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Research Article

A morphological study of the expression of the small G protein RhoA in resting and activated MDCK cells

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Abstract. The small G protein Rho subfamily controls several cellular events such as growth, movement, proliferation and differentiation by rearranging actin and cytoskeleton proteins. Most of these effects are mediated by the activation of growth factor and extracellular matrix molecule receptors, suggesting a role for Rho molecules in the transduction pathway of these receptors. Despite the importance of Rho peptides in fundamental cellular events, data on their subcellular immunolocalisation are sparse: here we investigated the expression and subcellular localisation of RhoA in resting (cultured on plastic) and activated (Matri-cell or hepatocyte growth factor) MDCK cells by immunoperoxidase and immunogold techniques. Resting MDCK cells contain detectable amounts of RhoA mainly localised in the cytoplasm; RhoA expression is significantly enhanced by Matri-cell substrates that promote translocation of RhoA at the membrane level. This enhancing effect is reduced after exposure to hepatocyte growth factor.

Key words. MDCK cells; RhoA; HGF; extracellular matrix; immunocytochemistry.

The Madin-Darby canine kidney (MDCK) cell line, derived from dog renal distal tubular cells, has been used extensively for numerous studies on cell attachment and cell functional properties [1]. Moreover, this cell line is widely used for studying the factors that govern the ordered assembly of epithelial cells into spatially organised multicellular units. MDCK cells generate tubule-like structures or spherical cysts under particular experimental conditions, for example when cultured with growth factors (GFs) such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor α (TGF-α) [2–4]. Much experimental evidence demonstrates that these GFs control in vitro MDCK cell properties [5, 6]. In particular, HGF, a glycoprotein secreted by mesenchyme-derived cells, induces membrane ruffling, centrifugal spreading and disruption of cell-cell contact in MDCK cells even if it does not appear to stimulate their growth [7].

GFs affect cells by linking specific receptors that in turn activate metabolic cascades, and also by recruiting many signalling molecules [8] among which G proteins play an important role. The Rho subfamily, a group of small G proteins, controls several GF-mediated cellular events such as in vitro cell growth [9], morphology [10] and motility [11]. All these GF-induced events depend on the rearrangement of actin and cytoskeleton proteins [12], suggesting cross-talk between the molecular systems that control cellular physiology and cell morphology.

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Despite the significant role of small G proteins, few data are available on the subcellular localisation of these molecules. Rho subfamily peptides have been found in nuclear, cytosolic or membrane fractions from cell and tissue extracts [13–15]; RhoA and RhoB have been observed with the immunogold technique in human fibroblasts [16] and in sea urchin sperm [17].

In the present study, the expression of constitutive RhoA was examined by immunohistochemical and immunoelectronmicroscopy techniques in resting and Matri-cell- and/or HGF-activated MDCK cells.

Materials and methods

Cells. MDCK cells, obtained from the Lombardia and Emilia Experimental Zooprophylactic Institute (Brescia, Italy), were cultured in Eagle's minimum essential medium (EMEM; Sigma, Milan, Italy). This medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Milan, Italy) and gentamicin (100 μg/ml; Gibco). The cells were grown almost to confluence in 25-ml plastic flasks and incubated in a 5% CO_2-humidified atmosphere at 37 °C. The cells were detached by treatment with trypsin-EDTA solution (Gibco) for 15 min, harvested and seeded onto 35-mm plastic dishes or similar dishes coated with extracellular matrix obtained from bovine cornea endothelial cells (Matri-cell dishes; P.B.I., Milan, Italy). The cells were dispensed in culture medium supplemented with or without 20 ng/ml HGF (Sigma) and incubated at 37 °C for 18 or 72 h.

