAVP; (▤) [^{3}H]AVP/10 μM SR 121463A.

N-terminally tagged V2 receptor was visible in both permeabilized (Fig. 4B) and unpermeabilized cells (Fig. 5A, upper panel). The signals obtained with cells expressing the C-terminally tagged V2 receptor were stronger than from those expressing N-terminally tagged V2 receptors. This result is in agreement with the immunoblot analysis showing weaker staining of the glycosylated bands of the N-terminally tagged V2 receptor compared with the C-terminally tagged V2 receptor.

Various membrane proteins are transported preferentially to either the apical or basolateral plasma membrane. For example, the adenosine A1 receptor [21] is almost exclusively found in the apical membrane of polarized cells and the α_{2A}-adrenergic receptor in the basolateral membrane [22]. We investigated the distribution of the c-myc–tagged V2 receptor in the subdomains of the plasma membrane of stably transfected MDCK II clones by laser scanning microscopy. Vertical sections of MDCK II clones revealed strong lateral staining (Fig. 5, lower panel). In addition to the lateral staining, faint staining of the apical membrane was visible. No basal staining was detectable in permeabilized cells grown on glass cover slips (Fig. 5 B, C, lower panel). To exclude the possibility that the antibodies added to the apical side of the cells grown on cover slips might fail to reach the receptors on the basal surface, the cells were grown on permeable filter supports. The addition of antibodies to both sides of the filter (corresponding to the basal and apical sides of the cell layer) did not result in staining of the basal membrane (Fig. 5A).

The separation of apical and basolateral subdomains is physically maintained by tight junctions. To exclude the possibility that the apical signal of the V2 receptor might be caused by incomplete polarization, we analyzed confluent MDCK II clones with anti–ZO-1 antibody for the presence of tight junctions. ZO-1, a 225-kDa polypeptide, is localized exclusively on the cytoplasmic face of the tight junctions in a variety of epithelia [23]. Figure 6 shows the horizontal (xy-scan, upper panel) and vertical sections (xz-scan, lower panel) of MDCK II clones grown on cover slips. The basal surface of the cells corresponds to the top of the xz-scan. A defined region of the lateral membranes close to the apical surface was stained strongly (compare lower panels of Fig. 6 and Fig. 5C), thereby confirming the integrity of the tight junctions. We also investigated V2 receptor localization in preconfluent MDCK II clones. In such subconfluent cultures (in which cells are not yet tightly associated), staining of the V2 receptor was homogeneous over the whole membrane, indicating no polarized localization (asterisk, Fig. 7). When adjacent cells formed cell–cell contacts, a strong staining in the plasma membrane between the cells was detectable, even before the cell layer became completely confluent (arrow ←, Fig. 7).

The L44P mutant V2 receptor has been identified in a family with X-linked NDI [24]. Functional characterization of the L44P mutant receptor in non-polarized COS.M6 and L*tk*$^-$ cells demonstrated that this mutant is retained within the endoplasmic reticulum [9]. To demonstrate that the L44P mutant V2 receptor is also trapped within a polarized cell, we used the TSA technique. Confluent monolayers of MDCK II clones stably expressing the L44P mutant receptor showed immunoreactivity within the cells, but not at the membrane (Fig. 4D). In contrast to the wild-type receptor (Fig. 4C), staining of the L44P mutant did not result in the typical honeycomb pattern. In conjunction with a lack of specific [^{3}H]AVP binding to membrane preparations of MDCK clones stably expressing the L44P mutant receptor, the data indicate that the transport defect is also manifested in a polarized cell.

