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# Spreading of differentiating human monocytes is associated with a major increase in membrane-bound CDC42

**(Ras/Rho/phorbol ester/membranes/macrophages)**

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**ABSTRACT** **In a search for a model cell system that might allow studies of the function of the Rho-related GTPase CDC42Hs in human cells, we measured the content and distribution of CDC42Hs in monocytes that were differentiating into macrophages. The total content of this protein increased 5- to 6-fold in phorbol ester-treated human monocytic THP-1 and U-937 cells and increased 13-fold in normal human blood monocytes. Moreover, membrane-associated CDC42Hs in these cells increased 13-fold and 30-fold, respectively, while cytosolic CDC42Hs increased only 3- and 6-fold. Measurements made specifically in U-937 cells showed that the increase in membrane CDC42Hs correlated closely with an increase in cell spreading. The changes in CDC42Hs in U-937 cells probably depended on increased mRNA translation and/or decreased protein degradation, since no change in CDC42Hs mRNA could be detected. Finally, the changes in CDC42Hs were relatively specific, since contents of the CDC42Hs-binding protein Rho-GDI and the Rho-related protein Rac2 were unaffected and no change in CDC42Hs occurred when the cells were stimulated by agonists that induce monocytes to differentiate into nonadherent cells. These findings show that marked changes in content and distribution of CDC42Hs occur when monocytes differentiate into macrophages, suggesting that membrane CDC42Hs may play a role in cell spreading.**

CDC42 proteins are closely related, low-molecular-weight GTPases that are present in a variety of eukaryotic cells (1, 2). They belong to the Rho family of Ras-related GTPases which also includes RhoA, -B, -C, and -G and Rac1 and -2 (3) and which has been implicated in the control of cytoskeletal organization (4). Like all Ras-related proteins, CDC42 proteins bind and hydrolyze GTP, are thought to cycle between a GTP-bound active and a GDP-bound inactive state, and are regulated by GTPase-activating proteins and guanine nucleotide exchange factors (5). Furthermore, like several other Ras-related GTPases, they contain a C-terminal, geranylgeranylated cysteine residue that can also be carboxylmethylated (6). These posttranslational modifications are thought to promote important protein–membrane and protein–protein interactions (7–9). In contrast to many other Ras-related GTPases, CDC42 proteins seem to be located mainly in the cytosol, where they are bound to Rho-GDI (Rho GDP-dissociation inhibitor; refs. 1, 10, and 11). How cytosolic CDC42 proteins translocate to membranes remains to be determined, but exchange of GTP for bound GDP, dissociation of CDC42 from Rho-GDI, and α-carboxyl methyl esterification of CDC42 may all be involved (11).

The function of CDC42 proteins has yet to be clarified, but CDC42Sc, the isoform of CDC42 that is present in *Saccharomyces cerevisiae*, has been shown to be required for bud-site assembly (12). Furthermore, two related proteins are also required, a protein geranylgeranyltransferase that catalyzes the prenylation of CDC42Sc and a guanine nucleotide exchange factor for CDC42Sc (13, 14). Two genes that encode human CDC42 (CDC42 *Homo sapiens*, CDC42Hs) separately complement mutations in the yeast CDC42Sc gene that cause defective budding (15, 16). These CDC42Hs genes encode protein isoforms that are identical except for an 8-amino acid segment at the C terminus and one additional conservative substitution (15, 16). Unfortunately, methods that allow the isoforms to be analyzed separately have yet to be developed. In addition, studies of the function of the CDC42 isoforms have been hindered by the lack of model cell systems that show marked changes in CDC42 expression that can be correlated with biological effects.

In this report we show that the differentiation of human monocytes into macrophages is accompanied by dramatic increases in the contents of total and membrane-associated CDC42Hs and that the increased content of membrane-associated CDC42Hs correlates closely with cell spreading. This establishes the differentiating macrophage as a suitable model system for studying the regulation and function of CDC42Hs in human cells.

## MATERIALS AND METHODS

**Materials.** RPMI 1640, fetal bovine serum, L-glutamine, and other chemicals were purchased from Sigma. U-937 cells were a gift from C. Wilson (Children's Hospital, University of Washington, Seattle). THP-1 cells were purchased from the American Type Culture Collection (Rockville, MD).

