Erratum

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Assessment of heterologous membrane protein polarity in transiently transfected MDCK cells

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Abstract

We have evaluated transient transfection of MDCK cells by the DEAE-dextran/chloroquine method as a rapid method for study of heterologous plasma membrane protein polarity. Transiently transfected cells reseeded onto permeable supports formed confluent monolayers with normal tight junctions and normal distribution of endogenous apical and basolateral surface markers. Transfected monolayers reseeded onto opaque polycarbonate filters attained cell heights 3 times greater than on transparent filters. Conventional and confocal immunofluorescence microscopy were used to assess polarity of transient expression of heterologous proteins previously defined in stably transfected cell lines as apical (DAF-CD55), basolateral (VSV-G), and nonpolarized (CD7) in distribution. Though each transiently expressed protein exhibited a polarity phenotype in most cells which resembled the stable phenotype, consistency of polarized localization was less than in stably transfected cells. Similar results were obtained by lipofection. We conclude that transient transfection of MDCK cells may be useful as a rapid screen, but is not sufficiently reliable for definitive assessment of heterologous membrane protein polarity.

Abbreviations: CD55-DAF – CD55-Decay-accelerating factor; DMSO – Dimethylsulfoxide; FBS – Fetal bovine serum; FITC – Fluorescein isothiocyanate; MDCK – Madin Darby canine kidney cells; PBS – Phosphate-buffered saline; TER – Transepithelial resistance; VSVG – Vesicular stomatis virus G protein.

Introduction

In order to generate and maintain distinct organelles, the cell must sort specific membrane and soluble proteins to their proper destinations with a high degree of fidelity. The search for targetting signals in the linear sequences of membrane proteins has been rewarding for those proteins which reside in intracellular organelles. Consensus sorting signals which are sufficient for proper targetting have been discovered for nucleus, mitochondrion, endoplasmic reticulum, lysosome and peroxisome. Only recently have apical and basolateral plasmalemmal sorting signals been elucidated (Rodriquez-Boulan and Powell, 1992), but among them no consensus sequences have emerged.

The process of experimentally defining a plasmalemmal sorting signal routinely requires the creation of a series of deletion, substitution, and point mutations in the cDNA encoding the protein under study, followed by the creation of clonal cell lines which stably express each mutant to be tested. Though directed mutagenesis can proceed with speed, selection and amplification of stably transfected, clonal cell lines usually requires months, with the attendant risk of interim phenotypic drift or selection.

Transient expression of heterologous membrane proteins has been applied to the study of epithelial membrane protein polarity using several expression

systems. After the discovery of polarized viral budding from epithelial cell monolayers (Rodriquez-Boulan and Powell, 1992, Rodriquez-Boulan and Sabatini, 1978), viral infection was used for many years to study membrane protein targetting until antibodies and plasmid expression vectors improved sufficiently to allow study of cDNA-transfected cells. More recently, infection with recombinant vaccinia virus has been applied to studies of viral membrane protein polarity (Hughey *et al.*, 1992. The polarized phenotypes detected by recombinant infection studies were confirmed by studies with native virus, with stably transfected cell lines, or by studies of the endogenous protein expressed at lower level. However, the delayed cytopathic effects of viral infection limits the time duration of these experiments.

We have evaluated transient transfection of MDCK cells as a more rapid and convenient method for the study of polarized expression of heterologous plasma membrane proteins. We tested the polarity of plasma membrane proteins previously shown in stably transfected cells to exhibit nonpolarized, apical, and basolateral localizations. Human CD7, a monotopic membrane protein expressed early in T-cell development prior to thymic maturation, and a candidate costimulatory molecule with T-cell antigen receptors (Aruffo and Seed, 1987; Ware *et al.*, 1889), is not targetted in MDCK cells, and exhibits a nonpolar localization (Haller and Alper, 1993). Decay-accelerating factor (DAF-CD55) is a phosphatidylinositol glycan-linked protein of the apical surface, which has been localized to caveolae (Lisanti *et al.*, 1993). Vesicular stomatitis virus G protein (VSVG), a monotopic, homotrimeric viral protein, was the first heterologous protein expressed from transfected cDNA shown to exhibit basolateral localization in polarized MDCK cells (Rodriguez-Boulan and Sabatini, 1978).

A recent report (Perez-Velazquez and Angelides, 1993) has suggested that transient transfection is a valid experimental approach for the study of heterologous membrane protein polarity, but comparison with the same heterologous proteins in stably transfected MDCK cells was not reported. Our studies addressed the efficiency of transient transfection of MDCK cells on coverslips, the preservation of the polarized epithelial phenotype by the transiently transfected cells, and the steady-state localization of the recombinant polypeptides. Though transient transfection of MDCK cells preserved their endogenous polar phenotype on permeable supports, the polarized expression of recombinant membrane proteins was less homogeneous than in clonal, stably transfected MDCK cells.

Methods

Cell culture

Low resistance (strain II) MDCK cells (gift of K. Matlin) were maintained in MEM, 10% fetal bovine serum (FBS), 2 mM glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37 °C, in a humidified 5% CO_2 atmosphere. Cells were fed 3 times per week and passaged weekly. Cells were used for experiments up to passage 30 after thawing from frozen stock. Tissue culture plasticware was from Falcon (Becton Dickinson); all cell culture reagents were obtained from Gibco/BRL. Chemicals were from Sigma unless otherwise mentioned.

For vectorial assays trypsinized cells from one 60 mm dish were seeded onto 6 24/25 mm filters or onto 24 6.5 mm or 10 mm filters. The filter types tested included a) 0.4 μm pore diameter Transwell® polycarbonate filters (Costar), b) 0.02 μm inorganic (Anopore®) filters (Nunc), and c) 0.4 μm Cyclopore® filters (Falcon).

Transepithelial resistance (TER) was measured with chopstick electrodes connected to a voltohmmeter (WPI) according to the protocol of the manufacturer. Monolayer inulin permeability was tested by addition of ^{14}C-inulin (1.2 μCi) to the growth medium in the cis compartment followed by timed sampling of the trans compartment. Bidirectional inulin fluxes were measured. Transepithelial hydrostatic pressures were maintained equal.