DISCUSSION

We established MDCK II clones stably expressing epitope-tagged V2 receptors for immunocytochemical analysis of polarized receptor expression. The functional properties (K_D, EC_{50}, B_{max}) of the c-myc–tagged (at the N- or C-terminus) wild-type V2 receptor expressed in MDCK II clones were the same as those of the untagged V2 receptor (Table 1). Similar results have been reported for N- or C-terminally HA-tagged V2 receptors (harboring a peptide derived from influenza hemagglutinin protein as epitope) expressed in non-polarized COS.M6, HEK293, or CHO-cells [6, 25, 26], and a polyoma virus

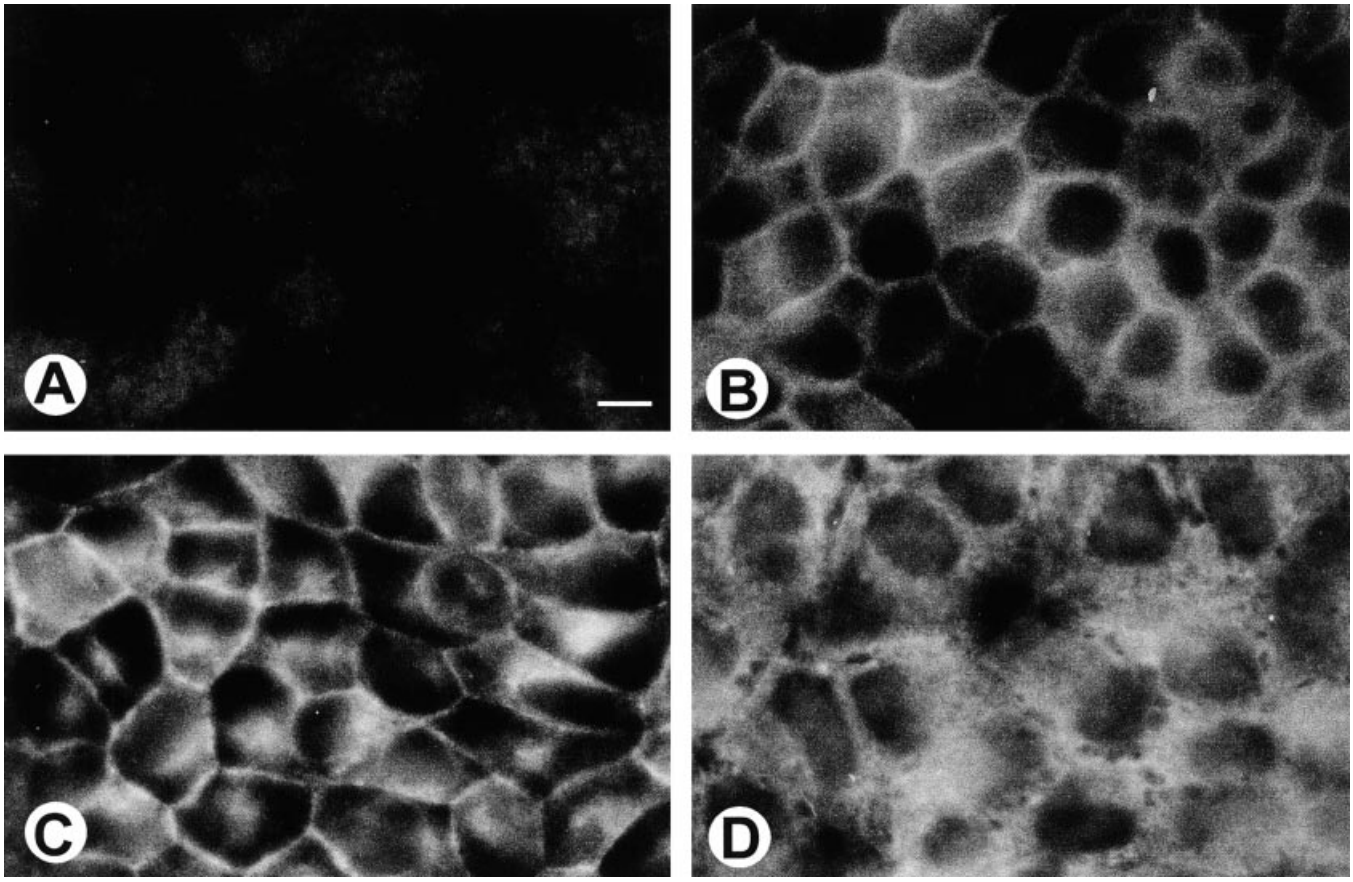

Fig. 4. Localization of the c-myc–tagged wild-type V2 receptor in confluent Madin-Darby canine kidney (MDCK) II clones. MDCK II clones were grown on glass cover slips, fixed, permeabilized, and processed with anti–c-myc antibody for epifluorescence microscopy. (*A*) V2 receptor without c-myc tag (302,000 sites per cell). (*B*) N-tagged V2 receptor (177,000 sites per cell). (*C*) C-tagged V2 receptor (156,000 sites per cell). (*D*) C-tagged L44P mutant of V2 receptor. Bar 10 μm.

medium T antigen epitope-tagged V2 receptor expressed in COS-7 cells [27]. Interestingly, immunoblot analysis with anti–c-myc antibodies of membranes derived from COS.M6 cells expressing either the C- or the N-terminally c-myc–tagged V2 receptor displayed prominent bands at approximately 37 and 75 to 85 kDa, which most likely represent the monomeric and dimeric immature V2 receptor, respectively (Fig. 1 B, D) [18]. Multiple bands of 45 to 60 kDa were easily detected, however, only in membrane preparations of COS.M6 cells expressing the C-terminally tagged V2 receptor (Fig. 1 B, D). These bands appear to represent the glycosylated V2 receptor. Sadeghi, Innamorati, and Birnbaumer also found bands at 45 to 55 kDa, which were assigned as the complex glycosylated V2 receptor [18]. Thus, it appears either that glycosylation of the V2 receptor masks the N-terminally fused c-myc epitope or, alternatively, that the c-myc epitope might hamper glycosylation. The former notion is supported by the work of Tsukaguchi et al [6]. Immunoprecipitation studies of membrane preparations of metabolically labeled CHO cells stably expressing the N-terminally HA–tagged V2 receptor yielded bands exclusively at 40 and 75 kDa, whereas immunoprecipitation of membranes of the same CHO clones after cell surface iodination yielded only a single band of approximately 55 kDa. It was suggested that only the 55 kDa band represents the glycosylated V2 receptor localized at the cell surface [6]. In these experiments, the N-terminally fully glycosylated HA-tagged V2 receptor was obviously also less efficiently recognized than the monomeric and dimeric immature receptors at 40 and 75 kDa.

In transiently transfected COS.M6 cells, c-myc–tagged V2 receptors were easily detectable by conventional immunocytochemistry. Saturation analysis of [^{3}H]AVP binding to intact COS.M6 cells revealed on average about 200,000 receptors per cell, that is, comparable to the MDCK II clones. However, considering that only approximately 5 to 10% of the transiently transfected cells showed high levels of expression, it is obvious that the receptor density in these cells is much higher (up to 20-fold) than indicated by saturation analysis. Interestingly, positive signals of transfected cells were more in-

