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Regulation of Scatter Factor/Hepatocyte Growth Factor Responses by Ras, Rac, and Rho in MDCK Cells

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Scatter factor/hepatocyte growth factor (SF/HGF) stimulates the motility of epithelial cells, initially inducing centrifugal spreading of cell colonies followed by disruption of cell-cell junctions and subsequent cell scattering. These responses are accompanied by changes in the actin cytoskeleton, including increased membrane ruffling and lamellipodium extension, disappearance of peripheral actin bundles at the edges of colonies, and an overall decrease in stress fibers. The roles of the small GTP-binding proteins Ras, Rac, and Rho in regulating responses to SF/HGF were investigated by microinjection. Inhibition of endogenous Ras proteins prevented SF/HGF-induced actin reorganization, spreading, and scattering, whereas microinjection of activated H-Ras protein stimulated spreading and actin reorganization but not scattering. When a dominant inhibitor of Rac was injected, SF/HGF- and Ras-induced spreading and actin reorganization were prevented, although activated Rac alone did not stimulate either response. Microinjection of activated Rho inhibited spreading and scattering, while inhibition of Rho function led to the disappearance of stress fibers and peripheral bundles but did not prevent SF/HGF-induced motility. We conclude that Ras and Rac act downstream of the SF/HGF receptor $p190^{Met}$ to mediate cell spreading but that an additional signal is required to induce scattering.

Regulated changes in actin polymerization and depolymerization are believed to act as the driving force for cell motility, and alterations in the organization of actin filaments accompany the induction of motility (10). Membrane ruffles and lamellipodia are located at the leading edges of motile cells, and their formation is rapidly stimulated by chemoattractants (41). In addition, motile fibroblasts contain fewer stress fibers than their nonmotile counterparts (7, 22, 50). Transformed cells in general are much more motile than their nontransformed counterparts; for example, Ras-transformed cells are highly motile, and microinjection of oncogenic Ras proteins stimulates cell motility within 8 to 10 h (26, 60). A role for endogenous Ras proteins in regulating motility is suggested by the observation that a dominant inhibitor of Ras, N17 Ras, inhibits the migration of endothelial cells in response to wounding (52).

Recent experiments suggest that other small GTP-binding proteins in addition to Ras could play a role in regulating cell motility. In Swiss 3T3 fibroblasts, the Ras-related proteins Rac and Rho have been shown to regulate actin reorganization induced by extracellular factors. Rac is specifically required for the formation of membrane ruffles, whereas Rho is required for the formation of stress fibers (42, 43). Microinjection of Ras proteins stimulates membrane ruffling, but at least in Swiss 3T3 cells, this is dependent on Rac; growth factor-induced membrane ruffling does not require Ras proteins (3, 43). In addition, microinjection of C3 transferase, an inhibitor of Rho function, inhibits the motility of Swiss 3T3 cells (57). C3 transferase has also been reported to inhibit the motility of neutrophils and of sperm (23, 53).

Scatter factor/hepatocyte growth factor (SF/HGF) is a glycoprotein secreted by cells of mesenchymal origin, and it stimulates the motility of several types of epithelial and endothelial cells, including the Madin-Darby canine kidney (MDCK) epithelial cell line (46, 55; for a review, see reference 15). It induces membrane ruffling and centrifugal spreading of epithelial cell colonies, disrupts cell-cell contacts, and is a chemoattractant but does not appear to stimulate the growth of MDCK cells (14, 54). SF/HGF has been reported to induce actin reorganization, including a decrease in stress fibers (11). It also induces MDCK cells to invade a collagen matrix and form branching networks of tubules (62) and stimulates blood vessel formation in vivo (9, 17). SF/HGF was independently characterized as a mitogen for cultured hepatocytes and subsequently for other cell types (29, 31, 33, 34). Purification, amino acid sequencing, and subsequent cDNA cloning of HGF and SF showed that they were identical (37, 61). SF/HGF is a 92-kDa heterodimer secreted as a single-chain, biologically inactive precursor (pro-SF/HGF) which is found predominantly in a matrix-associated form and is activated by proteolytic cleavage (30, 35). The heavy chain alone or a truncated variant of it can induce scattering of MDCK cells but is not mitogenic for hepatocytes, indicating that the two biological activities of SF/HGF can be dissociated (21).

The receptor for SF/HGF is the transmembrane tyrosine kinase $p190^{Met}$ encoded by the c-*met* proto-oncogene (6, 36, 37). Fibroblasts do not normally express $p190^{Met}$, but when transfected with human c-*met* cDNA, NIH 3T3 fibroblasts express functional receptors and respond to human SF/HGF with increased motility and invasion of extracellular matrices (16). Cells coexpressing both $p190^{Met}$ and SF/HGF are tumorigenic (44). $p190^{Met}$ is autophosphorylated in response to SF/HGF and has been shown to bind to a number of intracellular proteins containing *src* homology 2 (SH2) domains, including phosphatidylinositol 3-kinase, Ras-GTPase-activating protein, and phospholipase C-γ (2, 18, 40). Recently, it has been shown that SF/HGF activates Ras, increasing the level of Ras bound

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to GTP in cells by stimulating guanine nucleotide exchange (19).

We have used the potent motogenic activity of SF/HGF on MDCK cells to study the roles of Ras, Rac, and Rho proteins in regulating motility. We find that activation of both Ras and Rac is required for SF/HGF-induced motility, whereas an increase in active Rho inhibits motility.

MATERIALS AND METHODS

Cell culture and microinjection. MDCK cells were obtained from Flow Laboratories. Their behavior was not homogeneous, as some cells within the population were spontaneously motile in the absence of SF/HGF. A subclone which behaved as previously described for MDCK cells was therefore obtained by limiting dilution (56): it grew as discrete colonies when seeded at low density and responded to SF/HGF by centrifugal spreading and scattering. MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% bovine fetal calf serum. Cells for microinjection were seeded at 5×10^3 cells per well (diameter, 1.8 cm) on glass coverslips marked with a cross to facilitate localization of injected cells. After 3 days, cells were transferred to Dulbecco's modified Eagle's medium containing 5% fetal calf serum and colonies of between 8 and 64 cells were microinjected. For time-lapse videomicroscopy, all of the cells in a colony were injected. For analysis of actin organization, approximately 50% of the cells in each colony were injected with proteins as indicated together with 0.5 mg of rat immunoglobulin G (IgG) per ml as an inert marker protein to identify injected cells. Proteins were diluted in 50 mM Tris-Cl (pH 7.5)–100 mM NaCl–5 mM $MgCl_2$ immediately prior to microinjection.

The C3 transferase microinjected in these experiments was a different batch from that used previously (42), and the concentrations cannot be directly compared as they represent total protein and not active C3 transferase.

Pure recombinant human SF/HGF was a gift from G. Gaudino (University of Turin), and it was obtained from the supernatant of sf9 cells infected with a baculovirus carrying the human SF/HGF cDNA. Conditioned medium containing SF/HGF was obtained from Neuro2A cells transfected with the expression vector pBAT containing the cDNA encoding the 723-amino-acid form of human SF/HGF, as previously described (9). Conditioned medium was collected 3 days after transfection and titrated for SF/HGF activity in the scatter assay of MDCK cells. The conditioned medium contained serum, and the pro-SF/HGF secreted by Neuro2A cells was therefore proteolytically cleaved to form the active heterodimer (35). Control medium (CM) was conditioned medium from Neuro2A cells transfected with the vector pBAT without any insert.

Purification of recombinant proteins. H-Ras, RhoA, and Rac1 cDNAs were subcloned into the pGEX-2T vector and were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* (51). They were purified by binding to glutathione-agarose beads (Sigma Chemical Co.) and cleaved with thrombin to remove the glutathione *S*-transferase, as previously described (43).

Immunofluorescence. For immunofluorescence analysis, cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. Actin filaments were localized by incubating cells for 30 min with 0.5 μg of TRITC (tetramethyl rhodamine isothiocyanate)-labelled phalloidin (Sigma Chemical Co.) per ml, and cells containing rat IgG (the microinjection marker) were identified by incubation with 1:500 fluorescein isothiocyanate-conjugated goat anti-rat IgG (Sigma Chemical Co.). Vinculin was localized as previously described (42). The outer cells of microinjected colonies were analyzed for lamellipodia, membrane ruffles, and peripheral bundles. Cells were viewed with a Zeiss Axiophot microscope and photographed with Kodak T-MAX 400 film. All photographs were focused on the basal surfaces of the cells.