Plasmids and Transfections

CD7 cDNA (gift of A. Aruffo and B. Seed, 1987) was subcloned into pcDNA (Invitrogen) to yield pcDNA-CD7. The CD7-cDNA was also truncated at a unique internal EcoRI site, religated and subcloned into pCXM (gift of G. Yancopoulos, Davis *et al.*, 1991), yielding pCXM-CD7Δ. This mutation resulted in removal of the C-terminal 25 residues from the 39 amino acid cytoplasmic domain. pCB6G expressing VSVG was a gift of M. Roth (Brewer and Roth, 1991). pGD1 expressing DAF-CD55 was a gift of M. Lisanti (Lisanti *et al.*, 1989).

MDCK cells grown to 80–90% confluency in 60 mm dishes were transiently transfected with 1–

20 μg circular plasmid DNA by a modified diethylaminoethyl (DEAE)-dextran method (Sambrook *et al.*, 1989). The DEAE-dextran solution consisted of 140 mM NaCl, 50 mM TrisCl, 500 μg/ml DEAE-dextran (Pharmacia), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl; the pH was adjusted to 7.4 prior to sterile filtration. The plasmid DNA was diluted in this solution and kept at room temperature for 10 min before its addition to the cells for 3 hours at 37 °C. After three washes with serum-free MEM, cells were incubated 3 more hours at 37 °C with MEM, 2% FBS, 200 μM chloroquine. Monolayers were washed three times with serum-free MEM, allowed to recover from the chloroquine treatment in growth medium for 2 to 3 days with daily feeding, then trypsinized and seeded onto glass coverslips or permeable filter supports as described above. These cells were fed daily until experimental use, 5 to 15 days post-tranfection and 3 to 12 days post-transfer to coverslip or filter. Lipofection was performed using Lipofectamine (Gibco-BRL) according to manufacturer's instructions. Operational transfection efficiency was assessed by immunofluorescence analysis of CD7-positive (transfected) cells.

Antibodies

Mouse hybridoma supernatants against apical (gp135; Ojakian and Schwimmer, 1988) and basal (gp60; Herzlinger and Ojakian, 1984) marker proteins of MDCK cells (gifts of Dr. G. Ojakian) and were used at a 1:10 dilution in blocking buffer (1% bovine serum albumin (BSA) in PBS, 0.05% saponin). Mouse 3A1 hybridoma (ATCC HB-2) supernatant against an ecto-epitope of human CD7 was used neat for CD7 immunocytochemistry. Mouse hybridoma 3A1E-12H7 supernatant (Accurate Chemical and Scientific Corp) was used for comparison. Rabbit polyclonal anti-VSVG (gift of T. Kreis via K. Simons) and anti-DAF-CD55 ecto-domain (gifts of M. Lisanti and A. Nicholson) FITC, and Cy3-conjugated anti-mouse, anti-rat, and anti-rabbit IgG conjugated to Texas-Red, to FITC, and to Cy3 were obtained from Jackson Immunoresearch, and diluted 1:200 in blocking buffer.

Immunocytochemistry

Nonvectorial immunofluorescence was performed as described previously (Haller and Alper, 1993). For vectorial immunofluorescence, confluent monolayers on filters were washed with ice cold PBS containing 1mM $CaCl_2$ and 1mM $MgCl_2$ (PBS/CM) and vectorially incubated for 1–2 hours at 4 °C with primary antibody in the absence of saponin. Apical incubations used 100 μl hybridoma supernatant per 6.5 mm filter insert or 500 μl/24.5 mm tissue culture insert. For basal incubations, 6.5 or 10 mm filters containing 100 μl in the apical compartment were placed onto 100 μl drops of hybridoma supernatant on the inverted lid from a 24-well tissue culture plate. 24.5 mm filters containing 500 μl in the apical compartment were placed onto 300 μl drops of hybridoma supernatant on the lid from a 6 well plate. Filters were washed, fixed, quenched, and excised from their plastic supports. Excised filter segments were nonvectorially incubated with secondary antibody at a dilution of 1:50 in blocking buffer containing saponin.

The converse experiment was also performed. Live monolayers at 4 °C were incubated non-vectorially (i.e. from both apical and basolateral compartments) with anti-CD7 hybridoma supernatant followed by washing and vectorial incubation with secondary antibody, after which they were washed, fixed, quenched, and mounted. This experimental approach allowed staining of CD7 with different fluorophores on either cell surface of the same cell.

Conventional immunofluorescence microscopy was performed with an Olympus BH-2 photomicroscope. Single-channel confocal laser scanning immunofluorescence microscopy was performed with the Phoibos 1000 confocal system (Molecular Dynamics). The Cy3 fluorophore provided a signal intensity and excitation spectrum optimal for the system's argon laser. Stained filters were mounted in freshly prepared mowiol/glycerol under glass coverslips. When care was taken not to crush the cell monolayers (Bacallao *et al.*, 1990), cells were as tall as > 20 μm. Monolayers were viewed with sequential x-y sections stepping from apical to basal focal planes of the monolayer, as well as by transverse (x-z) tomographic sections. Cy3-staining of CD7 yielded sufficient signal intensity to allow detection of CD7, DAF-CD55, and VSVG in single tomographic x-z sections without 3-dimensional image reconstruction.

Immunodetection of CD7 polypeptide

Nonvectorial immunoprecipitation of CD7 to provide a positive control was performed with lysates of confluent MDCK-CD7 monolayers metabolically labeled to steady-state as previously described (Haller and Alper, 1993). Briefly, confluent monolayers in 100 mm tissue culture dishes were starved for Met and Cys for

8 hrs, followed by overnight incubation with 300 μCi ^{35}S-cysteine (NEN-Dupont, Boston).

Selective vectorial immunoprecipitation of CD7 from either the apical or basolateral surfaces was performed as described for confluent MDCK monolayers permanently transfected with CD7 (Haller and Alper, 1993). Transiently transfected and control monolayers on 24 mm filters were starved for Met and Cys for 1 hr, then metabolically labelled to steady state by overnight incubation with 110 μCi ^{35}S-cysteine in the basal tissue culture compartment. In some experiments, immunoprecipitation of metabolically labelled CD7 was combined with vectorial biotinylation using biotin-ε-aminocaproyl hydrazide (Calbiochem) as described by Brändli *et al.*, 1990). Cleared lysates from biotinylated cells were subjected to sequential avidin precipitation and anti-CD7 immunoprecipitation as previously described (Haller and Alper, 1993).

