J. Membrane Biol. 190, 175–187 (2002)
DOI: 10.1007/s00232-002-1034-4

The Journal of
Membrane
Biology

Polarized Expression of Shaker Channels in Epithelial Cells

J. Moreno, L.R. Cruz-Vera[1], M.R. García-Villegas, M. Cereijido
Department of Physiology, Biophysics and Neurosciences, [1]Department of Genetics and Molecular Biology, Center for Research & Advanced Studies, Av. Instituto Politécnico Nacional 2508, Col. Zacateco, México, D.F. 07300, México

Received: 25 April 2002/Revised: 5 August 2002

Abstract. The polarized distribution of ion channels into an apical *or* a basolateral domain is a fundamental feature of the transporting-epithelial phenotype. To study the molecular motifs of the channel that may serve as addressing signal(s), as well as the cellular mechanisms that interpret it and deliver the protein accordingly, we study the fate of transfected ShIR K^+ channels (a non-inactivating Shaker channel) tagged with an HA epitope, as well as several other deletants and mutants. Surface expression is triggered by Ca^{2+}-activated cell-cell contacts, through a cascade including a phospholipase C, a protein kinase C, and the cytoskeleton of actin and tubulin, and is partially impaired by suppressing N-glycosylation with tunicamycin. Using domain-specific biotinylation we show that the channel is delivered preferentially to the basolateral domain thanks to a segment between amino acids 571 and 613, and is retained on the membrane surface due to a region involving the last three amino acids (threonine, aspartic acid, valine, TDV) of the COOH terminal. Its association with the cytoskeleton seems to take the form of a scaffold comprising actin, α-actinin, β-tubulin, mLin7 and CASK. We also observe that membrane expression of ShIR channels depends entirely on its sequence of amino acids and the conformation that the molecule may adopt, but not on its ability to translocate K^+ across the membrane.

Key words: Transporting epithelia — K^+ channels — Shaker channels — Protein sorting — Membrane targeting — Polarity

Correspondence to: M. Cereijido; email: cereijido@fisio.cinvestav.mx

Introduction

More than half a century ago, the outside-to-inside transport of substances across epithelial cells, and the dendrites-to-synapse flow of electric current through the body of neurons obviously forecast that a day would arrive when those asymmetries would have to be accounted for by a polarized distribution of transporting mechanism in their plasma membrane. Yet membrane biologists were in for two big surprises. The first is that polarization is by no means an exclusive feature of membrane transporting mechanisms, but of most membrane features and functions, such as membrane particles, receptors, cell contacts, budding of viruses, etc. The second is that polarization is by no means restricted to epithelia and nervous cells, but is found to be a universal feature, from unicellular organisms to mammalian neurons (Cereijido et al., 1980 Cereijido, Shoshani & Contreras, 2000; Cereijido & Anderson, 2001).

Study of cell polarity has been greatly advanced by the use of cultured cell lines that retain a considerable degree of polarity, and by the fact that cells not only express their own proteins in polarized fashion, but also transfected ones of other animal species, including viruses (Rodriguez-Boulan, 1983; Rodriguez-Boulan et al., 1984; Lisanti et al., 1989). Study of the expression of foreign transfected proteins has the experimental advantage of allowing the use of mutants and deletants to detect and characterize the putative molecular signals and mechanisms involved (Matter & Mellman, 1994).

In previous studies we have shown with patch clamp methods that the strain of MDCK cells we use expresses six different types of channels, all of them absolutely polarized: four types of K^+ channels in the apical, a fifth in the basolateral, and a Cl^- channel also in the basolateral domain (Stefani & Cereijido, 1983; Bolivar & Cereijido, 1987; Ponce &

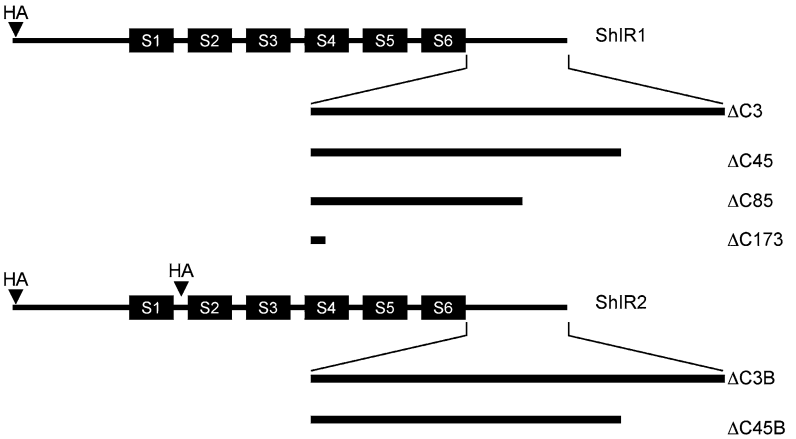

Fig. 1. Schematic representation of the different constructions used. Downward-pointing arrowheads indicate the position of epitope HA. Notice that constructions represented in top part have only one HA epitope, but those below have two.

Cereijido, 1991; Ponce et al., 1991a,b; Garcia-Villegas et al., 1996). We now study the mechanisms, signals and structures involved in the localized expression of K^+ channels. We resorted to the transfection of the ShIR channels, where IR stand for *I*nactivation *R*emoved, a deletant of the ShakerB channel (Hoshi, Zagotta & Aldrich, 1991), as well as other mutants and deletants specified below.

Here we find that channel ShIR is addressed to the basolateral domain due to a segment of 43 amino acids of the COOH terminus, located between aa 571 and 613, and is retained there through a PDZ-binding motif at the end of such terminus. The channel becomes part of a scaffold comprising proteins mLin-7, CASK, β-tubulin, actin and α-actinin. Surface expression depends on Ca^{2+}-activated cell-cell contacts that trigger a phosphorylation cascade involving PLC and PKC and the actin cytoskeleton.

A preliminary version of this work was presented in poster form (Moreno et al., 1999).

Materials and Methods

Constructs

The ShIR (non-inactivating, Δ6–46) and W434F (nonconducting) potassium channel cDNAs (derived from the *Drosophila* ShB1, Schwarz et al., 1988) were a kind gift from L. Toro and F. Bezanilla, respectively. The constructions pSHA and pWHA were obtained by amplifying the complete cDNA decoding the ShIR and W434F channel proteins by PCR to introduce an HA epitope (YPYDVPDYA) in the 5′ end using oligonucleotides H1: 5′-CCGGAATTCCATGGCCTACCCCTACGACGTGCCCGACT ACGCCGTTGCCCTGCGGGAGCAGCAC-3′ (the sequence of the HA tag is underlined), and 5′-CCGGAATTCTCAAACGTCG GTCTCGATACTAACGGC-3′. The PCR product obtained with *Pfu* polymerase (Stratagene) and flanked by *Eco* RI sites was cloned into pcDNA3 eukaryotic expression vector (Invitrogen). Therefore, the HA epitope was introduced between valine 2 and alanine 3. The protein products from these constructs were named ShIR1 and W434F1, respectively. Mutants ΔC173, ΔC85 and ΔC45 were constructed by introduction of stop codons (TAG or TGA) at positions, respectively: 1326 (amino acid 443), 1593 (amino acid 531) and 1713 (amino acid 571) using the QuikChang™ directed mutagenesis kit (Stratagene) and the respective two complementary oligonucleotides. The ΔC3 mutant was obtained by PCR introducing a stop codon in position 1836 (amino acid 614) using the H1 oligonucleotide and 5′-CCGGAATTCTCACTCGATACTAA CGGC-3′. We obtained a ShIR1 channel containing an extra HA epitope in the extracellular loop between phenylalanine 213 and lysine 214 at the loop between S1 and S2 domains (construct denoted as pSHA2) (Shih & Goldin, 1997). This version of the protein, called ShIR2, was obtained with the QuikChange kit and two complementary oligos. In all cases, pSHA construct was employed as template. ΔC3B mutant was generated as depicted for ΔC3 but using pSHA2 as template. ΔC45B mutant was obtained as described for ShIR2, but using ΔC45 as template. Constructs are schematized in Fig. 1. To obtain the in vitro transcribed RNA, full-length ShIR channel cDNA containing the HA epitope, ShIR1, was subcloned into the pBluescript KS^+ (Stratagene) (pBSHA). The correct introduction of both the HA epitopes and the stop codons were verified by dideoxynucleotide sequencing.

