Vol. 168, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1990 Pages 114-124

rho GENE AMPLIFICATION AND MALIGNANT TRANSFORMATION

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Received February 23, 1990

Our previous studies have shown that overexpression of the proto-oncogene rho in Rat-1 and 3T3 fibroblasts resulted in colonies that displayed altered growth regulation (8). In this report we demonstrate that the human rho gene is capable of eliciting neoplastic growth of an established cell line - Rat Rho1 DHFR, rat fibroblast cells that are stably transfected to overexpress the full-length cDNA rho A. The established transfectant lines grow to a high saturation density in monolayer cultures and, when maintained and postconfluence, developed dense foci. The overexpression of rho A cDNA altered not only the in vitro growth properties of Rat-1 cells but also their in vivo behavior. Injection of these transfected cells into athymic nude mice resulted in the formation of tumors. Introduction of rho A cDNA with the selectable dihydrofolate reductase marker into rat embryo fibroblasts resulted in the outgrowth of few rho transfected colonies. These colonies expressed high levels of rho cDNA and could proliferate for at least 40 generations. Our results show a positive correlation between overexpression of the human rho gene and tumorigenicity.

Emerging evidence indicates that ras proto-oncogenes are part of a much larger superfamily of genes. At least 15 cDNA and genomic clones have been identified by using probes derived from ras genes, which after sequence analysis have been found to encode small (about 21 Kd) guanine nucleotide binding proteins. Five genes, ral, R-ras, rap 1A, rap 1B, and rap 2, (1-5), are most closely related to ras with 50% amino acid

Abbreviations:
DHFR - Dihydrofolate reductase; G proteins - GTP binding proteins; REF - rat embryo fibroblast.

0006-291X/90 $1.50

homology. The rab genes (6) and the rho genes (7) share about 30% amino acids known to be essential for both guanine nucleotide binding and membrane localization. In humans, three classes of rho cDNA clones have been identified which differ by virtue of the presence of variable C-terminal domains. Despite the structural similarity between the rho and ras proteins, suggesting a similar mode of action, earlier studies have shown that the rho gene has effects that are quite distinct from the ras gene (8). For example, the activated H-ras gene is capable of focus formation and soft agar growth while the human rho A alleles as well as the variants carrying the same mutations as the activated H-ras are not capable of inducing these effects. However, overexpression of the normal rho A gene was found to alter growth regulation in established transfectants. In this regard, we have now extended this initial observation and tested the ability of human rho A to transform cells through a gene transfer approach. We found that human rho A cDNA expressed at high levels induced morphological transformation, sustained proliferation of early-passage of rat embryo cells and induced tumors in athymic mice.

Material and Methods

Plasmids

All constructions were done according to standard procedures (9). The human rho A cDNA was comprised of a 1.74 Kb EcoRI insert in the pSP65 plasmid. Construction of pJ3w/rho A expression vector has been described previously (8). The pEJ6.6 plasmid carried the Ha-ras oncogene from the EJ/T24 human bladder carcinoma cell line (10).

RNA and DNA Blot Analysis

Total cellular RNA was extracted from different cell lines using the guanidine thiocyanate procedure, followed by ultracentrifugation through a CsCl gradient (11). Total mRNA was run on a 1.2% formaldehyde-agarose gel and was transferred onto nitrocellulose or Zeta Probe membranes as described (9).

For Southern blot analysis, EcoRI-digested DNA was resolved by electrophoresis on agarose gels and was transferred onto nitrocellulose or Zeta Probe membranes as described (9). RNA and DNA blots were probed under conditions of high stringency by prehybridizing for 12 hours at 42°C in 50% formamide, 5 x SSC, 5 mM EDTA, 0.1% SDS, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidone, 12 mM Na_2HPO_4, 9 mM NaH_2PO_4, and 100 ug/ml of sonicated and denatured salmon sperm DNA.

