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Polarized cell surface expression of the green fluorescent protein-tagged vasopressin V2 receptor in Madin Darby canine kidney cells

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Abstract We have analyzed the polarized cell surface expression of the G protein-coupled vasopressin V2 receptor (V2 receptor) in Madin-Darby canine kidney (MDCK) epithelial cells by both conventional cell surface biotinylation assays and laser scanning microscopy of green fluorescent protein (GFP)-tagged receptors. Cell surface biotinylation assays with stably transfected filter-grown cells expressing alkaline phosphatase (PhoA)-tagged receptors demonstrated that the V2 receptor is located predominantly basolaterally at steady state, while minor amounts are expressed apically. Laser scanning microscopy of filter- and glass-grown MDCK cells stably transfected with a GFP-tagged V2 receptor confirmed that the receptor is expressed mainly basolaterally; within the basolateral compartment, however, the receptor was confined to the lateral subdomain. The results obtained with the GFP-tagged receptor are thus consistent with and refine those from the biotinylation assay, which does not discriminate lateral from basal membrane regions. Our data indicate that the GFP methodology may effectively supplement cell surface biotinylation assays in future studies of polarized receptor transport. We finally show that microinjection of a plasmid encoding the GFP-tagged V2 receptor into the nucleus of MDCK cells led to the same results as experiments with stably transfected cells. However, since there was no need for selecting stably transfected cell lines, the experiments were complete within hours. The microinjection technique thus constitutes a powerful single cell technique to study the intracellular transport of G protein-coupled receptors. The methodology may be applicable to any cell type, even to tissue-derived, primary cultured cells; coinjection of transport-regulating compounds should also be possible.

Key words: Vasopressin V2 receptor; Madin-Darby canine kidney cell; Polarized transport; Green fluorescent protein fusion

1. Introduction

The study of the intracellular trafficking of GPCRs is an important task since it is conceivable that the function of signal transduction cascades requires correct intracellular delivery of the proteins involved. In polarized epithelial cells the cell surface comprises apical and basolateral membrane compartments which are separated by tight junctions and differ in their lipid and protein composition [1]. Studies in MDCK epithelial cells demonstrated that GPCRs may be expressed predominantly either in the apical or in the basolateral compartment. Basolateral steady state expression was demonstrated for the α_{2A}- [2], α_{2B}- and α_{2C}-adrenergic receptors [3] and for the TSH, FSH and LH receptors [4]. Apical expression was shown for the A_1 adenosine receptor [5]. Sorting signals for apical or basolateral targeting of GPCRs are not well defined but it was recently shown for the FSH receptor that a tyrosine and a leucine residue in the intracellular C-terminus contribute to a motif which directs basolateral targeting [6].

The G protein-coupled V2 receptor mediates vasopressin-regulated water reabsorption in renal collecting duct epithelial cells. For this receptor, an expression at the basolateral (interstitial) rather than at the apical (luminal) side is to be expected. Immunocytochemical studies in rat kidneys indeed suggested a predominant basolateral localization of the receptor [7]. To establish a recombinant system for the investigation of the sorting mechanisms, we have assessed polarized V2 receptor transport in MDCK epithelial cells. Polarized protein transport in epithelial cells has hitherto been studied using surface biotinylation and immunofluorescence microscopy of cells grown on permeable filter supports. Although these methods are extremely useful they are complicated and time consuming since they require the establishment of stable cell lines expressing the protein of interest. For non-polarized cells, it was shown recently that GFP fusions provide an excellent tool for analyzing protein transport directly in living cells. Trafficking studies for GPCRs in these cell types include the β_2-adrenergic receptor [8,9], the CCKAR receptor [10] and the V2 receptor [11,12]. In this paper, we have also assessed whether the GFP methodology is applicable to the investigation of the polarized transport in MDCK epithelial cells and whether plasmid microinjection may constitute a single cell technique for these studies.