Expression in *Xenopus* Oocytes and Electrophysiological Recording

WT ShIR (cloned in pBluescript KS^+) and pBSHA were linearized with the *Not* I endonuclease and mRNAs obtained in a runoff assay using the T7 phage RNA polymerase (Invitrogen) (Sambrook & Russel, 2001). Stages V to VI oocytes were obtained by partial ovariectomy of adult female *Xenopus laevis* frogs (obtained from NASCO, Fort Atkinson, WI) anesthetized on ice. Harvested oocytes were shaken (60 rpm) for 60 min at room temperature in 1 mg/ml collagenase (Type II, Sigma) in ND-96 solution (in mM: NaCl, 2.0 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 5.0 HEPES, pH 7.5). Oocytes were left to recover overnight in ND-96 solution supplemented with 0.1 mg/ml gentamicin, 0.55 mg/ml pyruvate. Approximately 50 ng of transcripts obtained were injected into each oocyte. After 36 to 48 hr of incubation at 17°C in supplemented ND-96, potassium currents were recorded at room temperature using a two-electrode voltage clamp (Miledi, Parker & Sumikawa, 1983). The recordings were performed in ND-96 solution without supplements. The holding potential was −80 mV. All currents were recorded using p/4 subtraction to eliminate capacitive and linear leak currents (Bezanilla & Armstrong, 1977). Those oocytes expressing currents less than 50 nA during a depolarization to +30 mV were considered negative in respect to expression of functional ShIR channels. Currents were converted to conductance values using the equation: $G = I/(V-V_R)$, where G = conductance,

I = current, V = voltage, and V_R = reversal potential (assumed as −90 mV; Shih & Goldin, 1997). Conductance values were normalized to the maximum conductance and fit with a two-state Boltzmann equation: $G = 1/[1 + \exp(-0.03937 \cdot z \cdot (V - V_{1/2}))]$, where z = slope factor and $V_{1/2}$ = voltage for half-maximal depolarization. The values for the slope (k) and half maximal concfuctance ($V_{1/2}$) were averaged, and standard deviations were calculated for data from 12 to 15 oocytes.

Cell Culture and Transfection

MDCK cells were grown and maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (GIBCO, BRL), 2 mM L-glutamine$_s$ 100 U/ml penicillin, and 100 μg/ml streptomycin (In Vitro) at 37°C in 5% CO_2. Plasmids were purified with the Qiagen plasmid purification kit according to the manufacturer's instructions. Subconfluent MDCK cells were transfected using Lipofectamine 2000^{TM} (Invitrogen, Life Technologies) according to the manufacturer's instructions. For transient expression, cells were used at different times after transfection.

Immunoblots and Immunoprecipitation

For immunoblots of total cell extracts, transfected cells were washed three times with ice-cold phosphate-buffered saline solution (PBS) and extracted during 30 minutes with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.2% SDS, 1% Triton X-100, 1% NP-40 and a protease inhibitor cocktail including 1 mM PMSF and 10 μg/ml each of antipain, pepstatin A, and leupeptin). After a 10-min spin in a microfuge, the supernatant was recuperated and proteins were quantified with the BCA protein assay reagent (Pierce). For immunoprecipitation, cell extracts were precleared by incubation for 1 hr with fixed anti-mouse IgG-coupled *Staphylococcus aureus* cells (Tachisorb, Calbiochem), and cleared supernatants were immunoprecipitated with mouse anti-HA (Santa-Cruz Biotechnology) for at least 16 hr end-over-end rotation at 4°C. Immunoprecipitates were washed 6 times, two with lysis buffer, two with high-salt buffer (in mM: 500 NaCl, 50 Tris-HCl, pH 7.5, 5 EDTA, 1% Triton X-100) and two with low salt buffer (50 NaCl, 50 Tris-HCl, pH 7.5, 5 EDTA, 1% Triton X-100). After boiling in a buffer containing 80 mM DTT, 5.6% SDS, 16% glycerol, 50 mM Tris, pH 6.8, and 0.008% bromophenol blue, samples from total extracts and immunoprecipitates were subjected to electrophoretic separation and transferred to PVDF membranes. Blots were blocked with TBS (added with 1% Tween and 5% nonfat milk) and probed with the appropriate antibody. All proteins were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech). Band density was measured as follows: ECL plaques were acquired with the Foto/eclipse digital system from Fotodyne Inc. Pixel analysis was performed using the Collage (version 3.0) software from Dynamics Corp. Results are expressed as means ± SE; n depicts the number of observations. Densitometric values were subjected to a Student's t-test analysis.

Immunofluorescence

Glass coverslips containing MDCK-transfected cells were rinsed twice with PBS, fixed with 4% paraformaldehide during 20 min, rinsed with PBS and incubated with 3% fetal bovine serum in PBS for 30 min. Cells were treated for 1.0 hr with biotin-coupled anti-HA antibody. Monolayers were then rinsed 3 times with PBS for 5 minutes each, incubated with an FITC-labeled streptavidin (ZYMED) for 30 min, rinsed as above, mounted in Fluorguard (Bio-Rad) and examined with a confocal microscope (MRC-600, Bio-Rad, Hercules, CA).

Domain-specific Biotinylation

Apical and basolateral plasma membrane proteins were biotinylated as described by Gottardi, Dunbar and Caplan (1995). Briefly, confluent monolayers of MDCK cells on filters (Transwell; Costar) were washed thrice in ice-cold CM-PBS. 500 μg/ml sulfo-NHS-SS-biotin (Pierce), diluted from a 200-mg/ml stock in DMSO, was added to either apical (0.5 ml) or basolateral (1.5 ml) chambers. Surfaces not receiving biotin were incubated in CM-PBS alone. Filters were incubated 45 min on ice. Fresh buffer and biotin was applied and filters were incubated another 45 min. Biotinylation reactions were quenched by washing cells in five changes of CM-PBS containing 100 mM glycine. Filters were excised from plastic collars and monolayers solubilized by incubation for 1 hr at 4°C in lysis buffer. After immunoprecipitation as described, bound proteins were released from Tachisorb beads by boiling in 50 μl of SDS-containing buffer (1.5% SDS, Tris-HCl 50 mM, NaCl 150 mM) and diluted in 100 μl of lysis buffer. Therefore, we obtained two fractions in the immunoprecipitate: the membrane one, which is biotinylated and will bind to the neutravidin beads, and the cytoplasmic one, contained in the supernatant after exposure to neutravidin. After centrifugation, biotinylated proteins in the supernatant were precipitated with 100 μl of agarose-coupled neutravidin (Pierce). After washing as indicated, neutravidin-associated proteins were released by warming to 80°C in sample buffer, electrophoretically separated by SDS-PAGE, and transferred to PVDF membranes. Blots were incubated with rabbit anti-HA (Zymed) and with HRP-labeled goat anti-mouse IgG and proteins detected with ECL. To estimate the possible contamination of samples with the cytoplasmic fraction of the transfected channel protein, the neutravidin binding from immunoprecipitates of an intracellular control, β-catenin, was used in every biotinylation assay, indicating no signal in the immunoblot and therefore no biotin labeling.

Cell Surface Delivery of Newly Synthesized ShIR Channels

Filter-grown MDCK monolayers expressing ShIR channels were incubated for 45 min at 37°C in methionine- and cysteine-free DMEM. Cells were then pulsed for 45 min with methionine- and cysteine-free DMEM containing 200 μCi/ml ^{35}S-labeled methionine-cysteine (Promix, Amersham-Pharmacia Biotech). Monolayers were then washed twice with DMEM and chased for different times at 37°C in DMEM containing a 10-fold excess of unlabeled methionine and cysteine. At a given chase time, cells were chilled with two washes in ice-cold PBS at 4°C and subjected to cell surface biotinylation as described. Proteins were analyzed on 10% SDS-polyacrylamide gels.

For surface labeling with anti-HA, MDCK cells transfected with the extracellular epitope version of the ShIR channel (ShIR2) construct were blocked on ice with 3% BSA in PBS during 45 minutes, after which anti-HA (1:75) was added and exposed to cells for 60 min. Cells were washed with blocking solution (4 times) and lysed. After centrifugation at 10,000 × g, supernatants were incubated overnight with anti-mouse IgG-coupled Tachisorb. Immunoprecipitates were treated for SDS-PAGE and immunodetection as previously described.

Reagents

Cell culture reagents and oligonucleotides were obtained from GIBCO Laboratories (Grand Island, NY). Enzymes for DNA digestion and modification were from New England Biolabs. GF109203X was obtained from Calbiochem. Tunicamycin, cytochalasin D, colchicine and neomycin were obtained from Sigma (St. Louis, MO). The mLin-7 anti-serum was a kind gift from

Dr. B. Margolis at the Department of Biological Chemistry, University of Michigan Medical School.

Results

To facilitate the study of polarity we resorted to a ShIR1, a non-inactivating version of the Shaker channel (Bezanilla & Armstrong, 1977; Hoshi et al., 1991) tagged with an HA epitope. Therefore, the first control was to inject the in vitro transcribed ShIR and ShIR1 mRNA into *Xenopus laevis* oocytes to test their electrical performance. Table 1 lists the values of activation properties (k and $V_{1/2}$), indicating that the tagged version is essentially indistinguishable from the untagged channel.

Expression of the ShIR Channel in MDCK Cells Is Polarized

In spite of belonging to different animal species, MDCK cells express the ShIR channels and its mutants (Fig. 2). Yet only a fraction of the expressed channels reaches the membrane, indicating that the protein must be delivered and inserted in the membrane through a rate-limiting mechanism. According to immunofluorescence, the ShIR2 channel is preferentially expressed on the basolateral side of the membrane (Fig. 3, *left*). Only a few cells express the channel at a given time and do it clearly; weak expression was seldom detected. The domain-specific expression of the channel was confirmed by culturing ShIR1-transfected MDCK cells on Transwell filters, and labeling the protein in the apical or in the basolateral domains with the domain-specific biotinylation procedure described in Methods (Fig. 4, *left*).

