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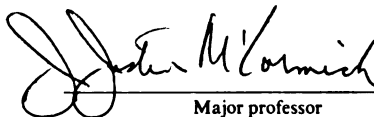
THE ROLE OF TWO RHO GTPASES, CDC42 AND RAC1,
IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

presented by

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of the requirements for

Ph.D. degree in Biochemistry and
Molecular Biology



Major professor

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**THE ROLE OF TWO RHO GTPASES, CDC42 AND RAC1,
IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS**

By

Kim-Hien T. Dao

A DISSERTATION

**Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

THE ROLE OF TWO RHO GTPASES, CDC42 AND RAC1, IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

By

Kim-Hien T. Dao

It is well established that carcinogenesis is a multistep process involving repeated selection and clonal expansion of cells that acquire the ability to proliferate and function uncontrollably as a result of genetic mutations. Increased activation of Rho GTPases, a subfamily of the Ras superfamily of small G-proteins, has been shown to confer cells with certain growth properties characteristic of malignant cells. However, the specific contribution of Rho GTPases to the malignant transformation of human fibroblasts has not been determined. My research emphasis has been to investigate the role of two members of the Rho subfamily, Cdc42 and Rac1, in the malignant transformation of human fibroblasts. Cdc42 and Rac1 were studied in parallel because they often act in the same pathways to regulate transcription of genes, cell cycle progression, cell differentiation, survival and apoptosis pathways, and membrane trafficking. For my studies, I chose two tumor-derived cell lines generated from the human fibroblast MSU1 cell lineage that display increased Cdc42 and Rac1 activity.

To investigate whether Cdc42 and Rac1 act as downstream mediators of oncogenic H-RAS, dominant-negative Cdc42 and/or Rac1 mutants were expressed in cells malignantly transformed by oncogenic H-RAS. Expression of dominant-negative mutant(s) in these cells significantly inhibited growth properties characteristic of the parental cell line, such as rapid cellular proliferation, focus-forming ability in culture, and tumor formation in athymic mice. These results

indicate that intact Cdc42 and Rac1 activity are required for oncogenic H-RAS transformation of human fibroblasts. Affymetrix GeneChip analysis was carried out to identify genes regulated by expression of oncogenic H-RAS *and* dominant-negative Cdc42 and Rac1 mutants. The mRNA expression of 28 genes was regulated in this manner. The endothelial PAS domain 1 (EPAS1) gene encodes a hypoxia-induced transcription factor and is one of nine genes that were dependent on intact Cdc42 and Rac1 activity for up-regulation by oncogenic H-RAS. Further analysis showed that secretion of vascular endothelial growth factor, a gene known to be up-regulated by EPAS1, was dependent upon Cdc42 and Rac1 activity.

To investigate the importance of endogenous, hyperactive Cdc42 and Rac1 in maintenance of growth properties characteristic of malignant cells, dominant-negative Cdc42 and/or Rac1 mutants were expressed in carcinogen-transformed cells that display a high level of Cdc42 and Rac1 activity. The activity of Cdc42 and Rac1 were determined by a p21-activated kinase pull-down assay, which utilizes the fact that the p21-binding domain only binds to activated, GTP-bound forms of Cdc42 and Rac1. The cells were infected with retroviruses carrying the cDNA encoding β -galactosidase, dominant-negative Cdc42, dominant-negative Rac1, or both mutants, and then selected with puromycin for the drug resistance marker. Three independent cell populations were generated for each cDNA, and the cells were injected subcutaneously into athymic mice. In addition, clonally-derived cell populations that express either a low or high level of the mutant(s) were established, as a more definitive test of the effect of dominant-negative mutant(s) expression on tumor formation in athymic mice.

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LIST OF ABBREVIATIONS

BCL-2, B-cell lymphoma 2
BPDE, benzo[a]pyrene diol epoxide
Cdc42, cell division cycle 42
CDK, cyclin-dependent kinases
cDNA, complementary DNA
Dbl, diffuse B-cell lymphoma
DH, Dbl homology
DNA, deoxyribonucleic acid
EPAS1, endothelial PAS domain 1
ERK, extracellular signal-regulated kinase
GAP, GTPase activating protein
GDI, guanine-nucleotide dissociation inhibitor
GDP, guanosine diphosphate
GEF, guanine-nucleotide exchange factor
G-protein, guanine-nucleotide binding protein
GST-PBD, glutathione-S-transferase-p21-binding domain
GTP, guanosine triphosphate
GTPase, guanosine triphosphate hydrolase
HRE, hypoxia response element
JNK, c-Jun amino terminal kinase
MAPK, mitogen-activated protein kinase
MEK, MAP/ERK kinase
NER, nucleotide excision repair
PAK, p21-activated kinase
PAS, period, aryl hydrocarbon receptor nuclear translocator, and single-minded
PBD, p21-binding domain
PH, pleckstrin homology
PI3K, phosphatidylinositol 3-kinase
PIP3, phosphatidylinositol (3,4,5)-bisphosphate
Rac, Ras-related C3 botulinum toxin substrate
Ral-GDS, Ral guanine-nucleotide dissociation stimulator
Rb, retinoblastoma
Rho, Ras homology
RIP1, Ral-interacting protein 1
SH2, Src homology region-2
SH3, Src homology region-3
SCS, supplemented calf serum
TBST, Tris-buffered saline containing 0.1% Tween-20
VEGF, vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor

INTRODUCTION

The presence of hallmark genetic defects in certain types of cancers and in familial cancer syndromes has led to the recognition that cancer is a genetic disease. Since then, there has been steady progress in studying genes involved in the multistep process of carcinogenesis. In the classical genetic description, two kinds of genes are involved, i.e., proto-oncogenes and tumor suppressor genes. Proto-oncogenes act as dominant-acting genes whereas tumor suppressor genes act as recessive-acting genes. Oncogenic activation of one allele of a proto-oncogene produces a cellular defect despite the presence of the other normal allele. In contrast, both alleles must be inactivated for a tumor suppressor gene to confer a cellular defect. Typically, the cellular genes involved in carcinogenesis are those that regulate cellular growth and differentiation, apoptosis pathways, DNA repair pathways, and telomere function (Cotran et al., 1999). Disrupted function of these genes contributes to the malignant transformation of cells if they confer a selective growth advantage, such as uncontrolled cellular proliferation, increased resistance to apoptosis signals, sustained angiogenesis, or enhanced invasive and metastatic potential (Hanahan and Weinberg, 2000). It has been estimated that 4-7 rate-limiting genetic defects are necessary to convert a normal human cell into a malignant cell (Renan, 1993). Thus, cancer cells have undergone progressive selection and clonal expansion such that they have acquired all the genetic changes necessary to confer the malignant phenotype.

Mutations in hundreds of genes could produce cellular defects that confer a selective growth advantage during the carcinogenesis process. However, only a small subset of these genes has widespread dysfunction in various types of human

cancers. An example of such genes is the RAS genes, which contain oncogenic mutations in approximately 30% of all human tumors (Bos, 1988 and 1989). This may be a significant underestimate because functional activation of Ras pathways may occur in the absence of mutations in the RAS genes themselves. Ras proteins are prototypes of a superfamily of small guanine-nucleotide binding proteins (Hall, 1990 and 1992b). By cycling between active, GTP-bound states and inactive, GDP-bound states, Ras proteins act as functional switches to regulate downstream signaling pathways. Activated Ras proteins activate three effector proteins, Raf, PI3K, and Ral-GDS, which constitute three major effector-mediated pathways involved in Ras function (reviewed in Crespo and Leon, 2000). All three pathways are required for RAS-transformed cells to exhibit malignant growth properties (Vojtek and Der, 1998). To gain insight into mechanisms of oncogenic RAS transformation of cells, many recent studies have focused on understanding the contribution of specific Ras effector pathways to the malignant transformation process.

Recently, it has been shown that Ras acts upstream of Cdc42 and Rac1 through phosphatidylinositol 3-kinase (Stephens et al., 1996; Aspenstrom, 1999). Some members of the Rho subfamily of GTPases were identified based on their biochemical and structural similarities to Ras proteins. However, the cellular functions of Rho GTPases are unique from that of Ras proteins (Hall, 1990 and 1992b). Cdc42, Rac, and Rho, three prototype members of this subfamily, induce morphologically distinct cytoskeletal structures in quiescent fibroblasts stimulated with growth factors (Ridley and Hall, 1992; Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995; Machesky and Hall, 1997). Cdc42 induces filopodia, Rac1 induces lamellipodia and membrane ruffles, and Rho induces stress fibers. Sequential formation of these structures led to the proposal that Cdc42 activates

Rac1, which then activates Rho. The Rho subfamily regulates other cellular functions including transcription, cell cycle progression, differentiation, survival and apoptosis pathways, and membrane trafficking (Van Aelst and D'Souza Schorey, 1997). Cdc42 and Rac1 act in parallel through p21-activated kinase to regulate some of these cellular processes (Bagrodia and Cerione, 1999). Three families of proteins regulate activity of Rho GTPases, the GTPase-activating proteins, the guanine-nucleotide dissociation inhibitors, and the guanine nucleotide exchange factors. Evidence that Cdc42 and Rac1 play a role in the malignant transformation of rodent fibroblasts comes from studies that show the transforming activity of oncogenic derivatives of guanine nucleotide exchange factors (Cerione and Zheng, 1996), the requirement of Cdc42 and Rac1 activity in oncogenic H-RAS-induced transformation (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997), and the involvement of p21-activated kinase in the induction of survival pathways (Tang et al., 2000; Jakobi et al., 2001). However, the specific contribution of Cdc42 and Rac1 to the malignant transformation of human fibroblasts has not been determined.

There are at least two possible mechanisms by which Cdc42 and Rac1 contribute to malignant transformation of cells. In cells that express the DBL oncogene, i.e., a truncated derivative of the Dbl proto-oncogene (Tang et al., 2000; Jakobi et al., 2001), which is a guanine-nucleotide exchange factor for Rho GTPases (Hart et al., 1991; Yaku et al., 1994), there is a dramatic increase in GTP-bound levels of Cdc42 and Rac1 due to an increase in exchange activity. Because the GTP hydrolysis rate is not concomitantly increased, ***a steady-state increase or static increase in Cdc42 and Rac1 activity*** is observed in these cells. In contrast, in cells that express the ABR oncogene (Chuang et al., 1995) or the RAS oncogene (Aspenstrom, 1999), an increase in exchange activity of Cdc42 and Rac1 is coupled

with an increase in GTP hydrolysis rates of these proteins. Therefore, the GTP-bound level of Cdc42 and Rac1 may not increase because there is rapid conversion of the GTP-bound states to the GDP-bound states. Thus, there is a ***dynamic increase in Cdc42 and Rac1 activity*** because there is increased cycling of the proteins between these guanine-nucleotide-bound states. Activation of Cdc42 and Rac1 by either of these two mechanisms plays a causal role in the malignant transformation of cells but the mechanism of activation probably influences their specific contribution to the malignant phenotype.

My dissertation research has been focused on investigating the role of Cdc42 and Rac1 in the malignant transformation of human fibroblasts. For my studies, I chose two tumor-derived fibrosarcoma cell lines generated from the non-tumorigenic MSU-1.1 human fibroblast cell strain. To generate such fibrosarcoma cell lines, morphologically transformed MSU-1.1 cells were isolated following expression of an oncogene or treatment with a carcinogen and then injected subcutaneously into athymic mice. Tumors from athymic mice were cultured to establish tumor-derived fibrosarcoma cell lines. PH3MT, was transformed by overexpression of the H-RAS oncogene (Hurlin et al., 1989) and is expected to have increased cycling of Cdc42 and Rac1 between active and inactive states. L210-6A/SB1, was transformed by BPDE carcinogen treatment (L. Milam, unpublished studies). These cells exhibit elevated levels of active, GTP-bound Cdc42 and Rac1 proteins. Dominant-negative Cdc42 and/or Rac1 mutants were expressed in both these cell lines, and the effect of these mutant(s) on the transformed phenotype of these cells was investigated.

The conclusions from the study with PH3MT cells are summarized here. Expression of dominant-negative Cdc42 and/or Rac1 mutants in cells malignantly transformed by oncogenic H-RAS inhibited rapid cellular proliferation, focus-forming

ability, and tumor growth in athymic mice. These results indicate that Cdc42 and Rac1 activity are required for oncogenic H-RAS-induced transformation of human fibroblasts. To determine the specific contribution of Cdc42 and Rac1 to oncogenic H-RAS transformation, Affymetrix GeneChip analysis was used to identify genes that are dependent on Cdc42 and Rac1 activity for up-regulation or down-regulation by oncogenic H-RAS. There were 28 genes that exhibited such regulation. One gene identified was EPAS1, a hypoxia-inducible transcription factor that displayed dependence on intact Cdc42 and Rac1 activity for up-regulation by oncogenic H-RAS. In turn, EPAS1 is known to up-regulate the vascular endothelial growth factor gene (Tian et al., 1997). Further analysis showed that, as expected, secretion of vascular endothelial growth factor into the growth medium is dependent on Cdc42 and Rac1 activity. These results reveal a mechanism by which H-RAS induces up-regulation of vascular endothelial growth factor, a protein that plays a major role in tumorigenesis and angiogenesis of solid tumors.

The dissertation is organized into three major parts. **Chapter 1** is a literature review of the current paradigm of carcinogenesis, multiple Ras pathways and their contribution to carcinogenesis, and Rho GTPases as important cellular mediators of malignant transformation. **Chapter 2** is a copy of a manuscript to be submitted to the journal *Nature Genetics*. It describes the results of research carried out with the PH3MT cell line. The title of the paper is "H-RAS-induced transformation of human fibroblasts is dependent on Cdc42 and Rac1 activity, as is up-regulation of EPAS1." This is followed by **Appendix A**, which describes the results of the research carried out with the L210-6A/SB1 cell line. Tumor studies for this work is currently in progress. **Appendix B** describes the results of PCR-Select cDNA subtractive hybridization analysis between MSU-1.1 cells and L210-6A/SB1 cells.

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CHAPTER I: LITERATURE REVIEW

A. Current paradigm of carcinogenesis

1. *Cancer is a genetic disease*

In 1914, T. Boveri published his theory that the accumulation of somatic mutations in cells causes the transformation of normal cells to cancer cells (Boveri, 1914). Support of his theory comes from investigation of hallmark chromosomal abnormalities present in certain leukemias and in Burkitt's lymphomas. It was discovered that such chromosomal abnormalities result in oncogene activation by balanced translocation (reviewed in Solomon et al., 1991). The finding that defects in gene structure and function are consistently present in inheritable forms of cancers, e.g., germline mutation of the retinoblastoma gene (Friend et al., 1986), further supported the theory that cancer originates from cells that harbor genetic defects. Such defects may result from point mutation, deletion, amplification of genes, recombination of DNA sequences, or rearrangement of chromosomes. These mutational events may be induced by chemical or physical agents or inherited in the germline. Since the discovery that cancer is a genetic disease, cancer research has been largely directed at understanding the genetic and molecular basis of cancer.

Under certain cellular contexts, the altered function of mutated genes may confer cells with a selective growth advantage (Hanahan and Weinberg, 2000). These genes can be categorized into four groups: genes involved in growth stimulation (proto-oncogenes), genes involved in growth inhibition (tumor suppressor genes), genes involved in apoptosis pathways, and genes involved in DNA repair pathways (Cotran et al., 1999). The intact function of genes that regulate apoptosis

pathways and DNA repair pathways is critical for preventing propagation of cells containing genetic damage. A further simplification can be made by describing genes in these two categories as proto-oncogenes or tumor suppressor genes depending on their ultimate gene function. Telomeres and telomerases will also be discussed. In the classical genetic description, proto-oncogenes have dominant-acting effects and tumor suppressor genes have recessive-acting effects (Barrett et al., 1986; Yuspa et al., 1994b). For example, an *activating mutation of one allelic copy of a proto-oncogene*, thereby converting it to an oncogene, produces a cellular defect despite the presence of the other normal allelic copy. In contrast, *tumor suppressor genes require inactivating mutations of both allelic copies* to produce a cellular defect. Generally, activating mutations in oncogenes result in a gain of function and inactivating mutations in tumor suppressor genes result in a loss of function.