Western blotting. Western blotting was employed to demonstrate the presence of RhoA protein in MDCK cells and to test anti-RhoA antibody specificity. In brief, subconfluent MDCK cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), homogenised in lysis buffer (10 mM Tris-HCl pH 7.5, 0.3 M saccharose, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF) and centrifuged for 10 min at 10,500 rpm. Equal protein amounts of the detergent-soluble fraction were boiled in sample buffer (50 mM Tris pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol and 0.025% bromophenol blue), separated on a 12% SDS-polyacrylamide mini-gel (Hoefer Instrument) and transferred to pure nitrocellulose membranes (Trans-Blot Transfer Medium; Bio-Rad, Milan, Italy) by a Hoefer Semiphor cell (Pharmacia Biotech, Milan, Italy). Blots were probed with polyclonal rabbit anti-RhoA or with blocked anti-RhoA immunoglobulins (1:1000 in PBS + 0.1% Tween 20 + 1% non-fat milk; Santa Cruz Biotechnology, Santa Cruz, Calif.) to test the specificity of RhoA immunoreaction. The primary antibody block was performed by adsorbing it with a tenfold excess of blocking peptide (Santa Cruz) for 2 h at room temperature. Signals were visualised by anti-mouse HRP-linked secondary antibody with enhanced chemiluminescence detection (ECL Western blotting analysis system; Amersham, Arlington Heights, Ill.).

Immunocytochemistry. After collection, MDCK cells were washed in PBS and fixed with 1% neutral buffered formalin (10 min), at 4 °C. The specimens were then permeabilised with a 10-min exposure to 0.2% Triton X-100 solution (Sigma). The samples were subsequently treated with a 3% H_2O_2 solution for 5 min to quench the endogenous peroxidase activity and with 5% swine serum (Dako, Milan, Italy)-PBS for 20 min at 37 °C to block non-specific reactivity. The samples were then incubated overnight (20 h) in a humidified chamber at 4 °C with the rabbit anti-RhoA polyclonal antibody (1:30 in 0.1% BSA-PBS; Santa Cruz Biotechnology). The detection protocol was carried out using biotinylated link antibodies and a streptavidin-peroxidase complex (LSAB kit; Dako). The reaction was developed by incubating samples in the substrate-chromogen solution [1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) containing 0.02% H_2O_2] for 5 min in the dark. Finally, the slides were mounted with Universal Mount (Research Genetics, Huntsville, Ala.) and observed with a DMRB Leica microscope using a $\times 40$ or $\times 100$ oil immersion lens. Between each step, slides were washed with PBS. All steps were performed at room temperature unless specified otherwise. Negative controls were obtained either by incubating the specimens with 0.1% BSA-PBS without the primary antibody or by using blocked primary antibody (see Western blotting).

Image analysis. The reacting surfaces and the degree of reactivity per cell were quantified with a Quantimet + image analysis system. Two series of ten microscopic fields were captured from each immunostained or control sample. After storage, the grey levels of immunostained sample pictures were compared with those from negative controls to ascertain the level above which the pictures were to be considered as the expression of the reacting products. Image analysis was performed taking into consideration only the positive cells; for each positive cell, the extension and the intensity of the reaction was calculated as the percentage of reacting surface in comparison to the total cell area and the mean level of grey intensity measured inside the positive area. For better expression of the distribution and the degree of reactivity, the final rate of immunoreactivity was calculated by the product of percentage of positive surface and the mean grey level of the reaction.

One-way analysis of variance (ANOVA) and Student's t-test were used to evaluate the statistical significance of the differences between the four different experimental conditions.

Immunoelectronmicroscopy. MDCK cells were fixed in situ at 4 °C by using a solution of 4% formaldehyde (freshly obtained from paraformaldehyde)-1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 60

min. The cells were harvested and postfixed with a 1% OsO_4 in 0.1 M phosphate buffer pH 7.2 for 60 min, washed in dH_2O, dehydrated with DMP, and finally embedded in Epon/Durcupan BEEM capsules no. 00 at 56 °C for 48 h. Ultrathin sections (20–30 nm thick) obtained by an Ultrotome Nova ultramicrotome (LKB, Bromma, Sweden) equipped with a diamond knife (Diatome, Biel, Switzerland), were placed on 400-square mesh nickel grids. Immunoreaction was then performed using 0.4 M PBS with 2.5% NaCl and at room temperature unless otherwise stated. The nickel grids, carrying the section face downwards, were placed on a droplet of saturated aqueous sodium metaperiodate solution in a humidified chamber for 30 min. After PBS washing, the sections were treated with 0.5 M NH_4Cl to reduce free aldehyde groups, which may cause undesired background staining. After PBS washing, the grids were then transferred to a droplet of 10% BSA-PBS for 15 min to prevent non-specific protein attraction. The incubation in rabbit polyclonal anti-RhoA antibody (Santa Cruz Biotechnology) diluted 1:400 in 1% BSA-PBS was performed overnight at 4 °C in a humidified chamber. The sections were then washed with 1% BSA-PBS to remove the unbound antibody and incubated with the appropriate gold (diameter 20 nm) conjugate secondary antibody (1:30 in 0.05% 100 Tween 20-PBS; Sigma). The grids were washed with PBS, fixed in 1% glutaraldehyde PBS for 3 min and slightly counterstained with uranyl acetate and lead citrate [18]. Observation was performed in a Jeol JEM-100SX transmission electron-microscope at 80 kV. Negative controls were carried out as described for the immunocytochemistry technique.