Table 1. ShIR channel activation parameters

	k	$V_{1/2}$ (mV)	n
ShIR	6.8 ± 0.89	−22.1 ± 3.11	12
ShIR1	7.7 ± 1.19	−21.6 ± 7.23	15

Normalized conductance data for individual recordings were fit with a two-state Boltzmann equation as described in Materials and Methods. ShIR1 depicts a modified ShIR channel with a non-apeptide HA epitope inserted between amino acids 2 and 3 of the channel protein.

The Expression of ShIR Is not Related to K^+ Permeability

Since epithelia form the barriers between higher organisms and the environment, they are exposed to large variations in the concentration of solutes. Yet most epithelial cells have the ability to adjust their specific permeability to a given molecular species in inverse proportion to its concentration (Cereijido et al., 1964; Contreras et al., 1992). On this basis one would expect that the cell would handle the ShIR channel that affects K^+-permeability in a different manner than one that does not influence this parameter. Accordingly, we tested channel W434F, a mutant of *Shaker*, in which a tryptophan at position 434 was replaced by a phenylalanine in ShIR. This mutation is located at the innermost end of the hydrophilic loop forming the pore of the channel, and abolishes its conductivity. Figure 4 (*right*) shows that the W434F1 mutation does not modify expression or polarity in the membrane.

The COOH-Terminal Domain of ShIR Channel is Responsible for the Basolateral Expression

Deletion of almost the whole COOH-terminal end, except for 6 residues located after the S6 transmem-

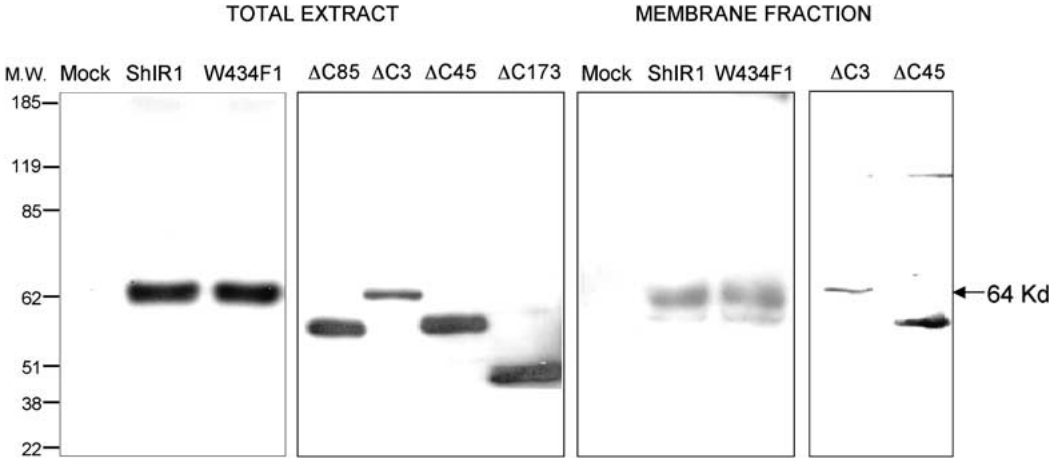

Fig. 2. Expression of transfected ShIR1 and W434F1 channels in MDCK cells. Both channels were tagged with HA epitope from influenza virus in the NH_2 terminus. Blots were probed with mouse anti-HA antibody. *Mock:* cells transfected with plasmid pcDNA3 without insert. First and second panels correspond to total cell extract, and third and fourth correspond to membrane-expressed fraction obtained with the biotinylation procedure described in Methods.

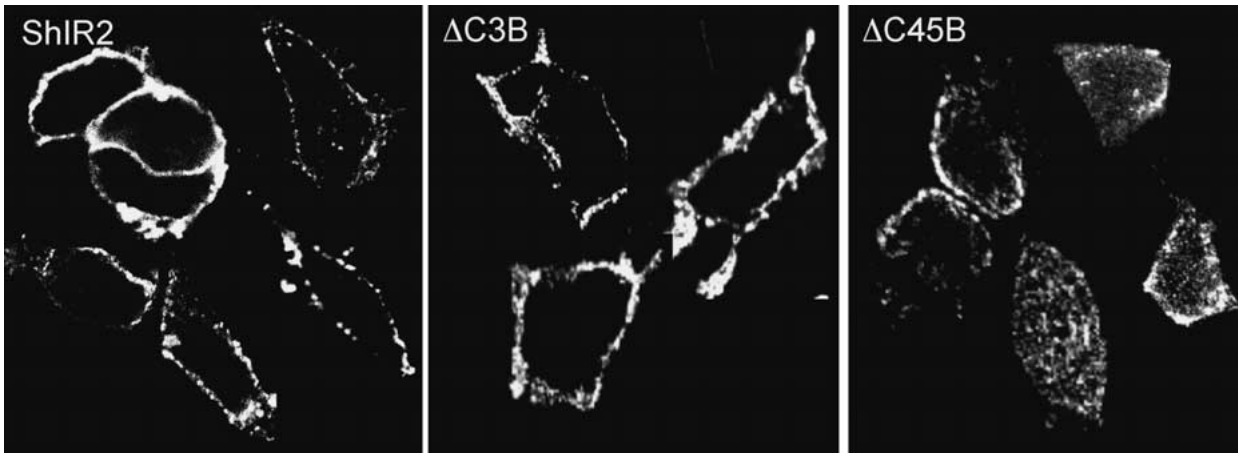

Fig. 3. Immunofluorescent images of MDCK cells expressing ShIR2 channels in the plasma membrane. Gallery of MDCK cells transfected with the indicated ShIR constructs (extracellular HA epitopes) containing ShIR channel protein on the lateral membrane domain, as observed with a biotinylated mouse anti-HA antibody, followed by an FITC-coupled streptavidin. *Left*: ShIR2; *center*: ΔC3B; *right*: ΔC45B.

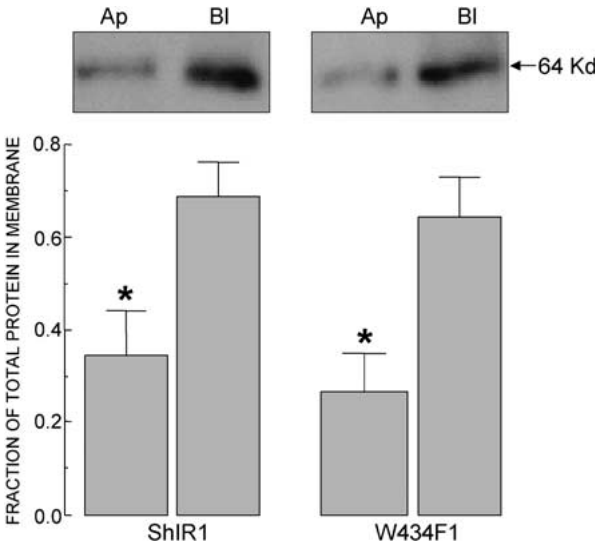

Fig. 4. Domain-specific expression of the transfected Shaker channels ShIR1 and W434F1. Confluent monolayers of MDCK cells were prepared in Transwell filters. Monolayers were treated with biotin on the apical (*Ap*) or on the basolateral (*Bl*) side. The channel was first precipitated with mouse anti-HA antibody. Immunocomplexes were dissociated by heating 5 min at 80°C in 1.5% SDS. Samples were then clarified with Neutravidin bound to agarose beads. Gels (8%) were transferred to PVDF membranes and blotted with rabbit anti-HA antibody and detected by ECL (*upper panel*). *Lower panel*: Band density was measured as described in Methods. Graphics show standard deviations. (*$p < 0.05$, $n = 4$).

brane segment (Fig. 5, ΔC173) does not prevent the membrane expression of the deletant ShIR1 channel, but causes a complete loss of polarity. The same phenomenon is observed with deletion of the last 85 (Fig. 5, ΔC85) and 45 amino acids (Fig. 3, *right* and Fig. 5, ΔC45). Yet, deletion of the last three amino acids of the COOH-terminal does not inhibit membrane delivery or basolateral polarity (Fig. 3, *center*; Fig. 5, ΔC3). Therefore, polarization depends on a domain localized between residues 571 and 613 of the COOH terminal.

Membrane Expression of ShIR Channel Depends also on Retention

The last four residues of the C-terminus (ETDV) of the Shaker (Kv1) channel subfamily constitute a PDZ binding domain contained in a set of membrane-retaining cortex proteins (Kim et al., 1995; Niethammer, Kim & Sheng, 1996; Garner, Nash & Huganir, 2000). The role of the last three residues of this sequence (TDV) in the residence time of the ShIR1 in the plasma membrane of MDCK cells was investigated in pulse-chase experiments, metabolically labeling the cells for 45 min with [^{35}S]methionine-cysteine and then chasing for different time periods, using cell surface biotinylation. This assay (Fig. 6) showed ShIR1 protein is rapidly transported and inserted into the plasma membrane. Yet deletion of TDV (ΔC3) markedly decreases its lifetime in the membrane (Fig. 6, *filled circles*). This result suggests that during its residence in the membrane surface, the ShIR1 channel associates with some stable structure. To investigate this possibility, we measured the fractions of channels that can be solubilized with or without the addition of 1.0% of Triton X-100 detergent. Figure 7 shows that virtually none of the ShIR1 or the ΔC3S deletant expressed at the membrane is free, but instead is bound to an insoluble component.

