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Rho GTPases ARE INVOLVED IN THE DEVELOPMENT OF TRANSFORMATION PHENOTYPE IN MAMMALIAN CELLS

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Small G-protein Ras has been found to play the pivotal role in the control of transducing signals for normal and transformed cell growth.(1) It is one of the most commonly mutated oncogenes. Cdc42, Rac and Rho of Ras superfamily have been suggested to be involved in transducing Ras signals for various cellular functions(1) and these Rho GTPases are thought to be downstream mediators of Ras functions.(2-4) The major functions of the Rho, Rac and Cdc42 GTPases are regulation of the rearrangement of actin cytoskeleton and modulation of the mitogen activated protein kinase cascades leading to transcriptional activation of genes.(1) Thereby these proteins control many biological activities. These evidences suggest that Rho GTPases produce two types of signals: regulating the actin cytoskeletal rearrangement and the induction of gene expression. But their clear molecular mechanisms are yet to be found.(5)

It is known that the activation of Ras stimutates Cdc42, Rac and Rho and each of these GTPase has independent signalling pathways. Rho is required for the formation and maintenance of stress fiber and focal adhesion, Rac regulates lamellipodia and membrane ruffle and Cdc42 regulates filopodia formation.(6) Ras can activate Rac, Cdc42 can also activate Rac. Therefore, the filopodia and lamellipodia are intimately associated. These observations indicate that there is significant cross-talk between GTPases of Ras and Rho subfamilies.

Cell adhesion and spreading are suggested to be the basic requirements of cell proliferation, which are possibly regulated by Rho GTPases. But very little is known about the mechanism of these biological processes. Besides this, the interaction of Rho family proteins with Ras family needs further investigation.

In these backgrounds, the present study has demonstrated the role of RhoA, Cdc42, and Rac1 GTPases in Ras induced transformational signaling of v-Ras-transformed-NIH 3T3 cells. Besides this, by inactivating these Rho, Rac and Cdc42, the role of these GTPases on spreading of cells was also studied.

To do these, the NIH 3T3 and v-Ha-Ras-transformed-NIH 3T3 cells were cultured in RPMI-1650 (Sigma) growth medium with 10% Fetal Bovine Serum (FBS) (GIBCO-BRL) and the transfectants in RPMI medium containing 10% FBS and G418 (400 μg/ml, Invitrogen).

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The RHG plasmid was constructed using rat p190 cDNA as a template. We have amplified an EcoRI/HindIII DNA fragment that contains a Kozak consensus sequence (GCCGCCACCATG) at the 5' end and encodes the COOH-terminus Rho GAP domain (residues 1186 to 1513) of p190 by PCR. The primers used were 5' GAA TTC GCC GCC ACC ATG GGG CGG 3' and 3' AAG CTT TTA AGA AGA CAA 5'. The amplified DNA product was inserted into pMV7 mammalian expression vector which carried a neomycin analogue. This plasmid was named RHG and purified by cesium chloride density gradients centrifugation.

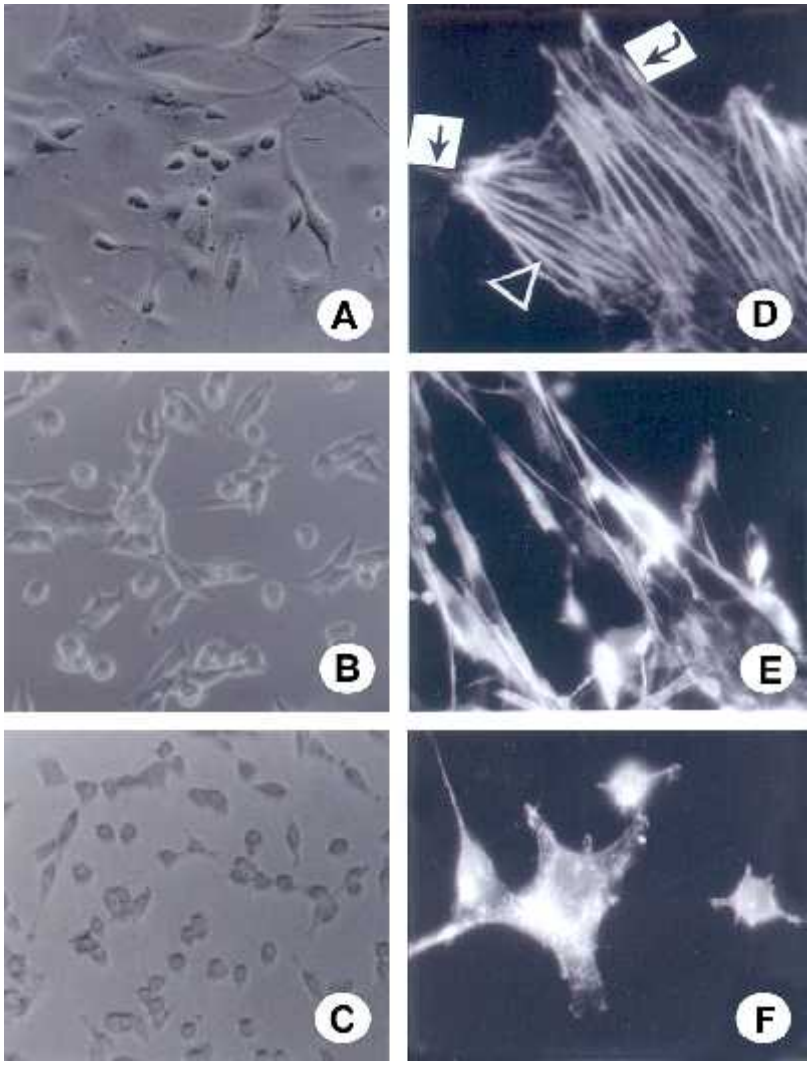
The oncogenic v-Ras-transformed-NIH 3T3 cells (5×10^5 cells/ml) were transfected by RHG plasmid using lipofectin reagent (GIBCO BRL kit) following the procedure as per instruction of the distributor. These cells were incubated overnight at 37°C. The medium was replaced by cell growth medium and incubated for 2 - 3 days. This medium was then replaced by RPMI, 10% FBS and 400 µg/ml G418 (a neomycin analogue medium) and the cells were allowed to grow for three weeks. The neomycin resistant colonies were picked up and re-cultured separately in RPMI supplemented with 10% FBS and G418 (400 µg/ml) medium.