Results

RhoA antibody specificity was confirmed by performing Western blotting and using negative controls obtained with RhoA blocking peptide; MDCK cells analysed by immunoblotting showed the presence of a protein corresponding to the molecular weight of the small G protein RhoA (fig. 1). Furthermore, no specific immunoreaction was obtained by omitting the anti-RhoA polyclonal antibody or by using it neutralised with the respective blocking peptide in the immunocytochemical methods carried out in the present investigation (fig. 2A, B).

Immunocytochemistry. Resting MDCK cells (grown on plastic dishes without HGF) were characterised by a typical adherent cell shape and most of the cells showed a reasonable reactivity in the cytosol particularly within cytoplasmic vesicles; variable immunoreactivity was also observed in short plasma membrane tracts (fig. 2C).

MDCK cells cultured on Matri-cell dishes displayed greater immunoreactivity than cells grown in the other conditions. Matri-cell-activated MDCK cells showed remarkable amounts of reactivity in the cytoplasm, both at the cytosolic and vesicle level, and the plasma membranes appeared to be strongly reactive (fig. 2D). In contrast, most MDCK cells cultured with HGF either on plastic or Matri-cell showed reactivity localised in the cytosol and within cytoplasmic vesicles closely resembling that observed in resting MDCK cells (fig. 2E, F). RhoA expression in MDCK cells treated with Matri-cell and/or HGF for either 18 or 72 h was essentially the same.

Image analysis. Image analysis data are summarised in figure 3, where data from three independent experiments are reported. The degree of immunoreactivity for cells cultured on plastic was 239.14 ± 15.45 without HGF and 169.34 ± 16.14 with HGF exposure. Cells grown on Matri-cell dishes displayed a level of immunoreactivity of 630.23 ± 14.18 without HGF and 158.91 ± 6.71 after HGF treatment. ANOVA and Student's t-test were used to evaluate the statistical significance of the differences between the four groups. Cells grown on Matri-cell expressed significantly more RhoA protein than cells cultured on plastic, plastic plus HGF and Matri-cell plus HGF ($p = 5 \times 10^{-6}$, $p = 3 \times 10^{-6}$,

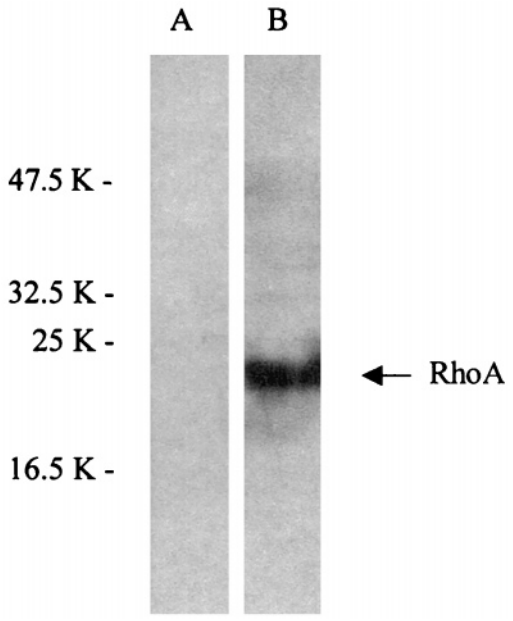

Figure 1. Western blotting analysis of RhoA expression in resting MDCK cells. Lane A, negative control for anti-RhoA antibody specificity obtained with the blocked primary antibody. Lane B, the blot probed with the anti-RhoA polyclonal antibody revealing the presence of RhoA protein.