The Membrane Fraction of ShIR Is Associated with the Actin Cytoskeleton in MDCK Cells

The obvious candidate to retain the channel at the membrane as described in the previous section was

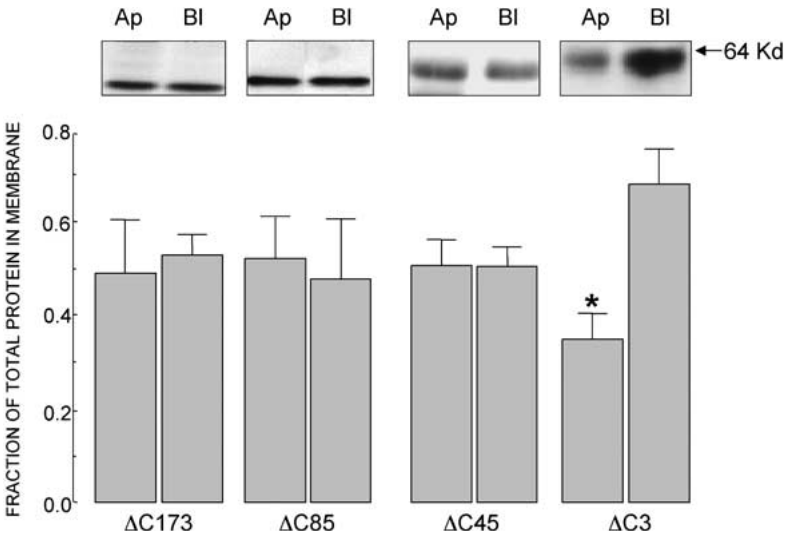

Fig. 5. Domain-specific expression of different deletants of the ShIR1 channel. Biotin was added to the apical (*Ap*) or to the basolateral (*Bl*) slide and cells were then processed as in Fig. 4. ΔC-nomenclature indicates the number of amino acids deleted on the COOH-end. Analysis was performed as indicated in Fig. 4. (*$p < 0.02$, $n = 5$).

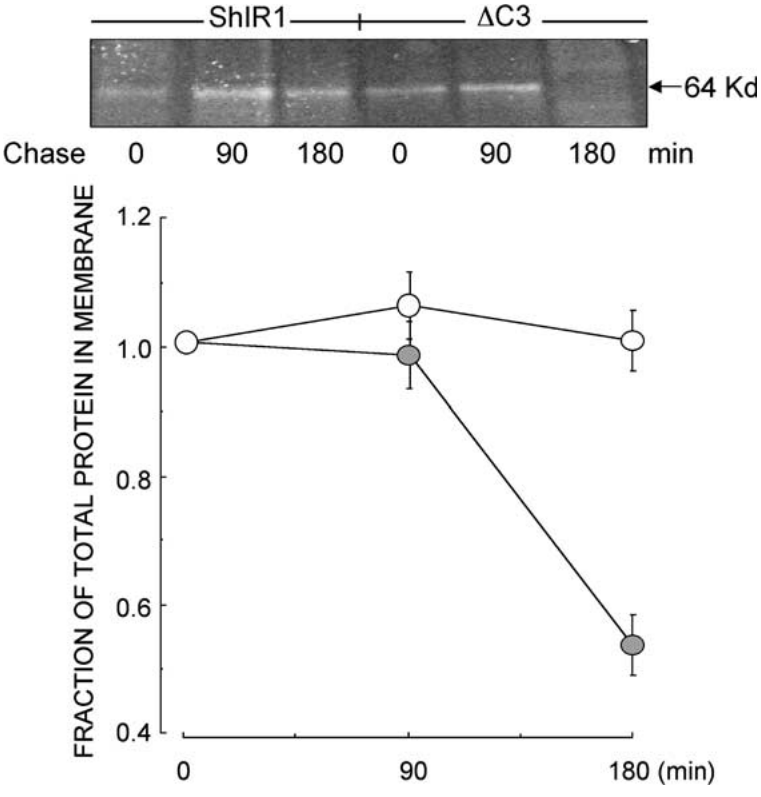

Fig. 6. Role of deletion of the last three amino acids (ΔC3) in the expression of ShIR1 channels. MDCK cells transfected with ShIR1 (*empty circles*) or with deletant ΔCS (*filled circles*) were pulsed with ^{35}S-methionine-cysteine for 45 min and biotinylated at the indicated times. Samples were processed as in Fig. 4.

the cytoskeleton. The observation that 12 μM cytochalasin significantly ($p < 0.01$, $n = 5$) decreases membrane expression of the channel (Fig. 8) demonstrates that the process requires the participation of microfilaments. Yet a sizable population of channels (~50%) still reaches the plasma membrane, indicating that some channels may reach the membrane through an independent way. Microtubules, in contrast, do not seem to be required, as 10 μM colchicine does not decrease the population of ShIR1 at the membrane (Fig. 8).

ShIR Channel May Participate in a Scaffold

To further analyze the association of the ShIR channels with the cytoskeleton, we used the extracellular epitope version of the channel ShIR2. We labeled those channels exposed at the membrane of MDCK cells transfected with pSHA2 plasmid with a biotinylated anti-HA antibody, then immunoprecipitated this fraction with Neutravidin, separated the peptides by SDS-PAGE and blotted the PVDF membrane-transferred proteins with a monoclonal antibody

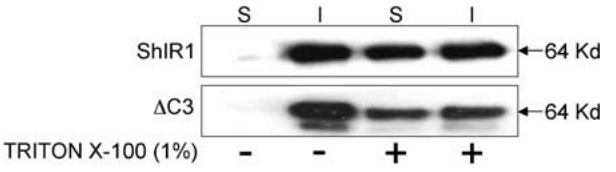

Fig. 7. Solubility of ShIR1 and ΔC3 channels. Transfected cells were lysed with a buffer containing no or 1% Triton X-100 for 10 min under vigorous stirring. Monolayers were then scraped and spun at 10,000 × *g* for 20 min. Soluble (*S*) and insoluble (*I*) fractions were adjusted to 200 μl, separated in SDS-PAGE and blotted as in Fig. 4.

against actin or α-actinin. Figure 9 reveals that these proteins were intimately associated with the channel, and that this association can be impaired by cytochalasin D. Figure 10 (*right*) suggests that actin and α-actinin, as well as β-tubulin, may be intimately assembling a scaffold with the ShIR2. Molecules such as mLin-7 and CASK are known to associate in a scaffold at the basolateral domain of epithelial cells (Borg et al., 1999; Straight et al., 2000). Figure 10 shows an intimate association between these molecules and ShIR channel. The association between ShIR2 and the different molecular species detected form a scaffold, including a cytoskeleton-linking molecule not yet described, possibly Mint-1 (Straight et al., 2000). The ΔC3B deletant is associated with all the described protein, but its co-immunoprecipitation ability is reduced almost to 50%. Blots on the *left* in Fig. 10 show that the surface anti-HA labeling procedure does not bind any of the listed molecules nonspecifically.

Glycosylation is Required for Delivery of ShIR to the Surface in MDCK Cells

Shaker K^+ channel protein is glycosylated on two asparagine residues, N (asparagine) 259 and N 263, in the first extracellular loop (Santacruz-Toloza et al., 1994). It was recently demonstrated that glycosylation dramatically increases stability and cell surface expression, without significantly affecting folding and assembly of the protein (Khanna et al. 2001). To study the role of glycosylation in the membrane expression of ShIR1 channels, we treated MDCK-transfected cells with tunicamycin, which prevents N-glycosylation by inhibiting the sialyltransferase of the endoplasmic reticulum (Duskin & Mahoney, 1982). MDCK cells treated with 12 μg/ml tunicamycin show that this drug does affect the channel in two aspects. The first is, of course, a decrease of molecular weight