2. Classification of cancer-related genes

a. Dominant-acting proto-oncogenes (e.g., RAS, MYC genes)

Proto-oncogenes encode proteins that usually promote cell growth and differentiation such as growth factors, growth factor receptors, proteins involved in signal transduction, nuclear transcription factors, and cyclins and cyclin-dependent kinases (reviewed in Dang et al., 1997; Todd and Wong, 1999; Prober and Edgar, 2001). A mutation that converts a proto-oncogene to its oncogenic derivative usually disrupts regulatory mechanisms in place to limit the protein at a certain level of activity (Todd and Wong, 1999). As a result, the proteins encoded by these oncogenes usually assume constitutively active protein conformations (e.g., H-RAS oncogene) (Feinberg et al., 1983), lose intrinsic domains that normally inhibit its

catalytic domains (e.g., DBL oncogene) (Eva and Aaronson, 1985), or lack functional domains required for negative regulation by other proteins. In other cases, there may simply be a marked increase in proto-oncogene expression as a result of one of the mutational events described earlier (e.g., Myc up-regulation) (Taya et al., 1984; Huber et al., 1985; Koda et al., 1985; Schwab et al., 1985).

The RAS gene is discussed further as an example of a proto-oncogene because activating mutations of RAS genes are frequently found in human and experimental tumors (Bos et al., 1987; reviewed in Bos, 1988). Ras is a small guanine-nucleotide binding protein (G-protein) that cycles between an inactive, GDP-bound state and an active, GTP-bound state (Hall, 1990 and 1992b). The Raf/MEK/ERK pathway, one of several major pathways stimulated by GTP-bound Ras, is a growth factor-stimulated pathway that induces transcription of genes involved in cell cycle progression and cell differentiation (Robinson and Cobb, 1997; reviewed in English et al., 1999). This pathway leads to up-regulation of cyclins (Fan and Bertino, 1997) and down-regulation of p27^{Kip1} (Rivard et al., 1999), which cooperatively induce cellular entry into S-phase and DNA synthesis. In cells that acquire an oncogenic or activating mutation of the RAS gene, there is overstimulation of Ras pathways without the normal dependence on upstream stimulatory inputs such as growth factor binding to its receptor (Balmain, 1985; Sekiya et al., 1985; Williams et al., 1985). This results in a *gain of function of Ras*, which causes uncontrolled cell growth and differentiation (Balmain, 1985; Sekiya et al., 1985; Williams et al., 1985). The contribution of the RAS genes to the malignant transformation of cells will be discussed in section I.B.

Another example of a proto-oncogene is the c-MYC gene, which encodes a basic-helix-loop-helix-zipper (bHLHZ) transcription factor (Dang et al., 1992; Torres

et al., 1992; Wechsler and Dang, 1992). It heterodimerizes with another bHLHZ transcription factor, Myc associated factor X (Max), in order to bind to CACGTG sites (E-boxes) present in the promoter region of genes that promote cell cycle progression and apoptosis pathways (Blackwood and Eisenman, 1991). The MYC gene is overexpressed in many human tumors, usually as a result of gene translocation or amplification (reviewed in Alitalo et al., 1983; Facchini and Penn, 1998). In Burkitt's lymphoma, the c-MYC gene, which is normally located at 8q24, is translocated to a chromosome region that contains the immunoglobulin heavy chain gene (14q32) (McFarlane et al., 1970; Warrens, 1974; Kaneko et al., 1980). The chromatin structure in this region favors highly active transcription. Cells with an increased steady-state level of Myc have sustained up-regulation of target genes that confer cells with uncontrolled cellular proliferation and differentiation (Koda et al., 1985; Schwab et al., 1985; Cole, 1986a and 1986b; Land et al., 1986). Evidence that the biological activity of Myc is linked to its transcriptional activity comes from studies that show mutations in the transactivation domain or in the bHLHZ domain abrogate the cellular defects induced by Myc overexpression (Kretzner et al., 1992a and 1992b).

b. Recessive-acting tumor suppressor genes (e.g., RB, p53 genes)

Tumor suppressor genes encode proteins that regulate a variety of cellular processes but their main regulatory input is to inhibit uncontrolled cell growth and differentiation (Geiser and Stanbridge, 1989; Macleod, 2000). The first tumor suppressor gene identified is the retinoblastoma (Rb) gene (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987), which encodes a nuclear protein that regulates cell cycle progression, cell differentiation, and p53-dependent and p53-independent

apoptosis pathways (reviewed in Nevins, 2001). Rb function is disrupted in retinoblastomas and frequently, in other human cancers, including osteosarcomas, small cell lung cancer, and breast and prostate carcinomas (reviewed in Nevins, 2001). Active, hypophosphorylated Rb binds to the E2F family of transcription factors and sequesters them from activating transcription (Flemington et al., 1993). The E2F family of transcription factors up-regulate genes that contain E2F sites in the promoter region and promote G1→ S transition in the cell cycle (Dyson, 1998; Nevins, 2001). More recent studies show that the Rb-E2F complex also actively represses transcription (Sellers et al., 1995; Weintraub et al., 1995). Furthermore, recruitment of histone deacetylase by the Rb-E2F complex promotes nucleosome assembly on the promoter, which prevents the transcriptosome from having access to DNA sequences (Brehm et al., 1998; Luo et al., 1998; Magnaghi Jaulin et al., 1998; Lai et al., 1999; Chen and Wang, 2000). Mutational or functional inactivation of Rb leads to unrepressed E2F-mediated transcription of genes that contribute to the malignant phenotype (reviewed in Nevins, 2001). Evidence that Rb acts as a tumor suppressor gene comes from several studies. First, re-introducing Rb into Rb-deficient cells impairs growth characteristics of the malignant phenotype (Huang et al., 1988). Second, in tumors that lack mutant Rb genes, functional inactivation of Rb occurs by a different mechanism such as hyperphosphorylation (Sherr, 1996). Third, transforming DNA viruses have been shown to carry DNA sequences that encode oncoproteins that bind to and inactivate the Rb protein; these include adenovirus E1A, SV40 large tumor antigen, and human papillomavirus E7 (DeCaprio et al., 1988; Dyson et al., 1989).

Retinoblastomas are tumors of retinal cells that contain two mutant copies of the Rb genes. Knudson described this phenomenon as a “two-hit” hypothesis

(Knudson, 1971). *Sporadic cases* are the result of two independent mutation events, one for each allelic copy of the Rb gene, occurring in the same retinal cell. In *familial cases*, the first genetic event is inherited from a parent with a germline mutation of the Rb gene. The second genetic event occurs spontaneously in cells already containing a single mutant allelic copy of the Rb gene. Therefore, two mutant copies of the Rb gene are present in both sporadic and familial cases of retinoblastoma. However, an important difference between sporadic and familial cases is that familial cases are nearly always associated with bilateral retinoblastomas and those patients that do survive this childhood tumor have a much higher risk of developing other types of cancers later in life, especially bone and soft tissue sarcomas, and malignant melanomas (reviewed in Nevins, 2001).

Another example of a tumor suppressor gene is the p53 gene, which is a key guardian against propagation of cells containing genetic damage. p53 acts as a transcription factor and induces expression of genes involved in cell cycle arrest (Hicks et al., 1991; Livingstone et al., 1992), apoptosis (Yonish Rouach et al., 1991; Shaw et al., 1992; el Deiry et al., 1994), and DNA repair (Marx, 1994). Both allelic copies of the p53 gene contain inactivating mutations in approximately 50% of all human tumors (Baker et al., 1989; Nigro et al., 1989; Hollstein et al., 1991). The p53 protein is normally expressed at a low level and has a short half-life but its protein level accumulates rapidly when DNA damage is detected (Jenkins et al., 1985; Williams and Wynford Thomas, 1989). DNA damage-induced accumulation of p53 occurs as a result of inactivation of murine double minute 2 (mdm2), a protein that targets p53 for ubiquitin-dependent degradation (Honda et al., 1997; Momand et al., 2000). Accumulation of p53 leads to up-regulation of p21WAF1 (Li et al., 1994; Shiohara et al., 1994; Steinman et al., 1994; Candeias et al., 1997; Wilson et al.,

1998), a protein that inhibits cyclin dependent kinase 2 (Chen et al., 1996; Lin et al., 1996) and cell division cycle 2 (Azzam et al., 1997). The result of such inhibition is cell cycle arrest. Depending on the extent of DNA damage, p53 may induce G1 arrest to allow DNA repair processes to take place or apoptosis to prevent propagation of cells with extensive damage (Hainaut and Hollstein, 2000). Thus, p53 is critical for maintaining genomic stability. Accumulation of p53 is also induced by other cellular signals such as critical shortening of telomeres, increased activation of oncogenes, and aberrant overexpression of tumor suppressor genes (Hainaut and Hollstein, 2000). Individuals that have the Li-Fraumeni syndrome inherit a mutant allelic copy of the p53 gene (Srivastava et al., 1990; Santibanez Koref et al., 1991; Toguchida et al., 1992). Consequently, they have a much higher predisposition of acquiring various types of cancers because the first “hit” is present in all somatic cells. A second “hit” occurring in the remaining normal allelic copy of the p53 gene results in loss of heterozygosity and complete loss of p53 function. The role of p53 as a tumor suppressor gene is further supported by the finding that overexpression of Mdm2 has oncogenic potential in human tumors (Momand et al., 1992; Oliner et al., 1992).

c. Genes regulating apoptosis pathways

Apoptosis, also known as programmed cell death, is a highly regulated process of cell death, which includes shrinkage of cells, nuclear condensation, degradation of nucleic acids and proteins, loss of membrane integrity, and cellular fragmentation (Cotran et al., 1999). The net activity of anti-apoptosis and pro-apoptosis pathways determines the fate of the cell confronted with extensive DNA

damage or unfavorable growth conditions. Disruption of this balance occurs, for example, if the genes involved in these pathways have mutational defects.

In B-cell lymphomas, up-regulation of the B-cell lymphoma 2 (BCL-2) gene occurs upon translocation of the gene to the 14q32 chromosome region, i.e., the same region involved in Burkitt's lymphoma (Cleary et al., 1986; Tsujimoto and Croce, 1986). Bcl-2 induces anti-apoptosis pathways by forming homodimers that displace bax proteins from the mitochondrial membrane (Oltvai et al., 1993; Yin et al., 1994). Bax, as a homodimer, is a channel-forming protein in the mitochondrial membrane that allows cytochrome C to be released into the cytoplasm (Manon et al., 1997; Jurgensmeier et al., 1998; Rosse et al., 1998). In turn, cytochrome C activates caspase-9, a protease that degrades cellular proteins as part of the apoptosis process (Li et al., 1997b). Up-regulating BAX gene expression is one mechanism by which p53 induces apoptosis (Miyashita et al., 1994; Selvakumaran et al., 1994; Zhan et al., 1994). Cells that overexpress bcl-2 are resistant to apoptosis signals even though there may be significant DNA damage present such as that caused by irradiation and chemotherapy. Although these Bcl-2-expressing cells are non-tumorigenic, they are more likely than normal cells to accumulate additional genetic changes that contribute to its malignant transformation. This is an example of a net gain in activity of anti-apoptosis pathways. Conversely, a net loss in activity of pro-apoptosis pathways may contribute to the malignant transformation process, e.g., a loss of bax activity (Hara et al., 1997; Krajewski et al., 1997; Colella et al., 1998; Gil et al., 1999).

Phosphoinositide 3-kinase (PI3K) is another gene that induces cell survival pathways (Kauffmann Zeh et al., 1997; Khwaja et al., 1997). Many receptor and soluble tyrosine kinases activate PI3K, which has both protein and lipid kinase

activity (reviewed in Vanhaesebroeck and Waterfield, 1999). PI3K phosphorylates inositol lipids at the third position in the inositol ring and generates active second messengers such as phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-bisphosphate (PIP3). These lipid products are involved in regulating signaling pathways that carry out the cellular functions of PI3K, including cell growth/survival, intracellular trafficking, and cellular motility (Vanhaesebroeck and Waterfield, 1999). Induction of cell survival is mainly through PI3K-mediated phosphorylation and activation of Akt (Franke et al., 1995; Datta et al., 1996; Kulik et al., 1997). Akt phosphorylates and inactivates Bad, which is a Bax-like protein that induces apoptosis signals (Datta et al., 1997). Thus, cells that have constitutively activated PI3K pathways are resistant to apoptosis. The role of PI3K in the malignant transformation of cells has been reported. Constitutively active PI3K mutants have been found in viral-induced (Aoki et al., 2000) and radiation-induced (Jimenez et al., 1998) tumors, and amplification and overexpression of PI3K have been found in ovarian (Shayesteh et al., 1999) and cervical cancers (Ma et al., 2000). Furthermore, the phosphatase and tensin homologue detected on chromosome 10 (PTEN) protein (Li et al., 1997a) is a lipid phosphatase that removes a phosphate on the active lipid product, PIP3, and acts as a negative regulator of PI3K function (Stambolic et al., 1998). Germline mutations of PTEN are associated with Cowden's disease. Individuals with this disease present with hamartomas and have an increased risk of developing breast cancers (Liaw et al., 1997). Mutation of the PTEN gene is frequently found in human tumors, especially in glioblastomas and cancers of the prostate, endometrium, and ovary (reviewed in Simpson and Parsons, 2001).

d. Genes regulating DNA repair pathways

DNA repair genes are essential for maintaining DNA sequence integrity (Lindahl, 1994; Ishikawa et al., 2001). DNA damage can result from exposure to environmental agents as well as from spontaneous errors during DNA replication. Such damage, if not repaired properly, could result in genetic defects that contribute to the malignant transformation of cells. Individuals with defective DNA repair genes have a strong predisposition of acquiring various types of cancers because the cells accumulate mutations at a much higher frequency compared with normal, repair-proficient cells (Lindahl, 1994). Two examples of defective DNA repair genes and their role in human cancers are discussed.

Defects in DNA mismatch repair genes are responsible for the most common form of hereditary colon cancer, i.e., hereditary nonpolyposis colon cancer (Fishel et al., 1993; Aaltonen et al., 1994; Bronner et al., 1994; Jiricny, 1994; Liu et al., 1994; Peltomaki, 1994). DNA regions containing short tandem repeat sequences are especially prone to frameshift mutations because of misalignment of repeats in the template and the newly synthesized strand during replication (Kim et al., 1994; Aquilina and Bignami, 2001). Loss of mismatch repair genes human MutL homologue 1 and MutS homologue 2 accounts for approximately 50% and 40%, respectively, of cases of hereditary nonpolyposis colon cancer (reviewed in Peltomaki, 2001). Individuals with germline mutations in one of those genes have increased susceptibility to colon cancer because, as described above, a somatic mutation or mitotic recombination event in the remaining normal allelic copy can eliminate the function of the gene, which results in profound defects in DNA

mismatch repair. Such cells are characterized with progressive accumulation of mutations throughout the genome (Peltomaki, 2001).