2. Materials and methods

2.1. Materials

Trypan blue was from Seromed (Berlin, Germany). Benzamidine,

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Abbreviations: BSA, bovine serum albumin; CCKAR, cholecystokinin receptor type A; FSH, follicle-stimulating hormone; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; LH, luteinizing hormone; LSM, laser scanning microscopy; MDCK, Madin-Darby canine kidney; NBT, nitro-blue tetrazolium; PBS, phosphate-buffered saline; PhoA, *E. coli* alkaline phosphatase; PMSF, phenylmethylsulfonyl fluoride; TMB, 3,3′,5,5′-tetramethylbenzamidine; TSH, thyrotropin; V2 receptor, vasopressin V2 receptor

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PMSF, protein A-Sepharose, TMB, trasylol and Triton X-100 were from Sigma (München, Germany). Sulfo-NHS-biotin was from Pierce (Rockford, IL, USA). Polycarbonate permeable filter supports (No. 3412, 24 mm in diameter) were from Costar (Bodenheim, Germany). The monoclonal peroxidase-conjugated anti-biotin antibody was from Dianova (Hamburg, Germany). Type IV collagen was from Becton Dickinson (Heidelberg, Germany). All other reagents were from Merck (Darmstadt, Germany). Plasmids pEU367.PhoA and pWT.GFP encoding PhoA- and GFP-tagged V2 receptors, respectively, have been described [13,12]. The anti-PhoA antiserum was a gift from Schering (Berlin, Germany). Madin-Darby canine kidney (MDCK) type II epithelial cells were a gift from K. Simons (Heidelberg, Germany).

2.2. Cell culture and transfection

Lipofectin transfection of MDCK epithelial cells and selection of stable cell lines was as described previously for L^{tk-} cells [14].

2.3. Cell surface biotinylation assay and immunoblots

The method described by Marmorstein and coworkers [15] was slightly modified: 1×10^6 MDCK cells stably expressing a PhoA-tagged V2 receptor (EU367.PhoA) were plated on polycarbonate filter supports and grown for an additional three days to allow the formation of a tight epithelial monolayer. Cells were rinsed twice with PBS-CM buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4); apical cell surface proteins were labeled with 0.7 ml and basolateral proteins with 1.5 ml sulfo-NHS-biotin solution (0.5 mg/ml in PBS-CM), respectively. Labelling proceeded for 30 min on ice and 100-μl samples were removed from the opposite side of the chamber in each case to determine biotin leakage (see below). Biotinylation reactions were quenched with 1 ml NH_4Cl (50 mM) and cells were washed 3 times with PBS-CM. Filters were excised and transferred into a reaction tube. Cells were lysed for 1 h on ice with 1 ml buffer A (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM Na-EDTA, 40 mM PMSF, 1 μg/ml trasylol, 100 mM benzamidine, pH 8.0). Insoluble debris was removed by centrifugation (20 min, 4°C, $47\,000\times g$), and the supernatant was supplemented with 8 μl anti-PhoA antiserum (1:125, final dilution) for immunoprecipitation. Samples were incubated on ice for 2 h, supplemented with 3.5 mg protein A-Sepharose and incubated further for 1 h on ice. Immunoprecipitated receptors were collected by centrifugation (3 min, $17\,000\times g$), washed once with buffer B (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 50 mM NaCl, 1 mM Na-EDTA, pH 8.0) and once with buffer C (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4). Pellets were solubilized in 50 μl Laemmli buffer, separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose as described [16]. Filters were blocked for 1 h with 0.5% Tween 20 in PBS and monoclonal anti-biotin antibodies were added for 2 h at room temperature (1:1000 in PBS/0.05% Tween 20/0.5% BSA). Filters were washed 3 times (10 min each) with PBS/0.05% Tween 20 and stained with 4-chloro-1-naphthol and H_2O_2 until immunoreactive protein bands became visible.