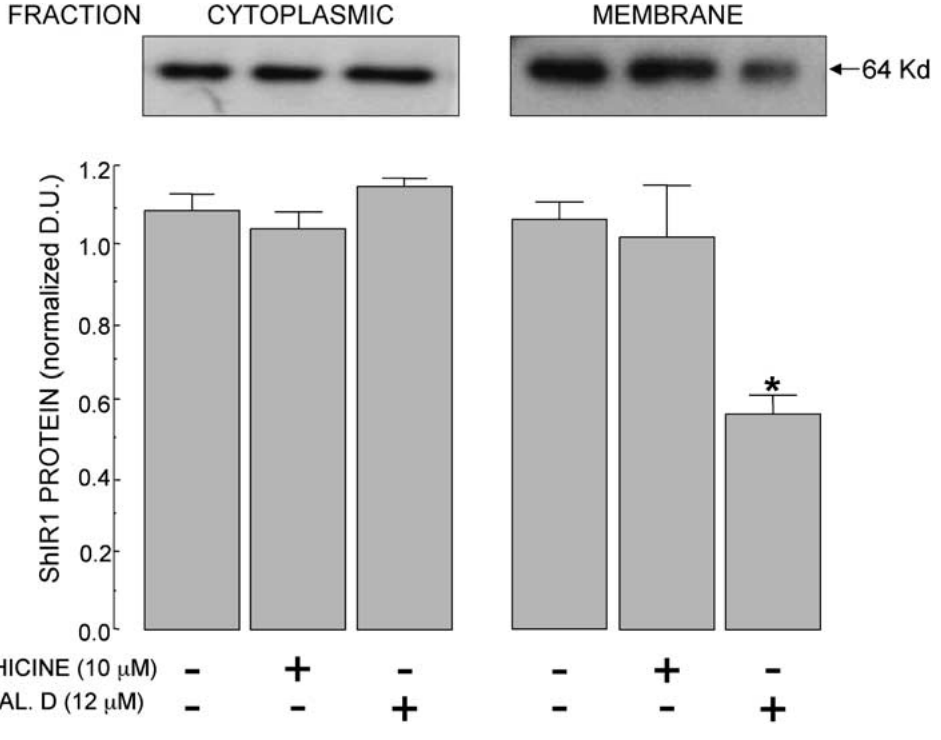

Fig. 8. Role of the cytoskeleton in the expression of ShIR1 channels. 6 hr after transfection, cells were trypsinized and plated in the presence of 10 μM colchicine (columns *2* and *5*) or 12 μM cytochalasin D (columns *3* and *6*) for 6 hr. Monolayers were then processed for biotinylation and blotted as in Fig. 4. Left and right groups of columns correspond to channels in the cytoplasm and the plasma membrane, respectively. Histograms show the densitometric values normalized to the mean calculated for the control. Analysis was performed as indicated in Fig. 4. (*$p < 0.01$, $n = 5$).

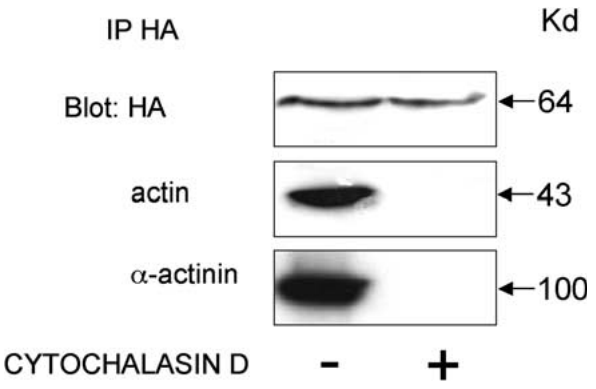

Fig. 9. Effect of 12 μM cytochalasin D on cytoskeleton-associated molecules (actin and α-actinin) co-immunoprecipitated with membrane-expressed ShIR2 channels. ShIR2-expressing MDCK cells were treated with biotinylated antibody against the HA epitope. Monolayers were then lysed and scraped, samples were precipitated with Neutravidin, separated in SDS-PAGE and blotted with the specified antibodies.

due to the elimination of glycosides (Fig. 11), and the second is a slight, yet significant ($p < 0.005$) decrease in the population of the channel in the membrane. Taken together, results in Fig. 11 reveal that glycosylation is required for membrane delivery, and may also affect the association— and thereby retention —at this position.

The Asymmetric Distribution of ShIR Is Part of an Overall Process of Polarization

Polarity is one of the two main features of the epithelial transporting phenotype, the other being the assembly and sealing of tight junctions (TJs) (Cereijido et al., 2000). Expression of both features depends on the presence of extracellular calcium, which acts on the extracellular domain of E-cadherin at cell-cell contacts (González-Mariscal et al., 1990; Contreras et al., 1992) that trigger a series of events involving at least two different G proteins, phospholipase C (PLC), protein kinase C (PKC), and calmodulin (Balda et al., 1991). This results in a polarized expression of Na^+, K^+-ATPase (Contreras et al., 1992) and K^+ channels (Talavera et al., 1995), as well as the establishment of TJs (González-Mariscal et al., 1990). Figures 12 and 13 show that the same mechanism operates in the expression of ShIR1 channels in the plasma membrane. Thus this expression depends on Ca^{2+} and cell-cell contacts (Fig. 12), and the inhibition produced by 100 μM Neomycin or 5 μM GF109203X (Fig. 13) indicates that it also relies

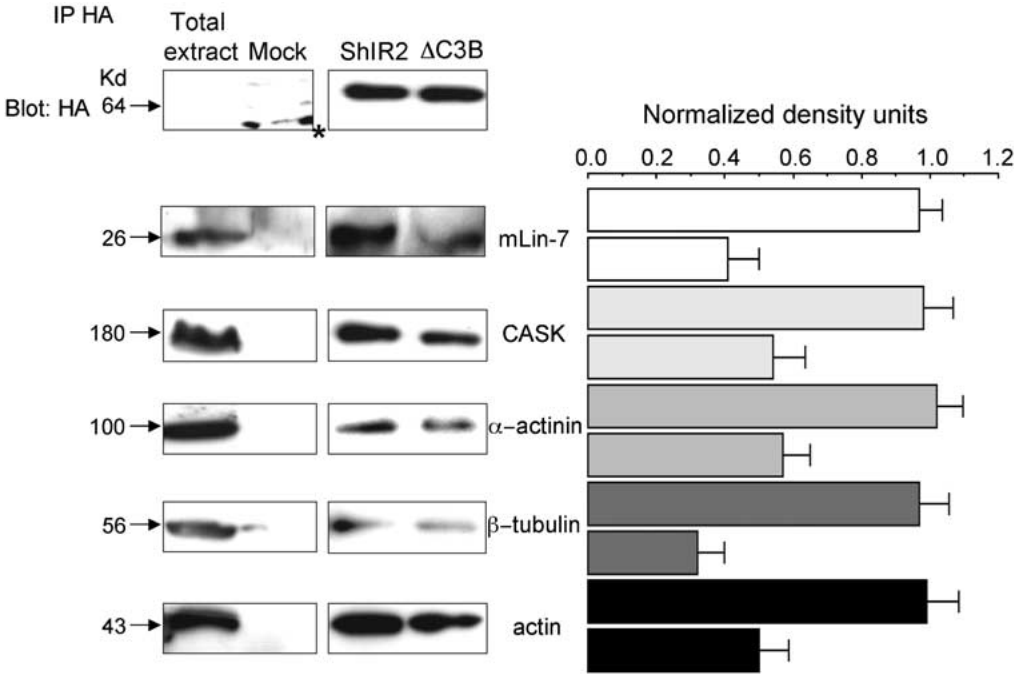

Fig. 10. Effect of deletion of the last three amino acids (TDV) of the COOH terminus (ΔC3B) on the association of ShIR2 channels to several protein species. Cells were processed as in Fig. 9, and membranes were blotted with the specified antibodies. The right column of immunoblots illustrates individual immunoprecipitations, and the histogram, averages of 3 measurements. Each pair of columns represents the average for Shir2 and ΔC3B, respectively. Immunoblots in the left column correspond to total cell extracts and to immunoprecipitations from mock-transfected cells, i.e., transfected with empty pcDNA3 plasmid. Asterisk indicates the IgG.

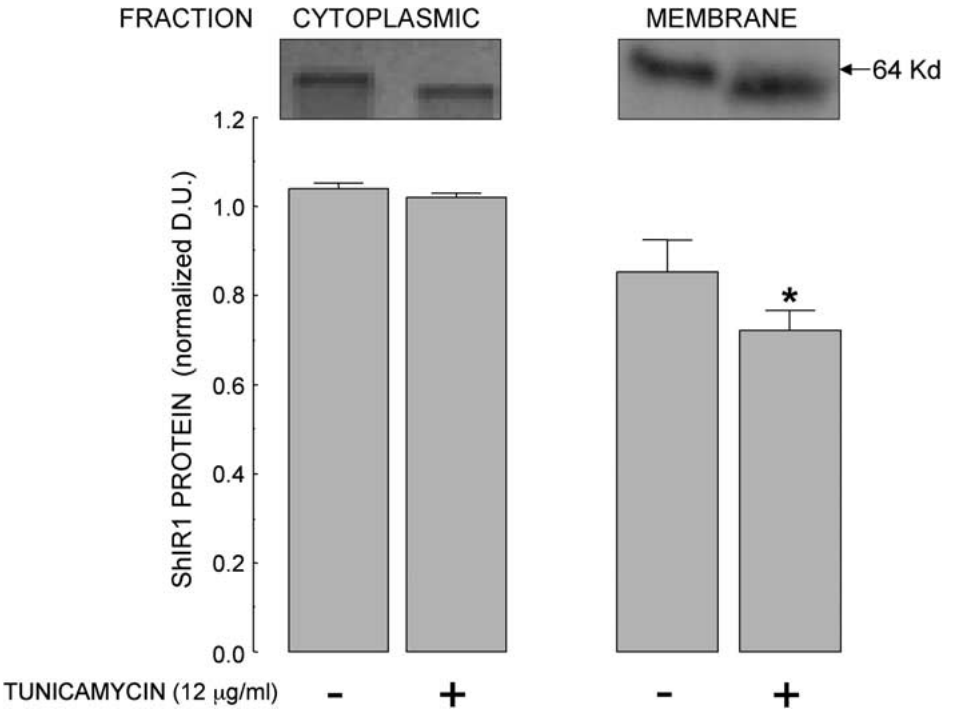

Fig. 11. Role of glycosylation in the expression of ShIR1 channels. 6 hr after transfection, cells were trypsinized and plated in the presence of 12 μg/ml tunicamycin. 12 hr later, cells were treated as in Fig. 8. Normalization was carried out as in Fig. 8 and analysis performed as indicated in Fig. 4. (*$p < 0.05$, $n = 3$).

on a cascade involving the activation of PLC and PKC signaling pathways.