Defects in genes involved in nucleotide excision repair (NER) are responsible for the autosomal recessive disorder xeroderma pigmentosum (XP) (Cleaver, 1990). Individuals with such defects are extremely susceptible to UV-induced skin cancers (Robbins et al., 1974). XP patients usually die from highly invasive and metastatic squamous and basal cell carcinomas, and malignant melanomas (Hashem et al., 1980; Cleaver et al., 1981). NER-deficient XP patients fall into seven complementation groups, XPA through XPG (reviewed in Berneburg and Lehmann, 2001). The genes encode proteins involved in NER, including damage-recognition proteins, helicases, and nucleases (Berneburg and Lehmann, 2001). This lack of repair accounts for the high frequency of sunlight-induced mutation in the cells of XP patients. In addition to these NER-deficient XP patients, there is one group of patients, designated as XP variants, whose cells are completely proficient in NER (Tung et al., 1996). Nevertheless, the patients are extremely prone to sunlight-induced skin cancer (Cleaver et al., 1981), and the cells exhibit a frequency of sunlight-induced mutations as high as other XP patients. The XP variant gene encodes DNA polymerase eta, which is capable of error-free DNA synthesis past UV-induced thymidine dimers (Masutani et al., 1999). Cells with defects in NER or DNA pol eta display genomic instability, a phenomenon that is known to accelerate the malignant transformation process (Maher et al., 1982; Patton et al., 1984; Tsujimura et al., 1990; Daya Grosjean et al., 1993).

e. Telomeres and telomerases

Telomeres are DNA sequences and protein complexes at the ends of chromosomes (Blackburn, 1991). Telomeres shorten with every replication cycle because the ends of chromosomes lack sequences preceding it for primed-DNA synthesis by DNA polymerase α and δ , a phenomenon described as the “end-replication problem (Levy et al., 1992).” This is an intrinsic mechanism of finite life-span cells to mark the number of cell divisions. After a finite number of cell divisions, normal cells undergo a process termed cellular senescence (Hayflick and Moorhead, 1961) because the telomeres have shortened to a critical length (Preston, 1997; Scherf and Mattei, 1992; Shay and Wright, 2001). Major chromosomal abnormalities such as end-to-end chromosome fusions signal the induction of cellular senescence (Hastie and Allshire, 1989; Ducray et al., 1999). Cells with finite life-span are less likely to accumulate *all the necessary genetic alterations* required to display the malignant phenotype (Hanahan and Weinberg, 2000).

Telomerase is a ribonucleoprotein complex, consisting of an RNA template (Shippen Lentz and Blackburn, 1990) and a catalytic subunit designated as telomerase reverse transcriptase (TERT) (Strahl and Blackburn, 1996; Cech et al., 1997; Lingner et al., 1997) as the core components. TERT catalyzes addition of telomeres by reverse transcription synthesis of hexameric TTAGGG repeats using an RNA template (Meyne et al., 1989; Morin, 1989). This prevents loss of DNA sequences that occurs with every DNA synthesis cycle. As expected, expression of TERT in normal human cells induces telomere elongation and stabilization, and such cells acquire the phenotype of cellular immortalization (Counter et al., 1994; Meyerson, 1998). Normal, finite-life span cells do not express telomerase at a high

level. In contrast, it has been estimated that 90% of human tumors have up-regulated telomerase, and that the remaining tumors have activated other mechanisms to acquire an extended or an infinite life-span (Ducray et al., 1999; Klingelhutz, 1999; Hahn and Meyerson, 2001; Kang and Park, 2001).

Some have described the acquisition of an extended or infinite life-span as a prerequisite or an early step in the multistep process of carcinogenesis (reviewed in Hanahan and Weinberg, 2000). It is now established that multiple genetic changes are required for cellular immortalization (Kuroki and Huh, 1993; McCormick and Maher, 1994). Cells that were infected with the SV40 virus or expressed SV40 T-antigen displayed extended life-span but eventually senesced after 20-30 doublings beyond the normal life-span (Wright and Shay, 1992). Within this population, a small number of cells acquired additional genetic change(s) that conferred cells with cellular immortalization but this occurred at a very low frequency. Shay and Wright estimated this frequency at one in every 30×10^6 human lung fibroblasts transfected with the SV40 T-antigen (Shay and Wright, 1989). Human cells can be immortalized by repeated treatment with carcinogens (e.g., γ -irradiation) or by expression of certain viral or cellular oncogenes (e.g., v-Myc) (McCormick and Maher, 1988; Shay et al., 1991; Bai et al., 1993; Kuroki and Huh, 1993). Hybridization of finite life-span cells and immortalized cells produces hybrid cells that display finite life-span, indicating that cellular immortalization is ultimately caused by recessive gene defects (Pereira Smith and Smith, 1981; Pereira Smith and Smith, 1983; Ryan et al., 1994). The disrupted function of p53 and Rb proteins is thought to play a causal role in cellular immortalization of certain cell types (Shay et al., 1991; Vojta and Barrett,

1995), and the involvement of telomerase has been noted above (Shay and Wright, 2001).

3. Multistep process of carcinogenesis

The focus so far has been on categories of genes that play a causal role in the malignant transformation process. It is important to emphasize that the normal function of these genes is required for various normal cellular functions. The aberrant function of these genes, however, may confer cells with defects that are selected for in the multistep process of carcinogenesis (Hanahan and Weinberg, 2000). The evolution of a normal cell to a malignant cell is a time-dependent process because multiple genetic alterations must accumulate in the same cell, i.e., cancer cells are typically monoclonal in origin (Hanahan and Weinberg, 2000). This is consistent with the observation that most human cancers occur with an age-associated incidence. Renan (1993) estimated that 4-7 genetic changes are necessary to transform a normal cell to that of a malignant cell. At each intermediate "step" the cell acquires an additional growth characteristic that gives the cell more cancer-like properties. Many genes, when functioning aberrantly, have the potential to contribute to the malignant transformation of cells. Although there may be near-infinite permutations of these affected genes in human cancers, there is a relatively limited set of acquired characteristics present in most cancer cells (Hanahan and Weinberg, 2000). In a review, Hanahan and Weinberg (2000) describe six of these characteristics: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The first model to consider carcinogenesis as a multistep process came from studies that repeatedly treated mouse skin with chemical agents (Boutwell, 1964; Hennings et al., 1990; Yuspa, 1994a). As a result of these studies, carcinogenesis was described as a process that occurred in three broadly defined stages: initiation, promotion, and progression. **Initiation** occurs by a single treatment with a mutagen, such as 7,12-dimethylbenz[a]anthracene (Hennings et al., 1990). Some of the progeny cells display phenotypic changes consistent with heritable genetic defects. An oncogenic mutation of the H-RAS gene is frequently found in such cells (Quintanilla et al., 1986; Bailleul et al., 1989; Nelson et al., 1992). However, the mutation spectrum is dependent on the specific target of the active metabolite of the mutagen. **Promotion** occurs by repeated stimulation of initiated mouse skin cells, for example, by wound infliction or 12-O-tetradecanoylphorbol-13-acetate treatment (Hennings et al., 1990). Papillomas typically appear over a few months, and about 50% of these transform to squamous cell carcinomas in about a year. Each papilloma is a clone of initiated cells that acquired an additional genetic event (Deamant and Iannaccone, 1987). The genetic event is thought to occur by epigenetic mechanisms because a single gene change is sufficient to produce papillomas (Greenhalgh et al., 1993) and tumor-promoting agents are typically not mutagens (Yuspa and Poirier, 1988). **Progression** occurs by spontaneous genetic events but can be accelerated by treatment with mutagens such as cisplatin (Hennings et al., 1990). Chromosomal aberrations occur frequently in the malignant transformation of papillomas to carcinomas (Yuspa, 1994a). Other studies show that loss of heterozygosity of the H-RAS (Yuspa et al., 1990), p53 (Boukamp et al., 1995; Dumaz et al., 1997), and transforming growth factor β genes (Bailleul et al., 1989;

Haddow et al., 1991) is also associated with malignant transformation. These studies demonstrate that carcinogenesis is multistep process, involving progressive accumulation of genetic defects that confer cells with more cancer-like properties.

In human cell carcinogenesis, Vogelstein et al. ((1988) and Fearon and Vogelstein, 1990) proposed a genetic model of colorectal carcinogenesis. The transformation of normal colorectal epithelial cells to colorectal carcinomas occurs in well-defined steps. At each intermediate step there is a molecular change that corresponds to a morphological change that promotes progression toward malignancy. Several genes that are involved in the process have been identified, e.g., loss of adenomatous polyposis coli, loss of global DNA methylation, oncogenic mutation of the RAS gene, and loss of tumor suppressor genes (e.g., p53) (Williams et al., 1993; reviewed in Baba, 1997). The successful characterization of colorectal carcinogenesis can be attributed to the fact that colorectal adenomas, the benign tumors that precede colorectal carcinomas, are more readily monitored *in situ* compared with other types of tumors. Individuals with familial adenomatous polyposis inherit a mutated APC in their germline and present with thousands of polyps at various stages of colorectal carcinogenesis (Kinzler et al., 1991). Examination of samples from these patients greatly facilitated identification of genetic and morphological changes at each intermediate stage. The pre-malignant states in most of the other types of common human cancers, e.g., lung, breast, and prostate carcinomas, are not as physically accessible or morphologically discrete. With the development of better detection and isolation strategies, the identification of genetic abnormalities responsible for other human cancers will become more feasible.

An ideal model for human carcinogenesis of a specific tumor type would consist of isogenic cell strains for each of the intermediate, pre-malignant states. However, most commonly, only the cancer cells from an individual are available. Normal cells of the same cell type, if available, are usually from a different individual, which introduces an added complication when determining whether the source of genetic alterations is due to the carcinogenesis process or due to genetic differences among individuals. The “multistep” part of the carcinogenesis process cannot be studied because the cells containing the genetic defect at each intermediate stage are not available. McCormick and colleagues established the human fibroblast MSU1 cell lineage as a model for carcinogenesis to address some of these limitations (Morgan et al., 1991).

The human fibroblast MSU1 cell lineage has been a valuable resource for the characterization of phenotypic and genotypic changes that occur at different stages of the carcinogenesis process (Figure 1). As described in the paper published by McCormick and colleagues (Morgan et al., 1991), the v-MYC oncogene was transfected in a normal human fibroblast cell line, LG1, established from the foreskin tissue of a male newborn. The vector containing the v-MYC oncogene also carried a neomycin resistance marker, which allowed for selection of cells that stably integrated the DNA into the genome. Clonally-derived cells that were neomycin resistant and expressing v-myc were isolated and propagated. All of the cell strains entered senescent crisis. However, in one flask, some viable cells were found among nondividing, senescent cells. These viable cells were maintained in cell culture and designated as **MSU-1.0**. After further characterization, it was determined that these cells display infinite life-span. More recently, increased telomerase activity has been detected in these cells compared with that in the parental cell line

LG1 or the v-myc-expressing precursor cells (Morgan et al., 1991). Since immortalized cells arose in only one flask of cells transfected with the v-MYC oncogene, this indicates that oncogenic v-MYC expression is not sufficient by itself to induce cellular immortalization. An additional genetic event, e.g., spontaneous activation of telomerase, is probably the change that allowed these cells to escape senescence. The immortalized MSU-1.0 cell strain does not display other characteristics that are typical of tumorigenic cell strains, such as growth factor independence and anchorage independence. Furthermore, subcutaneous injection of MSU-1.0 cells into athymic mice has never produced tumors, and karyotype analysis shows that MSU-1.0 cells do not contain any gross chromosomal aberrations. Attempts to malignantly transform this cell strain by treatment with carcinogens or expression of oncogenes have been repeatedly unsuccessful.

During the establishment of the MSU-1.0 cell strain a variant clonal derivative arose spontaneously in cell culture. This variant cell strain, designated as **MSU-1.1**, has a more rapid rate of cellular proliferation compared with that of the MSU-1.0 cell strain (Morgan et al., 1991). Studies show that MSU-1.1 cells display partial growth factor independence but does not display anchorage independence or form tumors in athymic mice. Interestingly, karyotype analysis shows that MSU-1.1 cells have two marker chromosomes that are products of translocation events as described (Morgan et al., 1991). It seems highly likely that one or more of these chromosomal aberrations, and perhaps other genetic events, represent the additional steps necessary to convert MSU-1.0 cells toward more advanced states of cellular transformation. Consistent with this finding is that, in contrast to MSU-1.0 cells, MSU-1.1 cells are more readily transformed to tumorigenic cell lines by treatment

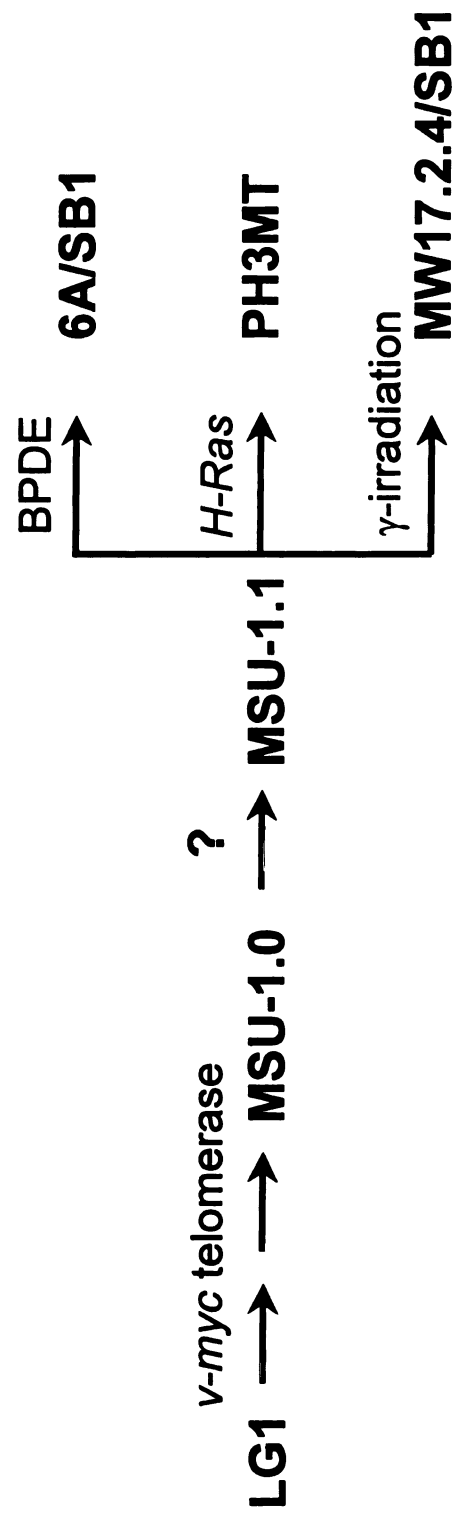
of MSU-1.1 cells with carcinogens (Yang et al., 1992; O'Reilly et al., 1998) or expression of oncogenes in MSU-1.1 cells (Fry et al., 1986; Hurlin et al., 1989; Lin et al., 1995). Figure 2 shows that two tumor-derived, fibrosarcoma cell lines transformed by γ -irradiation are capable of forming large colonies in soft agar, a growth property characteristic of tumorigenic cells. Establishment of tumor-derived, fibrosarcoma cell lines involves isolation of MSU-1.1 cells that formed foci after carcinogen treatment or oncogene transfection, followed by injection of these cells into athymic mice. These findings demonstrate that immortalization is not sufficient for the malignant transformation of cells. However, cells that acquire additional genetic changes, such as that which occurred in MSU-1.1 cells, may have increased potential for progression towards malignancy. Taken together, the MSU1 cell lineage, consisting of LG1 $\rightarrow$ MSU-1.0 $\rightarrow$ MSU-1.1 $\rightarrow$ tumorigenic derivatives, serves as an excellent model for the multistep process of carcinogenesis.

The advantages of using the MSU1 cell lineage as a model for studying carcinogenesis of human cells are summarized here (McCormick and Maher, 1994 and 1996). First, cell strains in this lineage are **isogenic**, i.e., they are sequentially derived from the original normal human fibroblast cell line, LG1. Direct comparisons can be made between LG1 and malignant derivatives to investigate genes that are involved in the malignant transformation of cells. Second, the availability of **two pre-malignant cell strains**, MSU-1.0 and MSU-1.1, allows one to address interesting questions. These include: 1) what genetic change(s) are involved in the acquisition of the immortalized phenotype (compare LG1 vs. MSU-1.0), 2) what additional genetic event(s) are necessary to convert immortalized cells to clonally-derived cell strains that display increased potential towards malignant transformation by

treatment with carcinogens or expression of oncogenes (compare MSU-1.0 vs. MSU-1.1), 3) what is the transforming potential of a carcinogenic agent or a putative oncogene (test in MSU-1.1)? Third, many **tumorigenic derivatives** of MSU-1.1 cells have been generated by various strategies, and the gene changes involved in their malignant transformation can be further characterized as either oncogenes (gain of function) or tumor suppressor genes (loss of function). Overexpression of putative tumor suppressor genes in these tumorigenic derivatives may also shed light on the proposed function of these genes. The relative importance of certain genetic defects in the carcinogenesis process can be determined by comparing among large panels of tumorigenic cell lines that have been generated in this laboratory. Finally, a major overall advantage of the MSU1 cell lineage is the potential to **characterize various phenotypic and genotypic changes** that occur during the malignant transformation of human fibroblasts. Such knowledge may provide not only valuable insight into the multistep process of carcinogenesis but also useful phenotypic and genotypic markers for detection of pre-malignant states. As seen with colorectal carcinogenesis, early detection is key for medical and surgical prevention of progression of precursor lesions to highly invasive and metastatic carcinomas.