2.4. Verification of epithelial integrity

Filter-grown cells were only used if transepithelial resistance was higher than 240 ohms/cm^2. For the biotin targeting assay, however, conventional transepithelial resistance measurements do not rigorously preclude biotin leak and thus we have developed an additional ELISA assay to assess for permeated biotin directly. 100-μl samples were taken after the biotinylation reactions from the opposite sides of the chambers and any permeated biotin was quantitated by its ability to label BSA. To this end, BSA-coated microtiter plates were supplemented with the 100-μl samples and incubated for 30 min at 4°C. Biotinylation was quenched with 200 μl NH_4Cl (50 mM) and microtiter wells were washed five times with PBS-CM. Monoclonal peroxidase-conjugated anti-biotin antibodies (100 μl, diluted 1:5000 in PBS-CM containing 0.5% BSA) were added and microtiter plates were incubated for 2 h at room temperature. Wells were washed five times with PBS-CM and biotinylated BSA was stained with 95 μl reaction buffer (200 mM citric acid, 200 mM K_2HPO_4, 0.01% H_2O_2) and 5 μl substrate solution (20 mM TMB in propanol) for 15 min at room temperature. Staining was stopped with 100 μl H_2SO_4 (1.5 M) and absorbance (450 nM) was read in an ELISA reader. Permeated biotin was calculated against a standard curve. Only those filters with a biotin leakage of less than 0.5% were used.

For cells grown on collagen-coated glass cover slips (and for filter-grown cells in addition), cell polarity and epithelial integrity was verified by LSM immunofluorescence detection of the zona occludens protein type 1 (ZO1) in the tight junctions which separate apical from basolateral membranes (*xy*- and *z*-scans; Andersen-Beckh, B., Dehe, M., Schülein, R., Liebenhoff, U., Wiesner, B., Rosenthal, W. and Oksche, A.; manuscript in preparation).

2.5. Visualization of GFP-tagged receptors in stably transfected MDCK cells grown on permeable filter supports or on collagen-coated glass coverslips

To visualize GFP-tagged receptors in cells grown on permeable filter supports, 1×10^6 cells were spread on a filter and grown for three days to allow the formation of an epithelial monolayer. Filters were washed three times with PBS, excised and transferred, cell side up, to a slide. A drop of PBS containing 0.05% trypan blue was added and filters were covered with a coverslip; GFP fluorescence was visualized with a Zeiss 410 invert laser scanning microscope ($\lambda_{exc}=488$ nm, $\lambda_{em}\geq515$ nm). The trypan blue fluorescence of the plasma membrane [12] was recorded on a second channel ($\lambda_{exc}=543$ nm, $\lambda_{em}\geq690$ nm), and its overlap with the GFP signal was computed. To visualize the GFP-tagged receptors of stably-transfected MDCK cells grown on collagen-coated glass coverslips, 3×10^5 cells were spread in a 35-mm diameter dish containing a coverslip and grown for three days. Coverslips were washed three times with PBS and transferred immediately into a self-made chamber (details on request). Cells were covered with 1 ml PBS and stained with 0.05% trypan blue for 10 min to allow the permeation of the fluorescence dye to the basolateral compartment. GFP and trypan blue fluorescences were recorded as described above.

2.6. Microinjection of plasmid DNA into glass-grown MDCK cells and visualization of GFP-tagged V2 receptors

For microinjection experiments, 3×10^5 MDCK cells were spread in a 35-mm diameter dish containing a collagen-coated coverslip and grown for three days. The GFP-tagged receptor cDNA was dissolved in HEPES buffer (5 mM NaCl, 135 mM KCl,100 μM EGTA, 10 mM HEPES, pH 7.3) and microinjected into the nuclei of the cells with an Eppendorf 5171 transjector (5–10 calculated copies/cell). Cells were grown for an additional 16 h to allow the expression of the GFP-tagged receptors. Coverslip processing, trypan blue staining and recording of GFP and trypan blue fluorescences were as described above.