Results

Evaluation of transfection efficiency

Cell lines vary in the efficiency with which they undergo transient transfection by given methods, in the efficiency with which their transcription apparatus responds to the transfected promoter, and in the stability of the heterologous protein once translated. Immunodetection of heterologous protein in transiently transfected cells reflects all these factors. Figs. 1c and 1d document CD7 immunofluorescence in fixed, permeabilized CD7-transfected MDCK cells grown on Cyclopore® filters.

The DEAE-dextran transfection protocol described above resulted in "operational" transfection of 33 ± 2.3% (s.e.m., n = 8), where "operational" refers to restriction of selected visual fields to those fields in which immunostained cells are present. Transfection efficiencies calculated from random visual fields ranged from 1–10%. Operational transfection efficiency increased with increasing input DNA, was twofold higher in cells on polycarbonate filters than in cells on coverslips, but did not vary between 4 and 7 days post-transfection. The transfection protocol did not alter the trypsin sensitivity of the cells on plastic when assessed one or two days following transfection.

CD7 expression persisted in filter-grown monolayers for 15 days post-transfection (the longest time evaluated) without progressive effect on cell density or population appearance. The transfected cells were usually larger (or more spread) than their nontransfected neighbors, and often altered in shape (Fig. 1c). The altered appearance of transfected cells led us to investigate modifications of the transfection procedure. The omission of chloroquine greatly reduced transfection efficiency. Lowering of the chloroquine concentration, or substitution by glycerol or DMSO shock also lowered the number of cells transfected by the DEAE-dextran procedure. Calcium phosphate and liposome-based procedures (Haller and Alper, 1993; Sambrook *et al.*, 1989) were also less efficient than DEAE-dextran/chloroquine for transient transfection of MDCK cells with CD7.

Preservation of the endogenous polarized phenotype in transfected MDCK cells

Table 1 shows that the TER across the transfected monolayers did not decrease, consistent with preservation of tight junctional barrier function. When measured early (2–6 days) post-transfection, TER actually increased by 2–3 fold in both CD7-transfected and in mock-transfected cells. However, when measured later (7–15 days) post-transfection, TER values were equal to those of control nontransfected MDCK monolayers of the same age post-seeding. Most TER measurements were performed with monolayers grown on Cyclopore® membrane. TER was similar when measured across monolayers grown on Anopore® and Transwell filters (data not shown). The transiently increased TER which followed exposure to the transfection procedure was accompanied temporally by a small increase in average cell area and decrease in confluent cell density, with an attendant decrease in junctional area for the monolayer (not shown).

Figure 2 shows a radioisotopic index of junctional permeability across transfected MDCK monolayers on Cyclopore® filters. ^{14}C-inulin was applied to the apical tissue culture compartment followed by timed sampling of the basolateral medium. The converse experiment of measuring the apical ^{14}C-activity after basal application of ^{14}C-inulin yielded similar results. The permeability of all MDCK-monolayers was approximately 2 orders of magnitude lower than that of empty filters during 21 hrs of observation. Transiently transfected monolayers and non-transfected monolayers showed no difference in inulin permeability. The assessments of marker protein distribution, together with the measurements of TER and transepithelial inulin flux, indicated preservation of functional inter-

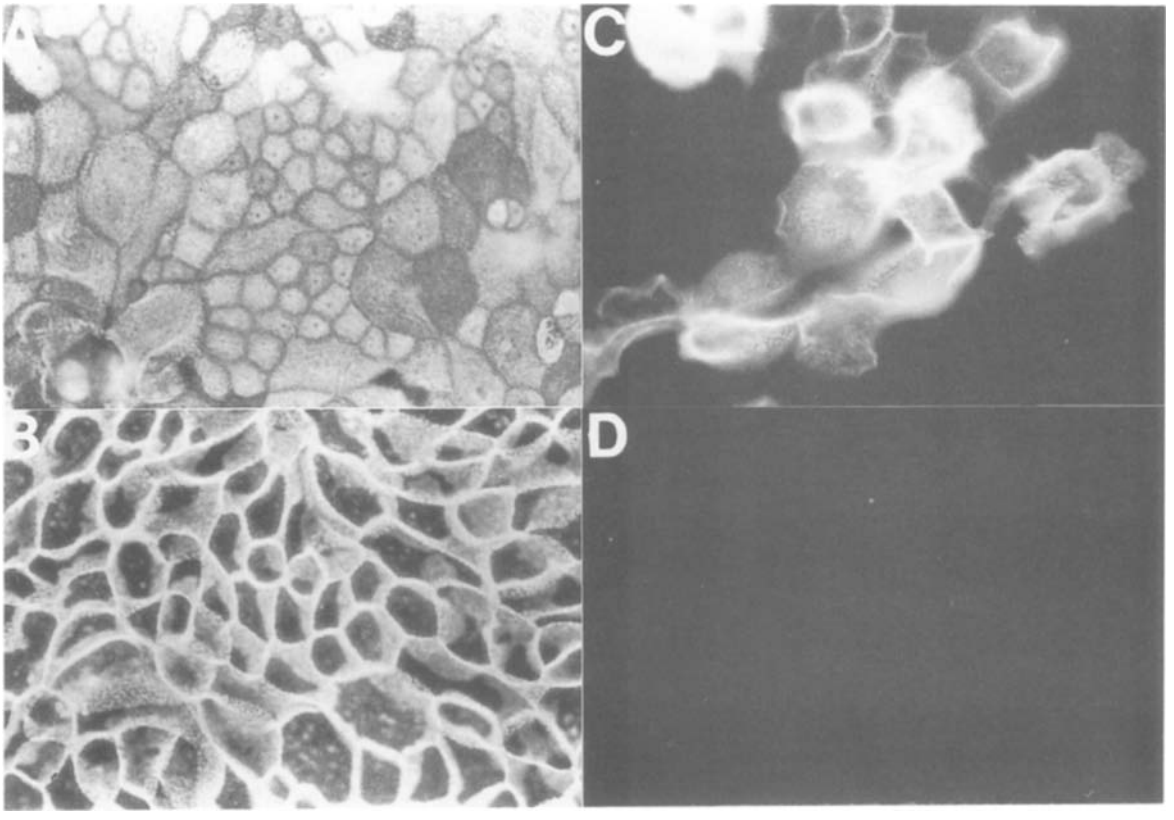

Fig. 1. Immunofluorescence analysis of fixed, permeabilized MDCK cells transiently transfected with CD7 cDNA, then seeded onto Cyclopore® filters. A: Staining of apical surface marker gp135; B: Staining of basolateral surface marker gp60; C: Staining of CD7; D: Texas Red-conjugated secondary antibody alone. Photographed through 40× objective.