Figure 1: Cell strains/lines of the human fibroblast MSU1 cell lineage. LG1 is a normal human fibroblast cell line established from the foreskin of a male newborn. After v-MYC oncogene transfection of LG1 cells, all of the clonal-derivative cell strains senesced but some viable cells were found in one flask (Morgan et al., 1991). These cells were isolated and designated as **MSU-1.0**. The MSU-1.0 cells display infinite life-span and have activated telomerase. A spontaneous variant, designated as **MSU-1.1**, arose in cell culture and grew faster than MSU-1.0 cells. These cells have two marker chromosomes resulting from translocation events and display partial growth-factor independence (Morgan et al., 1991). MSU-1.0 and MSU-1.1 do not form tumors when the cells are injected subcutaneously into athymic mice. **Malignant derivatives of MSU-1.1** are generated by treatment of MSU-1.1 cells with a carcinogen (e.g., γ -irradiation, BPDE) or expression of an oncogene in MSU-1.1 cells (e.g., H-RAS), followed by isolation of morphologically transformed cells and injection of these cells into athymic mice. The tumor-derived cells are established in cell culture to generate fibrosarcoma cell lines, e.g., L210-6A/SB1, MW17.2.4/SB1, and PH3MT. The additional genetic event(s) present in MSU-1.1 cells makes these cells more susceptible to carcinogen- and oncogene-induced transformation because attempts to transform MSU-1.0 cells by these same agents have been unsuccessful. As cell strains/lines of the MSU1 cell lineage become malignantly transformed, the cells acquire additional growth properties that are characteristic of cancer cells, including: ILS, infinite life-span; GFI, growth factor independence; FF, focus formation; AI, anchorage independence; Tu, formation of tumor in athymic mice.

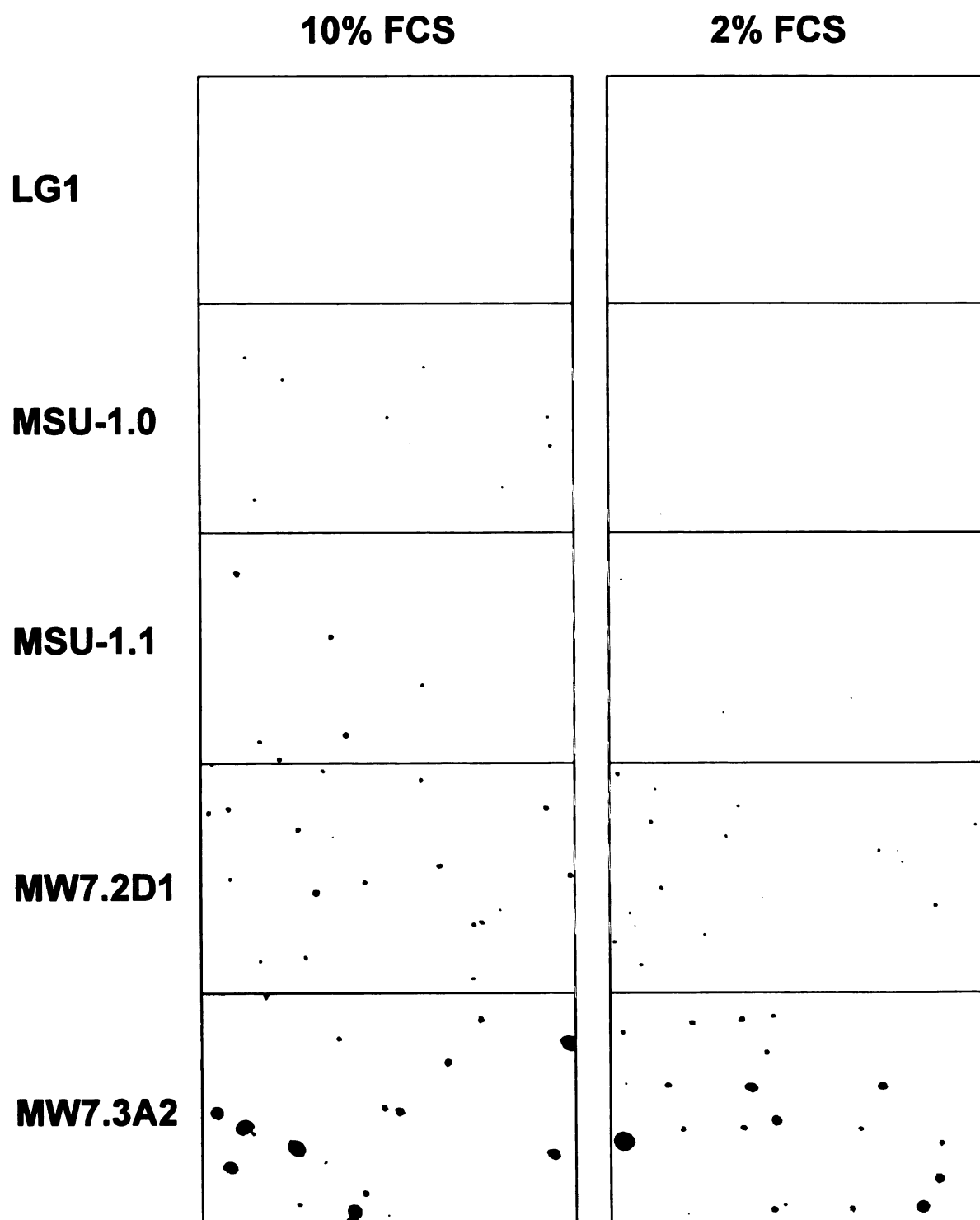
Figure 1



ILS	-	+	+	+
GFI	-	-	+	+
FF	-	-	-	+
AI	-	-	-	+
Tu	-	-	-	+

Figure 2: Anchorage independence status of cell strains/lines of the MSU1 cell lineage. Growth in semi-solid, agarose medium is characteristic of tumorigenic cells. Cell strains/lines of the MSU1 cell lineage were assayed for their ability to grow in such medium. As expected, the normal human fibroblast cell line, LG1, and its two pre-malignant, non-tumorigenic derivative cell strains, MSU-1.0 and MSU-1.1, did not form large colonies in medium supplemented with 2% or 10% fetal calf serum. In contrast, two MSU-1.1-derived, fibrosarcoma cell lines, MW7.2D1 and MW7.3A2, transformed by γ -irradiation (O'Reilly et al., 1998), formed large colonies in medium supplemented with low or high serum concentration.

Figure 2



B. RAS superfamily of small guanine-nucleotide binding proteins

1. *Ras GTPases and their cellular functions*

Ras is the prototype in the Ras superfamily of small G-proteins (Barbacid, 1987). Members of this superfamily are assigned into one of six subfamilies based on biochemical and structural characteristics. The six subfamilies include: Ras, Rho, Rab, Ran, Rad, and Arf (Valencia et al., 1991). G-proteins are guanine-nucleotide binding proteins. Unlike classic or heterotrimeric G-proteins, however, small G-proteins lack the regulatory β and γ subunits and consist of only the catalytic α subunit (Grand and Owen, 1991). All G-proteins have the intrinsic capacity to bind guanine nucleotides and hydrolyze GTP. This latter activity, i.e., the GTPase activity, is required for cycling between an active, GTP-bound state (ON) and an inactive, GDP-bound state (OFF). The capacity to cycle between these two states enables these proteins to function as molecular switches in the pathways they regulate. In the GTP-bound state, these proteins assume an active conformation that allows for subsequent binding and activation of various effectors. These effector-mediated pathways are essential for the cellular functions of G-proteins. At the cellular level, the more relevant determination of a G-protein activity is the ratio of GTP-bound to GDP-bound proteins, and *not* simply the absolute level of these guanine-nucleotide bound forms (Grand and Owen, 1991).

The Ras subfamily includes Ras, Rap, R-Ras, Ral, Rheb, and M-Ras (Bos, 1997), but only the Ras isoforms will be discussed here because of their importance in carcinogenesis. There are three RAS genes: H-RAS, K-RAS, and N-RAS. All Ras isoforms display a ubiquitous pattern of expression. Two families of regulatory

proteins regulate Ras activity, guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (reviewed in Takai et al., 2001). Ras proteins transduce signals from cell surface receptors to pathways that activate transcription of genes involved in regulating cellular growth and differentiation (Barbacid, 1987; Lowy and Willumsen, 1993). Various stimuli activate these receptors, including growth factors, cytokines, and hormones. Distinct roles are emerging for specific Ras isoforms. For example, mouse embryos deficient in K-Ras die *in utero* (Johnson et al., 1997; Koera et al., 1997), whereas mouse embryos deficient in N-Ras or H-Ras develop into phenotypically normal mice (Umanoff et al., 1995). Others have reported biochemical differences among Ras isoforms such as their capacity to activate Raf, a kinase in the Raf/MEK/ERK pathway (Yan et al., 1998; Voice et al., 1999; Walsh and Bar Sagi, 2001). Nevertheless, all RAS genes have been shown to play a causal role in the malignant transformation of human cells (Bos et al., 1987 and 1988).

2. Multiple pathways regulated by RAS genes

Active, GTP-bound Ras binds to and activates effector proteins through its effector-binding domain, some flanking residues, and switch II region (reviewed in Wojtek and Der, 1998). Three major Ras pathways have been described, these include the Raf/MEK/ERK pathway, the PI3K pathway, and the Ral-GDS/Ral GTPase pathway (Figure 3). Each of these will be discussed.

a. Ras/Raf/MEK/ERK pathway

The extracellular-signal regulated kinase (ERK) pathway is the prototype of the MAPK pathways (Robinson and Cobb, 1997). Briefly, when a ligand such as the

platelet-derived growth factor binds to its cell surface receptor, platelet-derived growth factor receptor, there is a protein conformational change that is transmitted to the intracellular domain of the receptor. As a result of this activation, the intracellular domain undergoes autophosphorylation at specific tyrosine residues. Proteins containing Src homology region-2 domains, which are involved in phospho-tyrosine recognition, then bind to the intracellular domain of the activated platelet-derived growth factor receptor. Grb2 is one such Src homology region-2-containing adaptor protein that fulfills this role for the ERK pathway. Activated Grb2 binds to SOS, a guanine nucleotide exchange factor (GEF) specific for Ras (Chardin et al., 1993). SOS activates Ras by binding to Ras and reducing its affinity for GDP, thereby facilitating GDP release. Subsequent loading of GTP results in active, GTP-bound Ras, which then activates a signaling cascade that consists of Raf (MAPKKK level) → MEK1 and MEK2 (MAPKK level) → ERK1 and ERK2 (MAPK level). The mechanism(s) responsible for Ras activation of Raf is not well understood but recruitment of Raf to the membrane is thought to be a major mechanism (Leevers et al., 1994). Activated Raf then activates MEK by phosphorylating specific serine residues on MEK. Activated MEK is a dual-specificity kinase that increases the kinase activity of ERK by phosphorylating key threonine and tyrosine residues (Robinson and Cobb, 1997). At the MAPK level, activated ERK1 and ERK2 then phosphorylate and activate cytoplasmic (e.g., SOS, MEK, Rsk) and nuclear (e.g., Elk-1, Ets-2, C/EBP, SMADS) targets that carry out the cellular functions of Ras (reviewed in Crespo and Leon, 2000). Some of these targets are transcription factors that up-regulate cyclins. Cyclin D1 is one such cyclin that is up-regulated by the Ras pathway, and one of its activities is to bind to and activate cyclin-dependent kinase 4/6 complex (Filmus et al., 1994; Arber et al., 1996; Amanatullah et al., 2001).

This activated complex then hyperphosphorylates Rb, thereby producing the inactive form of Rb, which has decreased binding affinity to the E2F family of transcription factors (Weinberg, 1995; Brehm et al., 1998). Release of E2F from Rb-E2F complexes promotes E2F-mediated up-regulation of genes involved in cell cycle progression.

b. PI3K-activated pathways

Active, GTP-bound Ras also activates phosphatidylinositol-3-kinase (PI3K) (Kodaki et al., 1994; Rodriguez Viciano et al., 1994), a kinase that is active on both lipid and protein targets. PI3K is composed of a regulatory p85 subunit and a catalytic p110 subunit. Certain isoforms of the catalytic subunit, i.e., p110 α and p110 β , bind to Ras-GTP through the effector domain of Ras proteins. There is evidence to support that this binding results in *in vivo* activation of PI3K and that PI3K mediates some of the transforming potential of oncogenic RAS (Rodriguez Viciano et al., 1994 and 1996; Fruman et al., 1998). Other upstream signals, such as receptor tyrosine kinases, soluble tyrosine kinases, and G-protein coupled receptors, activate PI3K independent of Ras (reviewed in Fruman et al., 1998). The **lipid kinase activity** of PI3K phosphorylates phosphoinositides into active second messengers, such as phosphatidylinositol (3,4,5)-triphosphate (PIP3). These active lipid products bind to the Pleckstrin homology (PH) domain present in Rho-specific GEFs and increase their activity by facilitating membrane localization and relieving the autoinhibitory effects of the PH domain (reviewed in Martin, 1998; Nimnual et al., 1998). As will be discussed below, this is one of the major mechanisms by which Ras acts as an upstream activator of Rho GTPases. The **protein kinase activity** of PI3K phosphorylates Akt (also known as protein kinase B), p70 ribosomal protein

S6, I κ B kinase, and other proteins (Toker and Cantley, 1997; Downward, 1998). Collectively, these PI3K targets regulate pathways involved in cell survival, glucose metabolism, mitogenesis, and cell motility. The best characterized of the PI3K pathways is the PI3K/Akt/BAD survival pathway. As noted above, PI3K is known to play a role in the malignant transformation of cells.

c. Ral-GDS/Ral GTPase/RIP1 pathway

Finally, active, GTP-bound Ras binds to and activates Ral-GDP dissociation stimulator (Ral-GDS), which acts as a GEF specific for Ral GTPases. Other Ral-GDS-like proteins such as Rgl, Rgl2, Rlf, are similarly activated by Ras (Hofer et al., 1994; Kikuchi et al., 1994; Wolthuis et al., 1996). Thus, Ras activates Ral in response to various stimuli, and studies have shown that Ral mediates some of the biological effects of Ras in mouse NIH3T3 fibroblasts (Downward, 1998; Toker and Cantley, 1997). Expression of dominant-negative Ral mutant in these cells partially impaired Ras-transformed characteristics. Ral GTPases, RalA and RalB, belong to the Ras subfamily, and regulate vesicle trafficking, cytoskeletal organization, and gene expression (Takai et al., 2001). Active, GTP-bound Ral activates phospholipase D and Ral-interacting protein 1 (RIP1). The latter is a GAP protein specific for Cdc42 and Rac1 GTPases, two members of the Rho subfamily (Cantor et al., 1995; Park and Weinberg, 1995). In this case, Ras inhibits activity of Cdc42 and Rac1 because the mechanism of regulation involves a GAP, a protein that stimulates intrinsic GTP hydrolysis rate of G-proteins.

Figure 3: Multiple Ras effector-mediated pathways lead to transcriptional activation of genes. There are at least three major effector proteins activated by GTP-bound Ras: Raf, phosphatidylinositol 3-kinase (PI3K), and Ral-guanine nucleotide dissociation stimulator (Ral-GDS) (Vojtek and Der, 1998). The signaling cascades regulated by these effectors lead to transcriptional activation of genes. The connection between the Ras pathways and the Rho GTPases, Cdc42 and Rac1, occurs by several mechanisms but the two major mechanisms are 1) PI3K-mediated generation of active lipid products that activate guanine nucleotide exchange factors (GEFs) specific for Cdc42 and Rac1, and 2) Ral-GDS activation of Ral GTPase, which binds to Ral interacting protein 1 (RIP1), a GTPase-activating protein specific for Cdc42 and Rac1. Coordinate regulation of Rho GTPases by these two mechanisms may induce rapid cycling of these proteins between their active and inactive states, such as that seen with the F28L functional mutants (Lin et al., 1997).