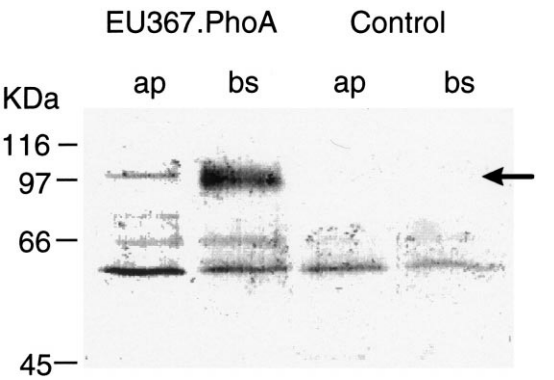

Fig. 1. Biotin targeting assay with stably transfected filter-grown MDCK cells expressing PhoA-tagged V2 receptors. The apical or basolateral surface of confluent cells was labeled with biotin. PhoA-tagged V2 receptors (EU367.PhoA) were immunoprecipitated with polyclonal anti-PhoA antibodies and receptors delivered to the apical (ap) or basolateral surface compartment (bs) were detected by SDS-PAGE-immunoblot analysis using monoclonal anti-biotin antibodies. Control samples (Control) were from apical (ap) or basolateral (bs) cell surface biotinylation reactions of untransfected MDCK cells. Each sample was derived from confluent cells from one 23-mm diameter filter support. The arrow indicates the specific 98-kDa band of EU367.PhoA. The immunoblot is representative of three independent experiments.