**Cell Culture and Cell Preparation.** U-937 and THP-1 cells were grown in RPMI 1640 with 2 mM L-glutamine and 10% fetal bovine serum at 37°C in a humidified 5% $CO_2$ atmosphere. Mononuclear cells were isolated from human blood by centrifugation in Ficoll/Hypaque (Seromed, Berlin; ref. 17). Monocytes were allowed to attach to culture dishes for 2 hr and nonadherent cells were removed by gentle washing. Adherent monocytes were cultured for 10–12 days in RPMI 1640 containing 20% human autologous serum and medium was changed every 3 days. For homogenization, adherent cells were first brought into suspension by trypsinization. Suspended cells [5–10 × $10^6$ per ml in ice-cold homogenization buffer containing 250 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA; 1 μg of pepstatin, leupeptin, and aprotinin per ml, and 0.2 mM phenylmethylsulfonyl fluoride] were sonified for 10 sec at 50 W with a Labsonic U (B. Braun, Melsungen, F.R.G.). Membranes and cytosol were separated

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; $Bt_2cAMP$, dibutyryl cyclic AMP; CDC42Sc and CDC42Hs, CDC42 of *Saccharomyces cerevisiae* and CDC42 of *Homo sapiens*, respectively.

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by centrifugation for 30 min at 100,000 × *g* in a Beckman TL-100 ultracentrifuge.

**Microscopy and Flow Cytometry.** Assays for substratum adherence were performed with cells grown on plastic culture dishes. Cell–cell adherence (which had a time course similar to that of cell–substratum adherence) was kept to a minimum by plating cells at low density (1–3 × $10^5$ cells per 60-mm-diameter dish). To determine the percentage of adherent cells, we removed nonadherent cells by gently washing the culture dish with warm phosphate-buffered saline and then removed the remaining, adherent cells by trypsinization. The percentage of spread cells was determined under a phase-contrast microscope. Adherent cells showing a broad, thin rim of cytoplasm surrounding the centrally located nucleus or completely flattened cells were defined as spread.

For immunofluorescence, cells on glass coverslips (18 mm × 18 mm, no. 1; Fisher Scientific) were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline and permeabilized for 10 min with 0.5% Triton X-100. Vinculin was detected with a monoclonal anti-vinculin primary antibody (Boehringer Mannheim) and a fluorescein isothiocyanate-conjugated rat anti-mouse IgG1 secondary antibody (Serotec, Oxford, England). Cells were examined with a Zeiss Axiophot microscope, and those showing vinculin concentrated in focal adhesion sites or podosomes (18) were defined as spread.

For flow cytometry, cells (2 × $10^5$ in 200 μl) were incubated for 30 min at 4°C in phosphate-buffered saline with a 1:100 dilution of fluorescein isothiocyanate-conjugated monoclonal anti-CD11b or isotype control (Coulter). Samples were washed and analyzed at $10^4$ cells per sample in a flow cytometer (Becton Dickinson). Cells showing significant specific fluorescence intensity were defined as positive.

**Antibodies and Western Blots.** Rabbit polyclonal antibodies were raised against peptides corresponding to amino acids 167–183 and 120–140 of the cDNA-predicted sequences of CDC42Hs. These amino acid sequences are identical in the two known isoforms of CDC42Hs except for positions 182 and 183 (15, 16). Antibodies were also prepared against peptides corresponding to amino acids 17–28 of the cDNA-predicted sequence of Rho-GDI (19). Synthetic peptides (Peptide Synthesis Facility, Howard Hughes Medical Institute, University of Washington) containing an additional N-terminal cysteine were coupled to maleimide-activated keyhole limpet hemocyanin (Pierce) according to the manufacturer's instructions. Immunogens were injected into New Zealand White rabbits and the antibodies generated were affinity-purified on a column containing the respective peptides coupled to SulfoLink gel (Pierce). Antibodies against peptides corresponding to amino acids 178–188 of Rac-2 were obtained from Santa Cruz Biotechnology.