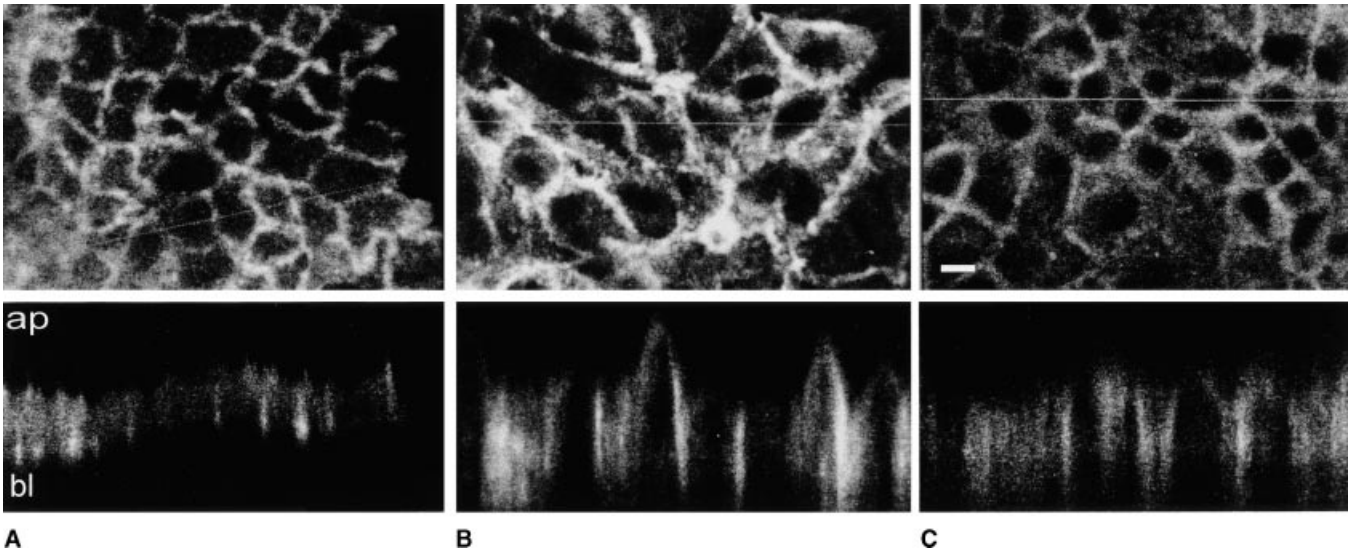

Fig. 5. Localization of c-myc–tagged wild-type V2 receptor in the subdomains of the plasma membrane of confluent Madin-Darby canine kidney (MDCK) II clones. MDCK II clones were grown to confluency on different supports. Cells were fixed and processed with anti–c-myc antibody for laser scanning microscopy. Shown are horizontal planes (xy-scan) of cells (upper panel) and vertical planes (z-scans) calculated at the indicated lines from 260 sections (lower panel). Abbreviations are: ap, apical surface; bl, basal surface. (*A*) N-tagged V2 receptor (940,000 sites/cell), filter support, cells not permeabilized. (*B*) N-tagged V2 receptor (940,000 sites/cell), glass cover slip, cells permeabilized. (*C*) C-tagged V2 receptor (431,000 sites/cell), glass cover slip, cells permeabilized. The extent of the lateral staining corresponds to the cell height. A, 12 μm high; B and C, 6 μm high.

tense for permeabilized than for unpermeabilized cells. The less intense signals obtained with unpermeabilized cells support the suggestion that the N-terminally tagged, glycosylated V2 receptors are less efficiently recognized by the anti–c-myc antibody than are the intracellularly located, immature V2 receptors (discussed earlier in this article). In addition, the presence of strong intracellular signals in permeabilized cells indicated a large number of intracellularly retained receptors [12]. In contrast, MDCK II clones stably expressing 150,000 N- or C-terminally c-myc–tagged V2 receptors per cell yielded no signal at either the cell surface or within the cell by conventional immunocytochemical analysis. These difficulties were overcome by applying the direct TSA technique. TSA was originally introduced to detect weak signals in solid-phase immunoassays [28, 29] and later adapted for immunohistochemistry at the light microscopic level [8, 30–32], the electron microscopic level [33], and for *in situ* hybridization [reviewed in 34, 35]. The procedure for signal amplification has been named differently by different authors and commercial suppliers: CARD [28], TSA (NEN, Köln, Germany), CSA (Dako, Hamburg, Germany), ImmunoMax [31], and TAT [32]. During our study with the V2 receptor, the use of TSA technique for the immunohistochemical localization of two other G protein–coupled receptors was published [36, 37]. We applied the TSA technique for the first time for the detection of a heptahelical membrane protein in stably transfected MDCK II clones. With this method, the lateral membranes of MDCK II clones were intensively stained and the apical membrane very faintly, suggesting a predominantly lateral expression of the V2 receptor (Figs. 4 and 5). In permeabilized MDCK II clones, the membrane staining was clearly dominant over the intracellular signals, suggesting, in contrast to COS.M6 cells, the presence of only a small number of intracellularly retained receptors. Thus, MDCK clones in combination with the TSA technique provide an excellent experimental system for the analysis of such V2 receptor mutants. In MDCK cells transfected with recombinant adenovirus harboring the wild-type V2 receptor cDNA, conventional immunocytochemistry was sufficient to detect V2 receptors. However, this approach yielded a pattern of heavily stained intracellular membranes, comparable to that found for transiently transfected COS.M6 cells [19]. The discrepancy between the results obtained with stably transfected MDCK clones (carrying probably only a single V2 receptor gene copy) and those transfected with recombinant adenovirus (with a high copy number) may arise from the higher level of expression in the adenoviral transfection, which may cause intracellular accumulation of V2 receptors.