Table 1. Transepithelial resistance (TER) and cell density of MDCK monolayers grown on 25 mm Cyclopore® filters (0.4 μm pore). "Early" measurements (2–6 days post-transfection) showed increased TER. "Late" measurements (7–15 days post-transfection) showed return to normal values with parallel increase in cell density

MDCK cells	Transepithelial resistance ($\Omega cm^2 \pm$ s.e.m., (n)		Monolayer cell density (cells/40x field ± s.e.m. (n=3))	
	Early	Late	Early	Late
Control	222±30 (9)	259±22 (6)	178±14	246±11
Mock-Tx	532±66 (15)	256±24 (7)	74±23	154±7
CD7-Tx	616±45 (25)	262±34 (8)	153±24	197±27

cellular tight junctions in monolayers of transiently transfected MDCK cells.

Figure 1 shows that the endogenous apical marker gp135 (a) and the endogenous basal marker gp60 (b) displayed normal polarized distributions in transfected cells grown on Cyclopore® filters. In Fig. 1b, gp60 immunofluorescence highlights those lateral membranes which are not orthogonal to the focal plane with a "drapery" appearance. The same polarized expression patterns were present in both transfected and in nontransfected cells, whether grown on transparent or on opaque Transwell filters (not shown).

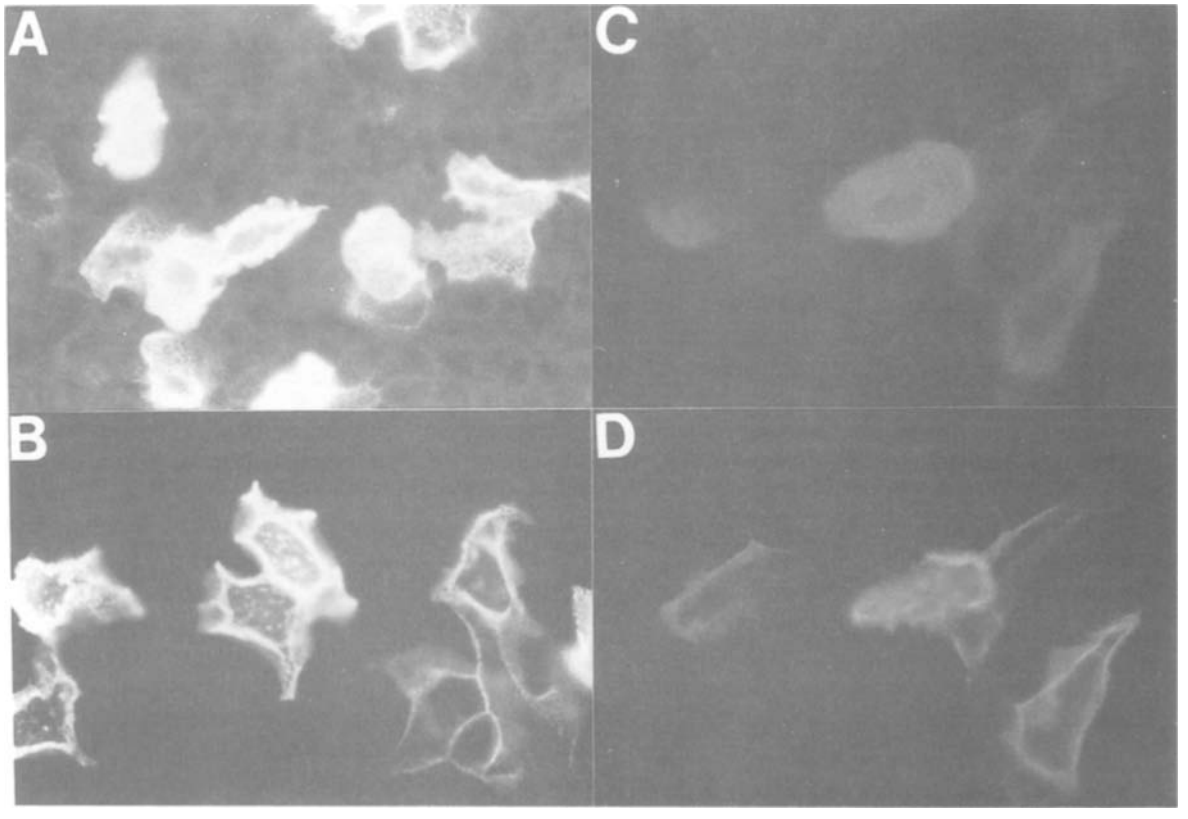

Fig. 3. Vectorial CD7 immunofluorescence of live, unfixed cells. Anti-CD7 hybridoma supernatant was added neat to the apical (A) or the basal (B) compartments of confluent filter-grown MDCK monolayers transiently transfected with CD7 cDNA. Cells were fixed and then incubated with Texas Red®-secondary antibody. Alternatively, anti-CD7 hybridoma supernatant was incubated neat in both apical and basal compartments of a single confluent filter-grown MDCK monolayer, then exposed vectorially to FITC-secondary antibody in the apical compartment (C) and to Texas Red-secondary antibody in the basal compartment (D). The same cells are stained apically and basolaterally. The plane of focus in panel C is that of the brighter central cell, while the peripherally situated fainter cells sit below the plane of focus. At their optimal planes of focus, they were as bright as the central cell.