Specificity of antibodies was tested on Western blots of recombinant glutathione *S*-transferase fusion proteins of CDC42Hs, Rac1, or RhoA (20). After SDS/12.5% PAGE (Mini-Protean II, Bio-Rad), proteins were electrophoretically transferred to Immobilon poly(vinylidene difluoride) membranes (Millipore) for 1 hr at 80 V in 20 mM Tris/192 mM glycine. Membranes were blocked for 1 hr in a 150 mM NaCl/20 mM Tris·HCl, pH 7.4/0.3% Tween/10% calf serum; then they were incubated for 1 hr with the primary antibodies (1:200 to 1:1000). After incubation for 45 min with peroxidase-labeled donkey anti-rabbit IgG (1:5000; Amersham), blots were developed with enhanced chemiluminescence (Amersham) and then exposed to x-Omat AR film (Kodak).

Specific bands were quantified by laser densitometry (Ultroscan XL, Pharmacia) using peak area integration according to the manufacturer's instructions. Values were expressed in arbitrary optical density units. The amount of cell material (typically from 3–8 × $10^5$ cells) and the exposure times (30 sec to 5 min) were chosen to yield absorbances that were in the linear range of the assay, as determined with known amounts of recombinant CDC42Hs protein or serial dilutions of U-937 cytosol. In any given experiment, material from equal numbers of cells was compared.

**Northern Blots.** RNA isolation and Northern blot analysis were performed as described (21). In brief, total cellular RNA was isolated and 10-μg samples were fractionated in a 1.2% agarose/formaldehyde gel and transferred onto a Nytran nylon membrane (Schleicher & Schull). Hybridization was performed for 2 hr at 68°C with QuickHyb hybridization solution (Stratagene) containing a $^{32}$P-labeled 1.0-kb CDC42 probe (4 × $10^6$ cpm/ml). The CDC42Hs cDNA (16) probe was prepared by priming with random hexamers and hence would probably not distinguish between the two known CDC42Hs isoforms. The membrane was washed three times for 20 min at 60°C with 0.1% SDS in 0.1× standard saline citrate and exposed for 8 hr to Kodak X-Omat AR film at −80°C. A $^{32}$P-labeled β-actin probe ($10^6$ cpm/ml) was used to monitor the quantity of RNA loaded on the gel.

## RESULTS

**Content of Total CDC42Hs Increases in Differentiating Human Monocytes.** A major goal of the present study was to determine whether the content of CDC42Hs in human monocytic cells increases when these cells differentiate into macrophages. To examine this question we took advantage of two immature monocytic cell lines, U-937 and THP-1, which grow in suspension but adhere to a culture dish, spread on its surface, and express macrophage differentiation markers in response to phorbol 12-myristate 13-acetate (PMA; refs. 18 and 22; M.A., unpublished observations). We stimulated the U-937 cells with PMA and demonstrated that >90% of the cells attached to a culture dish and spread, as determined by phase-contrast microscopy and immunostaining for the focal adhesion protein vinculin (data not shown). In addition, we measured the total cellular content of CDC42Hs by Western blot analysis and showed that it increased 5- to 6-fold in the PMA-treated cells (Fig. 1). We obtained similar results in experiments with PMA-treated THP-1 cells (data not shown)

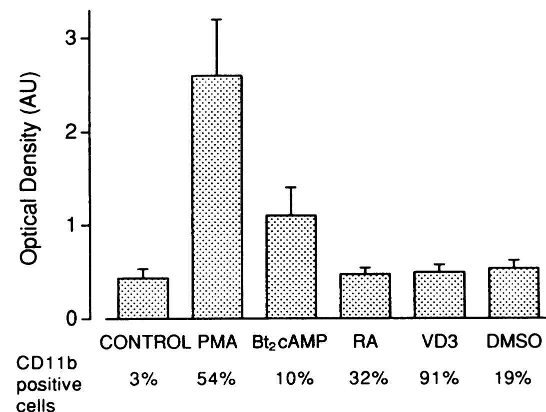


FIG. 1. Contents of CDC42Hs and CD11b in U-937 cells treated with various differentiation agents. U-937 cells were treated for 72 hr with or without 10 nM PMA, 500 μM dibutyryl cyclic AMP ($Bt_2cAMP$), 1 μM retinoic acid (RA), 10 nM 1γ,25-dihydroxyvitamin $D_3$ ($VD_3$) or 1.5% (vol/vol) dimethyl sulfoxide (DMSO). Total cell homogenates from equal amounts of cells were analyzed on Western blots with enhanced chemiluminescence detection; CDC42HS was quantified by laser densitometry, and the amounts present were expressed in arbitrary optical density units (AU), reflecting the integrated areas of peaks corresponding to the CDC42Hs bands. Each bar represents the mean ± SD of three experiments with 3–5 × $10^5$ cells performed in duplicate. The percentage of CD11b-positive cells was determined by flow cytometry. Measurements of CD11b in one experiment representative of two are shown.