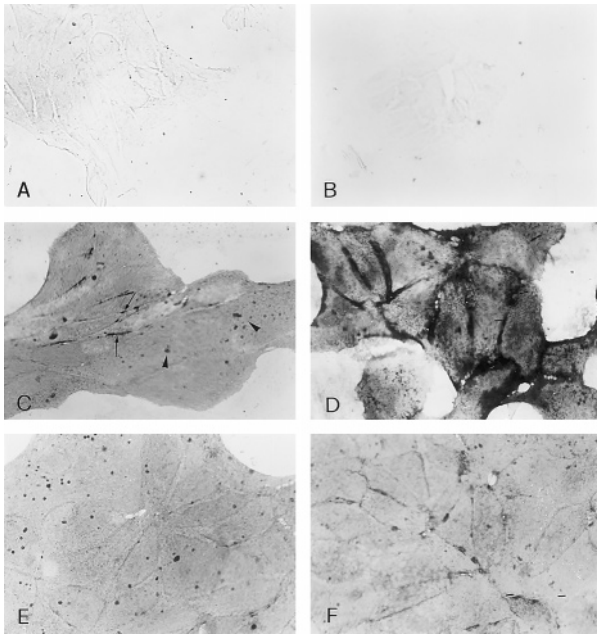

Figure 2. Indirect immunoperoxidase detection of RhoA distribution in MDCK cells cultured under different experimental conditions for 72 h. (*A*, *B*) MDCK cells grown on plastic dishes: negative control obtained by incubating the specimen without the primary antibody (*A*) or with blocked primary antibody (*B*). Magnification $\times 260$ (*A*), $\times 208$ (*B*). (*C*) MDCK cells grown on plastic dishes: RhoA is mainly present in the cytosol, where vesicles appeared particularly reactive (arrowheads). Variable immunoreactivity was also observed in short tracts of plasma membrane (arrows). Magnification $\times 208$. (*D*) MDCK cells cultured on Matri-cell dishes: preferential RhoA localisation is at the level of the plasma membrane. Magnification $\times 208$. (*E*, *F*) MDCK cells grown on plastic (*E*) or Matri-cell (*F*) dishes with HGF: the cells demonstrated reactivity mainly localised in the cytosol, similar to that observed in resting MDCK cells. Magnification $\times 208$.

$p < 10^{-6}$, respectively). The difference between the cells treated with HGF and the cells cultured on plastic dishes without HGF was also statistically significant ($10^{-2} < p \leq 10^{-3}$).

Immunoelectronmicroscopy. The ultrastructure of resting MDCK cells did not differ from that described elsewhere [19]. Subcellular protein detection carried out with the immunogold method enabled us to show a wide distribution of RhoA in various cellular compartments. Gold particles were mainly localised in the cytosol (fig. 4A, B), in certain microvilli, and within cytoplasm, either as isolated gold particles or clusters; however, very few gold labels were detectable at the plasma membrane level (fig. 4A). The nucleus was reactive: labelled particles were present in the more condensed chromatin and at the nuclear membrane (fig. 4B). Clusters of gold particles were often detected on each side of the nuclear membrane (fig. 4B), to give contiguous reactivity between the nucleus and related perinuclear cytoplasm. The nucleolus showed varying degrees of reactivity.

MDCK cells treated with HGF either on plastic or Matri-cell showed an immunolabelling picture similar to that observed in resting cells (fig. 4C). In contrast, the MDCK cells cultured on Matri-cell dishes without HGF showed enhanced expression of immunogold granules that were more frequently found along the peripheral plasma membrane (fig. 4D).

Discussion

The cellular and subcellular distribution of the small G protein RhoA was investigated in MDCK cells cultured on plastic- or Matri-cell-covered supports with or without HGF treatment; the results obtained by immunoperoxidase were analysed by computer-aided image analysis. Constitutive expression of RhoA was shown in resting MDCK cells, and it was found to be expressed at the plasma membrane, and in the cytoplasm and nucleus. RhoA localisation and the degree of its expression changed with experimental conditions: MDCK cells grown on Matri-cell showed the highest reactivity towards RhoA immunoglobulins with preferential RhoA localisation at the plasma membrane. HGF treatment lowered RhoA expression and prevented the Matri-cell-induced localisation of RhoA at the plasma membrane.