Immunocytochemical distribution of CD7 in transiently transfected, polarized MDCK cell monolayers

As shown in Fig. 1c, CD7 protein transiently expressed in polarized MDCK monolayers shows an immunostaining pattern which combines features of apical (Fig. 1a) and basolateral localization (Fig. 1b), and resembled the CD7 staining pattern of stably transfected MDCK cells (Haller and Alper, 1993). Omission of anti–CD7 antibody (Fig. 1d) provided a specificity control for CD7. CD7 immunostaining was also absent from mock transfected MDCK monolayers (not shown). The apparently nonpolarized CD7 expression was evaluated further by vectorial immunolocalization in transfected monolayers.

Figure 3 shows results of complementary vectorial immunolocalization experiments. Anti-CD7 antibody was added to unfixed, non-permeabilized transfected MDCK monolayers at 4 °C in either the apical (a) or basal compartment (b), followed by fixation/permeabilization and non-vectorial incubation of the filters with secondary antibody. In the converse experiment anti-CD7 antibody was added to both surfaces of unfixed, non-permeabilized transfected monolayers, followed after washing by addition of FITC-conjugated secondary antibody to the apical compartment (c) and Texas Red®-conjugated secondary antibody to the basal compartment (d) of the same filter assembly. Both approaches demonstrated the presence of CD7 at the apical, as well as the basolateral surfaces of the transfected MDCK cells. The latter experiment detected apical and basal expression of CD7

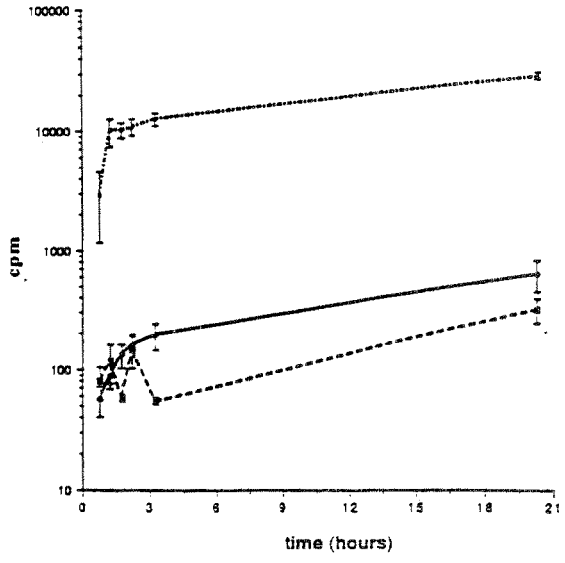

Fig. 2. Appearance of ^{14}C-inulin (cpm) in basal compartment at increasing times after addition to the apical compartment of MDCK monolayers grown on Cyclopore® filters. Empty filters (upper broken line, n=4); Non-transfected MDCK monolayers measured 4 days after seeding (solid line, n=6), MDCK monolayers 5 days after transient transfection with CD7 cDNA, 4 days after seeding (lower dotted line, n=6).

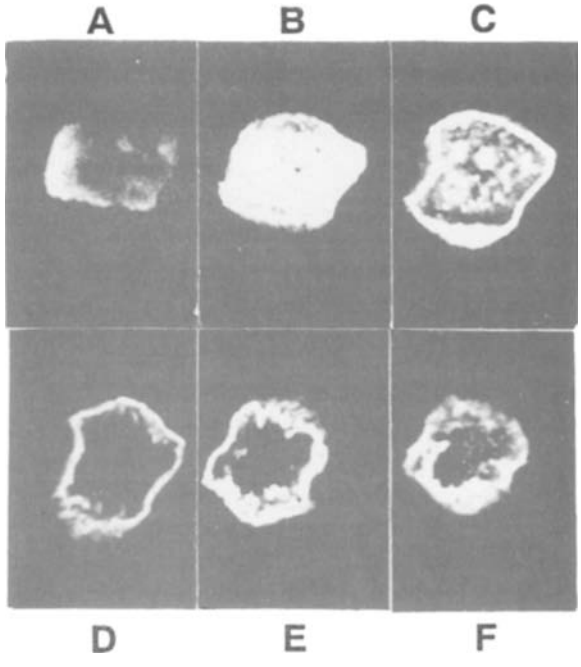

Fig. 4. Sequential confocal tomography x-y sections of CD7 immunofluorescence detected in an MDCK cell transiently transfected with CD7 cDNA and transferred onto a Transwell filter. Indirect immunofluorescence with Cy3-conjugated secondary antibody. Panels A through F progress from the apical surface to the basal surface at 4 μm intervals.

in the same cells. The apical stain in Fig. 3c was fainter than the basolateral stain in Fig. 3d because the FITC emission intensity was lower than that of Texas Red, the double exposure led to increased bleaching of FITC, and because of the absence of saponin during this supravital immunolabeling experiment. In addition, the apical surfaces of individual transfected cells resided in different focal planes.

In 12 randomly chosen 63× microscopic fields, there were 11.9 ± 0.8 (s.e.m.) CD7-positive cells per field of monolayers apically stained with anti-CD7 antibody, and 13.4 ± 1.0 CD7-positive cells per field of basally stained monolayers. In 8 fields from the converse vectorial experiment, monolayers apically stained with secondary antibody revealed 19 ± 1.2 CD7-positive cells per field, and monolayers basolaterally stained with secondary antibody revealed 18 ± 1.3 CD7-positive cells per field. These results supported the conclusion that most transfected MDCK cells expressed heterologous CD7 at both surfaces.

This finding was generally confirmed by confocal laser scanning immunoflorescent microscopy. Figure 4 shows an apical-to-basal series of selected x-y (horizontal) tomographic cuts from a single, fixed and permeabilized, CD7-expressing MDCK cell on a Transwell filter which had been non-vectorially incubated with anti-CD7 antibody. In this single cell, CD7 clearly is present in both apical and basolateral plasma membranes, with little or no detectable intracellular accumulation. The columnar height of CD7-expressing cells was no different from the height of nontransfected cells (not shown), suggesting that the transiently increased area of transfected cells evident by conventional microscopy (Table 1) reflected transiently increased cell volume.