Hybridization was performed for 24 hours at 50% in hydridization buffer containing the rho A probe. Following hydridization, the blots were washed for 2 hours at 65° with 2 x SSC and 0.1% SDS, and exposed to Kodak XAR-5 film with intensifying screens at -70°C.

DNA Probes: The rho A probe was comprised of a 1.74 Kb EcoRI-insert of the plasmid pJ3w-rho. Human rho A cDNA was cut at a single BalI site at codon 1488, generating a C-terminal specific fragment of 250bp. The 250bp of the untranslated region which was comprised of the BalI-EcoRI fragment was used for specific detection of the human rho A. DNA fragments were labeled to high specific activity (10^8-10^9 cpm/ug) according to the method of Feinberg and Vogelstein (12).

Cells and Transfections

Fischer rat fibroblasts (Rat-1) cells were obtained from the American Type culture collection. Rat-1 cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-Glutamine, 100 u/ml penicillin, and 100 ug/ml streptomycin, in a 37°C in a humidified atmosphere of 5% CO_2.

Transfections were performed as described elsewhere (8). For cellular transformation assays, 3T3 or Rat-1 fibroblasts were maintained in DMEM plus 10% fetal calf serum. Cultures of 4 x 10^5 fibroblasts in 60 mm dishes were transfected with 5 ug of plasmid DNA for monolayer focus assays. The cultures were trypsinized and plated onto six 100 mm dishes 16 hours after glycerol shock. Monolayer cultures were maintained for up to 21 days, with fresh medium being added every third day. Cells were stained with 0.5% (wt/vol) crystal violet (Sigma) in methanol-glacial acetic acid (3:1) and scored for focus formation.

For co-transformation and selection, 5 ug of plasmid DNA was mixed with 0.5 ug of pSV2 DHFR (8). Twenty-four hours after transfection, the cells were plated onto 100 mm dishes, methotrexate (Sigma) was added to 400 g/ml and 32 mM final concentrations respectively. Cultures were maintained for 2-3 weeks with the medium being changed every 3 days.

Transfection and Selection of REF Cells in Culture

Primary cultures of rat embryo cells were prepared from 12 to 14 day-old Fisher rat embryos after 2-4 days of growth in Dulbecco's modified Eagle's (DME) medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics as described by Land et al. (10). The cells were seeded at a density of 1 x 10^6 per 100-mm dish. Transfections were performed as described above with 75 ug REF carrier DNA, 10 ug of the plasmid DNA, and 2 ug of pSVv-myc cDNA. After 24 hours, the transfected cells were split in a ratio of 1:3 and cultures were refed every 4 days.

Assay for Colony Formation in Soft Agarose

For analyses of anchorage-independent growth, 5 x 10^4 cells were suspended in 5 ml of 0.35% low-melting-point agarose (Sea Plaque) and seeded into 60 mm culture dishes containing a 0.7% agarose base. All agarose suspensions were made in DME Medium containing 10% fetal calf serum. Plates were inspected by microscopy at regular intervals for 3 weeks.

Nude Mouse Tumorigenicity Assay

Actively growing cells were trypsinized, washed twice with PBS, and resuspended at a concentration of 2.5 x 10^7/ml. Approximately 1 x 10^6 cells were injected into the left and right flanks of 4-8 week old CD-1 athymic nude mice (Charles River Laboratory). Mice were examined one or two times per week for tumor formation and growth. Mice were sacrificed and tumors were excised aseptically for analysis as well as for establishment of cell lines.

Metabolic labeling of cells and immunoprecipitation.