Interest in this molecule involved in the signal transduction of various membrane receptors derives from the part played by Rho small GTP/GDP-binding proteins

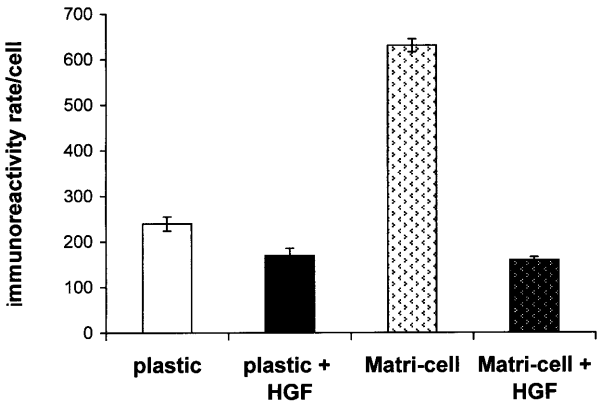

Figure 3. Image analysis of RhoA expression in MDCK cells cultured under different experimental conditions. RhoA reactivity was significantly higher in MDCK cells grown on Matri-cell dishes. Bars represent the average of immunoreactivity rates of three independent experiments $\pm$ SE (always less than 10% of the mean).

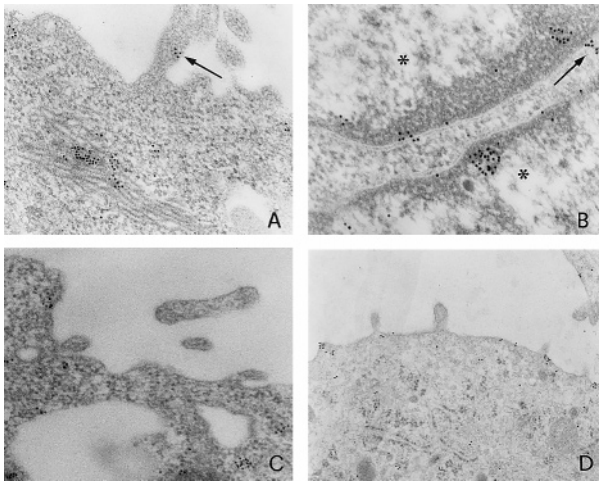

Figure 4. Immunoelectronmicroscopy for RhoA distribution in resting or activated MDCK cells. Paraformaldehyde 4%, glutaraldehyde 1%. OsO_4. Epon/Durcupan. (*A*, *B*) RhoA immunolocalisation in resting MDCK cells. (*A*) The reaction is widespread in the cytoplasm; clustered gold particles are present within the cytoplasm of microvilli and cytoskeleton structures; the plasma membrane is weakly reactive (arrow). Magnification × 30,000. (*B*) RhoA nuclear distribution: isolated or clustered gold particles are present within heterochromatin in the upper and lower part of the picture (*); gold markers are also present along the nuclear membrane and the cytoplasm (arrow). Magnification × 40,000. (*C*) RhoA immunolocalisation in HGF-activated MDCK cells cultured on Matri-cell. Isolated or clustered immunogold granules are found within the cytoplasm but no particles are present along the peripheral plasma membrane. Magnification × 28,000. (*D*) RhoA immunolocalisation in MDCK cells cultured on Matri-cell. Numerous gold particles are present along the peripheral plasma membrane. Magnification × 13,000.

in several biological processes among which the most relevant is cytoskeleton organisation during cell migration following GF and cytokine exposure. Fibroblasts stimulated by GF activate Ras, Rac1 and RhoA peptides suggesting the involvement of a G protein cascade after GF receptor activation [12, 20]; HGF also regulates MDCK cellular responses by Ras, Rac and RhoA pathways [21]. In particular, RhoA is required for stress fibre formation [12], disassembly and reassembly of which is observed in moving cells. HGF is able to induce cell migration by controlling various cellular processes including actin reorganisation and by decreasing stress fibre formation [22, 23].