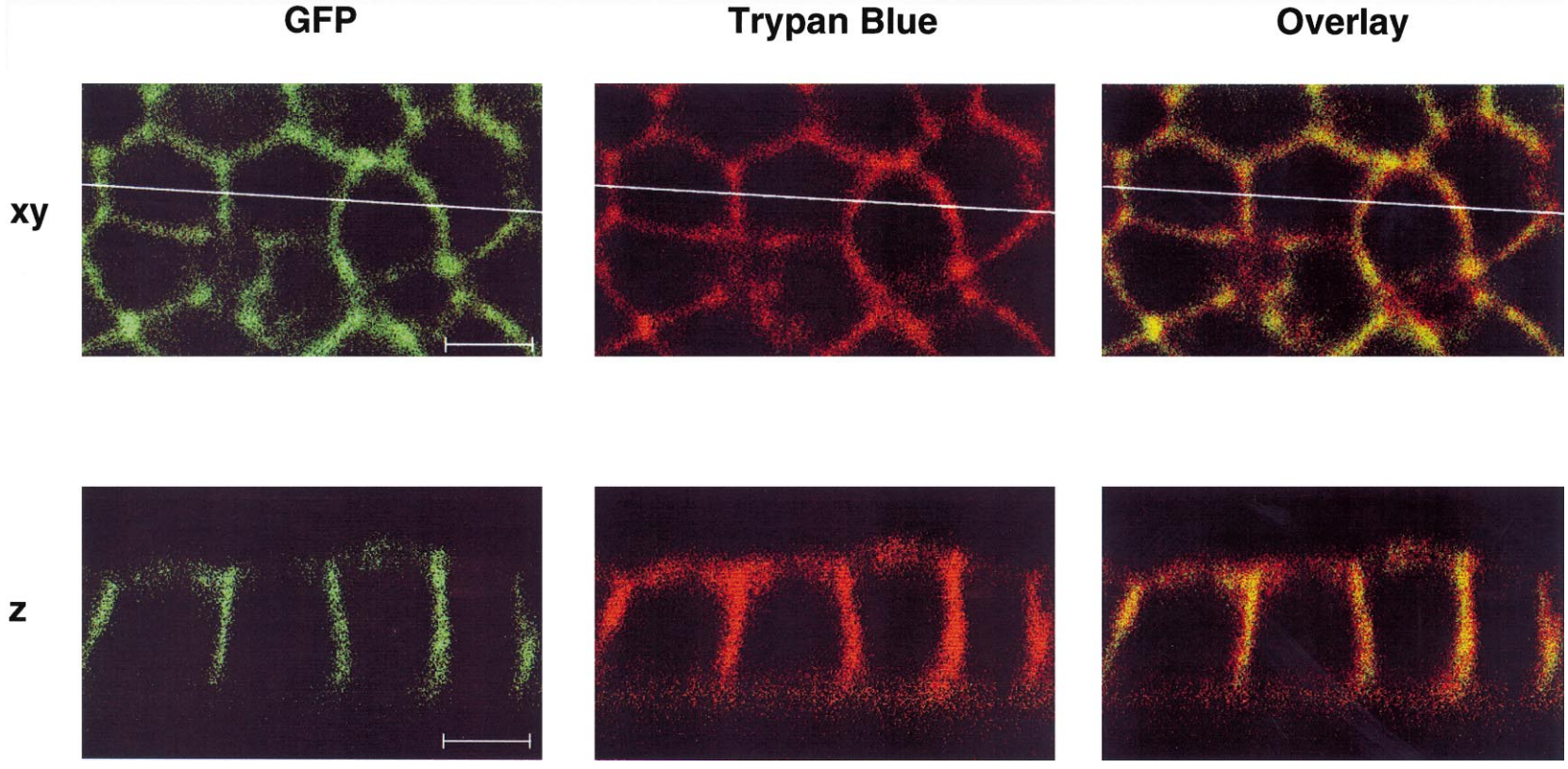

Fig. 2. LSM localization of GFP-tagged V2 receptors in live, stably transfected MDCK cells grown on permeable filter supports. Confluent cells were analysed by confocal laser scanning microscopy with *xy*-scans (upper panels) and with *z*-scans (lower panels) at the indicated lines. The GFP fluorescence is shown in green (left panels) and the cell surface trypan blue fluorescence of the same cells in red (central panels). GFP and trypan blue fluorescence signals were computer-overlayed (right panels; overlap is indicated by yellow areas). The apical membranes lie uppermost in the *z*-scans. Each photograph shows a representative scan. Scale bar = 10 μm.