Three examples of vectorially stained CD7-expressing cells are shown at higher magnification in Fig. 5. This figure demonstrates (a) apical staining with apically applied antibody, (b) basolateral staining with basally applied antibody, and (c) apicolateral staining with non-vectorial application. The intensity of CD7 immunostaining at the basal cell surface was usually lower than that at the lateral surfaces. Single x-z (transverse) sections of confluent monolayers 7 days post-transfection that were nonvectorially incubated with anti-CD7 antibody displayed surface staining that resembled the combined patterns of apical and baso-

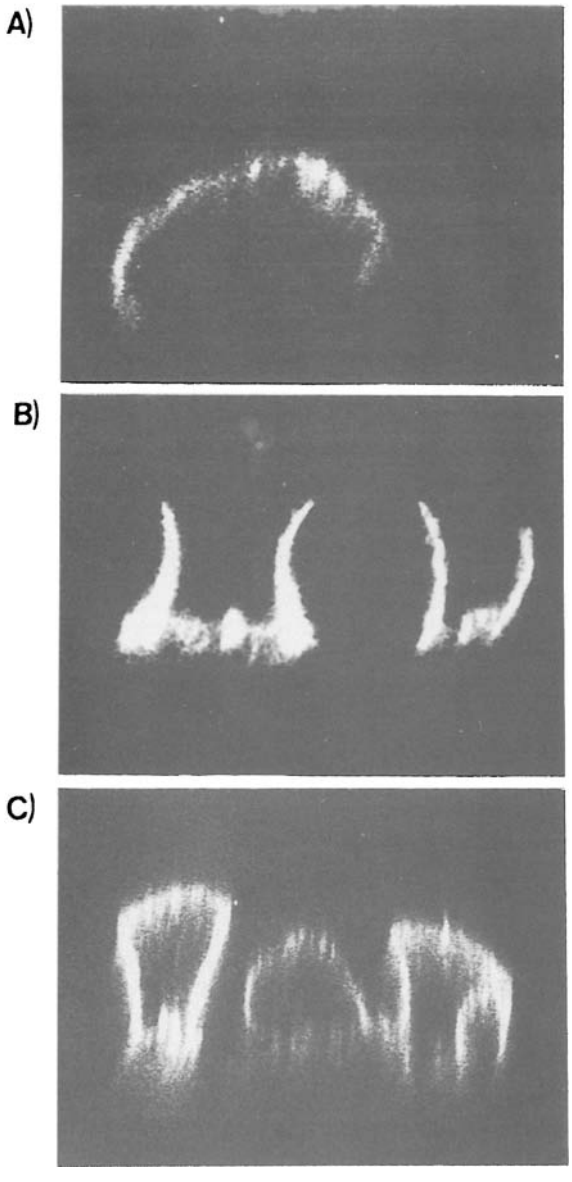

Fig. 5. Confocal x-z directions representing the varying CD7 surface distributions detected among MDCK cells on Transwell filters transiently transfected with CD7 cDNA. Indirect immunofluorescence with Cy3-conjugated secondary antibody. A: apicolateral distribution; B: basolateral distribution; C: apical and basolateral (circumferential) distribution.

lateral surface markers gp60 and gp135. However, x-z transverse confocal sections revealed both apical and basolateral CD7 expression in only about 2/3 of the evaluated cells, and basolateral expression only in 1/3 of the cells. There was no evident correlation between CD7 basolateral staining intensity and the absence or

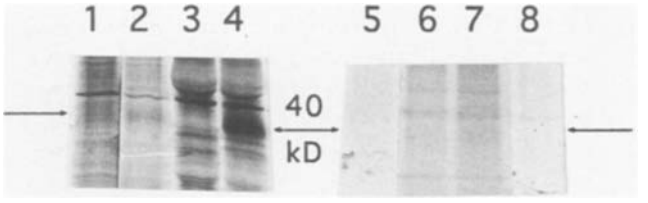

Fig. 6. Fluorograph of SDS-PAGE illustrating vectorial detection of CD7 polypeptide in live, transiently transfected, metabolically labeled, polarized MDCK cells on Transwell filters. Cells were incubated with anti-CD7 hybridoma supernatant in the apical (lane 1) or the basal compartments (lane 2), washed, then solubilized and precipitated with protein G-agarose. For comparison are shown metabolically labeled, nontransfected MDCK cells (lane 3) and stably transfected MDCK-CD7 cells (lane 4, Haller and Apler, 1993). Alternatively, cells transfected with CD7 (lanes 6,7) or mock-transfected (lanes 5,8) were biotinylated from the apical (lanes 5,6) or basal compartments (lanes 7,8) then sequentially avidin-precipitated and immunoprecipitated.

Table 2. Monolayer height in transiently transfected MDCK cells (7 days post-transfection, 5 days after seeding onto permeable supports). Fixed, permeabilized monolayers were subjected to indirect staining of CD7. Confocal x-z sections were evaluated for monolayer cell height. Adjacent CD7-positive and CD7-negative cells were of similar height

Filter Support	Cell Height [$\mu \pm$ s.e.m. (n)]	CD7 distribution	
		Circumferential	Basolateral
Anopore (pore: 0.02 μ)	8.6 $\pm$ 0.9 (7)	13	6
Cyclopore (pore: 0.45 μ)	7.0 $\pm$ 0.5 (7)	13	6
Transwell (pore: 0.4 μ)	21.1 $\pm$ 0.9 (10)	14	6

presence of detectable apical CD7 in single x-z sections.

The choice of filter support influenced the columnar phenotype of the transfected MDCK monolayers, but did not appear to alter the surface distribution of hererologous CD7 (Table 2). Monolayers grown on opaque Transwell filters were 3-fold taller than monolayers grown on transparent Anopore® or Cyclopore® filters.