Discussion

Polarity is one of the two basic characteristics of the epithelial transporting phenotype, the other being the establishment of tight junctions. This ability to express different transporting mechanisms in the two poles of the cell is crucial in several ways: (*a*) While the basolateral domain is mainly involved in house keeping, and therefore its properties are similar in different epithelia of the same animal species, the apical one depends on the physiological role of the cell type (e.g., a secreting or an absorbing cell). (*b*) The two poles face entirely different milieus: while the basolateral side is always in contact with interstitial fluid, the apical domain may be in contact with gastric juice, concentrated urine, sea or fresh water, etc. (*c*) While the inner milieu is remarkably constant, the outer environment is subject to drastic variations. Thus, a cell of the collecting tubule of the kidney of a dehydrated dog is bathed by a hypertonic solution that changes swiftly and drastically its tonicity upon hydration of the animal. (*d*) The expression of some proteins depends on the functional status of the organ. Thus, K^+ channel Kv4.3 is greatly diminished during pregnancy (Song et al., 2001). (*e*) The expression may also change with age, as it is the case with some K^+ channels in coronary smooth muscle (Marijic et al., 2001).

The mechanism underlying polarity depends on the capability of the cell to deliver and retain the peptides in a domain-specific manner. In keeping with the sundry scenarios described in the previous paragraph, the information available shows that the polarized expression of a protein does not depend on a couple of universal signals, one for the apical and another for the basolateral domain, but that there is a variety of signals and retention mechanisms that will take a considerable experimental effort to systematize in a conceptual framework. The present article is part of such an effort.

MDCK cells express at least 6 types of endogenous voltage-dependent channels, all of them in polarized fashion, but this polarization depends on the type of channel (Bolivar & Cereijido, 1987; Ponce et al., 1991a,b: Ponce & Cereijido, 1991). Actually, MDCK cells also polarize other types of membrane mechanisms, such as the Na^+, K^+-ATPase (Contreras et al., 1989), as well as microvilli, intramembrane particles, receptors, cell junctions, etc. (Cereijido et al., 1980). Yet, ion channels appear as suitable candidates for polarity markers, because experimental methods have reached the ultimate level of detection: patch clamp can individualize, characterize and tell apart a single channel. Furthermore, since preliminary results indicated that MDCK cells can also handle transfected channels

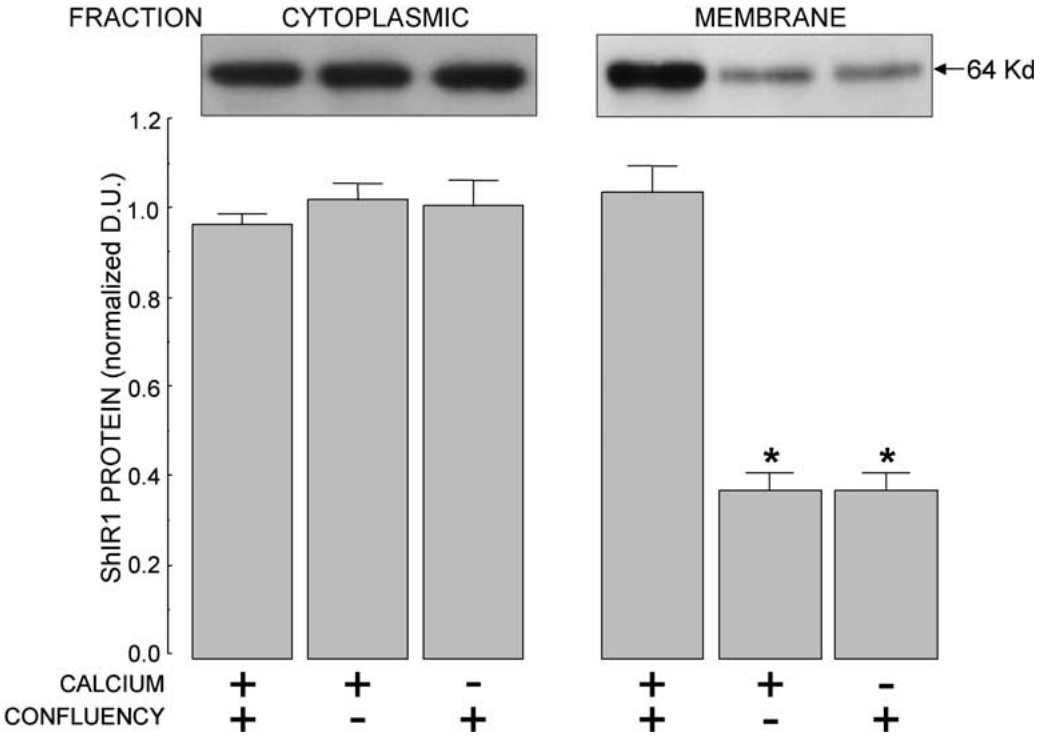

Fig. 12. Effect of Ca^{2+}-activated cell-cell contacts on the expression of ShIR1 channels in the surface of MDCK cells. 6 hr after transfection, cells were trypsinized and plated at confluence or at low density, in medium with or without 1.8 mM $CaCl_2$. 20 hr later, cells were biotinylated, and treated as in Fig. 8. Left and right groups of columns correspond to channels in the cytoplasm and the plasma membrane, respectively. Normalization was carried out as in Fig. 8 and analysis performed as indicated in Fig. 4. (*$p < 0.001$, $n = 3$).

from other animal species, and transfected channels offer the experimental advantage of allowing the use of mutants, deletants, epitope-tagged versions, etc., we resorted to ShIR and the other versions discussed above. Therefore, our first efforts were to show that MDCK cells do express ShIR, and do it in polarized fashion. The use of channels offers yet another advantage: a given property affected by deletions or mutations of the segment responsible for this property is not necessarily affected by modifications of molecular segments that fulfill other functions. Thus, Perozo et al. (1993) have shown that mutation in the putative pore region (W434F) completely abolishes ion conduction without affecting either kinetic or steady-state properties. In keeping with those observations, we observe that the W434F mutant does not affect polarity (Fig. 4).

Polarizing signals are not universal not only for all proteins of a given domain, but they change depending on the type of K^+ channel. Thus, the determinant controlling surface expression of Kv1 channels is localized to the highly conserved pore region (Manganas et al., 2001). CCD-IRK3 (mKir2.3) channel cloned from a cortical collecting duct is expressed on the basolateral membrane of MDCK cells (Le Maout et al., 1997) thanks to a signal comprising residues 431 to 442 (Le Maout et al., 2001). Fusion of its COOH-terminal structure onto CD4 is sufficient to express this chimera in the basolateral membrane (Le Maout et al., 2001). Accordingly, targeting of a given isoform of Kv3.2 channel depends on alternative splicing (Ponce et al., 1997). The segment of amino acids responsible for polarization may not always work as when incorporated in a chimeric protein. Such is the case of amino acids 536 to 666 of Kv2.1 channel, whose incorporation in an HA chimera does not restore the basolateral distribution displayed by the whole channel peptide despite the fact that its elimination alters its basolateral expression in MDCK cells (Scannevin et al., 1996). This suggests that the complete signal may be constituted by the tertiary structure of the channel oligomer rather than the primary sequence (Lim et al., 2000).

While the extent of ShIR channels expression is high enough to perform studies by immunoblotting, biotinylation, and immunostaining with florescent dyes, at any given time, the channel-expressing MDCK cells amount to only some 5–6%. Similar figures are usually interpreted as "weak" expression. Yet, as illustrated in Fig. 3, for a given cell, ShIR2 expression in by no means weak. "Weakness" seems to arise from the fact that, at a given moment, expression in some 95% of the cells is not detectable.