Confocal laser-scanning microscopy. Confocal laser-scanning microscopy was carried out with an Axioplan microscope (Zeiss, Oberkochen, Germany) aligned with a modified MRC 500 confocal visualization system (Bio-Rad, Hemel Hempstead, United Kingdom). The configuration and regulation of the laser illumination, the scanning microscope optics, the appropriate methods of alignment of the confocal optics, and the most accurate means of displaying the images have been described previously (12). The immunofluorescence filter sets and means of collecting images so that both low- and high-level signals are not lost have been described elsewhere (5), except that here each final image was the average of 120 passes of the laser illumination. Images of axial (*z*) sections were collected by scanning a single line and incrementing the stage by 0.195 μm between successive lines in the image. Conventional phase contrast images, in perfect registration with the confocal fluorescence images, were generated by relaying the transmitted laser illumination, collected with the phase contrast-configured condenser, to a photomultiplier tube in the confocal scanning head via a fiber-optic link.

Time-lapse videomicroscopy. Cells were filmed with a Nikon inverted microscope linked to a Panasonic camera and time-lapse video recorder. In each experiment, two adjacent colonies with similar numbers of cells and areas were chosen for microinjection and filming so that variations in colony behavior would be minimized as much as possible. The colonies contained between 20 and 50 cells, and all of the cells in each colony were microinjected. One colony was injected with the protein of interest, while the adjacent colony was injected with a control protein, usually rat IgG.

RESULTS

SF/HGF-induced changes in cell morphology and the actin cytoskeleton. MDCK cells proliferated as discrete colonies when sparsely seeded on glass coverslips (Fig. 1A) (56). Their response to purified SF/HGF or to conditioned medium containing SF/HGF (see Materials and Methods) was investigated 3 days after seeding, when the majority of colonies contained between 20 and 40 cells. As has been previously described (11, 56), cell colonies spread centrifugally during the first 4 to 6 h after the addition of SF/HGF so that the area covered by each cell and therefore by each colony increased between two- and threefold but cell-cell contacts were not broken (Fig. 1C and 2A). Many cells at the outer edges of colonies clearly showed a strong membrane ruffling response as observed by time-lapse videomicroscopy. In contrast, in cultures treated with CM (see Materials and Methods), cell areas did not increase significantly; the small increases in colony areas over the first 4 h were due to increases in cell numbers (Fig. 2A). Subsequent to cell spreading, the cells treated with SF/HGF showed a scattering response: cell-cell interactions decreased, some cells within each colony detached from their neighboring cells, and these cells assumed a shape resembling that of motile fibroblasts (Fig. 1E). The average motility of MDCK cells during this stage of their response to SF/HGF was approximately twice that of control cells (Fig. 3).

Changes in actin cytoskeletal organization following the addition of SF/HGF to MDCK cells have previously been reported (11) and are likely to be required for the motility response. To determine whether the small GTP-binding proteins Ras, Rac, and Rho regulate SF/HGF-induced actin reorganization, we first examined the distribution of actin filaments in MDCK cells over 16 h following SF/HGF addition. In unstimulated cells, stress fibers were located close to the basal membrane (Fig. 1D), and vinculin, a focal adhesion protein, was localized to the ends of these stress fibers where they attached to the basal membrane (data not shown). In addition, bundles of actin fibers, known as peripheral bundles, ran parallel to the outer membranes of many cells at the edges of colonies (e.g., see the arrow in Fig. 1D). Such actin bundles have been previously observed, both at the edges of epithelial cell colonies (11) and at the edges of wounds in epithelial cell monolayers (4, 28). These bundles were not homogeneous, and breaks and occasionally two distinct bundles were observed (Fig. 1D to F; the region around the arrow in Fig. 1D is shown at a higher magnification in Fig. 1F, lower part). By laser-scanning confocal microscopy, axial (*z*) sections clearly showed the localization of peripheral bundles at the basal edges of outer cells (Fig. 1F, upper part). Approximately 15% of the outer cells did not contain significant peripheral bundles (Fig. 4A), and in these cells, the plasma membrane protruded as a lamellipodium from the smooth outer boundary of the colony. Some cells had peripheral bundles which were partially disrupted or broken at one point along their outer membrane; these cells were not included in Fig. 4. Actin filaments were also located in the cortical actin network under the plasma membrane along cell-cell boundaries and in numerous microvilli on the ventral, or upper, surface of each cell (above the plane of the section in Fig. 1D).

During the first 4 h following the addition of SF/HGF, a large increase in the number of lamellipodia at the edges of cell colonies was observed (Fig. 1G and 4B). The outer edges of these lamellipodia often contained areas which stained strongly for actin filaments and which correlated with membrane ruffles in phase contrast micrographs (data not shown). Similar lamellipodia were also observed extending from the