Madin-Darby canine kidney II clones showed an intense labeling of the lateral membranes and faint signals at the apical membrane (Fig. 5). As apical membrane staining was also found (for both the N- or C-terminally tagged V2 receptor), we verified the normal polarization of the cells by staining for ZO-1, a tight junction-associ-

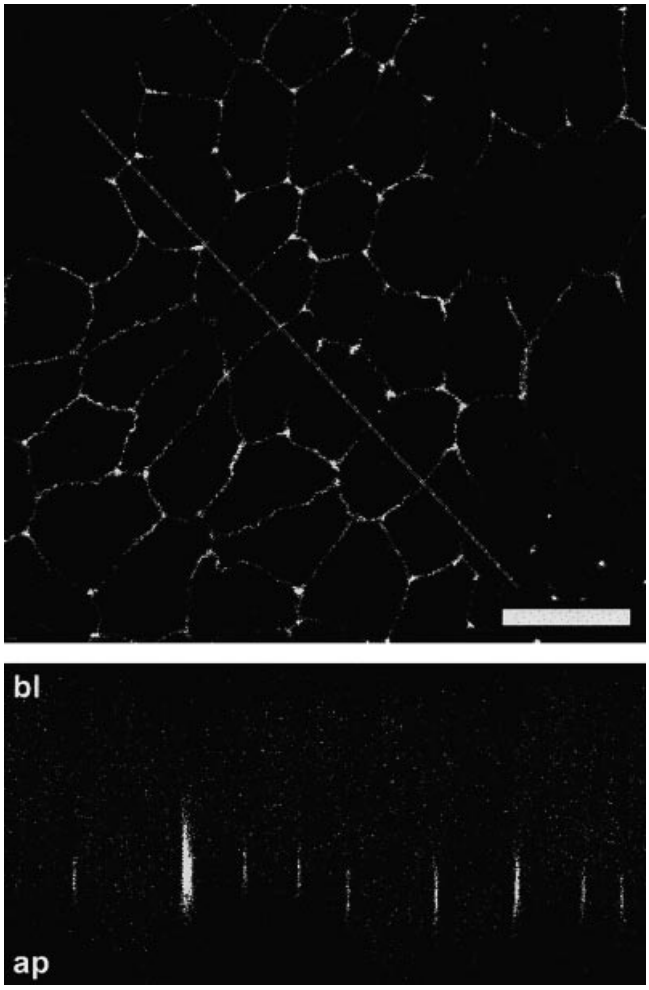

Fig. 6. Distribution of ZO-1 in Madin-Darby canine kidney (MDCK) II clones grown on glass cover slips. MDCK II clones expressing wild-type V2 receptor (302,000 sites/cell) were fixed, permeabilized, and processed with anti–ZO-1 antibody for laser scanning microscopy. Upper panel, horizontal plane (xy-scan). Lower panel, vertical plane (xz-scan). Bar 25 μm. Abbreviations are: ap, apical surface; bl, basal surface at the top of the scan.