Cells were labeled in DMEM minus cysteine (Amersham Corp.) for 12 h. Immunoprecipitation of p21 rho from labeled cells with antipeptide antiserum was performed as described previously (8). Portions of lysates containing 10^7 cpm of acid-insoluble incorporated [^{35}S]cysteine were incubated with 1 μg of the antiserum in a 0.5-ml reaction mixture. Immunoprecipitation samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Results

Transformation of Rat-1 cells by rho A cDNA

The established rodent fibroblast cell line Rat-1 was chosen as a recipient for transfected rho A cDNA. These cells display growth properties characteristic of normal fibroblastic cells (flat, density-dependent inhibition of growth, and anchorage dependence) and take up DNA efficiently. The plasmid pJ3w/rho A was introduced into Rat-1 cells by calcium phosphate-mediated transfection with pSV2 DHFR which confers resistance to methotrexate (8).

In our initial studies, we established rat fibroblast cell lines that stably expressed high levels of the human rho A protein. One of these cell lines, Rat Rho 1 DHFR (8) was chosen for further characterization of the changes that occurred with overexpression of rho A cDNA. Monolayer cultures were maintained at postconfluence for an extended period of time (10 days), with media changed every 3 days. The

plates were scored for transformed foci. Whereas the control cell line Rat-1 DHFR remained as a fairly uniform monolayer, after about 14 days the Rat Rho 1 DHFR cell line, which contained an amplified copy number of rho A cDNA and expressed high levels of human rho A mRNA (8), developed numerous dense foci. In further studies, several individual large colonies were randomly picked and established as clonal cell lines. All of these clones displayed the morphology of transformed cells and retained their anchorage independent growth. It is likely that these dense foci reflected genetic changes induced by the high level of the rho A p21 product. Therefore, total cellular DNA was isolated from some of these clones and examined for integrated copies of rho A sequences. Southern analysis of EcoRI-digested genomic DNA of foci derived Rat Rho 1 DHFR cells revealed the same pattern of hybridization of the human rho A probe as the parental cell line--the Rat Rho 1 DHFR (Fig. 1), including the presence of a 1.74Kb DNA fragment, indicative of rho A sequences in the genomic DNA of the transfected cell lines.

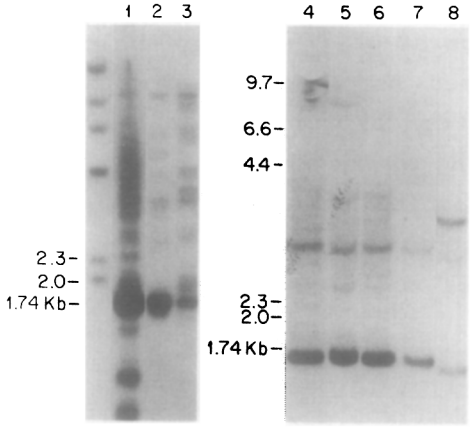


Fig. 1

Southern blot analysis of Rat Rho1 DHFR and Rat Rho1 DHFR foci cell lines.

Aliquots (10μg) of EcoRI-restricted genomic DNA from rho transfectants foci (lanes 2-8) as well as from Rat Rho1 DHFR (lane 1) were separated on a 1% agarose gel and transferred to nitrocellulose. The blot was probed with a ^{32}P-labeled 250-bp BalI-EcoRI-specific fragment for detection of the human rho A. Molecular sizes (in kilobases) as determined by migration of Hind III-digested lambda fragments are shown.

Tumorigenicity of rho A transfected lines

Table 1 summarizes the results of molecular analysis of the induced tumors. Representative clonal lines were tested for tumorigenicity in nude mice by inoculating 1 x 10^6 cells into nude mice and monitoring the mice for tumor appearance and growth. Tumors did occur with Rat Rho 1 DHFR cells, but only after about 40 days. With clonal cell lines, tumors (about 1cm in diameter) were apparent within 20 days of injection and rapidly grew to diameters exceeding 2cm at 30 days (Table 1). Untransfected cells Rat-1 cells used as controls did not form tumors under these conditions during 20 weeks. In contrast to the various rho A transfectants, ras-transformed cells formed tumors within 14 days of injection.