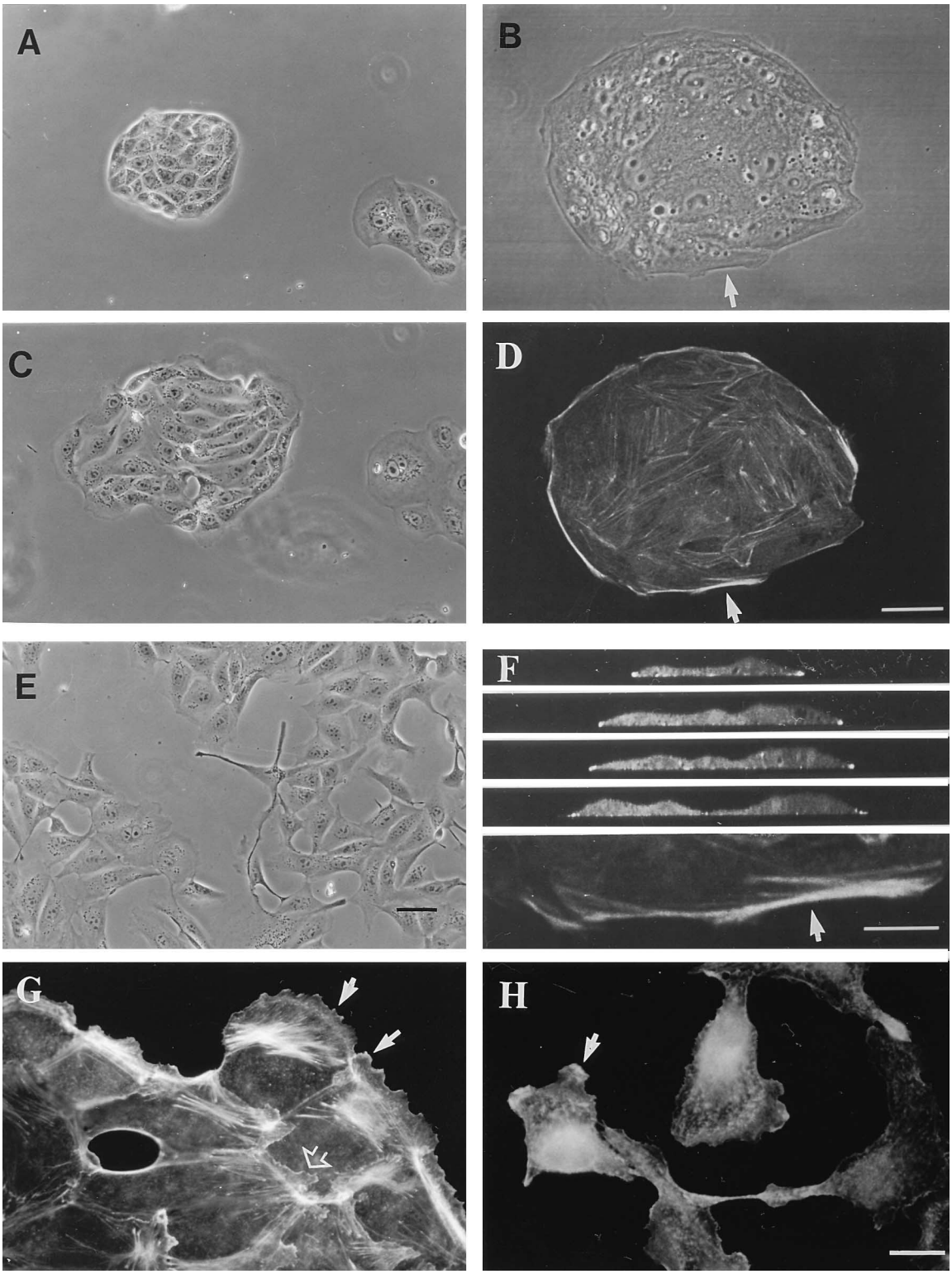

FIG. 1. SF/HGF induces colony spreading and actin reorganization. Phase contrast micrographs of a colony of MDCK cells are shown prior to the addition of SF/HGF (A and B) or at 4 (C) or 24 h (E) after the addition of SF/HGF. Fluorescence micrographs show actin filament organization in cells fixed before the addition of SF/HGF (D and F; same colony as shown in panel B) or after the addition of SF/HGF for 4 (G) or 16 h (H). In panel D, the arrow indicates a typical peripheral bundle, shown at a higher magnification in the lower part of panel F and by phase contrast microscopy in panel B. The upper part of panel F shows four different cross-sections (on the *z* axis) through the colony shown in panels B and D, and these indicate the localization of actin filaments, in particular the peripheral bundles at the edges of the colony. In panel G, the solid arrows indicate typical membrane ruffles at the outer edge of a colony and the open arrow indicates a membrane ruffle within a colony. In panel H, the arrow indicates a typical actin-rich lamellipodium. Actin filaments were revealed by staining with TRITC-labelled phalloidin. SF/HGF was added to a final concentration of 40 U/ml. The bar in panel E represents 50 μm and also applies to panels A and C; the bar in panel D represents 25 μm and also applies to panels B and D and the upper part of panel F; the bar in the lower part of panel F represents 10 μm; and the bar in panel H represents 20 μm and also applies to panel G.

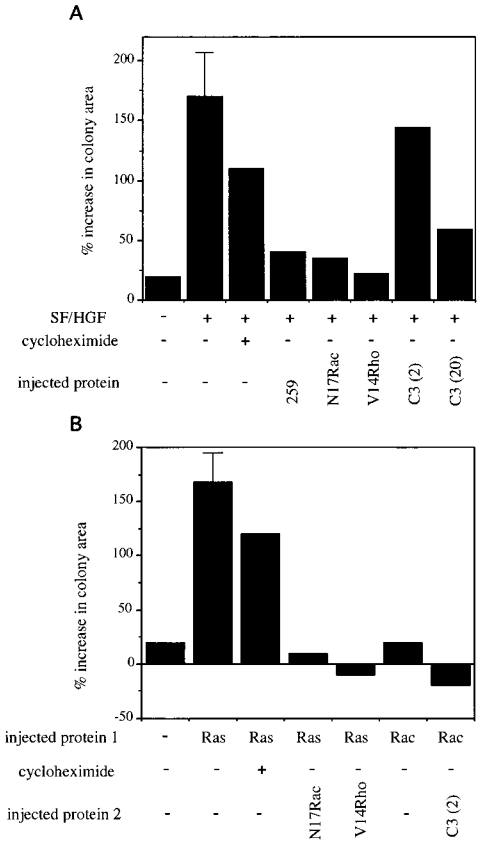

FIG. 2. Regulation of cell colony expansion by SF/HGF and Ras, Rac, and Rho proteins. Colony expansion was monitored by time-lapse videomicroscopy. (A) V12N17 Rac1 (N17Rac) and V14 RhoA (V14Rho) proteins were injected at 200 μg/ml, rat IgG was injected at 500 μg/ml, rat Y13-259 antibody (259) was injected at 10 mg/ml, and C3 transferase was injected at 2 μg/ml [C3 (2)] or 20 μg/ml [C3 (20)]. Fifteen minutes after injection, 40 U of SF/HGF per ml or an equivalent volume of CM was added. As indicated (+), 1 μg of cycloheximide per ml was added 15 min prior to the addition of SF/HGF. The colony area after 4 h was compared with the initial area prior to the addition of SF/HGF or CM. (B) V12 H-Ras (Ras), V12Rac1 (Rac), V12N17 Rac1 (N17Rac), and V14 RhoA (V14Rho) proteins were injected at 200 μg/ml, and C3 transferase was injected at 2 μg/ml [C3 (2)]. As indicated (+), 1 μg of cycloheximide per ml was added 15 min prior to the injection of V12 H-Ras. The colony area after 4 h was compared with the initial area immediately after microinjection. In each experiment for panels A and B, two colonies which were matched as closely as possible for cell number and initial area were chosen. All the cells in one colony were injected with the indicated protein, and for panel A, the cells in the control colony were injected with the buffer in which the proteins were diluted or with rat IgG, while for panel B, the cells in the control colony were injected with V12 H-Ras alone. The error bars shown for the response to SF/HGF alone (panel A) or for the response to V12 H-Ras injection (panel B) are included to indicate the extent of variation between independent experiments and represent the standard deviation from the mean ($n = 5$). Results with all other conditions are shown for a representative experiment of at least two independent determinations.

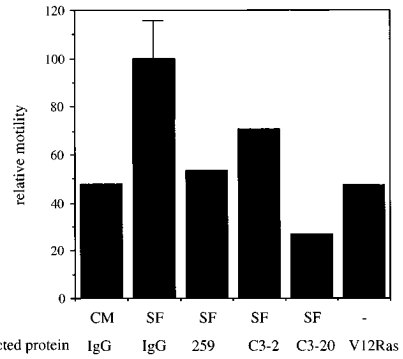

FIG. 3. Regulation of SF/HGF-induced motility associated with scattering of MDCK cells. Cell motility associated specifically with scattering and not spreading was measured by monitoring cells from 4 to 14 h after the addition of SF/HGF or CM or microinjection of proteins (by 4 h, the major phase of colony spreading induced by SF/HGF or V12 H-Ras was finished). All the cells in each colony monitored were microinjected with the indicated protein. SF/HGF at 40 U/ml (SF) or an equivalent volume of CM was added 15 min after microinjection, and the cells were monitored by time-lapse videomicroscopy. Ten cells, five at the outer edge and five from within the colony, were randomly selected from each colony, and the positions of their nuclei were marked every 2 h. When a cell divided, both progeny were monitored and the means of the distances they moved were taken. The final mean distance moved was calculated from the average distance moved by each of the 10 cells per hour. Rat IgG (IgG) or the rat Y13-259 antibody (259) was injected at 10 mg/ml, V12 H-Ras (V12Ras) was injected at 200 μg/ml, and C3 transferase was injected at 2 μg/ml (C3-2) or 20 μg/ml (C3-20). The error bar shown for control cells injected with IgG and treated with SF/HGF represents the standard deviation of the mean ($n = 4$). The other results are from single experiments and are representative of at least two independent experiments.

basal surfaces of cells within colonies, under adjacent cells (e.g., see the open arrow in Fig. 1G). Peripheral bundles were only rarely present where lamellipodia extended, and thus by 2 h after SF/HGF addition, approximately 50% of the outer cells had no peripheral bundles, compared with approximately 15% of the cells treated with CM (Fig. 4A). In addition, many cells contained short peripheral bundles which did not run contiguously along the full lengths of their outer membranes. During the first 4 h, distinct new stress fiber structures appeared in some cells within colonies (Fig. 1G). These structures disappeared between 8 and 16 h after SF/HGF addition, and by 16 h, cells contained very few stress fibers (Fig. 1H). Between 4 and 16 h after SF/HGF addition, breaks in cell-cell contacts appeared and cells at the outer edges of colonies began to detach. These cells had a polarized shape and extended lamellipodia (e.g., see the arrow in Fig. 1H).

The responses of MDCK cells to purified SF/HGF and to conditioned medium containing SF/HGF (see Materials and Methods) were indistinguishable whether they were analyzed by phase contrast microscopy or for actin filament organization. In the following experiments, conditioned medium was therefore used as a readily available source of SF/HGF.

Ras is required for SF/HGF responses and induces cell spreading. The receptor for SF/HGF, $p190^{Met}$, is a transmembrane tyrosine kinase, and many responses induced by activation of other tyrosine kinase receptors have been shown to require Ras function (20, 47). To determine whether Ras is required for SF/HGF responses, Ras activity was inhibited by microinjecting MDCK cells with a neutralizing antibody to Ras, the rat monoclonal antibody Y13-259 (13), and colonies of microinjected cells were observed by time-lapse videomicroscopy. SF/HGF-induced spreading was almost completely inhibited in colonies injected with Y13-259 compared with that in control colonies injected with the same concentration of rat IgG (Fig. 2A). In addition, the later scattering response was prevented, and the increased motility of SF/HGF-treated cells during the scattering stage was inhibited (Fig. 3). Analysis of actin organization showed that both the SF/HGF-induced increase in lamellipodia and the disappearance of peripheral bundles were inhibited in Y13-259-injected cells (Fig. 4A and B and Fig. 5A and B).