Immunodetection of surface CD7 polypeptide in transiently transfected MDCK cell monolayers

CD7 polypeptide detection in transiently transfected cells was more difficult than in stably transfected

MDCK-CD7 cells (Haller and Alper, 1993), consistent with overall transient transfection efficiencies of 1–10%. Nonetheless, long exposures of fluorographs from metabolically labeled transfected cells revealed CD7 from total cell lysates. Figure 6 shows biochemical detection of surface CD7 polypeptide in transiently transfected MDCK cells on polycarbonate filters by vectorial immunnoprecipitation. Lane 4 shows for reference immunoprecipitated CD7 from stably transfected MDCK-CD7 cells metabolically labeled to steady state (Haller Alper, 1993). Lane 3 shows normal MDCK cells processed in parallel. Lanes 1 and 2 show transiently transfected MDCK cells which had been exposed live to anti-CD7 antibody from the apical (lane 1) or the basal surface (lane 2), followed by lysis and direct precipitation with protein G. Both basolateral and apical surface CD7 bands were identified at the expected mobility of 40 kD. Vectorial biotinylation followed by sequential immunoprecipitation and avidin precipitation also detected CD7 polypeptide at apical (lane 6) and basolateral surfaces (lane 7), though the CD7 band intensities were weaker with this method. With both techniques, the basolateral CD7 band was more intense than the apical band, consistent with the greater basolateral membrane surface area in MDCK cells (Haller and Alper, 1993). CD7 could not be detected by immunoprecipitation of mock transfected cells with anti-CD7 (Fig. 6, Lanes 3, 5, 8) or by immunoprecipitation of CD7-transfected cells with irrelevant monoclonal antibody (not shown).

CD7 distribution in MDCK cells transiently transfected by coprecipitation of plasmid DNA with Lipofectamine® was indistinguishable from the CD7 distribution in cells transiently transfected by the DEAE / dextran technique (not shown).

Immunolocalization of heterologous apical and basolateral membrane proteins in transiently transfected MDCK cells. To evaluate more fully the utility of transient transfection of MDCK cells in rapid monitoring of membrane protein polarity, we examined the polarity of heterologous proteins previously defined as apical or basolateral polypeptides in stably transfected MDCK cells. DAF-CD55 is a phosphatidylinositol glycan-linked polypeptide known to localize to apical membranes of stably transfected MDCK cells (Lisanti *et al*, 1989). As shown in Figs. 7a and 7b, though most transiently transfected cells localized DAF-CD55 apically, a minority localized DAF-CD55 laterally, with little apical expression (Figs. 7c and 7d).

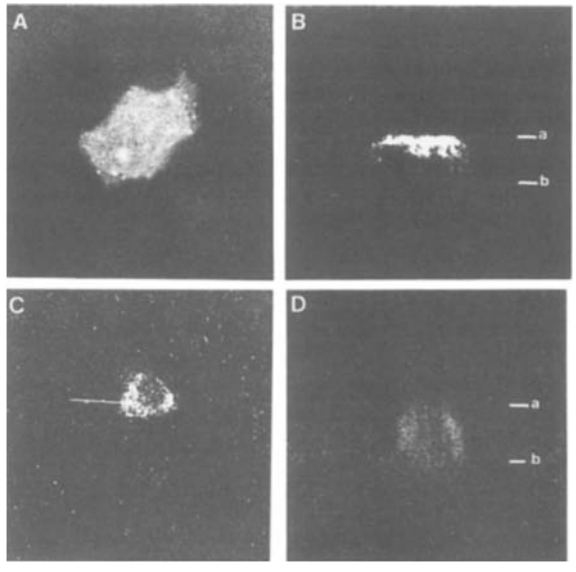

Fig. 7. Confocal immunofluorescent micrographs of representative MDCK cells transiently transfected with DAF-CD55 cDNA, plated onto Transwell filters, and viewed in an x-y plane at the apical surface (A) or midway through the cell (C), or viewed in an x-z plane (B,D). Apical (a) and basal (b) membrane locations are indicated. Most cells localized DAF-CD55 apically (A,B), but in occasional cells the protein was localized laterally (C,D). Cell heights were 17.5 μm (B) and 15 μm (D).

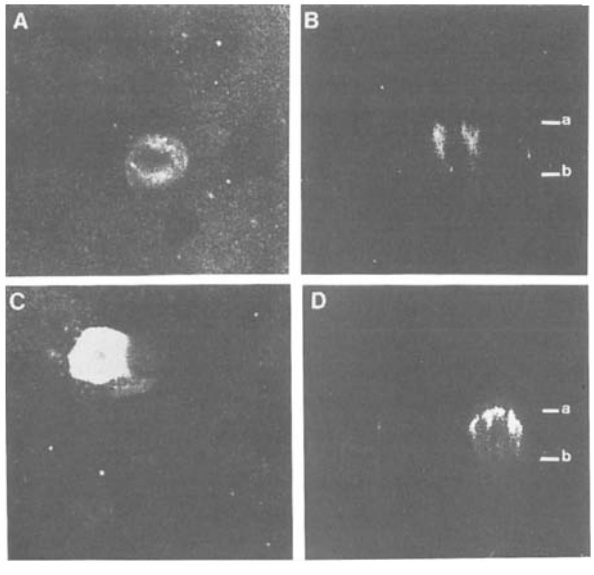

Fig. 8. Confocal immunofluorescent micrographs of representative MDCK cells transiently transfected with VSVG cDNA, plated onto Transwell filters, and viewed in an x-y plane midway through the cell (A) or at the apical surface (C), or viewed in an x-z plane (B,D). Apical (a) and basal (b) membrane locations are indicated. Similar numbers of cells localized VSVG laterally (A,B) and apically (C,D). Cell heights were 18 μm (B,D).

In contrast, vesicular stomatitis virus G protein (VSVG) is a basolateral glycoprotein in stably transfected MDCK cells (Brewer and Roth, 1991). As shown in Figs. 8a and 8b, though some transiently transfected cells localized VSVG laterally as expected, some also localized VSVG preferentially to the apical surface (Figs. 8c, d). The distribution of VSVG displayed no greater fidelity in MDCK cells transfected by the Lipofectamine® technique, since 60% of transfected cells exhibited basolateral or basolateral plus intracellular staining, whereas 40% of transfected cells exhibited intracellular staining alone or apical plus intracellular staining.