Rho A Sequences and RNA Expression in rho A Induced Tumors

To evaluate the necessity of retention of the transfected rho A sequences and their expression in the latter stages of tumorigenesis, tumors arising from inocula of rho A transfectants were established as cell lines and were examined for exogenous rho A DNA sequences and rho A mRNA levels. The rho A tumors retained these sequences, as was determined by the presence of the human rho A fragment in Southern blot analysis of the induced tumor DNA (Fig. 2A). Northern blot analysis of

Table 1: Phenotypes of Rat Rho1 DHFR Transfected Cells

Cell Line	Morphology	Tumorigenicity[a]	Tumor Appearance (days)
Rat-1 DHFR	Flat	0/5	
Rat Rho1 DHFR	Dense	4/5	35-43
Rat Rho1 DHFR 1	Dense, piled	5/5	15-20
2	Dense, piled	5/5	18-22
3	Dense, piled	5/5	15-25
4	Dense, piled	5/5	20-25
5	Dense, piled	4/5	20-25

[a]Data are expressed as number of tumors observed/number of animals injected.

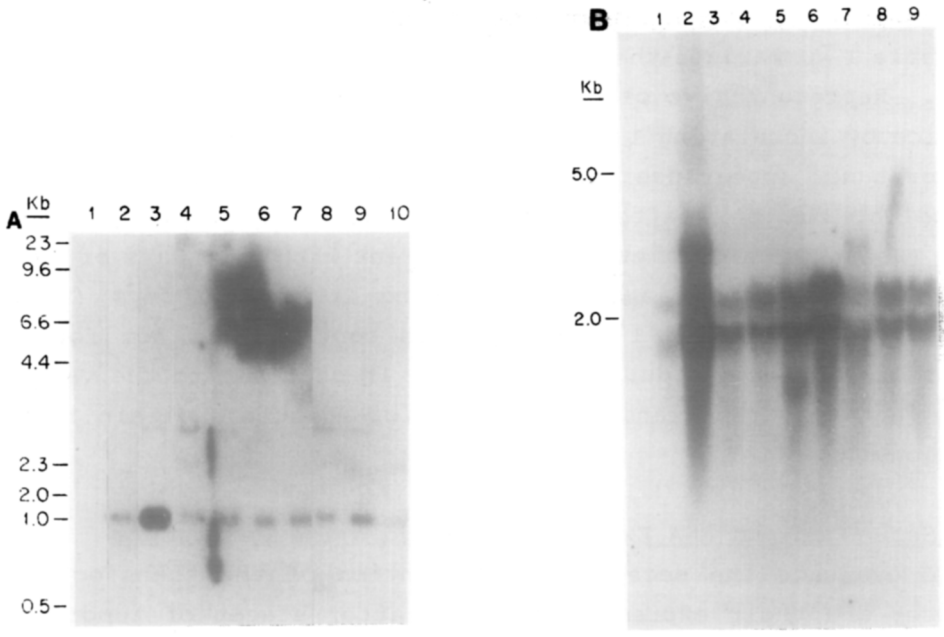


Fig. 2

Southern and Northern blot analyses of rho induced nude mouse tumors.

A Aliquots (10μg) of EcoRI-restricted genomic DNA from rho A induced nude tumor cell lines (lanes 2-10) as well as from Rat-1 cells DNA as a control (lane 1) were resolved on a 1% agarose gel and transferred to nitrocellulose. The blot was probed with a ^{32}P-labeled 600-bp XbaI-EcoRI-specific fragment for detection of the human rho A. Molecular sizes (in kilobases) as determined by migration of Hind III-digested lambda fragments are shown.

B rho A cDNA transcripts in rho induced tumorigenic cell lines (lane 1-9). Approximately 15 μg of total RNA was extracted from the indicated Rho1 DHFR foci, separated on a formaldehyde-containing 1% agarose gel and transferred to nitrocellulose. Blots were hybridized with a ^{32}P-labeled 600-bp XbaI-EcoRI-specific fragment.

tumor derived cell line RNA revealed specific expression of mRNA of rho A (Fig. 2B). Taken together, these data indicate retention and expression of the transfected rho A sequences in the tumors.