To determine whether constitutively activated Ras could induce a response similar to that induced by SF/HGF, the recombinant protein V12 H-Ras, with an activating mutation of amino acid 12 from Gly (G) to Val (V), was injected into MDCK cells. Colonies of cells injected with V12 H-Ras spread

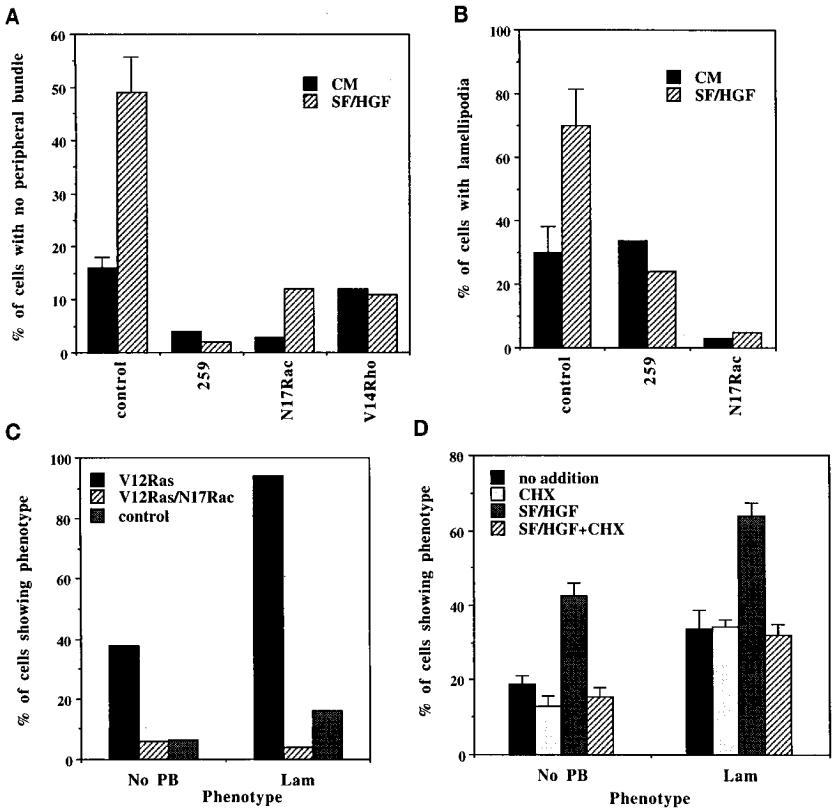

FIG. 4. Changes induced by SF/HGF and Ras, Rac, and Rho proteins in the actin cytoskeleton of MDCK cells. The rat Y13-259 antibody (259) at 10 mg/ml or V12 H-Ras (V12Ras), V12N17 Rac1 (N17Rac), or V14 RhoA (V14Rho) protein at 200 μg/ml together with rat IgG at 0.5 mg/ml was injected into approximately 50% of the cells in several colonies. For panels A and B, 40 U of SF/HGF per ml or an equivalent volume of CM was added 15 min after the final injection. For panel C, the cells were either injected with V12 H-Ras (solid bars) or coinjected with V12 H-Ras and N17 Rac1 (hatched bars). For panels A, B, and C, the cells were fixed after 2 h, except that in the case of N17 Rac1 (A and B), cells were fixed 4 h after the addition of SF/HGF. In panel D, the effect of cycloheximide (CHX) on SF/HGF-induced actin reorganization is shown. Cells were treated with 1 μg of cycloheximide per ml for 1 h prior to the addition of SF/HGF and fixed after 4 h. In all experiments, cells were stained with fluorescein isothiocyanate-labelled goat anti-rat IgG to identify injected cells and with TRITC-conjugated phalloidin to reveal the localization of actin filaments. For panels A, B, and C, all the outer cells from each colony containing injected cells were scored for the absence of peripheral bundles (PB) and the presence of lamellipodia (Lam) (e.g., see Fig. 1D and G); control cells represent the uninjected cells from these colonies. Between 50 and 100 microinjected cells were scored for each condition. For panel D, 100 outer cells were scored for peripheral bundles and lamellipodia. In panels A and B, the results for control cells are mean values ± standard errors of three independent experiments. In panel D, the results are mean values ± standard errors of three or four independent experiments. All other results are representative of at least two independent experiments.

rapidly within the first 4 h (Fig. 2B and Fig. 5E and F), and membrane ruffles were observed on outer cells (data not shown). Analysis of the actin cytoskeleton following injection of V12 H-Ras revealed that it induced changes similar to those induced by SF/HGF. Within 30 min of injection, new lamellipodia extending both from outer cells in colonies and, at the basal surfaces of cells within colonies, beneath adjacent cells were observed (shown at 1 h in Fig. 5C [e.g., see the closed and open arrows, respectively]; see also Fig. 4C). V12 H-Ras also stimulated the disappearance of peripheral bundles (Fig. 4C; also compare the cells marked with a closed arrow and an arrowhead in Fig. 5C) and induced reorganization of stress fibers (data not shown). The early responses to SF/HGF were therefore mimicked by V12 H-Ras. Unlike SF/HGF, however, V12 H-Ras did not subsequently induce disruption of cell-cell junctions and scattering, although the protein was stable, as it was clearly detected by immunofluorescence at 24 h after injection (data not shown). In addition, when the initial spreading response was complete, the motility of V12 H-Ras-injected cells was not significantly greater than that of uninjected cells (Fig. 3). It therefore appears that although Ras function is required for SF/HGF-induced scattering, it is not sufficient.

Inhibition of SF/HGF responses by cycloheximide. The observation that V12 H-Ras mimicked early but not late responses to SF/HGF implied that the scattering response required activation of another, Ras-independent, intracellular signalling pathway by SF/HGF. The time delay of 4 to 6 h before scattering was observed suggested that it might involve changes in gene expression. Cycloheximide, an inhibitor of protein synthesis, has been previously reported to inhibit SF/HGF-induced scattering (45), and we therefore studied the effects of cycloheximide in more detail. Cycloheximide slightly inhibited cell spreading induced by either SF/HGF or microinjected V12 H-Ras after it was added to MDCK cells either 15 min (Fig. 2) or 1 h (data not shown) before treatment. Subsequent cell scattering was completely inhibited, as monitored by time-lapse videomicroscopy. Initially, SF/HGF induced actin reorganization in cycloheximide-pretreated cells, but by 4 h after SF/HGF addition, these changes were reversed (Fig. 4D). In this assay, cycloheximide was equally potent at 1 μg/ml and 0.1 μg/ml but had no significant inhibitory effect at 0.01 μg/ml. These results suggest that one or more labile proteins are required to maintain the actin reorganization and the motile response induced by SF/HGF. It was therefore not possible to determine whether new protein expression was required spe-