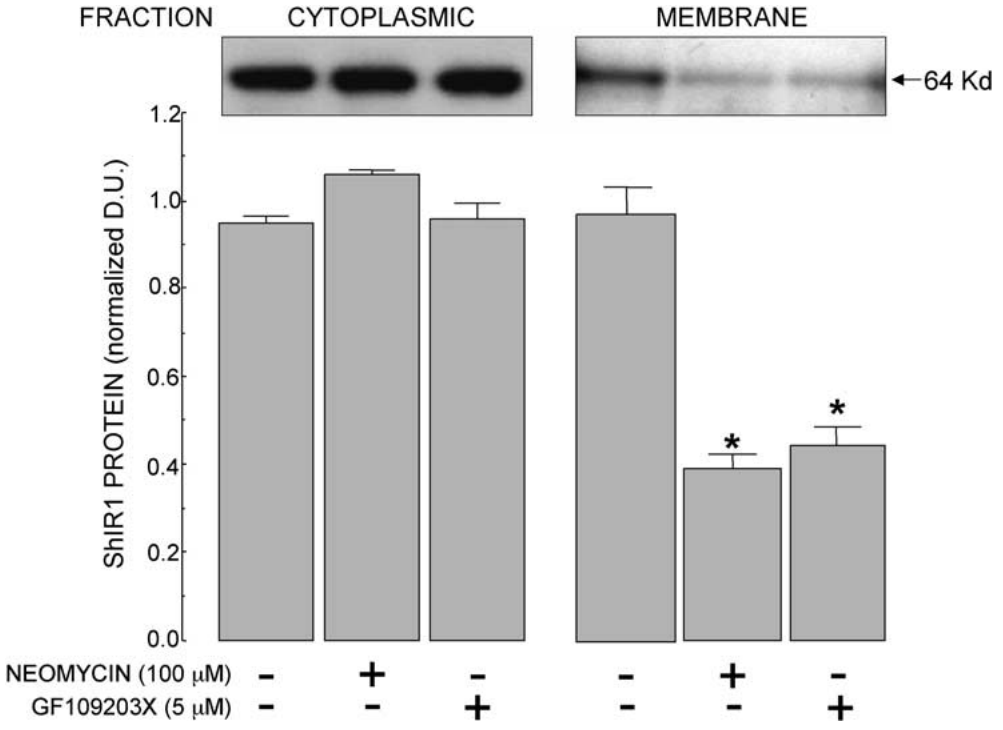

Fig. 13. Role of PLC and PKC in the expression of ShIR1 channels. Cells were transfected, trypsinized and replated as in Fig. 8, except that during the last incubation, they were treated with 100 μM neomycin or 5.0 μM GF109203X to inhibit PLC and PKC, respectively. Six hours later, cells were biotinylated and processed as in Fig. 4. Normalization was carried out as in Fig. 8 and analysis performed as indicated in Fig. 4. (*$p < 0.001$, $n = 4$).

Therefore, expression appears as an all-or-nothing phenomenon. This cannot be attributed to cell heterogeneity, as the MDCK batch of cells that we use has been cloned upon arrival to our laboratory.

Our results show that the expression of Shaker channels in MDCK cells depends on cell-cell contacts activated by Ca^{2+}, a cascade of reactions mediated by PLC and PKC, the cytoskeleton, and glycosylation. The signal that determines the basolateral expression appears to be located at the COOH-terminal, from the aminoacids 571 to 613. The putative sorting signal identified in ShIR does not share any resemblance to any known sorting signal described for basolateral proteins (Mostov, 1993; Aroeti & Mostov, 1994; Matter & Mellman, 1994; Matter, 2000). However, it is noteworthy that the ShIR sorting determinant is located on the intracellular cytoplasmic COOH-terminal portion of the channel protein, the same domain described to contain the sorting determinant of other voltage-dependent potassium channels (Le Maout et al., 1996; Scannevin et al., 1996; Le Maout et al., 2001). Yet the position of the Shaker in the membrane is not the only characteristic that conditions its fate in this location, because the deletion of the last three amino acids at the COOH-end clearly alters its residence time, suggesting that its membrane stability is enhanced by an association with subcortical proteins. Such is the case for numerous K^+ channels (Burke et al., 1999; Jugloff et al., 2000).

This result is in keeping with experiments of co-immunoprecipitation with actin, α-actinin, and β-tubulin, showing that ShIR2 probably forms a scaffold with these molecular species (Figs. 8 and 9). We found that mLin-7, CASK, tubulin, α-actinin and actin interaction is only partially influenced by the elimination of the last three amino acids in the COOH-terminus, since there is only half the amount of this proteins co-immunoprecipitating with the ΔC3 mutant. P.A. Welling and colleges have described a similar case for Kir2.3 channels, since the substitution of the entire PDZ binding domain by alanines attenuates to about one-fourth of control levels the association ability of the mutant channels (Le Maout et al., 2001; Olsen et al., 2002). mLin-7 also associates with receptors such as the epithelial γ-aminobutyric acid (GABA) transporter BGT-1 (Perego et al., 1999) and indirectly to the N-type Ca^{2+} channel through its association with Mint-1 (Maximov, Sudhof & Bezprozvanny, 1999) among other proteins. The fact that deletion of the PDZ domain-binding motif (ΔC3B) does not abolish the association between mLin-7 and the channel may be explained by a putative indirect interaction of the ShIR with a molecule also bound to the heterotrimer mLin-7, CASK and Mint-1. This, of course, implies a binding domain of the channel

protein different from the PDZ binding motif. These associations would enable voltage-gated potassium channels from the Shaker subfamily to participate in such supra-molecular structures, and thereby play an important role in signal transduction in epithelial cells.

We also observe that the polarized distribution of Shaker channel is not affected by abolishing its conductance through mutation W434F. This was somewhat unexpected because, in keeping with the diversity of conditions met by epithelial cells that we summarized above, many epithelial cell types have the ability to adjust their specific permeabilities by adding or subtracting translocating mechanisms to their plasma membrane (Cereijido et al., 1973, 1996; Contreras et al., 1992).

We thank Dr. Gerardo Contreras, Catalina Flores, Rosana Florentine, Alma Monroy and María Luisa Roldán for their efficient and pleasant help and Elizabeth del Oso for secretarial assistance. We also acknowledge the economic support of the National Research Council of Mexico (CONACYT) and of the Deutsche Forschungs Gemeinschaft via Dr. Olaf Pongs (Zentrum für Molekulare Neurobiologie der Universität Hamburg). J.M. was recipient of a doctoral fellowship from CONACYT.

References

Aroeti, B., Mostov, K.E. 1994. Polarized sorting of the polymeric immunoglobulin receptor in the exocytotic and endocytotic pathways is controlled by the same amino acids. *EMBO J.* **13:**2297–2304

Balda, M.S., Gonzalez-Mariscal, L., Contreras, R.G., Macias-Silva, M., Torres-Marquez, M.E., Garcia-Sainz, J.A., Cereijido, M. 1991. Assembly and sealing of tight junctions: Possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J. Membrane Biol.* **122:**193–202

Bezanilla, F., Armstrong, C.M. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* **70:**549–566

Bolívar, J.J., Cereijido, M. 1987. Voltage and Ca^{2+}-activated K^+ channel in cultured epithelial cells (MDCK). *J. Membrane Biol.* **97:**43–51

Borg, J.P., Lopez-Figueroa, M.O., de Taddeo-Borg, M., Kroon, D.E., Turner, R.S., Watson, S.J., Margolis, B. 1999. Molecular analysis of the X11-mLin-2/CASK complex in brain. *J. Neurosci.* **19:**1307–1316

Burke, N.A., Takimoto, K., Li, D., Han, W., Watkins, S.C., Levitan, E.S. 1999. Distinct structural requirements for clustering and immobilization of K^+ channels by PSD-95. *J. Gen. Physiol.* **113:**71–80

Cereijido, M, Anderson, J.M. 2001. Tight Junctions. CRC Press, Boca Raton, FK

Cereijido, M, Contreras, R.G., García-Villegas, M.R., González-Mariscal, L., Valdés, J. 1996. Epithelial Polarity. *In:* Epithelial Transport: a guide to methods and experimental analysis. N.K. Wills, L. Reuss, S.A. Lewis editors), pp. 49–69. Chapman & Hall, London

Cereijido, M., Ehrenfeld, J., Meza, I., Martinez-Palomo, A. 1980. Structural and functional membrane polarity in cultured monolayers of MDCK cells. *J. Membrane Biol.* **52:**147–159

Cereijido, M., Herrera, F.C., Flanigan, W.J., Curran, P.F. 1964. The influence of Na concentration on Na transport across frog skin. *J. Gen. Physiol.* **47:**879–893

Cereijido, M., Moreno, J.H., Reisin, L, Boulan, E.R., Rotunno, C.A., Zylber, E.A. 1973. On the mechanism of sodium movement across epithelia. *Ann. N. Y. Acad. Sci.* **204:**310–324

Cereijido, M., Shoshani, L., Contreras, R.G. 2000. Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. *Am. J. Physiol.* **279:**G477–G482

Contreras, R.G., Avila, G., Gutierrez, C, Bolivar, J.J., Gonzalez-Mariscal, L., Darzon, A., Beaty, G., Rodriguez-Boulan, E., Cereijido, M. 1989. Repolarization of Na^+-K^+ during establishment of epithelial monolayers. *Am. J. Physiol.* **257:**C896–C905

Contreras, R.G., Miller, J.H., Zamora, M., González-Mariscal, L., Cereijido, M. 1992. Interaction of calcium with plasma membrane of epithelial (MDCK) cells during junction formation. *Am. J. Physiol.* **263:**C313–C318