[illegible]

3. Contribution of RAS genes to malignant transformation

The finding that the RAS genes are mutated in approximately 30% of all human tumors suggests that the Ras pathways must broadly contribute to the malignant transformation process (Bos, 1988 and 1989). In certain cancers, such as colorectal and pancreatic cancers, this frequency is even higher, 50% and 90%, respectively. Others have suggested that the Ras pathways may be important for development of most, or perhaps even all human cancers because the functional activation of the Ras pathways may occur in the absence of mutations of the RAS genes themselves (Hanahan and Weinberg, 2000). The pleiotropic effects of oncogenic RAS at the cellular level underscore their importance in human cancers.

Constitutively active Ras proteins activate multiple downstream Ras pathways independent of ligand-induced activation of upstream receptors such as receptor tyrosine kinase, soluble tyrosine kinase, and G-protein coupled receptor. Cells that express such proteins are **self-sufficient in their growth signals**, i.e., they do not need exogenous growth factors to drive cellular growth (Hanahan and Weinberg, 2000). Constitutive activation of Raf/MEK/ERK pathway is a major mechanism by which oncogenic RAS induces rapid cellular proliferation (Marais and Marshall, 1996). The mechanism involves up-regulation of cyclin D1 (Filmus et al., 1994), which promotes progression of cells through the G1 phase in the cell cycle (Liu et al., 1995). Cells that pass the G1/S checkpoint are committed to progress through S phase. Cyclins, which are up-regulated by growth-promoting stimuli at specific phases of the cell cycle, phosphorylate and activate constitutively expressed cyclin-dependent kinases (CDKs). The cyclin/CDK complexes, for example, cyclin

D/CDK4, cyclin D/CDK6, and cyclin E/CDK2, phosphorylate and inactive Rb, and promotes its release from members of the E2F family of transcription factors (Filmus et al., 1994). Unbound E2F transcription factors then activate transcription of genes involved in cell cycle progression such as cyclins.

Interestingly, the maximal level of cyclin D1 expression occurs during the late stages of G1 even though the activity of the ERK pathway has significantly diminished (reviewed in Crespo and Leon, 2000). This implies that other mechanism(s) may also increase cyclin D1 expression. Gille and Downward (1999) investigated the activation of PI3K as one such a mechanism, and found that PI3K activity is indeed required for cyclin D1 up-regulation and for S-phase entry of quiescent NIH3T3 fibroblasts. They further show that this is mostly dependent on Akt activation and less so on Rac activation. The Ral-GDS-like factor also strongly activates cyclin D1 transcription and they suggest that this could be through Rlf-mediated up-regulation of c-fos expression. These studies indicate that multiple Ras pathways cooperate to mediate Ras-induced cell cycle progression.

Expression of oncogenic RAS in cells disrupts the balance between survival and apoptosis pathways, and in certain cellular contexts, this confers cells with **increased resistance to apoptosis pathways**. Such cells are more likely to accumulate additional genetic changes because the overstimulated survival pathways oppose apoptosis signals that may have been induced by DNA damage (Hanahan and Weinberg, 2000). Evidence to date implicates a major role of the PI3K/Akt/Bad pathway in Ras-induced cell survival signals (Toker and Cantley, 1997; Downward, 1998). This pathway promotes phosphorylation and inactivation of Bad, a protein that promotes apoptosis. Other lines of evidence further support a

role of the PI3K pathway in promoting cell survival. Expression of the RAS oncogene in epithelial cells prevents anoikis of cells upon detachment from cell-cell and cell-ECM contacts, indicating that Ras pathways are involved in opposing apoptosis signals (Khwaja et al., 1997). Anoikis is a distinct cellular morphology that occurs in cells undergoing apoptosis. They further show that the oncogenic RAS inhibition of anoikis is a PI3K-dependent process. PI3K also activates Rho GTPases by generating active lipid products such as PIP3 (Vanhaesebroeck and Waterfield, 1999). Active Cdc42 or Rac1 then activates p21-activated kinase, which regulates survival and apoptosis pathways (reviewed in Van Aelst and D'Souza Schorey, 1997). Activation of PAK in certain cellular contexts confers cells with increased resistance to apoptosis (Jakobi et al., 2001; Schurmann et al., 2000). Expression of oncogenic RAS in cells transformed by oncogenic MYC abrogates the well-documented Myc-induced apoptosis of such cells by activating PI3K survival pathways (Kauffmann Zeh et al., 1997). Thus, Myc and Ras act cooperatively in cells to promote resistance to apoptosis signals.

Cells that express oncogenic RAS have increased secretion of vascular endothelial growth factor (VEGF), which plays a major role in inducing angiogenesis and promoting tumor growth (Rak et al., 1995). Splice variants of the VEGF gene show tissue-specific expression and different biological and biochemical activity (reviewed in Clauss, 2000). When secreted, VEGF acts on endothelial cells to promote vascular permeability and angiogenesis (Senger et al., 1993). There are two VEGF receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Fik-1), although VEGFR-2 appears to mediate most of the biological effects of VEGF (Robinson and Stringer, 2001). Several mechanisms are known to

modulate activity of VEGF and its receptor, including VEGF binding to VEGFR-1 and sequestering VEGF from activating VEGFR-2 (Robinson and Stringer, 2001), neuropilin-1 acting as a co-receptor for VEGF to enhance its binding to VEGFR-2 receptor (Soker et al., 1998; Gluzman Poltorak et al., 2000), and heparan sulphate proteoglycans binding to certain VEGF isoforms and their receptors (Houck et al., 1992; Tessler et al., 1994; Gengrinovitch et al., 1999) to modulate their biological activity. However, the specific Ras signaling pathway(s) involved is only beginning to be understood. In a recent report, Arbiser et al. (1997) show that Ras induces up-regulation of VEGF through two distinct pathways, the Raf/MEK/ERK pathway and the PI3K pathway. These pathways lead to the activation and/or up-regulation of hypoxia-inducible factor 1 α (HIF-1 α), which is a transcription factor for genes containing the hypoxia response element (HRE) in their promoter region, such as that present in the VEGF gene (Forsythe et al., 1996; Maxwell et al., 1997). However, various cell types differ in their dependence on these two pathways, e.g., up-regulation of HIF-1 α in fibroblasts is dependent on the ERK pathway whereas in epithelial cells the PI3K pathway is required (Arbiser et al., 1997).

Cancer cells that express oncogenic RAS have **enhanced invasive and metastatic potential**. Invasion is a process that requires disruption of cell-cell and cell-ECM interactions and degradation of barriers such as the basement membrane (Hernandez Alcoceba et al., 2000). Invasion is a prerequisite of metastasis because the cells must seed the hematologic or lymphatic circulation to reach distant organs (Cotran et al., 1999). Once cells extravasate from the circulation, they are immediately challenged with a tissue microenvironment different from the primary tumor site. Successful establishment of metastases occurs if such cells acquire the

ability to adapt to competing growth stimulatory and inhibitory signals, hypoxic growth conditions, and limited tissue space for tumor growth. The systemic effects of metastases account for the majority of deaths in human cancers (Hernandez Alcoceba et al., 2000).

Expression of oncogenic RAS in tumor cells promotes their invasion across multiple barriers and their establishment as metastases by up-regulating genes that promote adaptation to a foreign tissue microenvironment. VEGF increases vascular permeability by acting directly on endothelial barriers and stimulating endothelial proliferation in a disorderly manner (Dvorak et al., 1991; Kondo et al., 1993; Ellis et al., 2000). Hepatocyte growth factor and its receptor, Met, signal through Ras pathways to increase cellular proliferation, motility, and invasiveness (Ridley et al., 1995; Webb et al., 1998; Ueoka et al., 2000). Moreover, this is a PI3K-dependent process (Bardelli et al., 1999; Dong et al., 2001). Oncogenic RAS-transformed cells adapt to limited tissue space by increasing expression of various proteases, such as urokinase and its receptor uPAR (Brunner et al., 1989; Paciucci et al., 1998), cathepsins (e.g., cathepsin B, C, and L) (Chambers et al., 1992; Zhang and Schultz, 1992; Silberman et al., 1997), and matrix metalloproteinases (e.g., MMP-2, MMP-9) (Giambernardi et al., 1998; Reddy et al., 1999; Liu et al., 2000; Moon et al., 2000). Down-regulation of tissue inhibitor of metalloproteinases has also been reported (Chambers and Tuck, 1993). Thus, the widespread dysfunction of Ras in human tumors is likely to reflect its ability to confer cells with multiple growth characteristics of the malignant phenotype.

C. Cdc42 and Rac1, two members of the Rho subfamily of GTPases

1. *Rho GTPases and their cellular functions*

In the mid- to late-1980s, there was a major effort to identify proteins that function in an analogous manner to that of Ras G-proteins (Aspenstrom, 1999). RhoA (Ras homology) was one such protein isolated and characterized by biochemical analyses (Hall, 1990 and 1992b). Although RhoA shares 30% amino acid homology to Ras, this conservation is predominately in domains involved in guanine nucleotide binding and GTP hydrolysis. This was the first line of evidence that Rho is a new prototype of a family of Ras-related proteins (Hall, 1990 and 1992b). Multiple isoforms of Rho were subsequently identified (reviewed in Aspenstrom, 1999). Two new members of the Rho subfamily that share approximately 50-60% amino acid homology with Rho and contain a domain unique to Rho proteins, the Rho insert, were identified and named Cdc42 (cell division cycle 42) and Rac (Ras-related C3 botulinum toxin substrate).

Cdc42 was first isolated in studies that characterized temperature-sensitive budding defects in mutant strains of *Saccharomyces cerevisiae*. In one such mutant strain, the budding defect was due to a non-functional, mutant Cdc42 protein (Adams et al., 1990). G25K was the first Cdc42 isoform isolated from tissues of higher eukaryotes, i.e., from bovine brain and human placenta tissues (Polakis et al., 1989; Munemitsu et al., 1990). Subsequently, the human homolog of the yeast Cdc42 was isolated from a human placenta cDNA library using G25K sequences as the labeled oligonucleotide probe (Shinjo et al., 1990). G25K expression is relatively limited to brain tissue whereas Cdc42 is more ubiquitous in its expression pattern.

Structural divergence between G25K and Cdc42 proteins is present from amino acid 163 to 191, which suggests that these two isoforms are alternative splicing products of a single gene (Johnson, 1999).

The Rac isoforms, Rac1 and Rac2, were isolated from a differentiated HL-60 cDNA library using a labeled oligonucleotide probe that hybridizes to a highly conserved region in all Ras-related proteins, for example, the FDTAGQEDYD sequence in human placenta G25K protein (Didsbury et al., 1989). Simultaneously, another group purified Rac1 from membrane fractions of human platelets using columns containing beads cross-linked with GTP γ S (a non-hydrolyzable GTP analog) or α -³²P-labeled GTP (Polakis et al., 1989). Rac1 is ubiquitously expressed in tissues whereas Rac2 displays rather hematopoietic-specific tissue expression.

The most established function of the Rho subfamily is their ability to alter organization of the actin cytoskeleton (Kozma et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Machesky and Hall, 1997). Through microinjection and transfection studies in mouse fibroblasts, it has been shown that Rho induces formation of stress fibers and clustering of focal adhesion complexes, Cdc42 induces formation of filopodia or microspikes, and Rac induces formation of lamellipodial extensions and membrane ruffles. The sequential occurrence of these cytoskeletal structures formed the basis for early speculation that these proteins act in a hierarchical pathway (Nobes and Hall, 1995). For example, because treatment of quiescent fibroblasts with growth factors induces formation of filopodial structures, followed by lamellipodial structures, Cdc42 has been described as an upstream activator of Rac. Using similar analyses, it was reported that Cdc42 activates Rac, which then activates Rho. More recent studies report that this hierarchical

arrangement, however, does not apply to the other cellular functions regulated by the Rho subfamily (reviewed in Van Aelst and D'Souza Schorey, 1997).

Cdc42 and Rac proteins share more structural homology (70% amino acid homology) with each other than with Rho proteins (50-60% amino acid homology). This finding is consistent with the observation that Cdc42 and Rac1 act in parallel in several effector-mediated pathways in which a similar role for Rho has not been found. Because of their similarity in structure and function, the contribution of Cdc42 and Rac1 to the malignant transformation process should be investigated simultaneously. My research is directed at understanding the role of Cdc42 and Rac1 in the malignant transformation of human fibroblasts. Therefore, the remainder of this literature review will cover topics most relevant to this focus.

In addition to their role in actin cytoskeleton reorganization, Cdc42 and Rac1 regulate diverse cellular functions including transcription, cell cycle progression, cell differentiation, survival and apoptosis pathways, and membrane trafficking (Van Aelst and D'Souza Schorey, 1997; Takai et al., 2001). Many reports indicate that these cellular processes are highly dependent on coordinate rearrangement of the actin cytoskeleton. Defects in regulation of Cdc42 and Rac1 activity are implicated in human diseases, for example, in developmental (e.g., Aarskog-Scott Syndrome) and immunological disorders (e.g., Wiskott-Aldrich Syndrome), and in certain types of malignancies (e.g., leukemias) (Johnson, 1999).

2. Biochemical and structural features of Cdc42 and Rac1

Similar to the Ras proteins, Cdc42 and Rac1 proteins cycle between two states, the inactive, GDP-bound state and the active, GTP-bound state (Hall, 1990 and 1992b). Although the domains required for their biochemical activity are arranged in a similar configuration to that found in Ras proteins, there are important differences between Ras proteins and Cdc42 and Rac1 proteins. Presumably, these differences account for the unique cellular functions of Cdc42 and Rac1, and the unique contribution of these proteins to malignant transformation. There are four functional domains required for the biochemical activity of Cdc42 and Rac1 proteins. These include 1) guanine nucleotide binding and GTP hydrolysis domains, 2) an effector binding domain, 3) a CAAX domain, and 4) a Rho insert domain. Each of these functional domains will be discussed.

a. Guanine nucleotide binding and GTP hydrolysis domains

Small G-proteins and heterotrimeric G-proteins share significant amino acid homology in domains involved in guanine nucleotide binding and GTP hydrolysis (Hall, 1990 and 1992b) but there is very low amino acid homology in regions outside of these domains. This strongly suggests that there are common structural requirements for effective guanine nucleotide binding and GTP hydrolysis. One of these structural requirements is the presence of a groove in the protein that is formed by multiple domains and required for efficient magnesium ion binding (Menard and Snyderman, 1993; Zhang et al., 2000). Tethering of a magnesium ion by specific residues in this groove stabilizes the protein conformation such that the G-protein is locked in a GDP-bound state. Disruption of this protein conformation

promotes release of GDP due to decreased GDP binding affinity. GEF, one of three major families of regulatory proteins, is known to bind to small G-proteins and disrupt their binding affinity for the magnesium ion (Zhang et al., 2000). This facilitates passive GTP loading because the cellular concentration of GTP exceeds GDP by approximately ten-fold. The intrinsic GTPase activity of G-proteins is also another biochemical activity requiring the same domains involved in guanine nucleotide binding and GTP hydrolysis. In the GTP-bound state, the GTPase activity is required to hydrolyze GTP to GDP and inorganic phosphate. This completes the cycle and the G-protein is returned to an inactive, GDP-bound state. Small G-proteins are characterized by the rate of their intrinsic GTPase activity, e.g., Ras isoforms are “slow” GTPases whereas Cdc42 and Rac1 are “fast” GTPases (Hall, 1990 and 1992b).