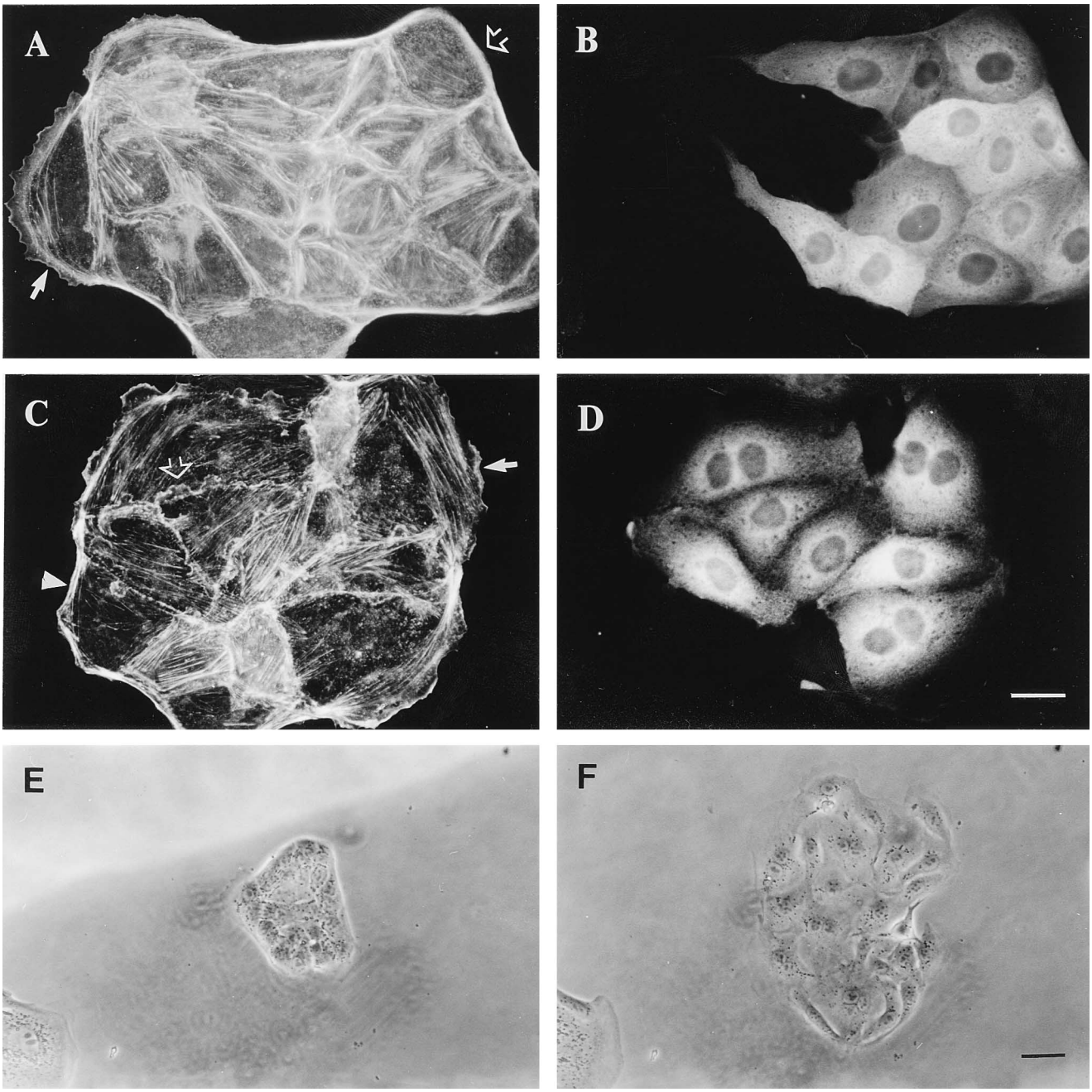

FIG. 5. Ras induces membrane ruffling and is required for SF/HGF-induced actin reorganization. Fluorescence micrographs of MDCK cells injected with Y13-259 antibody at 10 mg/ml and stimulated with 40 U of SF/HGF per ml for 2 h (A and B) or injected with V12 H-Ras at 200 μg/ml and rat IgG at 0.5 mg/ml and fixed after 1 h (C and D) are shown. Phase contrast micrographs of an MDCK cell colony prior to injection (E) and 4 h after injection of all cells with V12 H-Ras at 200 μg/ml (F) are shown. Actin filaments were localized by staining with TRITC-labelled phalloidin (A and C), and microinjected cells were detected with fluorescein isothiocyanate-conjugated goat anti-rat IgG (B and D). In panel A, the solid arrow indicates a representative uninjected cell and the open arrow indicates an equivalent injected cell. In panel C, the solid arrow indicates a typical membrane ruffle on an injected cell and the arrowhead indicates a peripheral bundle in an equivalent uninjected cell. The open arrow indicates a membrane ruffle on an injected cell in the center of a colony. The bar in panel D represents 20 μm and also applies to panels A to C; the bar in panel F represents 50 μm.

cifically for scattering in addition to the continued synthesis of this labile protein.

Rac is required for SF/HGF- and Ras-induced membrane ruffling and colony spreading. Both SF/HGF and V12 H-Ras induce membrane ruffling and lamellipodium formation, and we have previously shown that Rac is required for both growth factor- and Ras-induced membrane ruffling in fibroblasts (43). To determine whether Rac is similarly involved in the response to SF/HGF, MDCK cells were microinjected with a dominant inhibitory Rac protein, V12N17 Rac1, in which amino acid 17 is mutated from Thr (T) to Asn (N). The initial spreading (Fig. 2A) and membrane ruffling (data not shown) responses to

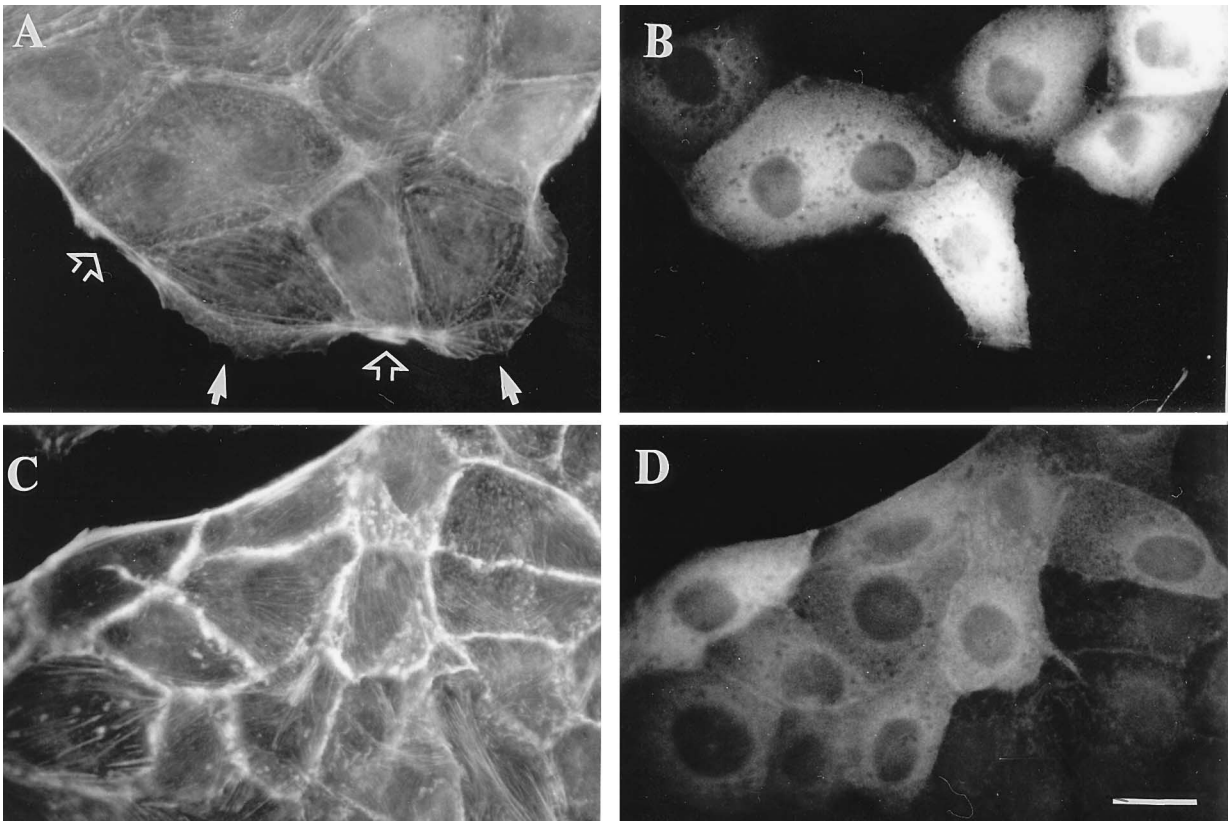

FIG. 6. V12N17 Rac1 inhibits SF/HGF-induced actin reorganization. Cells were microinjected with V12N17 Rac1 and rat IgG and stimulated with 40 U of SF/HGF per ml for 1 h (A and B) or injected with V12 Rac1 and rat IgG and fixed after 2 h (C and D). V12N17 Rac1 was injected at 200 μg/ml, and rat IgG was injected at 500 μg/ml. Actin filaments were localized by staining with TRITC-labelled phalloidin (A and C), and microinjected cells were detected with fluorescein isothiocyanate-conjugated goat anti-rat IgG (B and D). In panel A, the open arrows indicate peripheral bundles in two injected cells and the solid arrows indicate two uninjected cells lacking peripheral bundles and with extending lamellipodia. The bar in panel D represents 20 μm and applies also to panels A to C.

SF/HGF were almost completely inhibited in cell colonies injected with V12N17 Rac1, as observed by time-lapse videomicroscopy. V12N17 Rac1 also inhibited the extension of lamellipodia and the disappearance of peripheral bundles (Fig. 4A and B; compare microinjected cells marked with open arrows with uninjected cells marked with solid arrows in Fig. 6A). Similarly, V12N17 Rac1 inhibited V12 H-Ras-induced cell spreading and actin reorganization (Fig. 2B and 4C). Even in control cells treated with CM, V12N17 Rac1 induced a decrease in the background level of lamellipodia and an increase in the percentage of cells containing peripheral bundles (Fig. 4). The inhibitory effect of V12N17 Rac1 was transitory, lasting approximately 4 h (data not shown), consistent with our previous observations with fibroblasts, for which we reported that the active half-life of microinjected V12N17 Rac1 was approximately 3 h (43).