Our experiments in resting MDCK cells (on plastic without HGF) using immunocytochemical and immunogold techniques demonstrated the subcellular localisation of constitutive RhoA: this protein is expressed most strongly in the cytosol, inside cytoplasmic vesicles and, to a lesser extent, along the plasma membrane, in agreement with the literature [13, 24]. Studies carried out on MDCK cells microinjected with expression vectors containing genes encoding Rho family peptides showed the presence of relevant amounts of RhoA and RhoC in the cytosol and of RhoB at the plasma membrane level as well as inside cytosolic vesicles by immunofluorescence and cellular fractionation techniques [13]. Furthermore, RhoA was also detected by the immunogold technique within the nucleus, mainly localised within the etherochromatin, and along the nuclear membrane of the MDCK cells under study; sometimes there was a cross-bridge localisation of gold clusters across the nuclear membrane to reveal contiguous nuclear and cytoplasmic reactivity. This picture might be the morphological pattern showing RhoA transmembrane migration from the cytoplasm to the nucleus and vice versa. G proteins likely act as signal transducers inside the nucleus as well [14, 25]: specific RhoA, but not RhoB nuclear localisation has been shown by Western blot on isolated nuclei [14], and RhoA-dependent phospholipase D activity has been detected inside the nucleus of an MDCK cell subclone [14].

The immunocytochemical methods and quantification by image analysis demonstrated RhoA expression and localisation dependent on the experimental condition used. MDCK cells grown on Matri-cell without HGF showed the highest RhoA expression and the most extensive RhoA localisation along plasma membranes. It is interesting to note that the subcellular localisation of Rho proteins corresponded to their metabolic form: inactive (GDP-bound) and active (GTP-bound) forms of Rho proteins show subcellular cytosol and membrane localisation, respectively [24–29]. Thus, MDCK cells cultured on Matri-cell and bearing RhoA mostly localised along their plasma membranes might contain the highest expression of RhoA in its active form. The high RhoA level detected in Matri-cell-grown MDCK cells might be explained by the low susceptibility to endogenous proteolytic degradation of the active, membrane-linked RhoA form: Desrosiers and coworkers [30] demonstrated that the GTPγS-bound RhoA remains in a conformational form that strengthens its attachment to the plasma membrane preventing proteolytic digestion. Another explanation for Matri-cell-induced RhoA increase might be that integrin receptor stimulation up-regulates this protein. Studies on Matrigel basement membrane matrix suggest that this substrate may influence gene expression in adult rat hepatocytes [31] as well as increase casein gene expression in mouse mammary epithelial cells [32]. RhoA is likely translocated to the plasma membrane following the activation of integrin receptors by extracellular matrix molecules contained in the Matri-cell substrate, e.g. fibronectin and laminin, in accordance with previous observations [26, 27]. Miyamoto and coworkers [28] have shown that simple integrin aggregation triggers 20

signal transduction molecules, including RhoA, at the plasma membrane level. The high expression of RhoA protein mainly found along the plasma membrane of Matri-cell-activated cells described here accords with recent studies carried out on rat MM1 hepatoma cells: overexpression of RhoA facilitates its translocation from the cytosol to the plasma membrane where it is activated and, consequently, a threshold level in RhoA expression is postulated for the RhoA activation cascade in each cell [33].

After HGF stimulation, RhoA expression in MDCK cells cultured on plastic or Matri-cell dishes decreased. This might have been due to down-regulation of protein synthesis [23] or to increased endogenous degradation of Rho protein. Following addition of HGF, a decrease in active, membrane-linked RhoA is particularly likely, in agreement with the decrease in RhoA activity following HGF treatment described by Ridley and coworkers [21]. Consequently, the level of inactive, cytosolic RhoA may increase and its susceptibility to endogenous proteolytic degradation become enhanced. Addition of HGF to MDCK cells cultured on Matri-cell dishes also interfered with the cellular localisation of RhoA, leading to its localisation mainly in the cytosol: under this experimental condition, the Matri-cell-induced localisation of RhoA at the plasma membrane is prevented by HGF. However, for MDCK cells grown on plastic dishes, HGF appears not to modify cellular RhoA localisation; under these conditions, RhoA is probably present mainly in the non-active form and therefore the inhibitory effect of HGF is negligible.

In conclusion, the results of this investigation demonstrate constitutive RhoA distribution in the MDCK cell line and show, from a morphological point of view, that interaction between the extracellular environment and cells can modulate the cellular expression and redistribution of the signal transducer RhoA.

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