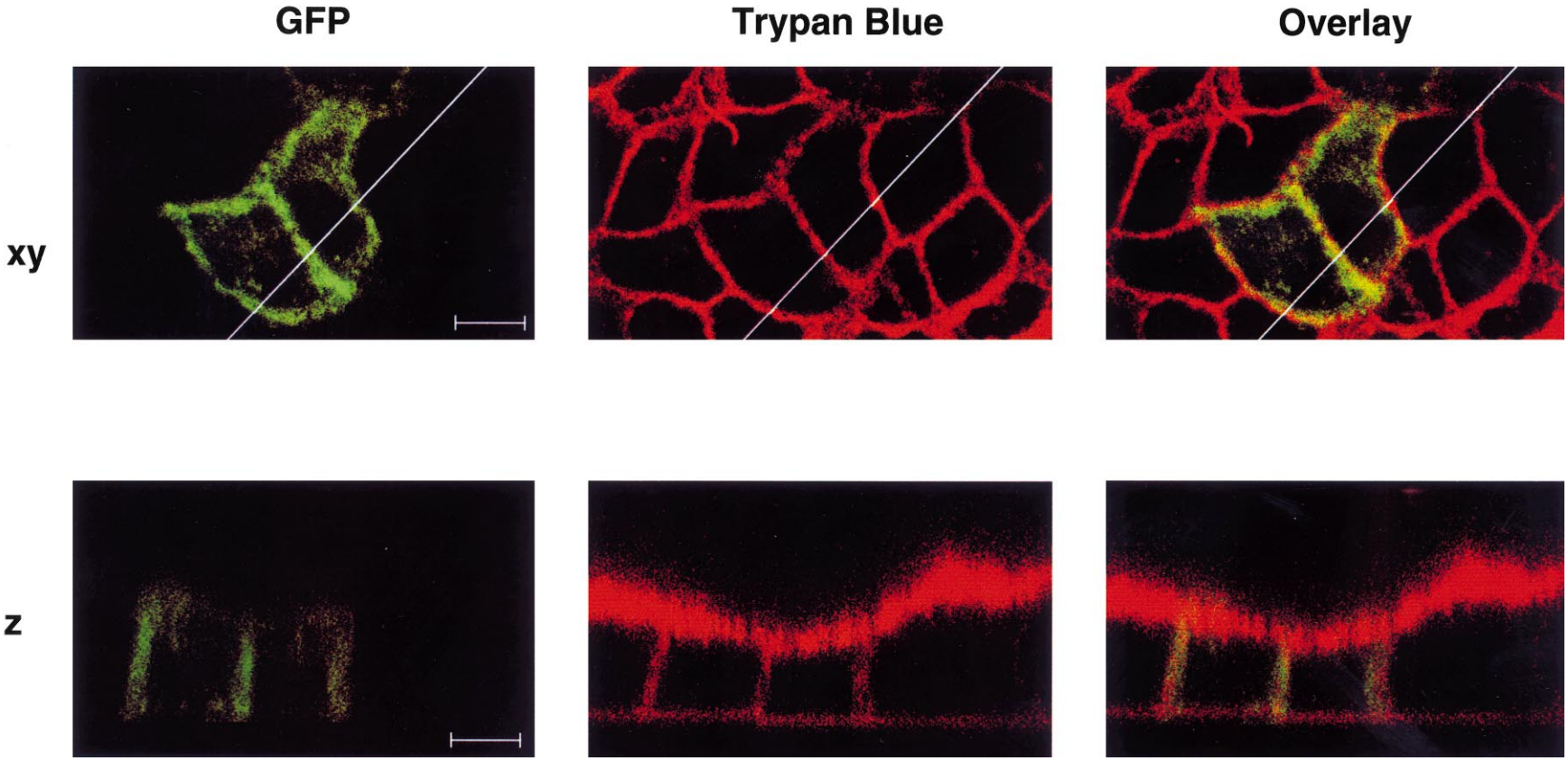

Fig. 3. LSM localization of GFP-tagged V2 receptors after microinjection of cDNA into the nuclei of glass-grown MDCK cells. Confluent cells were analysed by confocal laser scanning microscopy with *xy*-scans (upper panels) and with *z*-scans (lower panels) at the indicated lines. GFP fluorescence is shown in green (left panels) and trypan blue fluorescence of the same cells in red (central panels). Note that GFP fluorescence is detectable only in the case of the two cells which were microinjected, whereas trypan blue fluorescence is present in every cell. GFP and trypan blue fluorescence signals were computer-overlayed (right panels; overlap is indicated by yellow areas). The apical membranes lie uppermost in the *z*-scans. Each photograph shows a representative scan. Scale bar = 10 μm.

3. Results

3.1. A cell surface biotinylation assay reveals a predominantly basolateral expression of a PhoA-tagged V2 receptor at steady state

To assess for the polarized expression of the V2 receptor, we first carried out a classical biotin targeting assay. MDCK cells stably expressing a PhoA fusion to residue K367 of the V2 receptor (i.e. the entire receptor lacking only the four C-terminal residues) were used. We have previously shown that the PhoA-tagged receptor has pharmacological properties similar to that of the untagged receptor [13]. Cells were grown on permeable filter supports and apical or basolateral plasma membrane proteins were labeled with biotin. Tightness of the epithelial monolayer was verified by quantitation of the biotin leakage to the opposite side of the filters (see Section 2 for details). V2 receptors were immunoprecipitated with anti-PhoA antibodies and biotinylated V2 receptors in the apically or basolaterally labeled samples were detected on immunoblots using anti-biotin antibodies (Fig. 1). A prominent immunoreactive protein band with an apparent molecular mass of 98 kDa, representing the glycosylated PhoA-tagged V2 receptor [13], was present after basolateral biotinylation. Minor amounts of this band were also detectable in the sample from apical biotinylation. This protein band was absent in control cells. Two minor immunoreactive proteins with apparent molecular masses of 66 and 60 kDa were present in both the apical and the basolateral samples, but also in those of control cells, thus representing unspecifically stained proteins. This experiment demonstrates that the V2 receptor is located predominantly basolaterally in MDCK cells at steady state. Minor amounts are expressed apically.

3.2. A GFP-tagged vasopressin V2 receptor is expressed predominantly in the lateral membrane of stably-transfected MDCK epithelial cells at steady state

In contrast to biochemical techniques, the GFP methodology allows the observation of protein trafficking in living cells. To confirm the results obtained for the V2 receptor by the cell surface biotinylation assay, we established a stably-transfected MDCK cell line expressing a GFP-tagged V2 receptor. We have used a GFP fusion to K367 of the V2 receptor (i.e. to the same residue as for the PhoA fusion) whose pharmacological properties have previously been shown to be similar to those of the untagged receptor (pWT.GFP) [12]. The cells were grown on permeable filter supports and the GFP fluorescence signals were localized by laser scanning microscopy in living cells at the apical and/or basolateral membrane with *xy*- and *z*-scans (Fig. 2, left panels, in green). The entire cell surface (i.e. apical+basolateral membranes) of the same cells was identified by the use of trypan blue (Fig. 2, central panels, in red). Computer overlays of GFP and trypan blue fluorescence signals allow a more defined localization of the receptors to the apical and/or basolateral compartment (Fig. 2, right panel, colocalization is indicated by yellow areas).