Since endogenous Rac proteins were required for MDCK cell spreading, membrane ruffling, and disruption of peripheral bundles, we wished to determine whether constitutively activated Rac1 could itself induce these responses. MDCK cells were therefore injected with recombinant V12 Rac1 protein, but colonies showed no increases in membrane ruffling or cell spreading (Fig. 2B). Examination of the actin cytoskeleton following V12 Rac1 microinjection revealed that it did not significantly stimulate the formation of lamellipodia or disruption of peripheral bundles, although it did induce an increase in cortical actin within 15 min of injection (shown at 2 h in Fig. 6C). These results indicate that, unlike V12 H-Ras, activated Rac1 by itself is not sufficient to mimic early SF/HGF responses.

Inhibition of SF/HGF responses by V14 RhoA. As described above, one of the long-term changes observed in the actin cytoskeleton of cells treated with SF/HGF was a decrease in stress fibers. We have previously shown that recombinant Rho proteins stimulate stress fiber formation after they are injected into fibroblasts (38) and that injection of MDCK cells with an expression vector encoding RhoA stimulates stress fiber formation (49). We therefore investigated the effect of activated Rho on the responses to SF/HGF. As expected, microinjection of recombinant V14 RhoA protein into MDCK cells stimulated stress fiber formation within 5 min, and the new fibers were close to the basal surface (shown after 15 min in Fig. 7A). In colonies of cells injected with V14 RhoA, the initial spreading response to SF/HGF or to V12 H-Ras was completely inhibited (Fig. 2 and 7E and F) and motility was considerably impaired (data not shown). Analysis of Rho-injected cells by immunofluorescence over 16 h following the addition of SF/HGF revealed that they remained in small, tightly adherent patches and had a high density of stress fibers while uninjected adjacent cells spread and eventually detached from the Rho-injected cells (Fig. 7C and D). These results suggest that the presence of abundant stress fibers can inhibit cell spreading and motility.

Effects of inhibiting Rho function on responses to SF/HGF and V12 H-Ras. Since the introduction of activated Rho inhibited cell spreading and scattering, it is possible that reducing

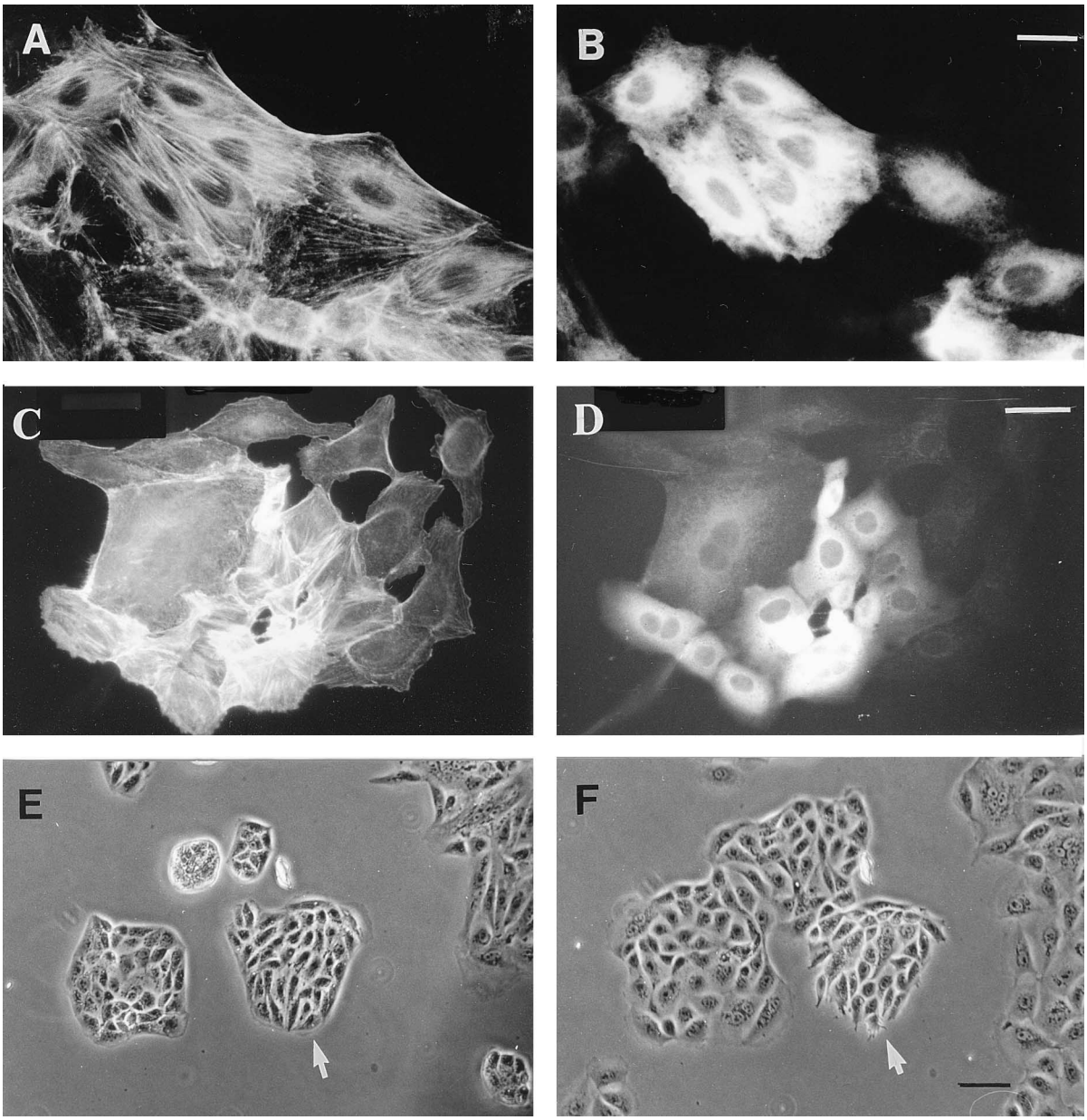

FIG. 7. Rho induces stress fiber formation and inhibits SF/HGF-induced motility. Fluorescence micrographs of cells injected with V14 RhoA at 200 μg/ml and rat IgG at 500 μg/ml and either fixed after 15 min (A and B) or stimulated with 40 U of SF/HGF per ml and fixed after 16 h (C and D) are shown. Actin filaments were localized (A and C) and microinjected cells were detected (B and D) as described in the legend to Fig. 2. Phase contrast micrographs of a colony of cells immediately after injection with V14 RhoA (E) and the same colony 4 h after the addition of SF/HGF (F) are shown. The injected colony is indicated by arrows in panels E and F. The bar in panel B represents 20 μm and also applies to panel A, the bar in panel D represents 40 μm and also applies to panel C, and the bar in panel F represents 50 μm.

the activity of endogenous Rho proteins could be an important part of the response to SF/HGF. Rho function can be inhibited in cells by the introduction of C3 transferase, an exoenzyme produced by *Clostridium botulinum* which specifically ADP-ribosylates and inactivates Rho proteins (1, 38, 48). C3 is also able to ribosylate the Rho-related proteins Rac and CDC42Hs in vitro but is at least 100-fold less active on these proteins (42). When C3 was microinjected into MDCK cells, it rapidly induced the disappearance of stress fibers and peripheral bundles, thus indicating that the formation of both of these structures is regulated by endogenous Rho proteins. By 15 min, no stress fibers or peripheral bundles were detected in over 95% of the cells injected with C3 at 2 μg/ml (Fig. 8C and D). In addition, after 1 to 2 h, cell boundaries became very irregular, with thin processes extending outwards, and many cell-cell contacts were disrupted (Fig. 8A and B). A titration of C3 activity showed that at 0.08 μg/ml it was less active and that at 0.016 μg/ml it had little effect on the actin distribution in cells, as determined 2 h after microinjection.