and verified that the changes in CDC42Hs were associated with the monocyte–macrophage differentiation pathway by demonstrating that the content of CDC42Hs increased 13-fold in normal human blood monocytes that were allowed to differentiate into macrophages for 11 days in culture (data not shown, but see below).

To evaluate the specificity of the changes in content of CDC42Hs, we stimulated U-937 cells with PMA and measured the contents of two CDC42Hs-related proteins, the small GTPase Rac2, which is the closest known homologue of CDC42Hs in mammalian cells (3), and Rho-GDI, a protein that binds CDC42Hs and other Rho-like GTPases in the cytosol (10, 11, 23). The fact that these proteins did not increase (Fig. 2) suggested that the PMA-induced changes in content of CDC42Hs may have been relatively selective.

We also stimulated U-937 cells with $Bt_2cAMP$, retinoic acid, 1$\alpha$,25-dihydroxyvitamin $D_3$, or dimethyl sulfoxide. Each of these substances induced some degree of cell differentiation as revealed by an increased expression of the phagocyte integrin CD11b (24), but there was no correlation between the degree of CD11b expression and cellular levels of CDC42Hs (Fig. 1). Moreover, $Bt_2cAMP$ was the only one of the substances that caused cell changes similar to those observed for PMA: >90% of the $Bt_2cAMP$-treated cells attached to the culture dish, 20–30% of the attached cells spread out, and the attached cells showed a 2- to 3-fold increase in total content of CDC42Hs. These results suggest that the cellular content of CDC42Hs increases when differentiating monocytic cells adhere to a culture dish and spread, but not when they remain in suspension.

**Subcellular Distribution of CDC42Hs Changes in Differentiating Monocytes.** In undifferentiated U-937 cells only 5–10% of the total cellular CDC42Hs was bound to membranes, the remainder being located in the cytosol. However, when these cells were induced to spread by PMA, the relative content of membrane-bound CDC42Hs increased to 30%, reflecting a 15-fold increase in total membrane-bound CDC42Hs and a 3-fold increase in cytosolic CDC42Hs (Fig. 3 *B* and *C*). Comparable results were obtained for THP-1 cells (data not shown) and normal human blood monocytes (Fig. 4), though the relative content of membrane-bound CDC42Hs in the blood monocytes increased from 30% to 60% when they were allowed to differentiate into macrophages for 11 days in culture, reflecting a 30-fold increase in membrane-bound CDC42Hs and a 6-fold increase in cytosolic CDC42Hs. These results provide evidence that the differentiation of monocytes into macrophages is associated with a large increase in membrane-bound CDC42Hs.

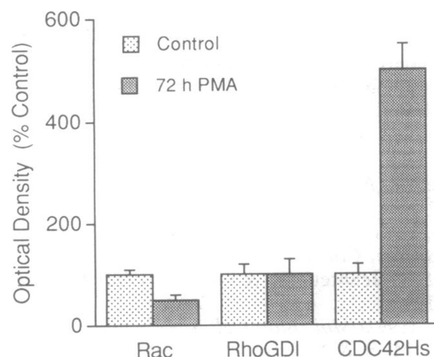


FIG. 2. Contents of Rac2, Rho-GDI, and CDC42Hs in control and PMA-treated U-937 cells. U-937 cells were treated for 72 hr without (light bars) or with (dark bars) 10 nM PMA and total cell homogenates from equal numbers of cells were analyzed as described for CDC42Hs in Fig. 1. Each bar represents the mean ± SD of three experiments performed in duplicate.