To investigate the role of Rho, Rac and Cdc42 GTPases in Ras cellular transformation, the constructed RHG plasmid was transfected into v-Ras transformed NIH 3T3 cells (V12, an oncogenic mutant of Ras) to inactivate Rho, Rac and Cdc42 proteins. This RHG plasmid contains p190 GAP domain at C-terminus. This domain stimulates the intrinsic GTPase activity of Cdc42, Rac and Rho and thereby attenuates their signal transducing activity.[7]

It was observed here that the NIH 3T3 cells were flat, well-spread (cytoplasmic area quite prominent), polygonal in shape, well-adherent in nature and showed cell-to-cell attachment (Fig. A). Rhodamine-conjugated-phalloidin staining revealed that these cells possesed finger-like protrusions (filopodia) and sheet-like membranous projections (lamellipodia) containing actin filaments. These cells also displayed very conspicuous actin stress fibres traversed from one end to another in the cytoplasmic area (Fig. D).

The role of the Rho GTPases on v-Ras-transformed-NIH 3T3 cells was observed *in vitro* after inactivating these GTPases by overexpressing RHG. Two different stably expressing transformed cell lines were found which were phenotypically completely new. These were clone 1 (Figs. B and E) and clone 2 (Figs. C and F).

Clone 1 cells were elliptical in shape (Figs. B and E). These were more flat (Figs. B and E) than spherical (Figs. C and F), but not as flat as NIH 3T3 cells (Fig. A). They possessed less cytoplasmic portion (Figs. B and E). As a consequence, the size of the transfectant became smaller than the NIH 3T3 cells. Due to this, the shape of this transfectant was significantly different from the NIH 3T3 cells (Figs. A and D).



Figs. A-F: Immunostaining showing morphologically transformed RHG overexpressor derived from v-Ras transformed NIH 3T3 cells. Phase contrast image of NIH 3T3 cells (A) (control), RHG transfectant clone 1 (B) and RHG transfectant clone 2 (C) showing RHG expressors that failed to re-spread. Rhodamine-conjugated-phalloidin staining showing normal NIH 3T3 cells (D) stress fibre (Arrowhead), filopodia (arrow) and lamellipodial protrusions (bent arrow). RHG clone 1 (E) and RHG clone 2 (F) cells were devoid of these actin filament structures.

These findings indicate that overexpressing RHG causes a gross change in cell morphology of NIH 3T3 cells. Rhodamin-conjugated-phalloidin staining showed that in this RHG transfectant the actin stress fibres, lamellipodia and filopodial protrusions were absent (Fig. E). This observation indicates that because of the absence of these structures, the cells failed to re-spread and had tendency to round

up (Fig. E). The cells had contracted morphology with increased height and refractile appearance (Fig. E).

Most of the clone 2 cells were spherical (80%) and possessed very little cytoplasmic area (Figs. C and F) in comparison to NIH 3T3 cells (Fig. A). These cells were greatly reduced in size and possessed completely different morphology than the NIH 3T3 cells and v-Ras-transformed-NIH cells. These cells showed a rim of cytoplasm just around the nucleus (Figs. C and F). Though the cells were monolayered, they could not be brought to the same focus possibly due to contraction of cytoplasm. In rhodamine-conjugated-phalloidin staining the RHG transfectants were found to be devoid of detectable filopodia, lamellipodia and stress fibres (Fig. F).

To see whether these reduced-size transfectants (both elliptical and spherical) re-spread or not, these transfectants were allowed to grow for longer period in growth medium. Even then morphologically these RHG transfectants remained unchanged (Figs. B, C, E, F). It indicates that these cells failed to re-spread (Figs. B, C, E, F).

It has been reported earlier that activation of Cdc42, Rac and Rho induce filopodia, lamellipodia and stress fibre, respectively.[6] However, the normal NIH 3T3 cells possessed these structures (Fig. D). These observations suggest that RhoA, Rac1 and Cdc42 GTPases are quite active in NIH 3T3 cells. In contrast, these structures were absent in the RHG transfectants (Clones 1 and 2). It indicates that over-expressing RHG inhibits the activation of Cdc42, Rho and Rac thereby they are unable to polymerize actin filaments and show a different cell morphology.

These findings lead to the conclusion that over-expressing RHG blocks the signals mediated through Cdc42, RhoA and Rac1 in Ras signalling pathway that is involved in development of transformed phenotype. These results indicate that the RHG domain which stimulates the intrinsic GTPase activity of Rho GTPases also attenuate their signal transducing activity. These observations also indicate that over-expression of RHG inhibits the development of v-Ras-transformed phenotype of NIH 3T3 cells and Cdc42, Rho and Rac are involved in the development of transformation phenotype in mammalian cells.

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