C3-injected cell colonies did not spread or show increased motility (data not shown), indicating that a reduction in stress fibers and cortical actin is not sufficient to induce an SF/HGF-like response. The effects of C3 on the responses to SF/HGF and V12 H-Ras were investigated both by monitoring cell

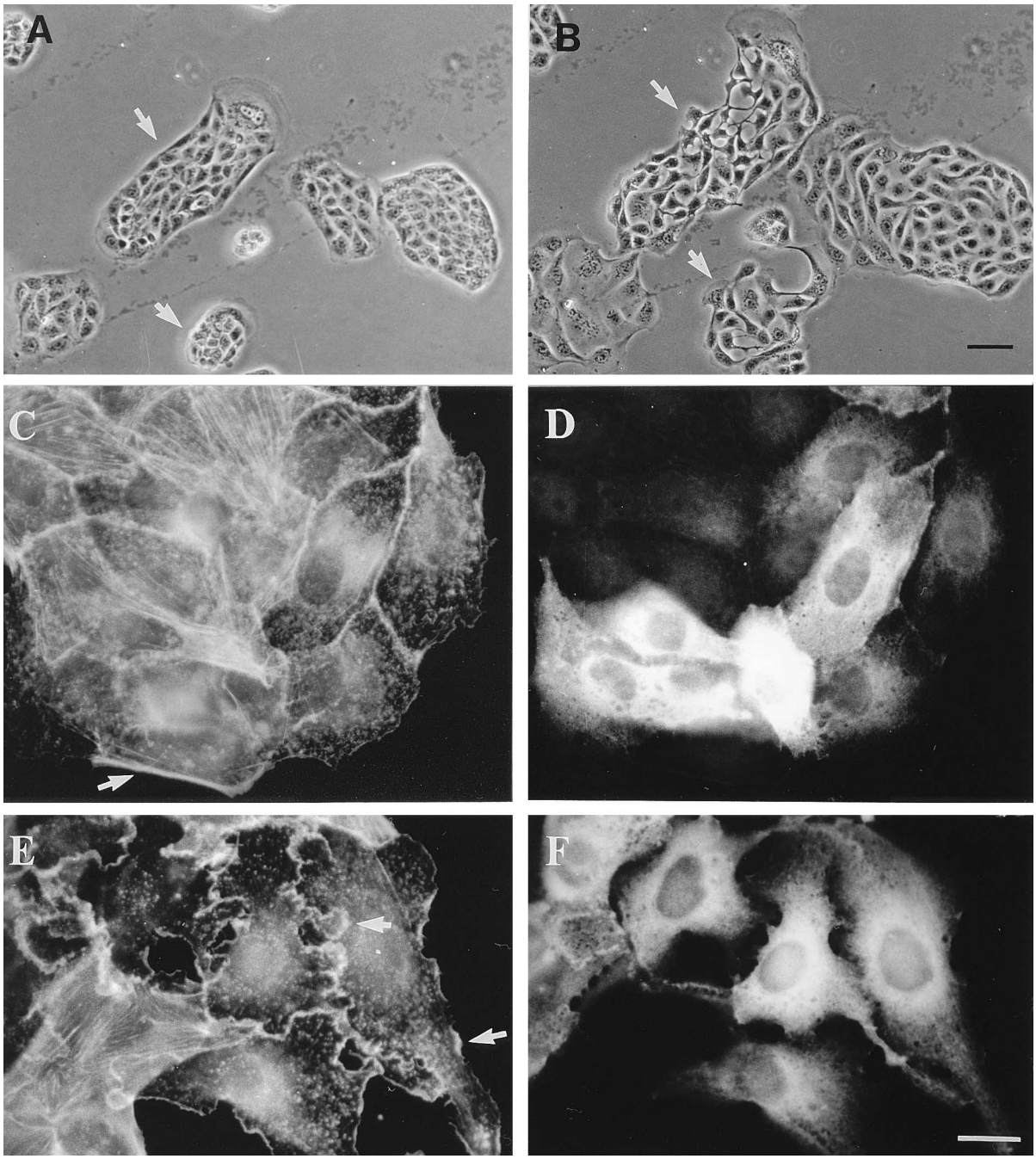

FIG. 8. C3 transferase inhibits the formation of stress fibers and peripheral bundles but not membrane ruffles. Phase contrast micrographs of two colonies of MDCK cells immediately after injection with C3 transferase (A) and of the same colonies 4 h after the addition of SF/HGF (B) are shown. The two injected colonies are indicated by arrows in panels A and B. Fluorescence micrographs of cells injected with C3 transferase and rat IgG and fixed after 15 min (C and D) or coinjected with C3 transferase, V12 H-Ras, and rat IgG and fixed after 1 h (E and F) are shown. C3 transferase was injected at 2 μg/ml, rat IgG was injected at 500 μg/ml, and V12 H-Ras was injected at 200 μg/ml. Actin filaments were localized (C and E) and microinjected cells were detected (D and F) as described in the legend to Fig. 2. In panel C, the arrow indicates a peripheral bundle in an uninjected cell, whereas surrounding injected cells lack peripheral bundles and stress fibers. Uninjected cells (top left of panel C) contain stress fibers. In panel E, the arrows indicate membrane ruffles on injected cells. The bar in panel B represents 50 μm and applies also to panel A, and the bar in panel F represents 20 μm and also applies to panels C to E.

motility and by observing changes in the actin cytoskeleton. At concentrations at or below 2 μg/ml, C3 had little effect on the initial centrifugal spreading response to SF/HGF (Fig. 2A) or V12 H-Ras (Fig. 8A and B). When SF/HGF was added to cells injected with C3 at 2 μg/ml, the cells lacked detectable stress fibers or peripheral bundles, but SF/HGF could still stimulate membrane ruffling and lamellipodium formation, as observed both by time-lapse videomicroscopy and through localization of actin filaments (data not shown). Similar results were observed in cells coinjected with V12 H-Ras at 200 μg/ml and C3

at 0.2 μg/ml (Fig. 8E and F), indicating that inactivation of Rho did not inhibit these responses to SF/HGF and V12 H-Ras.

In contrast to cells injected with C3 at 2 μg/ml, cells injected with C3 at 20 μg/ml were considerably impaired in their spreading response (Fig. 2A). The motility of cells during the scattering stage was also inhibited by C3 at 20 μg/ml but only slightly reduced by C3 at 2 μg/ml (Fig. 3). The concentrations of C3 affecting cell motility, therefore, titrated out at considerably higher levels than those affecting the actin cytoskeleton, with substantial inhibition requiring an approximately 100-fold higher concentration of C3 (20 μg/ml) than that for maximal reduction in stress fibers (less than 0.2 μg/ml). It is therefore possible that, at this high concentration, C3 acts less specifically and not only inhibits Rho function but also that of other related proteins.

DISCUSSION

The motile response of MDCK cells to SF/HGF can be divided into two stages: the first lasts 4 to 6 h and involves centrifugal spreading of cells so that the surface area of each cell and therefore of each colony increases, while the second stage begins after 4 to 6 h and involves the breaking of cell-cell contacts, leading to the scattering of cells from colonies (56). Ras has been shown to mediate many cellular responses to extracellular signals (20, 47), and we have shown here that it is also required for both the spreading and scattering responses to SF/HGF. The SF/HGF receptor, $p190^{Met}$, is a tyrosine kinase, and SF/HGF has recently been reported to activate Ras proteins by stimulating guanine nucleotide exchange (19). Microinjection of MDCK cells with constitutively activated H-Ras protein induced a spreading response resembling that to SF/HGF, but this did not lead to scattering, implying that the scattering response requires another signal transmitted by $p190^{Met}$ in addition to that leading to Ras activation.

Regulated changes in actin microfilament organization underlie cell motility (10), and scattering of MDCK cells is indeed prevented by cytochalasin B, which inhibits actin polymerization (45). We have therefore monitored early changes in the actin cytoskeleton, from 0 to 4 h after stimulation with SF/HGF or microinjection of V12 H-Ras, as another method to compare the effects of these two treatments. Three types of actin reorganization were observed in response to both SF/HGF and V12 H-Ras: the extension of lamellipodia and formation of membrane ruffles, the disappearance of peripheral bundles in many cells at the outer edges of colonies, and an overall reduction in stress fibers. Membrane ruffles and lamellipodia are found at the leading edges of motile cells and are believed to play a fundamental role in migration (10, 50). Our previous work showed that in Swiss 3T3 fibroblasts, early membrane ruffling induced by extracellular factors is dependent on endogenous Rac proteins (43), but this finding did not address the role of Rac during longer-term responses to factors inducing cell motility. The present results now show that Rac is required for motility-associated ruffling and lamellipodium extension in MDCK cells and that Rac function is essential for the motility of cells during colony spreading. It has been reported that in KB cells, another epithelial cell line, Rho but not Rac is required for membrane ruffling induced within 10 min of SF/HGF addition (37a). Therefore, the signalling pathways regulating early membrane ruffling may differ from those regulating longer-term responses and the precise roles of Rac and Rho may vary among different cell types. It is interesting in this respect that microinjection of activated Rac1 by itself was insufficient to induce lamellipodium formation or membrane ruffling in MDCK cells, whereas it is able to stimulate membrane ruffling in Swiss 3T3 fibroblasts. This suggests that although Rac function is required for membrane ruffling, an additional signal is necessary in MDCK cells. V12 H-Ras can provide this second signal, as it induces Rac-dependent membrane ruffling and spreading, and one possibility is that changes in protein phosphorylation resulting from activation of the MAP kinase pathway are required (25).