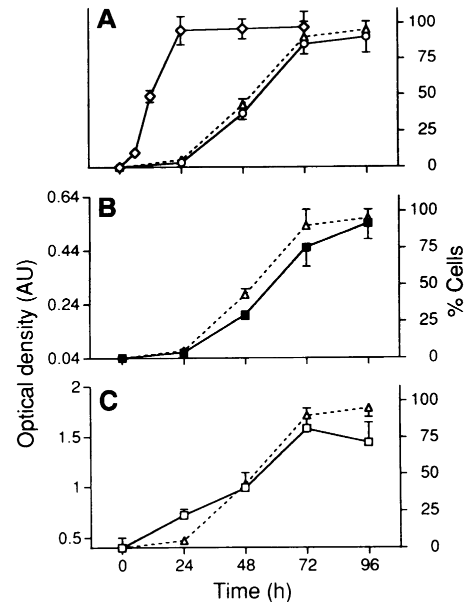


FIG. 3. Time courses of cell adherence, cell spreading, and increases in membrane-bound and cytosolic CDC42Hs in PMA-treated U-937 cells. U-937 cells were treated with 10 nM PMA for the indicated times and then analyzed. (*A*) Percentages of adherent (◇) and spread (△) cells grown on culture dishes, determined by phase-contrast microscopy, and percentage of spread cells (○) grown on glass coverslips, determined by immunostaining for vinculin. (*B*) Comparison of data for cell spreading (△) from *A* with content of membrane-bound CDC42Hs (■) in 8 × $10^5$ cells. (*C*) Comparison of data for cell spreading (△) from *A* with content of cytosolic CDC42Hs (□) in 8 × $10^5$ cells. Each value represents the mean ± SD of three experiments.

**Time Course of Accumulation of Membrane-Bound CDC42Hs Correlates with That of Cell Spreading.** To obtain further information about the relation between CDC42Hs and cell spreading, we compared the time courses of cell adherence, cell spreading, and accumulation of membrane-bound and cytosolic CDC42Hs in PMA-treated U-937 cells. Cells began to adhere to the culture dish as early as 3 hr after the addition of PMA, whereupon adherence increased until it became maximal at 24 hr (Fig. 3*A*). In contrast, cell spreading became detectable only after the first 24 hr of incubation. The proportion of spread cells then increased at a linear rate for the next 48 hr until it reached a plateau. The time course of the change in cell spreading exactly paralleled the time course

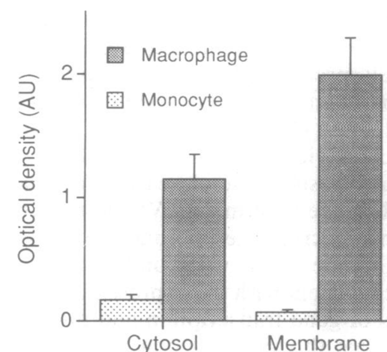


FIG. 4. Cytosolic and membrane-bound CDC42Hs in normal human monocytes and macrophages. Human blood-derived monocytes were prepared and allowed to differentiate into macrophages for 11 days in culture. Cytosol and membranes from 5 × $10^5$ monocytes (light bars) and macrophages (dark bars) were prepared by ultracentrifugation and analyzed as described for Fig. 1. Each bar represents the mean ± SD of three experiments. AU, arbitrary units.

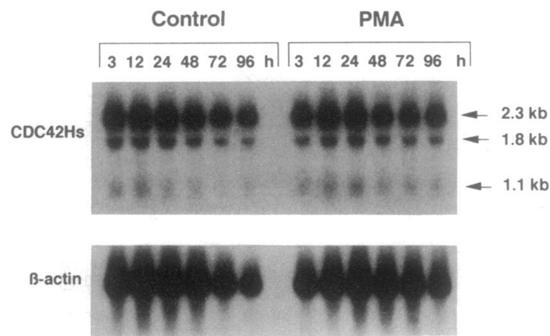


FIG. 5. CDC42Hs mRNA in undifferentiated (control) and PMA-treated U-937 cells. Total cellular RNA was isolated at the indicated times from control U-937 cells or U-937 cells that had been treated for 72 hr with 10 nM PMA. Then 10-$\mu$g samples of the total RNA were analyzed for CDC42Hs mRNA or $\beta$-actin mRNA. One experiment representative of three is shown.

of the increase in membrane-bound CDC42Hs but correlated less well with the time course of the increase in cytosolic CDC42Hs (Fig. 3 *B* and *C*). In three separate experiments an increase in cytosolic CDC42Hs was already evident 24 hr after the addition of PMA. Furthermore, two comparable time-course experiments with PMA-treated THP-1 cells yielded similar results (data not shown).