The *xy*-scans revealed a honeycomb pattern for the GFP signals, which overlapped with the trypan blue signal. These signals thus represent receptors which are transported to the cell surface. Minor GFP signals were detected inside the cells, presumably representing transport intermediates en route to the cell surface. The *z*-scans revealed minor GFP signals which overlapped with apical trypan blue signals. The majority of the GFP signals colocalized with basolateral trypan blue signals. Within the basolateral compartment, however, the overlap was detected exclusively laterally; no overlap was seen with basal trypan blue signals. These results are consistent with those obtained for PhoA-tagged V2 receptors by biotin targeting assays (the lateral confinement within the basolateral compartment cannot be resolved with biotin targeting assays, see Section 4). They demonstrate that the V2 receptor is located predominantly in the lateral membranes at steady state. In addition, the data show that GFP fusions are suited to study the polarized transport of the V2 receptor.

We next assessed whether the lack of basal V2 receptors may be caused by the use of the permeable polycarbonate filter supports. MDCK cells stably transfected with the GFP-tagged V2 receptor were spread on collagen-coated glass coverslips and grown to a tight monolayer. The results obtained for cells grown on glass coverslips were identical to those obtained with filter supports (data not shown; see also the microinjection experiments with glass-grown cells, Fig. 3). Thus, the lack of basal V2 receptors seems to be independent of the adhesion matrix of the cells. These results also show that glass coverslips can be used instead of filter supports to study the polarized transport of the GFP-tagged V2 receptor.

3.3. Microinjection of GFP-tagged receptor cDNA into the nuclei of MDCK cells grown on glass coverslips constitutes a single cell method to study V2 receptor transport

A single cell method suitable to study polarized protein transport has not been available. Microinjection of a GFP-tagged receptor cDNA and subsequent monitoring of the GFP fluorescence may serve to fill this gap. To address whether this technique is applicable, MDCK cells were grown on collagen-coated glass coverslips with reference grids. Plasmid pWT.GFP, encoding the GFP-tagged V2 receptor cDNA, was microinjected into the nuclei of cells grown to confluence. After growth for an additional 16 h, polarized V2 receptor expression was monitored as described above with *xy*- and *z*-scans (Fig. 3). The results obtained by the microinjection technique were comparable to those from stably transfected cells (see Fig. 2). Again, the GFP and trypan blue fluorescence signals overlapped predominantly at the lateral membranes and to a minor extent at the apical membrane. No overlap was detected at the basal membrane. The apical membranes are stained more deeply with trypan blue than the basal and lateral membranes. This arises from the fact that the tightness of the epithelial monolayer impairs access of the dye to the basal and lateral membranes. Longer staining periods (up to 10 min) increase basolateral signals but also lead to a further increase in apical staining. Our data show that the microinjection methodology is applicable to the study of polarized protein transport.

4. Discussion

In this paper we have described the polarized cell surface expression of the G protein-coupled V2 receptor in MDCK epithelial cells. We have introduced the GFP methodology to the investigation of polarized protein transport and have assessed whether plasmid-microinjection might constitute a single cell technique to study polarized sorting.