It has recently been reported that in a keratinocyte cell line, SF/HGF-induced cell motility is not inhibited by microinjection of N17 Rac1 or N17 Ki-Ras but is inhibited by C3 transferase (58). The apparent discrepancies between these results and ours are probably due to the use of different experimental approaches and the measurement of different parameters. In the keratinocyte study, cell dissociation, or scattering, is observed, but no direct measurements of cell motility are included. In addition, it is notable that antibodies to the cell-cell adhesion molecules E-cadherin and P-cadherin were added to the keratinocytes in addition to SF/HGF to obtain the observed cell dissociation. It is possible that the presence of the antibodies bypasses a requirement for endogenous Ras or Rac proteins in SF/HGF signalling; indeed, MDCK cells treated with antibodies to E-cadherin dissociate and acquire invasive properties in the absence of SF/HGF (3a). Furthermore, we too observe inhibition of motility after a high concentration of C3 transferase is microinjected but have shown that this dose is supramaximal in terms of inducing changes in actin organization. Finally, it should be noted that N17 Rac1 has a short half-life of approximately 3 h following microinjection (43), and it is therefore not possible to analyze long-term effects of this protein on cell motility.

The observation that microinjection of V14 RhoA inhibited motility during both the spreading and scattering stages of the response to SF/HGF provides evidence that a high level of stress fibers and focal adhesions can actually antagonize cell motility. This has previously been suggested by the observation that highly motile or transformed cells have very few stress fibers (8, 22). Through its action in regulating the formation of focal adhesions (42), Rho is also likely to be involved in altering the interaction of cells with the extracellular matrix through integrins. Presumably, a large number of cell-substratum contacts and a dense network of stress fibers are not conducive to cell movement, and decreasing the activity of Rho proteins could therefore be one mechanism for enhancing cell motility. Indeed, two of the changes in actin reorganization induced by SF/HGF—the disappearance of peripheral bundles and the overall decrease in stress fibers—can be mimicked by inhibiting Rho function in cells. However, it is unlikely that SF/HGF is simply reducing Rho activity, as new stress fiber structures are transiently formed in some cells during the spreading stage. In addition, SF/HGF-induced disruption of peripheral bundles is not due to direct inhibition of Rho but also involves Rac and Ras, as disruption is prevented in cells injected with V12N17 Rac1 or with the Y13-259 anti-Ras antibody.

The fact that motility could be stimulated in C3-injected MDCK cells lacking stress fibers and peripheral bundles indicates that neither of these structures is essential for cell migration. Much higher concentrations of C3 (20 μg/ml) were required to inhibit cell motility substantially than to eliminate stress fibers and peripheral bundles (less than 0.2 μg/ml). Interestingly, cells injected with C3 at 20 μg/ml showed some increased motility for 1 to 2 h following SF/HGF addition (data not shown). These results suggest either that stress fiber formation is much more sensitive to Rho activity than is motility or that at this high concentration, C3 is acting less specifically and gradually inactivating other Rho-related proteins in addi-

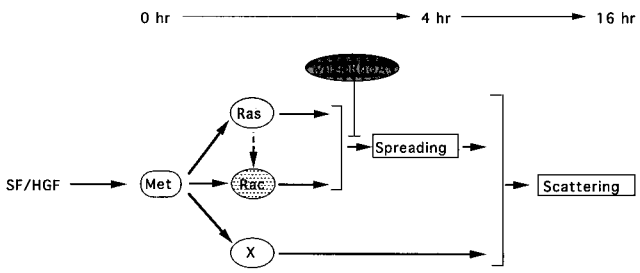

FIG. 9. A model for the regulation of SF/HGF responses by Ras, Rac, and Rho. SF/HGF activates $p190^{Met}$, which stimulates an increase in the levels of Ras-GTP and Rac-GTP, each of which provides a signal required to induce motility associated with colony spreading. Either the increase in Rac-GTP is achieved independently of Ras, or endogenous Ras acts upstream to induce the increase (dashed arrows). Activated Rho can inhibit cell motility, both during spreading and during scattering. The scattering of cells requires the signals involved in spreading and an additional signal (X) which is not induced by Ras.

tion to Rho; for example, C3 can inefficiently ribosylate Rac and CDC42Hs in vitro (42). It is therefore important to measure C3-induced effects carefully and relate them to changes in actin organization before assigning them specifically to inhibition of Rho.

In addition to inducing changes in actin reorganization, microinjection of C3 transferase at 2 μg/ml led to the breaking of cell-cell contacts between MDCK cells. Recently, other experiments have suggested that Rho might regulate cell-cell contacts in platelets and in B lymphocytes (32, 59). It is therefore possible that in epithelial cells, Rho is required for the maintenance of cell-cell contacts and therefore that a decrease in Rho activity could contribute to the SF/HGF-induced scattering response. Alternatively, C3 transferase-induced loss of stress fibers and focal adhesions might indirectly lead to destabilization of cell-cell contacts. Investigating the localization of proteins, such as E-cadherins and desmosomal proteins, involved in cell-cell contacts in C3-injected cells may further elucidate a role for Rho in regulating these interactions. It is clear from our comicroinjection experiments, however, that a combination of V12 H-Ras-induced spreading and C3-induced loss of cell-cell contacts is insufficient to induce scattering and that other factors must be involved.

On the basis of the results reported in this paper, we propose a molecular model for the involvement of Ras, Rac, and Rho proteins in regulating responses to SF/HGF in MDCK cells (Fig. 9). Activation of $p190^{Met}$ by SF/HGF leads to Ras and Rac activation, each of which generates signals required for colony spreading, including membrane ruffling and lamellipodium extension. These changes in Ras and Rac activity also contribute to the later scattering response, but scattering requires one or more additional signals, X. Both responses can be inhibited by introducing constitutively activated Rho protein, which induces the formation of stress fibers. SF/HGF has been shown to induce an increase in the level of Ras bound to GTP in MDCK cells (19), and direct measurement of the levels of Rac bound to GTP in cells will similarly be required to determine whether SF/HGF indeed alters its activity. In the SF/HGF response, we cannot at present distinguish whether Rac activity is altered independently of Ras or whether Ras normally acts upstream to activate Rac (dashed arrows in Fig. 9). In Swiss 3T3 fibroblasts, however, we have shown that Rac functions independently of Ras to regulate growth factor-induced membrane ruffling (43).

Our results indicate that an additional signal induced by SF/HGF but not by V12 H-Ras is required for scattering. The time delay between addition of SF/HGF and scattering suggests that this signal could involve new gene transcription. This could include genes encoding proteins regulating cell-cell contacts or proteases which degrade the extracellular matrix. Indeed, SF/HGF has been reported to stimulate the expression of urokinase-type plasminogen activator in MDCK cells, which can activate plasminogen to form plasmin, a protease capable of degrading certain matrix components (39). In contrast, no change in the expression or phosphorylation state of E-cadherin, a major cell-cell adhesion molecule in epithelial cells, was observed in MDCK cells following SF/HGF addition (62). It is possible, however, that SF/HGF regulates the expression of E-cadherin-associated cytosolic proteins such as the catenins, which could alter cell-cell adhesion (24). Further studies comparing the effects of Ras, Rac, and Rho proteins with those of SF/HGF on the localization of proteins involved in cell-cell contacts, such as E-cadherin, desmosomal proteins, and keratins (27), should provide more information on the role of these GTP-binding proteins in scattering. Ras function is required for scattering, but whether this is only because it is essential for SF/HGF-induced membrane ruffling or because it induces other signals is not known. The inability of constitutively activated Ras to stimulate scattering of MDCK cells suggests that, unlike SF/HGF, it would not induce an invasion of the extracellular matrix (62). This is consistent with the idea that other changes in addition to oncogenic activation of Ras are required to allow cells of epithelial origin to invade and metastasize.

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