**Increase in CDC42Hs Protein Cannot Be Explained by Increased Transcription.** To investigate the molecular mechanism responsible for the increase in CDC42Hs protein during spreading of monocytes, we compared CDC42Hs mRNA levels in untreated and PMA-treated U-937 cells. Northern blot analysis revealed that at least one CDC42Hs gene was strongly expressed in both types of cells (Fig. 5). One strong and one weaker band hybridized with the CDC42Hs probe at 2.3 kb and 1.8 kb, respectively. In addition, a relatively faint band was seen at 1.1 kb. Treatment of U-937 cells with PMA caused no change in intensity of any of these bands at any time point. These results indicate that the increase in CDC42Hs protein that accompanies monocyte spreading is not caused by increased gene transcription.

## DISCUSSION

The results support the following conclusions. (*i*) The conversion of human monocytic cells into macrophage-like cells is accompanied by a substantial increase in CDC42Hs protein but not by an increase in CDC42Hs mRNA. (*ii*) The increase in CDC42Hs protein is associated with a change in the distribution of CDC42Hs from the cytosol to cell membranes. (*iii*) The increase in membrane-bound CDC42Hs correlates closely with cell spreading. Though these conclusions depend mainly on experiments that were done with PMA-stimulated monocytic cell lines, experiments with differentiating normal human monocytes yielded even more striking results.

The molecular basis of the increase in cellular CDC42Hs remains largely to be determined. We do not know whether monocytes and macrophages normally express more than one CDC42Hs gene. That we found three transcripts for CDC42Hs is consistent with the expression of several genes, but intricacies of gene transcription and mRNA processing might also explain this phenomenon (25). We do not know whether the increase in CDC42Hs content that accompanied monocyte spreading depended mainly on an increased rate of CDC42Hs mRNA translation or a decreased rate of CDC42Hs degradation. With regard to the latter possibility, the membrane-bound form of CDC42Hs might conceivably have been stabilized by interactions with other membrane proteins, since protein–protein interactions have been shown to prevent several other membrane proteins from degradation (26, 27).

The mechanisms that cause CDC42Hs to translocate from the cytosol to membranes in spreading monocytes have yet to be clarified. Studies of other cell systems have suggested that the cytosolic form of CDC42Hs contains bound GDP, lacks an $\alpha$-carboxyl methyl group, and is largely present as a 1:1 complex with Rho-GDI (10, 11). Therefore, translocation of cytosolic CDC42Hs to membranes may conceivably require dissociation of the CDC42Hs/Rho-GDI complex, replacement of the bound GDP with GTP, and $\alpha$-carboxyl methyl esterification of the C-terminal geranylgeranylcysteine of CDC42Hs. Tests of these and other possibilities should be feasible with the differentiating monocyte system.

The monocyte system may also allow studies of the function of CDC42Hs in animal cells. The fact that differentiating human monocytes show a dramatic increase in the relative content of membrane-bound CDC42Hs that correlates with cell spreading (Fig. 3*B*) suggests that membrane-bound CDC42Hs may in some way contribute to the spreading process. For example, membrane-bound CDC42Hs might conceivably promote clustering of integrins in the ventral surface of the plasma membrane and thus strengthen the linkage between extracellular matrix proteins and the interior cytoskeleton. Such a role might be compatible with the role of CDC42Sc in promoting the assembly of bud components in yeast. It might also be consistent with the suggested involvement of Rho proteins in the activity of the leukocyte integrin LFA-1 (28), the known roles of Rho and Rac proteins in promoting cytoskeletal organization (29, 30), and the demonstrated effect of guanosine 5′-[$\gamma$-thio]triphosphate on cell spreading in *Xenopus* fibrobasts (31).

To obtain further information about the role of CDC42Hs in cell spreading, the following question should be addressed. Is CDC42Hs mainly bound to the plasma membrane? Does it codistribute with integrins or other proteins that promote cell–substratum attachment? Do interventions that affect cell spreading influence the translocation of CDC42Hs between membranes and the cytosol? Does the experimental "knock out" of active CDC42Hs affect the spreading process? These are all questions that might profitably be examined with the differentiating monocyte system.

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