Selective cell surface biotinylation assays with filter-grown

cells stably transfected with a PhoA-tagged V2 receptor revealed that the receptor is expressed mainly basolaterally, and to a minor extent apically, at steady state. This technique does not discriminate between the lateral and basal membranes of the basolateral cell surface compartment. A preferential transport to either of these subdomains thus could not be resolved. Localization of GFP-tagged V2 receptors in stably transfected MDCK cells grown on both permeable filter supports and glass coverslips confirmed that the V2 receptor is present mainly at the basolateral surface at steady state. In addition, these experiments showed that the majority of receptor protein is confined to the lateral subdomain; no receptor protein was detectable in the basal plasma membrane. The lateral confinement of the V2 receptor in glass- or filter-grown MDCK cells was also observed in a classical immunofluorescence study investigating naturally occurring V2 receptor mutants causing nephrogenic diabetes insipidus (Andersen-Beckh, B., Dehe, M., Schülein, R., Liebenhoff, U., Wiesner, B., Rosenthal, W. and Oksche, A.; manuscript in preparation). These results are somewhat surprising since there are no known structural barriers separating the lateral from the basal membranes. However, previous immunofluorescence experiments with glass- and filter-grown MDCK cells expressing α_{2A}- [2] and with filter-grown MDCK cells expressing α_{2B}- and α_{2C}-adrenergic receptors [3] demonstrated their exclusively lateral location within the basolateral compartment. From the *z*-scans of glass-grown MDCK cells stably transfected with FSH, TSH and LH receptors presented by Beau and coworkers [4], the same conclusion may be drawn although this was not emphasized by the authors. Lateral confinement was also observed in filter-grown cells for other membrane proteins, e.g. the Na^+, K^+-ATPase [17] and CD44 [18]. One might criticise that the apparent lateral confinement observed in these classical immunofluorescence experiments may be due to the inaccessibility of the basal membrane for antibodies. At least for the V2 receptor this possibility can now be excluded, as our data were directly derived from the localization of autofluorescence signals. It is also unlikely that the lateral confinement within the basolateral compartment relies on the adhesion matrix for the basal membrane, since our results for cells grown on glass coverslips or on permeable filter supports were comparable. Therefore our data suggest that basolaterally sorted GPCRs are indeed transported only to the lateral subdomain of this surface compartment. It is, however, possible that some factor essential for basal receptor expression is still not present either in our MDCK cell culture system or in the others cited above.

Both the biotin targeting assay with filter-grown cells expressing PhoA-tagged V2 receptors and the localization studies with filter- and glass-grown cells expressing GFP-tagged V2 receptors demonstrate that minor amounts of the receptor are present in the apical membrane at steady state. These results are in agreement with an immunohistochemical study which also revealed apical signals in renal collecting duct epithelial cells, where the V2 receptor occurs naturally [7]. Therefore, in the case of the V2 receptor, the recombinant MDCK cell system seems to reflect the physiological situation in the collecting duct.

Although the results obtained by the biotin targeting assay and the GFP methodology were consistent for the V2 receptor, an influence of the GFP moiety on the polarized transport of other membrane proteins cannot yet be excluded. The detection of such an influence should depend on the strength of the sorting signals of the proteins of interest and additional studies are needed to clarify whether the GFP methodology is applicable to all membrane proteins. The great advantage of the GFP methodology for the study of polarized transport is that protein sorting can be studied in living cells. In addition, problems of unspecific signals and of the accessibility of membranes to antibodies, which may complicate immunofluorescence experiments, do not arise. Difficulties might occur when substantial amounts of the protein under study are located intracellularly. Surface and intracellular fractions would be detected simultaneously in this case and it may be difficult to distinguish between them. The problem should be largely overcome by visualization of the cell surface with trypan blue and overlay of GFP and trypan blue fluorescence signals [12].

By microinjection of plasmids encoding a GFP-tagged receptor cDNA into the nucleus of confluent, glass-grown MDCK cells, the same results were obtained as with stably transfected cells. Microinjection thus constitutes a powerful single cell method to monitor GPCR transport in general, since the studies should be easily extended to any desired cell type and even to tissue-derived primary cultured cells. Furthermore this technique is very time saving since there is no need for the development of stable cell lines and experiments are completed within hours. Most importantly, GPCR transport may not only be monitored but also easily manipulated by this methodology. Co-expression of other transport-relevant proteins may be achieved by simple co-injection of the requisite plasmids. Co-injection of antisense oligonucleotides and other regulating compounds should also be possible. For polarized transport, it would also be interesting to see whether plasmid microinjection, whereby direct DNA transfer causes induction of protein expression, would allow time-course recording of protein sorting.

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