b. Effector binding domain

The high degree of homology between Cdc42 and Rac1 is consistent with the finding that they bind to and activate some common effector proteins (Bishop and Hall, 2000; Boettner and Van Aelst, 1999). They do, however, have some structural differences in their respective effector-binding domains. The effector or switch I domain region of Cdc42 and Rac1 is an extended β 2-strand/loop structure that forms a significant proportion of one face of the protein (Johnson, 1999). Effectors and regulators bind to particular subdomains of the extended structure, and in some cases, multiple effectors and regulators compete for the same subdomains. Effector proteins are activated by changes in their protein conformation that relieve autoinhibitory interactions, for example, between the regulatory domain and the catalytic domain (Bishop and Hall, 2000; Boettner and Van Aelst, 1999). Such

protein conformational changes are induced upon binding to GTP-bound forms of Cdc42 or Rac1.

c. CAAX domain

All small G-proteins contain a CAAX domain (cysteine, aliphatic, aliphatic, and any residue X) at the most distal carboxy-terminus (C-terminus), which is essential for post-translational modification and subsequent membrane localization (Heyworth et al., 1993; Ziman et al., 1993). Post-translational modification in this case is a sequence of steps that includes prenylation of the cysteine residue, proteolytic cleavage of the last three amino acids (AAX), and carboxyl methylation of the cysteine residue. Addition of the lipid moiety to the C-terminus is required for insertion of small G-proteins into the inner leaflet of the plasma membrane. As expected, there is a dramatic shift in the subcellular localization of Cdc42 and Rac1 proteins, from particulate cellular fractions to soluble cellular fractions, when the CAAX domain is mutated or deleted (Ziman et al., 1993). Proteins lacking a functional CAAX domain have markedly reduced capacity to activate downstream pathways involved in their cellular functions. This is largely due to that fact that stimulators of Cdc42 and Rac1 activity, i.e., GEFs, are localized to the plasma membrane and require the close proximity of Cdc42 and Rac1 in order to exert their regulatory effects (reviewed in Whitehead et al., 1997).

d. Rho insert domain

The Rho insert is present in all members of the Rho subfamily (Hall, 1990 and 1992b). The Rho insert forms an α helix and has been shown to be important for Cdc42 and Rac1 activity in several ways. First, this domain has been shown to be

an effector-binding domain for IQGAP, a well-characterized effector for Cdc42 (McCallum et al., 1996). It is possible that other effectors of Cdc42 and Rac1 require this domain, in addition to the effector-binding domain, for the most efficient binding and activation. Second, when the Rho insert in Cdc42 was replaced with H-Ras sequences that are not homologous to the Rho insert, the chimeric protein had reduced sensitivity to Rho GDI (guanine nucleotide dissociation inhibitor), a regulator of Cdc42 activity, even though the binding affinity was not altered significantly (Wu et al., 1997). These results suggest that the Rho insert mediates some of the effects of Rho GDI and possibly other regulators of Cdc42 and Rac1. Ras proteins, which lack the equivalent Rho insert domain, do not interact with GDI proteins. Third, in a more recent study, deletion of the Rho insert abrogated the transforming potential of a fast-cycling, constitutively active Cdc42 mutant (L28-Cdc42) in NIH3T3 mouse fibroblasts (Wu et al., 1998). However, activation of two downstream pathways of Cdc42 and Rac1, the JNK (jun N-terminal kinase) and PAK (p21-activated kinase) pathways, was unaffected by this deletion. These results indicate that the activity of Cdc42 and Rac1 depends on multiple but specific domains in Cdc42 and Rac1. Finally, some reports have suggested that the Rho insert influences the intrinsic GTPase rates of Cdc42 and Rac1 (reviewed in Van Aelst and D'Souza Schorey, 1997).

In summary, Cdc42 and Rac1 have functional domains dedicated to its multiple biochemical activity. In order to function as molecular switches, these proteins have intrinsic GTPase activity for cycling between active, GTP-bound states and inactive, GDP-bound states. When bound to GTP, the proteins assume an active protein conformation, which is conducive for effector binding and activation and subsequent effector-activation of downstream pathways that carry out the

cellular functions of Cdc42 and Rac1. Two other important biochemical characteristics include the CAAX domain for subcellular localization to the plasma membrane and the Rho insert domain for its role in modulating effector binding, action of regulators, and GTPase activity.

3. Regulation of Cdc42 and Rac1 activity

Cdc42 and Rac1 are regulated by direct and indirect mechanisms. The direct mechanisms involve three families of proteins that directly bind to Cdc42 and Rac1 proteins and alter either affinity for guanine nucleotides or intrinsic GTPase activity (Van Aelst and D'Souza Schorey, 1997). Collectively, these regulatory proteins, which include GTPase activating protein (GAP), guanine nucleotide dissociation inhibitor (GDI), and guanine nucleotide exchange factor (GEF), affect the rate of cycling between active and inactive states. The integrity of this regulation is critical because the timeliness and appropriateness of Cdc42 and Rac1 activity are essential for normal cellular function and tissue development. GDI and GAP are inhibitory proteins, whereas GEF are stimulatory proteins. The molecular mechanisms by which these proteins act on Cdc42 and Rac1, thereby regulating their activity, will be discussed. The indirect mechanisms are not as well defined but they regulate the overall activity of Cdc42 and Rac1 by exerting their influence on the direct mechanisms (Kjoller and Hall, 1999). It is well documented that defects in either of these regulatory mechanisms cause dysfunction of Cdc42 and Rac1 in a variety of cell types.

a. GTPase-activating protein (GAP)

GAPs enhance intrinsic hydrolysis of GTP to GDP and inorganic phosphate, i.e., conversion of Cdc42 and Rac1 proteins from their active states to their inactive states. The prototype GAP is p50RhoGAP, which was originally isolated from cell extracts by affinity purification with recombinant GTP-bound Rho (Lancaster et al., 1994). This particular GAP has activity towards Rho, Cdc42, and Rac1 proteins.

Over 13 GAPs have been isolated and characterized, and they differ in their ability to inactivate various members of the Rho subfamily (Van Aelst and D'Souza Schorey, 1997). GAPs share a highly conserved GAP domain that spans approximately 140 amino acids. Despite their low amino acid sequence homology overall, p50RhoGAP and Ras-GAP have similar three-dimensional structures. Most notable is the arrangement of key catalytic arginine residues. When these residues were replaced with alanine residues, such mutant GAP proteins exhibited markedly reduced GAP activity towards their targets (Zhang et al., 1999). The positive charges contributed by the arginine residues stabilize the negative charges of the transition state, which is formed upon interaction of a catalytic glutamine residue (e.g., Q61-Cdc42 and Q61-Rac1) with the oxygen atoms of GTP. Thus, GAPs enhance intrinsic GTPase activity of Cdc42 and Rac1 by binding to these proteins and stabilizing their transition states. Furthermore, the presence of multiple protein-protein and protein-lipid binding domains in GAPs suggests that GAPs have additional biochemical activity (Gamblin and Smerdon, 1998; Zhang and Zheng, 1998; Zalcman et al., 1999).

b. Guanine-nucleotide dissociation inhibitor (GDI)

Another family of regulatory proteins that directly inhibits Cdc42 and Rac1 activity is the GDI proteins (Longenecker et al., 1999; Olofsson, 1999; Zalcman et al., 1999). The first one discovered is the ubiquitously expressed Rho GDI, which is active on Rho, Cdc42, and Rac1 proteins (Fukumoto et al., 1990; Adra et al., 1993). At least two other GDIs have been characterized (Lelias et al., 1993; Scherle et al., 1993). As their name suggests, the GDI proteins inhibit guanine nucleotide dissociation from Rho GTPases. In theory, these proteins could inhibit GDP or GTP

dissociation to maintain the Rho subfamily of proteins in their existing guanine nucleotide-bound states. However, recent studies show that GDI proteins preferentially inhibit GDP dissociation from GDP-bound Rho GTPases over GTP dissociation from GTP-bound Rho GTPases (Longenecker et al., 1999; Read and Nakamoto, 2000; Scheffzek et al., 2000). This may simply be a direct result of its preferential binding to GDP-bound and prenylated forms of the Rho GTPases. Thus, Rho GTPases are stabilized in their inactive, GDP-bound states upon binding to GDI proteins. By inhibiting dissociation of GDP, exchange for GTP cannot occur. As noted earlier, there is some evidence to support that the Rho insert domain modulates the sensitivity of Rho GTPases to the action of GDIs.

Some *in vitro* studies report that weak interactions occur between GDI proteins and GTP-bound Rho GTPases, and that these interactions inhibit intrinsic and GAP-stimulated GTPase activity (Hart et al., 1992; Chuang et al., 1993). In this scenario, the Rho GTPases remain in active, GTP-bound states. It is not clear whether these weak interactions are significant *in vivo*. GDI proteins also influence translocation of Rho GTPases between the cytosol and the plasma membrane. In unstimulated cells, Rho GTPases exist predominately in the cytosol in their GDP-bound forms as complexes with GDI proteins (Bilodeau et al., 1999; Hoffman et al., 2000; Longenecker et al., 1999; Read and Nakamoto, 2000). Stimulation of cell-surface receptors by certain extracellular factors such as platelet-derived growth factor, insulin, and bradykinin, induces release of GDI proteins. One mechanism for this release involves proteins of the ezrin, radaxin, and moesin (ERM) family (Hirao et al., 1996; Maeda et al., 1999; Menager et al., 1999). When a protein of the ERM family binds to a GDI complexed with a GDP-bound Rho GTPase, the affinity of the GDI protein for GDP-bound Rho GTPase is significantly reduced. Finally, there may

be significant redundancy of GDI regulation of Rho GTPases; for example, deletion of a GDI gene in embryonic cells produced macrophages with only subtle defects in superoxide production (Guillemot et al., 1996), a Rac-specific pathway.

c. Guanine-nucleotide exchange factor (GEF)

GEF proteins are the only class of proteins known to activate, as their main regulatory input, activity of Rho GTPases. It has been reported that their mechanism of activation involves induction of protein conformational changes that decrease affinity of Rho GTPases for magnesium ion (Pan and Wessling Resnick, 1998; Zhang et al., 2000). This results in unloading of GDP and loading of GTP, i.e., guanine nucleotide exchange. At least 16 GEF proteins have been identified to date and they display different specificities toward members of the Rho subfamily (Van Aelst and D'Souza Schorey, 1997). The first one discovered was the diffuse B-cell lymphoma (Dbl) oncogene. Eva and Aaronson (1985) isolated the Dbl oncogene from a human diffuse B-cell lymphoma cDNA library by characterizing genes that transformed NIH3T3 cells. An interesting historical fact is that the Dbl oncogene was discovered several years before its target small G-proteins were discovered. The biochemical activity of the Dbl oncogene was not realized until sequence comparisons revealed a high homology to the *S. cerevisiae* Cdc24 gene, an exchange factor for Cdc42 (Hart et al., 1991). Biochemical analysis shows that Dbl has GEF activity on all three Rho GTPases and that the Dbl homology (DH) domain is responsible for this activity (Aghazadeh et al., 1998). Moreover, this domain is essential for the oncogenic properties of the Dbl oncogene (Zhu et al., 2000).

The pleckstrin homology (PH) domain is another domain present in GEF proteins that are specific for members of the Rho subfamily (Bender et al., 1996;

Zheng et al., 1996; Ma and Abrams, 1999). The PH domain is ~100 amino acid motif originally found in pleckstrin, a substrate for protein kinase C. The PH domain is frequently found in proteins that are involved in signal transduction or cytoskeletal organization. Current evidence suggests that the PH domain is involved in two basic interactions, i.e., protein-lipid and protein-protein interactions. Some of these interactions account for the observation that diverse extracellular signals activate Cdc42 and Rac1 pathways. Several reports have shown that overexpression of GEFs proteins lacking the PH domain in mouse fibroblasts failed to transform such cells (Zheng et al., 1996; Olson et al., 1997; Qian et al., 1998; Glaven et al., 1999; Russo et al., 2001). The transforming capacities, however, were restored with the addition of membrane-targeting domains. This is the most convincing evidence that the PH domain is required for membrane localization. The PH domain also acts as an autoinhibitory domain (Bi et al., 2001). Full activation of GEF proteins requires relief from this autoinhibitory effect, which occurs by inducing conformational changes of the PH domain. The phosphoinositol lipid, PIP3, is one such signaling molecule that has this capacity (Van Aelst and D'Souza Schorey, 1997). Therefore, PIP3 stimulates the catalytic activity of GEF proteins. Other signaling molecules may also function similarly to relieve PH domain-induced autoinhibition of GEF activity. The role of the PH domain in protein-protein interaction is not as well established. Finally, tandem arrangement of the DH domain and PH domain is generally a signature feature of GEFs specific for the Rho subfamily (Whitehead et al., 1997).

In summary, the GAP, GDI, and GEF families of proteins directly regulate specific biochemical aspects of Rho GTPases. As noted earlier, these regulatory proteins generally affect the affinity for guanine nucleotides or the intrinsic GTPase

activity, thereby influencing the cellular ratio of GTP-bound to GDP-bound forms. This ratio dictates activity level of Rho GTPases. One of the most intriguing features of these regulatory proteins is the significant redundancy observed within each family of regulatory proteins (Van Aelst and D'Souza Schorey, 1997). It is not clear why there are so many regulatory proteins within the same family since they fulfill the same biochemical activity. Furthermore, within each family of regulatory proteins there are multiple domains involved in protein-protein and protein-lipid interactions, which increases potential for crosstalk between Rho subfamily pathways and other signaling pathways (Van Aelst and D'Souza Schorey, 1997). Many have suggested that these characteristics allow Rho GTPases to regulate diverse cellular processes. This is further supported by the observation that there is typically low sequence homology outside of the domains that are characteristic of each family of regulatory proteins. For example, Dbl and Vav are both members of the Rho-specific GEF family and are capable of the same basic catalytic activity but Dbl lacks SH2 and SH3 domains, whereas Vav has one SH2 domain and two SH3 domains (Bustelo, 1996; Aghazadeh et al., 1998). Thus, Dbl and Vav proteins differ not only in their tissue-specific expression patterns but also in their protein-binding partners. This suggests that Dbl and Vav proteins are activators of Rho GTPases under specific cellular contexts.

The indirect mechanisms of regulating Cdc42 and Rac1 activity include effects of upstream growth factor and cytokine pathways, subcellular localization, cross-talk and feedback from other signaling pathways, formation of multimolecular complexes, competition for effectors and/or regulators, and tissue-specific expression patterns (Van Aelst and D'Souza Schorey, 1997). These are mechanisms in which the protein(s) involved do not *directly* affect the affinity for

guanine nucleotides or the intrinsic GTPase activity of Cdc42 and Rac1 proteins. Nevertheless, they strongly influence the ratio of GTP-bound to GDP-bound Cdc42 and Rac1 levels, usually by exerting their action on the direct mechanisms. It is beyond the scope of this literature review to discuss each of these regulatory mechanisms, however, some of the most important examples have been or will be highlighted in other sections.

4. Effector-mediated pathways of Cdc42 and Rac1

As noted above, the main cellular functions mediated by Rho GTPases include regulation of cytoskeleton organization, gene transcription, cell cycle progression, cell differentiation, survival and apoptosis pathways, and membrane trafficking (Van Aelst and D'Souza Schorey, 1997). When stimulated by certain extracellular or intracellular stimuli, Rho GTPases activate various effectors that are involved in regulating these cellular pathways. In most cases, these effectors act in signaling cascades to activate further downstream protein components. The cellular functions of Cdc42 and Rac1 can be simplified into two bifurcating pathways based on their effector proteins, i.e., those involved in organization of the actin cytoskeleton and those involved in regulation of transcription pathways. This analysis assumes that all the other cellular functions rely on the intact function of one or more of these same effectors. There are both common and unique effectors that mediate the cellular functions of Cdc42 and Rac1. Some of these effectors share a binding motif, designated as Cdc42/Rac1 interactive binding (CRIB) motif (Zhang et al., 1997). Among the effectors that participate in both pathways, the p21-activated kinases (PAKs), a family of serine/threonine kinases (Knaus and Bokoch, 1998), have received the most attention recently and will be discussed in some detail. The

cellular processes regulated by Cdc42 and Rac1 are highly dependent on signaling through PAK.

a. Organization of the actin cytoskeleton

The first cellular function assigned to members of the Rho subfamily is their involvement in mediating formation of actin cytoskeletal structures in fibroblasts (Knaus and Bokoch, 1998). When fibroblasts are treated with a growth factor or microinjected with the V12-H-Ras oncoprotein, there is rapid formation of cytoskeletal structures in an order that suggests Cdc42 activates Rac1, which then activates RhoA. As expected, microinjection of dominant-negative N17-Rac1 protein prior to the same treatment abolishes sequential formation of these cytoskeletal structures. The cytoskeletal changes induced by Cdc42 and Rac1 have been linked to various cellular processes, including protein scaffolding, cell motility, cytokinesis, cell shape modeling, cell-cell and cell-extracellular matrix contacts, and microenvironment sensing (Aspenstrom, 1999; Johnson, 1999). Some of these cellular processes require activation of PAKs (Knaus and Bokoch, 1998).