Establishment of Rat Embryo Cells in Culture by rho A cDNA

In an attempt to establish long-term cultures of early-passage rat embryo cells, we transfected the rat embryo cells with the rho A

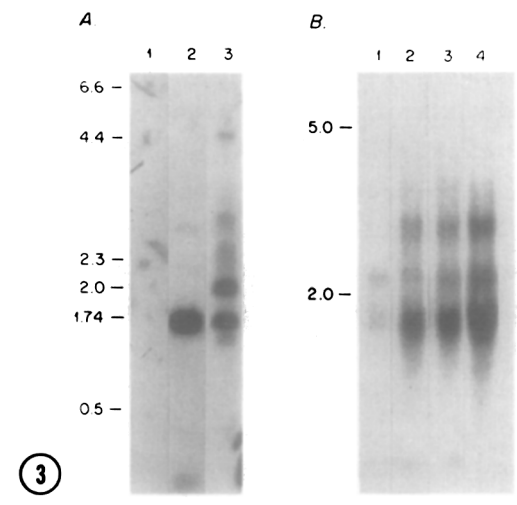
expression vector pJ3W under the SV_{40} promoter. The cells were then passaged at a ratio of 1:2 twice a week. Upon further passaging, the cells altered their growth properties and ceased to proliferate.

Reasoning that normal cells in the cultures might inhibit the proliferation of transfected cells expressing rho A, we then used an alternative strategy. Cells were co-transfected with the rho A expression vector and with the plasmid pSV2/DHFR, conferring methotrexate resistance. Approximately 48 hrs later, methotrexate was added to the culture medium, and the cells were then maintained in the selection medium. Three weeks after transfection, a few colonies of methotrexate resistant cells were clearly visible. Two cell lines were derived from 10 isolated cell clones. These cell lines have been established in long-term culture for more than 40 generations and then ceased to proliferate. The cells displayed a fusiform morphology, differing from the less refractile, flat morphology of REF cells (data not shown). To analyze rho A sequences in the transfected cells, DNA from each clone was digested with EcoRI restriction endonuclease and analyzed by electrophoresis in agarose. We used the 250bp of BalI-EcoRI fragment specific for the detection of rho A sequences as the molecular probe. The results indicated that both clones carried high copy numbers of human rho A cDNA, the 1.74 Kb fragment (Fig. 3), and Northern blot analysis of RNA revealed high expression of rho A mRNA (Fig. 3A). Elevated amounts of the rho p21 were observed in both clones compared to normal REF (Fig. 4). However, these cells were not tumorigenic in nude mice, did not form foci and did not grow in soft agar.

It therefore appears that the sustained proliferation of rat embryo cell line cotransfected with rho A cDNA and pSV2/DHFR, resulted probably from the amplified numbers of rho A copies.

Cooperation of myc with rho A

Rat embryo cells can be established in culture by a variety of protooncogenes and oncogenes including myc and adenovirus E1a. The malignant transformation of REFs requires the cooperative actions of an overexpressed c-myc gene and a mutant ras oncogene. In order to evaluate coopertion of rho A with the myc gene in the Rat-1 and REF transformation assays, assayed by development of dense foci, transfection experiments with both genes were performed. pSVv-myc, which contains the myc gene (10), was neither able to transform REFs or Rat-1 alone nor cooperate with rho to transform Rat-1 or REFs at a detectable level in our assay. Thus, there did not appear to be an interaction between myc and rho A in our system.