Duksin, D., Mahoney, W.C. 1982. Relationship of the structure and biological activity of the natural homologues of tunicamycin. *J. Biol. Chem.* **257:**3105–3109

Garcia-Villegas, M.R., Valdes, J., Reyes, G., Moreno, J., Cortes, N., Contreras, R.G., Cereijido, M. 1996. Morphogenesis of the epithelial cell transporting phenotype: synthesis and distribution of ion channels. *Braz. J. Med. Biol. Res.* **29:**581–587

Garner, C.C., Nash, J., Huganir, 2000. PDZ domains in synapse assembly and signalling. *Trends Cell Biol.* **10:**274–280

González-Mariscal, L., Contreras, R.G., Bolivar, J.J., Chavez-de-Ramirez, B., Cereijido, M. 1990. The role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* **259:**C978–C986

Gottardi, C.J., Dunbar, L.A., Caplan, M.J. 1995. Biotinylation and assessment of membrane polarity: caveats and methodological concerns. *Am. J. Physiol.* **268:**F285–F295

Hoshi, T., Zagotta, W.N., Aldrich, R.W. 1991. Two types of inactivation in *Shaker* K channels: effects of alterations in the carboxyl-terminal region. *Neuron* **7:**546–556

Jugloff, D.G., Khanna, R., Schlichter, L.C., Jones, O.T. 2000. Internalization of the Kv1.4 potassium channel is suppressed by clustering interactions with PSD-95. *J. Biol. Chem.* **275:**1357–1364

Khanna, R., Myers, M.P., Laine, M., Papazian, D.M. 2001. Glycosylation increases potassium channel stability and surface expression in mammalian cells. *J. Biol. Chem.* **276:**34028–34034

Kim, E., Niethammer, M., Rothschild, A., Jan, Y.N., Sheng, M. 1995. Clustering of Shaker-type K^+ channels by interaction with a family of membrane-associated guanylate kinases. *Nature* **378:**85–88

Le Maout, S., Brejon, M., Olsen, O., Merot, J., Welling, P.A. 1997. Basolateral membrane targeting of a renal-epithelial inwardly rectifying potassium channel from the cortical collecting duct, CCD-IRK3, in MDCK cells. *Proc. Natl. Acad. Sci. USA* **94:**13329–13334

Le Maout, S., Sewing, S., Coudrier, E., Elalouf, J.M., Pongs, O., Merot, J. 1996, Polarized targeting of a *Shaker*-like (A-type) K(+)-channel in the polarized epithelial cell line MDCK. *Mol. Membr. Biol.* **13:**143–147

Le Maout, S., Welling, P.A., Brejon, M., Olsen, O., Merot, J. 2001. Basolateral membrane expression of a K^+ channel, Kir 2.3, is directed by a cytoplasmic COOH-terminal domain. *Proc. Natl. Acad. Sci. USA* **98:**10475–10480

Lim, S.T., Antonucci, D.E., Scannevin, R.H., Trimmer, J.S. 2000. A novel targeting signal for proximal clustering of the Kv2.1 K^+ channel in hippocampal neurons. *Neuron* **25:**385–397

Lisanti, M.P., Caras, I.W., Davitz, M.A., Rodriguez-Boulan, E. 1989. A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelialcells. *J. Cell Biol.* **109:**2145–2156

Manganas, L.N., Wang, Q., Scannevin, R.H., Antonucci, D.E., Rhodes, K.J., Trimmer, J.S. 2001. Identification of a trafficking determinant localized to the Kv1 potassium channel pore. *Proc. Natl. Acad. Sci. USA* **98:**14055–14059

Marijic, J., Li, Q., Song, M., Nishimaru, K, Stefani, E., Toro, L. 2001. Decreased expression of voltage- and Ca(2+)-activated K(+) channels in coronary smooth muscle during aging. *Circ. Res.* **88:**210–216

Matter, K. 2000. Epithelial polarity: sorting out the sorters. *Curr. Biol.* **10:**R39–R42

Matter, K, Mellman, I. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* **6:**545–554

Maximov, A., Sudhof, T.C., Bezprozvanny, I. 1999. Association of neuronal calcium channels with modular adaptor proteins. *J. Biol. Chem.* **274:**24453–24456

Miledi, R., Parker, I., Sumikawa, K. 1983. Recording of single gamma-aminobutyrate- and acetylcholine-activated receptor channels translated by exogenous mRNA in *Xenopus* oocyte. *Proc. R. Soc. Lond. B. Biol. Sci.* **218:**481–484

Moreno, J., Monroy, A.O., Fiorentino, R., García-Villegas, M.R., Cereijido, M. 1999. Polarized expression of Shaker K^+ channels in MDCK cells. *Mol. Biol. Cell* **10**(suppl.):519

Mostov, K. 1993. Protein traffic in polarized epithelial cells: the polymeric immunoglobulin receptor as a model system. *J. Cell Sci. Suppl.* **17:**21–26

Niethammer, M., Kim, E., Sheng, M. 1996. Interaction between the C terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J. Neurosci.* **16:**2157–2163

Olsen, O., Liu, H., Wade, J.B., Merot, J., Welling, P.A. 2002. Basolateral membrane expression of the Kir 2.3 channel is coordinated by PDZ interaction with Lin-7/CASK complex. *Am. J. Physiol.* **282:**C183–C195

Perego, C., Vanoni, C., Villa, A., Longhi, R, Kaech, S.M., Frohli, E., Hajnal, A., Kim, S.K., Pietrini, G. 1999. PDZ-mediated interactions retain the epithelial GABA transporter on the basolateral surface of polarized epithelial cells. *EMBO J.* **18:**2384–2393

Perozo, E., MacKinnon, R, Bezanilla, F., Stefani, E. 1993. Gating currents from a nonconducting mutant reveal open-closed conformations in Shaker K^+ channels. *Neuron* **11:**353–358

Ponce, A., Bolívar, J.J., Vega, J., Cereijido, M. 1991a. Synthesis of plasma membrane and potassium channels in epithelial (MDCK) cells. *Cell Physiol. Biochem.* **1:**195–204

Ponce, A., Cereijido, M. 1991. Polarized distribution of cation channels in epithelial cells. *Cell Physiol. Biochem.* **1:**13–23

Ponce, A., Contreras, R.G., Cereijido, M. 1991b. Polarized distribution of chloride channels in epithelial cells. *Cell Physiol. Biochem.* **1:**160–169

Ponce, A., Vega-Saenz de Miera, E., Kentros, C., Moreno, R., Thornhill, B., Rudy, B. 1997. K^+ channel subunit isoforms with divergent carboxy-terminal sequences carry distinct membrane targeting signals. *J. Membrane Biol.* **159:**149–159

Rodriguez-Boulan, E. 1983. Polarized assembly of enveloped viruses from cultured epithelial cells. *Methods Enzymol.* **98:**486–501

Rodriguez-Boulan, E., Paskiet, K.T., Salas, P.J., Bard, E. 1984. Intracellular transport of influenza virus hemagglutinin to the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* **98:**308–319

Sambrook, J., Russel, D.W. 2001. *Molecular Cloning: Laboratory Manuals.* Cold Spring Harbor Lab. Press.

Santacruz-Toloza, L., Huang, Y, John, S.A., Papazian, D.M. 1994. Glycosylation of shaker potassium channel protein in insect cell culture and in Xenopus oocytes. *Biochemistry* **33:**5607–5613

Scannevin, R.H., Murakoshi, H., Rhodes, K.J., Trimmer, J.S. 1996. Identification of a cytoplasmic domain important in the polarized expression and clustering of the Kv2.1 K^+ channel. *J. Cell Biol.* **135:**1619–1632

Schwartz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N., Jan, L.Y. 1988. Multiple potassium-channel components are produced by alternative splicing at the Shaker locus in Drosophila. Nature **331:**137–142

Shih, T.M., Goldin, A.L. 1997. Topology of the Shaker potassium channel probed with hydrophilic epitope insertions. *J. Cell Biol.* **136:**1037–1045

Song, M., Helguera, G., Eghbali, M., Zhu, N., Zarei, M.M., Olcese, R., Toro, L., Stefani, E. 2001. Remodeling of Kv4.3 potassium channel gene expression under the control of sex hormones. *J. Biol. Chem.* **276:**31883–31890

Stefani, E., Cereijido, M. 1983. Electrical properties of cultured epithelioid cells (MDCK). *J. Membrane Biol.* **73:**177–184

Straight, S.W., Karnak, D., Borg, J.P., Kamberov, E., Dare, H., Margolis, B., Wade, J.B. 2000. mLin-7 is localized to the basolateral surface of renal epithelia via its NH(2) terminus. *Am. J. Physiol.* **278:**F464–F475

Talavera, D., Ponce, A., Fiorentino, R., González-Mariscal, L., Contreras, R.G., Sánchez, S.H., García-Villegas, M.R., Valdés, J., Cereijido, M. 1995. Expression of potassium channels in epithelial cells depends on calcium activated cell-cell contacts. *J. Membrane Biol.* **143:**219–226