PAKs participate in signaling cascades that regulate actin cytoskeleton reorganization (Eby et al., 1998), survival and apoptosis pathways (Tang et al., 2000), mitogen-activated protein kinase (MAPK) pathways (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Zhang et al., 1995), and NADPH oxidase pathway (Prigmore et al., 1995; Freeman et al., 1996). Cdc42 and Rac1 act upstream in these pathways by activating PAKs. Three isoforms of PAK have been identified, α -PAK (68 kD), β -PAK (65 kD), and γ -PAK (62 kD). α -PAK is expressed in brain and spleen, and β -PAK is expressed only in brain. γ -PAK is ubiquitously expressed in tissues. The important structural features of PAKs are three proline-

rich motifs, a p21-binding domain (PBD), a region rich in acidic residues, and a catalytic domain (Guo et al., 1998). The regulatory domain is located at the amino-terminus (N-terminus) and consists of the first three structural features. The catalytic domain is located at the carboxy-terminus (C-terminus) and is responsible for the kinase activity of PAK.

Proline-rich domains are commonly binding sites for SH3 domain-containing proteins. Most notable of such proteins that interact with PAKs are Nck and PIX proteins (Bokoch et al., 1996; Manser et al., 1998; Kim et al., 2001). Nck is an adaptor protein that contains both SH2 and SH3 domains, thereby directly linking cell surface receptors and soluble tyrosine kinases to the PAKs. The name PIX comes from the identification of this protein as a PAK-interacting exchange factor. It mediates some of the actin cytoskeletal changes induced by Cdc42 and Rac1.

Sequence analysis of the PBD shows that within this domain there is a CRIB domain, which is the minimal consensus sequence required for Cdc42 and Rac1 binding. Binding of PAK to Cdc42 or Rac1 is most efficient when Cdc42 and Rac1 are in their active, GTP-bound states. Upon binding to GTP-bound Cdc42 or Rac1, the autoinhibitory protein conformation of PAK is disrupted (Tu and Wigler, 1999). This 'opens' the kinase domain or catalytic domain of PAK. Activated PAK then undergoes autophosphorylation on multiple serine and/or threonine sites present in the catalytic domain. The specific sites are slightly different among PAK isoforms. Autophosphorylated PAKs have up to a 300-fold increase in phosphotransferase (kinase) activity towards their substrates (Manser et al., 1995; Peter et al., 1996). Some studies show that PAKs are also activated by addition of membrane targeting domains at the C-terminus (Lu et al., 1997; Daniels et al., 1998; Lu and Mayer,

1999). Thus, direct interaction of PAK with Nck proteins or specific lipids facilitates membrane targeting and increased activation of PAK.

b. Induction of survival and apoptosis pathways

Recent reports describe a direct link between γ -PAK and caspase-induced apoptosis. When cells are exposed to various stress stimuli, such as ultraviolet radiation, caspase-induced proteolytic cleavage of γ -PAK result in two protein fragments, an N-terminus regulatory fragment (28 kD) and a C-terminus catalytic fragment (34 kD) (Bokoch, 1998; Rudel et al., 1998; Walter et al., 1998). Physical separation of the regulatory domain from the catalytic domain produces a functionally similar effect when the active, GTP-bound form of Cdc42 or Rac1 binds to the regulatory domain of PAKs. Proteolytic cleavage of γ -PAK and generation of these fragments parallel the time-course induction of apoptosis in Jurkat cells (Rudel et al., 1998). Other studies report that PAK is required to stimulate c-Jun amino terminal kinase (JNK) pathway in these same cells (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Zhang et al., 1995). Expression of dominant-negative versions of PAK abolishes formation of apoptotic bodies in Jurkat cells and delays onset of Fas ligation-induced apoptosis (Faure et al., 1997). Thus, PAK induces apoptosis pathways.

The cellular context, under which PAK functions, however, is critical in altering its influence on survival and apoptosis pathways. Contradicting cellular responses have been observed among various cell types and stress stimuli. In *Xenopus* oocytes, caspase-induced proteolytic cleavage and activation of PAK result in *stimulation* of survival pathways and *modulation* of cell cycle arrest pathways (Faure et al., 1997). Other examples of PAK-induction of survival pathways have

been reported in studies with mouse fibroblasts. In one study, expression of dominant-negative versions of PAK in mouse fibroblasts increased its sensitivity to oncogenic RAS-induced apoptosis (Gallick et al., 2000, Tang et al., 2000). This indicates that intact PAK pathways are required to subvert oncogenic RAS-induced apoptosis. In another study, overexpression of wildtype PAK in mouse fibroblasts increased survival of such cells when exposed to various stress stimuli, such as ultraviolet radiation, growth factor deprivation, or interleukin treatment (Jakobi et al., 2001; Roig and Traugh, 2001).

c. Regulators of gene expression

PAKs are activators of the three MAPK pathways, i.e., ERK, p38, and JNK pathways. Cdc42 and Rac1 are known to cooperate and enhance proliferative signals induced by the Ras/Raf/MEK/ERK pathway (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Zhang et al., 1995). Part of this effect comes from the stimulatory action of PAKs on two ERK pathway proteins, Raf and MEK. Activated PAK phosphorylates Raf at specific residues, which increases Raf kinase activity on MEK1 and MEK2. Activated PAK also phosphorylates specific residues on MEK1 and increases its sensitivity to Raf activation.

In addition to the ERK pathway, activated PAKs regulate two other MAPK pathways, the p38 and JNK pathways (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Zhang et al., 1995). The p38 and JNK pathways have functionally similar upstream kinases at the MAPKKK and MAPKK level, however, the kinases involved are not as well characterized. Furthermore, the kinase levels at which PAKs exert their influence on the p38 and JNK signaling cascades have not been completely determined. Studies so far show that PAKs phosphorylate one or

more upstream signaling components of the p38 and JNK pathways to activate these pathways. Like the ERK pathway, the final signaling output of the p38 and JNK pathways is activation of specific transcription factors. The serum response factor pathway and the nuclear factor κ B pathway are two other transcription factor pathways that are also activated by Cdc42 and Rac1 (Westwick et al., 1997; Foryst Ludwig and Naumann, 2000).

d. Role in membrane-trafficking processes

Several studies provide evidence of a role for Cdc42 and Rac1 in membrane-trafficking processes including phagocytosis, endocytosis, and secretory vesicle transport (reviewed in Van Aelst and D'Souza Schorey, 1997). These cellular processes require coordinate cytoskeletal rearrangements by Rho GTPases. The reader is referred to the referenced review paper for further information.

e. Cellular context of Cdc42 and Rac1 function

As briefly discussed, the level of Cdc42 and Rac1 activity and their cellular functions are also influenced by indirect mechanisms of regulation. Some of these are apparent in the discussion of PAKs as effectors of Cdc42 and Rac1. Various stress- or receptor-initiated stimuli give rise to different cellular responses even though common signaling components may be activated (reviewed in Van Aelst and D'Souza Schorey, 1997). For example, fibroblasts exposed to UV light had activated Cdc42 and Rac1 proteins, which induced apoptosis pathways through the activation of PAKs. However, when fibroblasts were treated with growth factors, PAK was also activated but there was activation of cell proliferation and survival pathways. Thus, additional factors must dictate the balance between Cdc42- and Rac1-induced

apoptosis and survival pathways in response to various stimuli. Factors that affect the balance between apoptosis and survival pathways include subcellular localization of proteins, multimolecular complex formation, tissue-specific expression patterns, and competing signaling pathways, especially when they involve upstream or downstream components of these pathways (Van Aelst and D'Souza Schorey, 1997). At a more global level, there may be cross-talk and feedback from other signaling pathways, e.g., the ERK pathway. Thus, effector-mediated pathways of Cdc42 and Rac1 are coordinately regulated pathways, rather than distinct pathways that produce specific cellular responses appropriate for the cellular context.

5. Contribution of CDC42 and RAC1 genes to malignant transformation

a. Constitutively active Cdc42 and Rac1 mutants

Since many parallels exist among Ras and Rho GTPases, there was early speculation that activating mutations of the Rho subfamily of genes could display similar oncogenic potential to that demonstrated with the RAS genes. However, overexpression of Rho GTPases carrying mutations analogous to those found in oncogenic RAS, e.g., G12V and Q61L, in various cell types had only weak and inconsistent transforming potential of rodent fibroblasts (Hall, 1990 and 1992b). One explanation for this difference is that Rho GTPases are regulated more extensively by inhibitory proteins than Ras GTPases (Van Aelst and D'Souza Schorey, 1997). In the case of the Rho subfamily, the significant redundancy of negative regulators, which include members of the GAP and GDI families, may impart an overriding limitation on the activity level of Rho GTPases (Mackay and Hall, 1998; Van Aelst and D'Souza Schorey, 1997). In contrast, Ras has only a few GAPs and no equivalent GDIs, which would act as negative regulators of Ras activity. Thus,

oncogenic RAS activity is not modulated to the same extent as Rho GTPases. Another possible explanation is that Rho GTPases have higher intrinsic GTPase activity than that demonstrated with Ras GTPases (Hall, 1990 and 1992b). A higher intrinsic GTPase activity translates to a more rapid conversion of active, GTP-bound forms to inactive, GDP-bound forms of the Rho subfamily. This results in a relatively brief and measured activation of downstream Rho subfamily pathways in response to either upstream activation by various stimuli or constitutively active mutants of Rho GTPases. In contrast, Ras has been described as a “slow” GTPase because it remains in the activated, GTP-bound state much longer. Therefore, activating mutations of RAS genes give rise to more persistent activation of Ras pathways.

Furthermore, unlike RAS genes, which contain an activating mutation in approximately 30% of all human tumors (Bos, 1988 and 1989), there are no reports of human tumors containing an activating mutation in RHO genes. For the reasons described above, cells with constitutively active mutations of RHO genes probably display only subtle “selectable” phenotypes. The finding that the Rho subfamily is more extensively regulated, however, does not eliminate the potential of their effector-mediated pathways as important pathways in the malignant transformation of cells. Dysfunction of these pathways is more likely to occur by more robust mechanisms that exceed the influence of negative regulators of Rho GTPases.

b. Oncogenic derivatives of the Dbl family of GEFs

Although constitutively active mutant Cdc42 and Rac1 proteins do not function as oncoproteins, many studies report that members of the Dbl family of GEFs, which directly activate Cdc42 and Rac1 activity, mediate oncogenic

transformation of cells when expressed as truncated derivatives. Three examples of oncogenic GEFs will be discussed.

The **DBL** oncogene was originally isolated from a human diffuse B-cell lymphoma cDNA library because of its capacity to transform NIH3T3 cells to focus-forming cells that were tumorigenic in athymic mice (Eva and Aaronson, 1985). The gene that induced this phenotype was identified as a truncated, oncogenic derivative of the DBL gene. Other GEFs specific for the Rho subfamily have also been identified in similar function-based screens (Cerione and Zheng, 1996).

The **Bcr-Abl** fusion protein is a byproduct of the hallmark chromosomal aberration, the Philadelphia chromosome, found in greater than 90% of chronic myeloid leukemias and in some acute lymphocytic leukemias (Cortez et al., 1995; Arlinghaus, 1998; Faderl et al., 1999). The breakpoint cluster region (Bcr) protein has domains characteristic of both GEF and GAP proteins, which is an example of a bifunctional regulatory protein specific for Rho GTPases (Diekmann et al., 1991; Chuang et al., 1995). Abl protein is a tyrosine kinase. It is not exactly clear why the fusion of these two proteins is required for leukemogenesis, i.e., generation of leukemia cells. However, there is evidence to support that the individual biochemical activity of Bcr and Abl are greatly enhanced as a fusion protein, possibly because of the partial or complete disruption of autoinhibitory domains from chromosomal breakage and/or the potential for non-physiological multimolecular complexes (Chuang et al., 1995). Generation of non-physiological multimolecular protein complexes could place two proteins in close proximity, allowing one protein to activate another protein, when normally the proteins do not bind or interact.

The **tumor invasion and metastasis-1** oncogene was isolated using a similar strategy that led to the discovery of the DBL oncogene (Habets et al., 1994).

Investigators were looking specifically for genes that conferred an invasive phenotype when introduced into a non-invasive lymphoma cell line. Other studies provide further evidence of a role of Cdc42 and Rac1 in increasing motility and invasiveness of a variety of tumor cell types (Michiels et al., 1995; Keely et al., 1997; Banyard et al., 2000; Evers et al., 2000; Schmitz et al., 2000).

These three examples illustrate that the Dbl family of GEFs is a family of proto-oncogenes that promote tumor generation and tumor progression when converted to oncogenic derivatives, usually by truncation of autoinhibitory domains. In summary, because members of the Dbl family of GEFs are activators of Cdc42 and Rac1 activity, and their oncogenic potential is well documented, Cdc42 and Rac1 must regulate effector-mediated pathways that contribute to the malignant transformation of human cells.

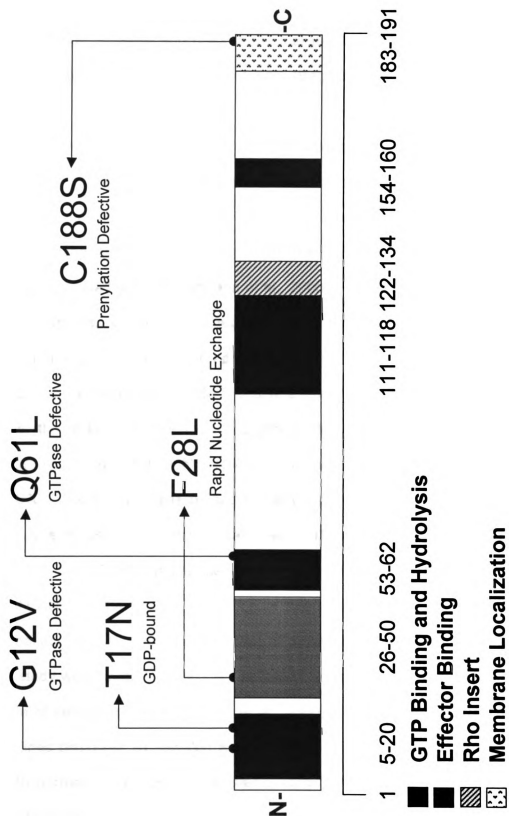
c. Cdc42 and Rac1 as mediators of oncogenic H-RAS transformation

Several studies show that Rho GTPases are required for oncogenic H-RAS transformation of rodent fibroblasts (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997). In these studies, they overexpressed dominant-negative mutant, N19-Rho, N17-Cdc42, or N17-Rac1, i.e., mutants analogous to the well-characterized dominant-negative Ras mutants, to inhibit endogenous activity of the specific Rho GTPase (Figure 4). Dominant-negative mutants are constitutively in their inactive, GDP-bound states. However, the mutant proteins retain the ability to bind to GEF and some effector proteins. Therefore, when a specific mutant is expressed in cells, they limit or sequester the availability of GEF and effector proteins from their endogenous counterpart, thereby inhibiting the function of the specific Rho GTPase in such cells. Expression of these mutants in rodent

fibroblasts malignantly transformed by oncogenic RAS differentially impaired various transformed growth characteristics, including anchorage independence and growth factor independence (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997). For example, expression of N17-Cdc42 in Rat1 fibroblasts only partially inhibited Ras-induced growth factor independence, whereas expression of N17-Rac1 strongly inhibited this transformed phenotype. Thus, although Cdc42 and Rac1 proteins share some signaling pathways, it is generally accepted that they contribute uniquely to oncogenic H-RAS transformation of cells. The most direct evidence that Cdc42 and Rac1 act downstream of Ras pathways comes from studies showing the involvement of phosphoinositide-3-kinase (PI3K) (Bi et al., 2001). As discussed above, Ras activates PI3K by binding to the catalytic subunit of PI3K. The active lipid products generated by PI3K bind to the PH domains present in Rho-specific GEFs and enhance their localization to the plasma membrane. Thus, through lipid activation of GEFs, Ras acts as an upstream activator of Cdc42 and Rac1 pathways (Cerione and Zheng, 1996; Whitehead et al., 1997; Aspenstrom, 1999). Activation of Cdc42 and Rac1 pathways is necessary for the full oncogenic potential of mutant RAS genes.