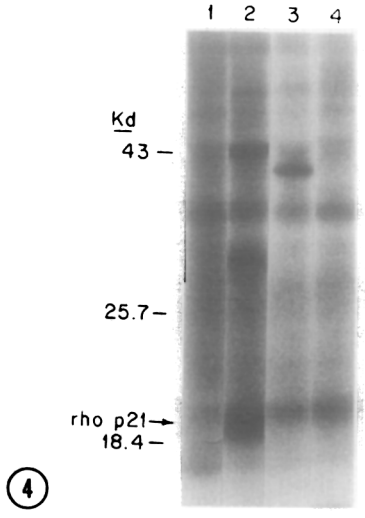


Fig. 3

Detection of transfected rho A in REF cell lines.

A DNA was extracted, cleaved with EcoRI and analyzed as described. Intensity of bands at 1.74 Kbp reflects the copy number of human rho A. Lane 1: untransfected rat embryo cells; Lanes 2-3: REF transfected clones.

B Abundance of rho RNA in established rat embryo cells. RNA was isolated and analyzed in an agarose gel (20 μg per lane) as described. Lane 1- normal rat embryo cells. Lane 2-3: REF transfected clones. Lane 4: Rat Rho1 DHFR cell blots were hybridized with a 600-bp XbaI-EcoRI ^{32}P-labeled rho A cDNA.

Fig. 4

Immunoprecipitation of rho proteins. Rat Rho 1 DHFR and REF cell lines were metabolically labeled with [^{35}S] cysteine, and lysates were prepared and subjected to immunoprecipitation with preimmune serum followed by immunoprecipitation with the rho peptide antiserum. Lane 1 - REF cells; lane 2 - Rat Rho 1 DHFR; lane 3-4: REF transfected clones.

Discussion

Although the rho gene is related to the ras gene, (7) formal evidence for its oncogenic potential has been lacking. Here we have demonstrated that rho sequences can contribute to the tumorigenicity of Rat-1 cells. Transfection of a rho A construct specifically induced

tumorigenicity in these cells when rho expression was high. The established cell lines from the rho A mediated tumors exhibited a transformed morphology when established in culture. These results indicate that there is a longer latency period required for rho A associated tumor formation compared to the ras transformed cells. Additional secondary events probably contribute to the tumorigenicity of rho A expressing cells. The results of our experiments also demonstrate that the tumorigenicity of Rat rho transfected cell lines was correlated with amplification and expression of rho A cDNA.

Two basic conditions were required to sustain the long-term proliferation of rat embryo cells: the absence of adjacent normal cells and a high expression level of the rho encoded p21 protein. In heterogeneous cell cultures, normal cells adjacent to transfected cells can be eliminated by selective killing with a cytotoxic drug which spares only the cells that have acquired a drug resistance marker cointroduced with the proto-oncogene (13). The levels of rho p21 expression in proliferating cells was far higher (> 10 times) than the levels observed in normal REF indicating that the abundant expression of rho can sustain the long-term proliferation of rat embryo cells for about 40 generations.

Several reports indicating that amplification of oncogenes may be a common occurrence in tumors have recently appeared. Amplification of c-myc, and genes related to c-myc, have been detected in tumor cell lines and also in many primary human tumors (14-15). In human mammary cancers, amplification of the gene c-erbB2/HER is correlated with a higher malignant phenotype and poor prognosis (16). Several other proto-oncogenes, including c-abl, N-ras and K-ras, have been found to be amplified in a variety of tumor cell lines as well as in some primary neoplasms (17). In these cases, gene amplification was always associated with increased expression of the corresponding proto-oncogene product. It is very likely that increased amounts of normal proto-oncogene proteins may alter the basic regulatory controls of cell proliferation.

The observation that multiple copies of the human rho A proto-oncogene induced malignant transformation of Rat-1 cells should lead to studies to determine whether rho gene amplification occurs in unmanipulated human tumors.

Acknowledgments

We would like to thank Dr. P. Madaule and R. Axel for providing the rho A clone cDNA. H. Avraham was supported by Rothschild and Fulbright-Hays fellowships.

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