Discussion

These studies were undertaken with the objective of testing transient transfection as a means of facilitating and accelerating the study of polarized expression of heterologous membrane proteins and their mutants in polarized MDCK cell monolayers. The results demonstrate that transient transfection with DEAE-dextran can lead to expression of heterologous proteins in polarized MDCK cells on permeable supports for up to two weeks. Moreover, levels of transient expression sufficed for immunochemical detection of metabolically labeled polypeptide at either cell surface (Fig. 6). However, the uniformity of heterologous protein polarity in transient transfectants was less than that reported for stable transfectants. This was most apparent when analyzed by confocal x-z sections. Whereas vectorial immunostaining and en face microscopy suggested that CD7 was uniformly nonpolarized (Figs. 1, 3), confocal x-z sections showed that 1/3 of transfected cells appeared to localize CD7 only to the lateral membranes (Table 2). The "apical" protein DAF-CD55 was (conventionally) apically situated in most cells examined by confocal x-z sections, but a small number showed preferentially lateral or circumferential staining (Fig. 7). The "basolateral" protein VSVG was (conventionally) basolaterally localized in only 1/3 of the cells examined, but the remainder exhibited nonpolar or preferentially apical staining (Fig. 8).

Transiently transfected MDCK monolayers retained their ability to form tight junctions and establish plasmalemmal polarity with respect to endogenous marker proteins. The most highly columnar MDCK phenotype was achieved on polycarbonate filters. The transfection procedure led to a transient elevation in TER across the MDCK cell monolayer. The decreased cell number in early times post-transfection is consistent with the antiproliferative effects of chloroquine (Zeilhofer *et al.*, 1989; Inyang *et al.*, 1990). However, the correspondingly larger average cell size, if attended by a corresponding decrease in junctional area per confluent monolayer, was insufficient to account for the 2.5-fold increase in TER. Curiously, chloroquine has been reported to increase MDCK dome formation upon shift from 10% to 0.2% serum (Slaughter *et al.*, 1982), suggesting additional possible effects of increased cell differentiation.

CD7 is unusual as a heterologous transmembrane protein which is not sorted in polarized, stably transfected MDCK cells (Haller and Alper, 1993). Therefore, the consequences of the transfection procedure, in particular the use of chloroquine, require consideration in view of the reported effects of chloroquine on sorting of secreted proteins. Chloroquine was used at 0.2 mM for 3 hrs in the CD7 transfection experiments, a concentration twice that routinely used as a lysosomotropic agent. In previous reports, chloroquine reduced protein secretion rates and abolished basolateral secretory targetting (Caplan *et al.*, 1987), or redirected basally secreted protein apically (Parczyk and Kondor-Koch, 1989). Chloroquine and other lysosomotropic weak bases retarded rates of protein appearance at the plasma membrane (Parczyk and Kondor-Koch, 1989; Mattey *et al.*, 1990), but did not alter membrane protein sorting or steady-state distribution (Caplan *et al.*, 1987; Matlin, 1986). Most importantly, in all these studies, the chloroquine was present for the duration of the biosynthetic pulse-chase or labeling experiments, and the effects of lysosomotropes were fully reversible. Transfected protein localization in the present studies was assessed at least 48 hrs following brief exposure to chloroquine. Since measured endogenous indices of MDCK cell polarity remained intact, it is unlikely that the nonpolarized CD7 distribution was secondary to a generalized disruption of membrane protein sorting caused by earlier exposure to chloroquine. (Haller and Alper, 1993). This conclusion was further strengthened by the finding that neither CD7 nor VSVG proteins exhibited any greater fidelity of sorting in MDCK cells transiently transfected with these cDNAs by the Lipofectamine® technique, in which they were never exposed to chloroquine. Moreover, the increased cell size which followed the exposure to chloroquine during the DEAE/dextran transfection procedure was not observed following the Lipofectamine® procedure, which nonetheless resulted in low-fidelity sorting of

heterologous proteins. Thus, the altered cell size need not have contributed to the observed mis-sorting.

The possibility of a masked polar targetting signal or an active targetting signal for nonpolarized delivery in the CD7 polypeptide was addressed in a preliminary experiment by transient transfection of MDCK cells with a truncated cDNA encoding a CD7 molecule lacking the 24 carboxyterminal residues from the 39 amino acid-long cytoplasmic tail. Immunostaining of the truncated CD7 in MDCK cells was indistinguishable from that of the full length protein (not shown), suggesting that no such signals reside in this portion of the protein.

The 30% frequency of basolateral CD7 expression in the absence of apical CD7 expression observed in single x-z confocal tomographic sections differed from the high coincidence of apical and basolateral CD7 expression observed in the conventional microscopic analysis of vectorially immunolabeled monolayers. This difference most likely reflects low density of apical CD7 expression in those cells below the threshold of detection for single x-z cuts. Alternatively, the variable phenotype of CD7 polarization could be due to sequellae of the transient transfection procedure. These sequellae might include sufficient overexpression of the heterologous membrane protein in individual cells to promote "mis-sorting" (Matter *et al.*, 1992). The apical localization of DAF-CD55 in most but not all transfected cells, and the even less consistent basolateral localization of VSVG, are at least compatible with this possibility.

In summary, MDCK cells can be transiently transfected without evident attenuation of their endogenous polarized phenotype on permeable filter supports. Transfection efficiency and heterologous protein expression level can suffice for subsequent morphological and immunochemical analysis of the heterologous polypeptide. However, polarity of heterologous protein localization is not consistently preserved among all transfected cells when compared to the localizations previously described in stably transfected cells.

Recently, the polarized expression of $GABA_A$ receptor $\alpha 1$ and $\beta 1$ subunits was demonstrated in MDCK cells transiently transfected with Lipofectin in glass coverslips (Perez-Velazquez and Angelides, 1993). These interesting results, showing that $\beta 1$ subunit could redirect $\alpha 1$ subunit from the basolateral to the apical membrane, have not yet been confirmed in stably transfected cells/or on permeable supports. Our results suggest caution in extrapolating results from transient polarity studies to stable polarized cell lines or to polarized cell in situ. Though transient transfection may have a useful role for the initial rapid screening of heterologous wild-type and mutant protein polarity, creation of stable transfectants remains the required method for definitive analysis of heterologous protein targetting in polarized MDCK cells.

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