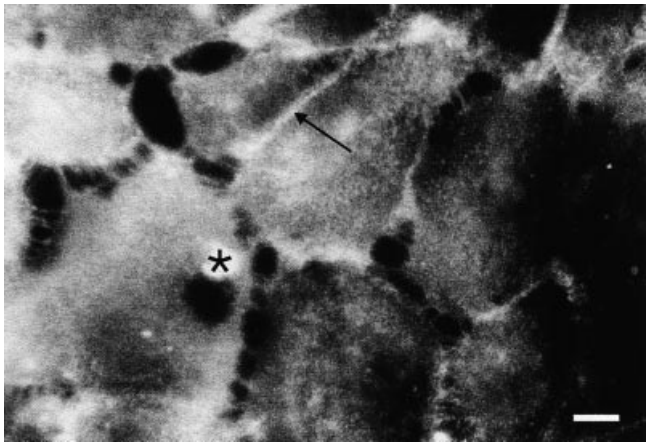

Fig. 7. Subconfluent Madin-Darby canine kidney (MDCK) II clones: Localization of the c-myc–tagged wild-type V2 receptor. Subconfluent MDCK II clones expressing N-tagged V2 receptor (940 000 sites/cell) were grown on glass cover slips and were fixed and processed with anti–c-myc antibody for epifluorescence microscopy. Symbols are: (*) staining of the whole plasma membrane; (←) staining at the cell–cell contact (Bar 10 μm).

ated cytoplasmic protein [23]. ZO-1 was detected exclusively in the apical region of the lateral membranes (Fig. 6), thus indicating a tight, polarized monolayer. These results were recently confirmed histocytochemically using V2 receptors fused at the C-terminus to the green fluorescent protein and biochemically by biotinylation experiments [38]. When MDCK clones were not confluent and thus not polarized, as evidenced by gaps between the cells, the V2 receptor was expressed over the whole cell (Fig. 7).

The existence of an apical signal in the confluent, polarized MDCK II clones is in agreement with immunocytochemical data obtained from the rat renal collecting duct, in which V2 receptors were also observed to a minor extent at the apical surface [2]. Interestingly, in our MDCK II clones, no signals were seen in the basal subdomain (Fig. 5). Similar results were obtained for the α_{2A} adrenergic receptor expressed in MDCK cells [39]. This is in contrast to the staining of the basal membranes observed in the rat renal collecting duct [2]. The reason for the differences observed between cultured cells and kidney tissue sections is unclear. One explanation might be that cells cultured on Petri dishes in general display more focal adhesion plaques than those cultured on an authentic substratum or those in the collecting ducts *in situ.* The strong expression of focal adhesion plaques might interfere with receptor distribution into the basal subdomain. Thus, the total receptor number in the basal membrane may lie under the detection limit. Alternatively, the access of the antibodies to the basal subdomain may be reduced, although this is less likely, as experiments with MDCK II clones grown on filter supports (in which antibodies were applied from both the basal and apical sides) did not result in basal staining. The apical membrane was faintly stained, independent of the culture support used.

The NDI-causing L44P mutant V2 receptor was localized only within the cell (Fig. 4D). No signal in the plasma membrane was found. As the pattern of immunoreactivity resembles that obtained with rhodamine 6G chloride, a fluorescent marker that enriches within mitochondria and the endoplasmic reticulum [12], we assume the mutant to be trapped within the endoplasmic reticulum. The lack of specific [^{3}H]AVP binding to either intact MDCK II clones expressing the L44P mutant or to membrane preparations of these cells indicates misfolding as the cause of retention. This notion is supported by the demonstration of only core-glycosylated L44P mutant

V2 receptors in transiently expressing COS.M6 cells, indicating retention in a pre-Golgi compartment [9].

Our results suggest that in conjunction with the tyramide signal amplification technique and anti-epitope antibodies, MDCK II clones represent a suitable polarized system for the analysis of V2 receptor transport. Demonstration of V2 receptor mutants within the endoplasmic reticulum, lysosomes, Golgi, or other compartments of polarized cells will afford subsequent characterization of transport defects in more detail.

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