Figure 4: Functional mutants of the Rho subfamily of GTPases. Multiple domains are responsible for activity of Rho GTPases: guanine nucleotide binding and GTP hydrolysis domains, effector binding domain, Rho insert domain, and membrane localization domain (Johnson, 1999; Takai et al., 2001). The Rho insert is unique to members of the Rho subfamily. Dominant-negative mutants, T17N and C188S, are defective in GTP-binding and prenylation, respectively, but retain the ability to bind to GEFs and certain effector proteins. Dominant-active mutants G12V and Q61L are defective in GDP binding and GTP hydrolysis, whereas dominant-active mutant F28L (Lin et al., 1997) is defective only in GDP binding. Thus, the F28L mutant displays rapid cycling between active, GTP-bound state and inactive, GDP-bound state because the GTPase activity remains intact.

Figure 4



d. PAK as the major effector mediating transformation of cells

The contribution of Cdc42 and Rac1 to the malignant transformation of cells is thought to be mainly through activation of PAK (Bagrodia and Cerione, 1999; Gallick, 2000; Roig et al., 2000; Tang et al., 2000). First, cells that express oncogenic H-RAS have increased sensitivity to apoptosis unless there is additional modulation of other pathways. PAK has been shown to counteract the apoptosis signals induced by oncogenic RAS (Tang et al., 2000). One such mechanism is by PAK-mediated phosphorylation and activation of Bad, a Bax-like protein that antagonizes apoptosis pathways (Schurmann et al., 2000). In such a scenario, the oncogenic RAS-transformed cells acquire a balance of increased stimulation of Ras pathways without increased sensitivity to apoptosis signals. These studies suggest that oncogenic RAS transformation of cells is dependent on PAK activation.

Second, the level of GTP-bound Rac3 protein, as detected by an affinity-precipitation assay, is elevated in highly proliferative human breast cancer cell lines (Mira et al., 2000). The assay utilizes the finding that the p21-binding domain (PBD), located at the N-terminus of PAK, only interacts with GTP-bound Cdc42 and Rac1 isoforms, and not their GDP-bound forms (Benard et al., 1999). Such an assay allows for estimation of the relative proportion of total Cdc42 and Rac1 in their active, GTP-bound forms, i.e., the activity level of these proteins. The cell lines tested in this study were divided into two groups based on the absence or presence of a rapid rate of cellular proliferation (Mira et al., 2000). The highly proliferative group of cell lines displayed an elevated level of active, GTP-bound Rac3 protein compared with normal human breast cell lines. They further identified the PAK effector pathway as the essential pathway for mediating this transformed phenotype.

Third, in a recent report investigators overexpressed wildtype γ -PAK in BALB3T3 mouse fibroblasts and determined the effect on the survival of such cells exposed to stress stimuli such as serum starvation, UVC exposure, and IL-2 treatment (Jakobi et al., 2001). The cells displayed enhanced survival compared with the same cells not expressing exogenous γ -PAK and increased resistance to apoptosis signals normally activated by such stress stimuli. Such cells may be more susceptible to malignant transformation because they have lost a major mechanism that prevents propagation of cells containing DNA damage.

e. Summary

There are two major themes that summarize the role of Cdc42 and Rac1 pathways in the malignant transformation of cells. First, **dysfunction of Cdc42 and Rac1 pathways is caused by defective regulation**, and not by mutations of CDC42 and RAC1 genes themselves. In theory, the regulation of Cdc42 and Rac1 activity could be disrupted in several ways to cause an overall increase in Cdc42 and Rac1 activity. However, increased activity of positive regulators, such as members of the Dbl family of GEFs, is the only known mechanism that has been documented in human tumor cells (Cerione and Zheng, 1996; Whitehead et al., 1997). Conversion of GEF proto-oncogenes to oncogenic derivatives occurs by truncation of key regulatory domains in GEF proteins. The result is constitutive activation of Cdc42 and Rac1 and their downstream pathways. A net loss of negative regulators, such as GAP and GDI proteins, could also cause an overall increase in Cdc42 and Rac1 activity. Therefore, these genes could be thought of as tumor suppressor genes because they negatively regulate Cdc42 and Rac1 activity. However,

inactivating mutations in neither GAP nor GDI genes have been found in human tumors.

Second, evidence to date supports that **Cdc42 and Rac1 fulfill a permissive or supporting role**, rather than a causal role, in the malignant transformation of cells. Although expression of constitutively active mutants of Cdc42 or Rac1 in rodent fibroblasts is only weakly transforming (Hall, 1990 and 1992b), there is a consistent requirement for Cdc42 and Rac1 activity in maintenance of the malignant phenotype induced by oncogenic H-RAS (Prendergast et al., 1995; Qiu et al., 1997). Thus, increased Cdc42 or Rac1 activity alone is not sufficient to induce the malignant transformation of cells but the pathways regulated by Cdc42 and Rac1 are required for establishment and maintenance of the malignant phenotype.

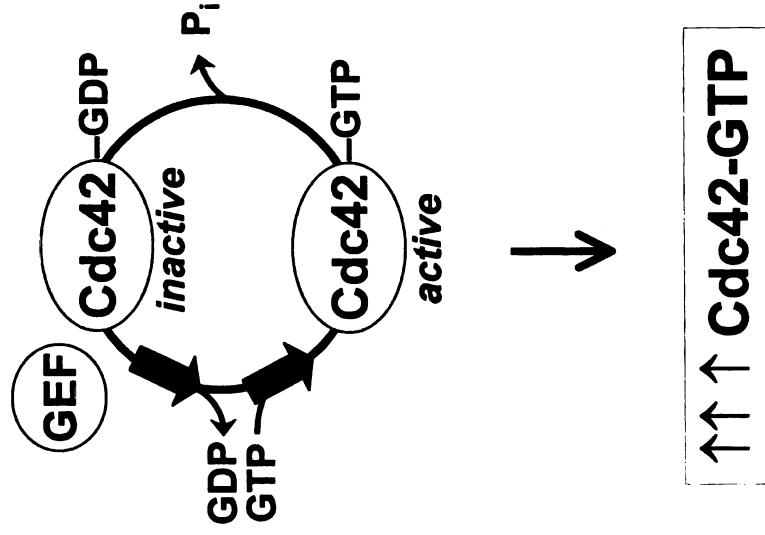
Newly characterized mutants of Cdc42 and Rac1, i.e., the F28L mutants, may shed light on the full transformation potential of Cdc42 and Rac1. G12V and Q61L mutants are constitutively GTP-bound, *GTPase-defective* mutants. In contrast, F28L mutants are constitutively GTP-bound, *GTPase-competent* mutants (Lin et al., 1997; Johnson, 1999). Because they are GTPase-competent, F28L mutants retain the ability to return Cdc42 and Rac1 to their GDP-bound states. Reloading of GTP occurs rapidly because the conformation of such mutants favors GTP binding over GDP binding. Therefore, F28L mutants of Cdc42 and Rac1 display increased exchange activity AND increased GTPase activity. Such mutants have been described as fast-cycling mutants. In contrast, G12V and Q61L mutants display a more static or steady-state increase in Cdc42 and Rac1 activity because they are locked in their GTP-bound states. Such mutants display only increased exchange activity. Some studies report that F28L mutants are more effective transforming agents than G12V and Q61L mutants because of these differences (Johnson, 1999).

This led to the speculation that the full oncogenic potential of Cdc42 and Rac1 is dependent on increased rate of cycling of these proteins and not simply increased level of GTP-bound forms. Further investigation into the significance of dynamic (F28L) vs. static (G12V and Q61L) increases in Cdc42 and Rac1 activity could also offer insight into the mechanistic basis for bifunctional regulators such as the Bcr protein, which contains both GEF and GAP domains (Chuang et al., 1995) (Figure 5). Bcr has the potential to increase rate of cycling of Cdc42 and Rac1 by exerting similar biochemical effects to that observed with F28L mutants. Ras activation of Cdc42 and Rac1 potentially operates through similar mechanisms because the influence of Ras is through activation of both positive (e.g., GEFs) and negative regulators (e.g., GAPs) of Cdc42 and Rac1 (Mackay and Hall, 1998).

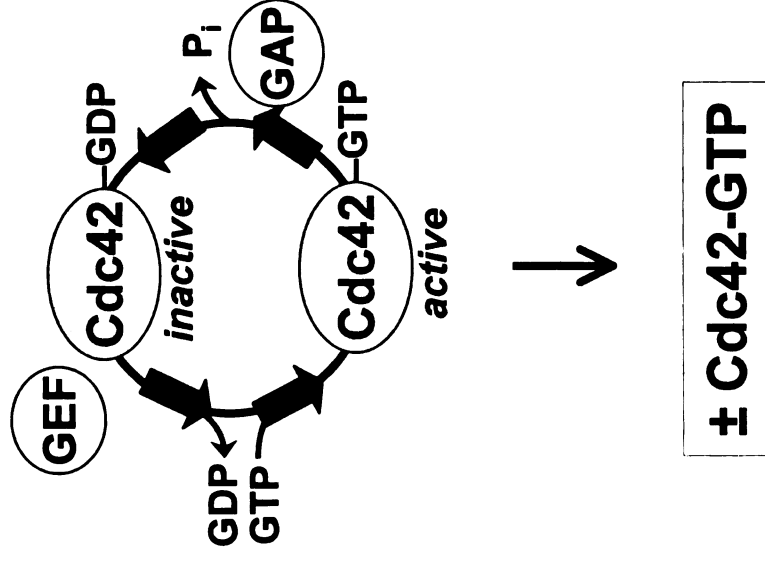
Figure 5: Cdc42 and Rac1 contribute to malignant transformation of cells by at least two possible mechanisms. A static increase in Cdc42 or Rac1 activity, i.e., an increase in exchange activity of Cdc42 or Rac1, occurs when proteins are activated by truncated, oncogenic derivatives of GEFs specific for the Rho GTPases (e.g., Dbl oncogene). A dynamic increase in Cdc42 or Rac1 activity, i.e., an increase in exchange activity coupled with an increase in GTP hydrolysis rate, occurs when the proteins are activated by bifunctional regulators such as Abr, which has both GEF and GAP action on Cdc42 and Rac1, and by Ras proteins, which activate both GEFs and GAPs for Cdc42 and Rac1. The transforming potential of increased Cdc42 and Rac1 activity may be greater when occurring by dynamic mechanisms than by static mechanisms.

Figure 5

e.g., GEF oncprotein



e.g., Ras activation and ABR



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**CHAPTER 2: H-RAS-INDUCED TRANSFORMATION OF HUMAN FIBROBLASTS
IS DEPENDENT ON CDC42 AND RAC1 ACTIVITY, AS IS UP-REGULATION OF
EPAS1**

**H-RAS-induced transformation of human fibroblasts is dependent on
Cdc42 and Rac1 activity, as is up-regulation of EPAS1**

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ABSTRACT

Expression of a dominant-negative mutant Cdc42 or Rac1, or both, in human fibroblasts malignantly transformed by oncogenic H-RAS strongly inhibited the characteristics of such malignant cells, i.e., substantially reduced their rate of proliferation, markedly reduced their ability to form foci, and significantly reduced their ability to form malignant tumors in athymic mice, indicating that these transformed characteristics are dependent on functional Cdc42 and Rac1 signaling pathways. Inhibiting Rac1 prevented focus formation even more effectively than inhibiting Cdc42. Affymetrix GeneChip analysis revealed 28 genes whose expression was regulated by oncogenic RAS and dominant-negative Cdc42 and/or Rac1. Among the genes up-regulated by H-RAS and dependent upon functional Cdc42 and Rac1 was EPAS1, a transcription factor for VEGF. Indeed, expression of dominant-negative Cdc42 and Rac1 markedly reduced the level of VEGF secreted into the growth medium.

INTRODUCTION

Ras proteins are small G-proteins that cycle between two forms, an active GTP-bound state and an inactive GDP-bound state, to regulate cellular growth, differentiation, and survival in response to extracellular stimuli, such as growth factors and hormones (Grand and Owen, 1991). Intact extracellular-signal regulation of the Ras pathways is critical for normal cellular responses and processes (Morrison et al., 1988; Gale et al., 1993). Oncogenic mutations in the RAS genes disrupt this important regulation, and cells that have acquired such mutations display growth characteristics typical of cancer cells, e.g., reduced dependence on growth factors, enhanced angiogenesis, and increased invasive and metastatic potential (reviewed in Hanahan and Weinberg, 2000).

In approximately 30% of human tumors, the RAS gene contains an oncogenic mutation, and the frequency is even higher in colorectal (50%) and pancreatic (90%) cancers (Bos, 1988 and 1989). However, these figures are thought to underestimate the contribution of aberrant Ras signaling pathways to human cancers because there may be functional activation of Ras in the absence of a mutation in the RAS gene itself (Hanahan and Weinberg, 2000). For example, expression of the activated form of Raf, a mediator in the Ras/Raf/MEK/ERK pathway, is sufficient to induce some of the growth characteristics that are associated with oncogenic H-RAS transformation (Oldham et al., 1996). The finding that activated Raf induces only a partial complement suggests that other Ras-stimulated pathways are required to achieve the full oncogenic potential of H-RAS (Vojtek and Der, 1998). Several studies show that oncogenic H-RAS transformation of rodent fibroblasts requires the activity of Rho GTPases, a subfamily of the Ras superfamily of small G-proteins (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997). When the

investigators expressed dominant-negative mutant versions of Rho GTPases to block activity of their endogenous homologues in H-RAS-transformed rodent fibroblasts, they found that various H-Ras-specific growth characteristics were significantly impaired. These data indicate that Rho GTPases act as downstream mediators of Ras-induced transformation of rodent fibroblasts.

Rho GTPases activate effectors involved in regulating actin cytoskeleton reorganization, transcriptional activation, and other signaling pathways (Hall, 1990 and 1998; Van Aelst and D'Souza Schorey, 1997). Microinjection into rodent fibroblasts of Cdc42, Rac1, or RhoA proteins, representing three prototype members of the Rho subfamily, induces a sequence of morphological changes, caused by rapid actin cytoskeleton reorganization that is consistent with a linear hierarchical pathway, i.e., Cdc42 → Rac1 → Rho (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). However, this linear relationship does not hold for the other cellular processes regulated by Rho GTPases. For example, Cdc42 and Rac1 proteins act in parallel to regulate transcription in the p38 and c-jun N-terminal kinase pathways (Coso et al., 1995; Minden et al., 1995), nuclear factor κ B pathway (Sulciner et al., 1996; Perona et al., 1997), and serum-responsive factor pathway (Hill et al., 1995). Recent studies indicate that Cdc42 and Rac1 contribute to the malignant transformation of cells by regulating these pathways (Mackay and Hall, 1998; Van Aelst and D'Souza Schorey, 1997).

The present study shows that Cdc42 and Rac1 act as downstream mediators of oncogenic H-RAS transformation of human fibroblasts. Expression of either the dominant-negative Cdc42 or dominant-negative Rac1, or both mutants (Hurlin et al., 1989), in human fibroblasts malignantly transformed by a transfected H-RAS

oncogene impaired several H-RAS-induced growth characteristics including rapid cellular proliferation, focus-forming ability, and tumor formation in athymic mice. Affymetrix GeneChip analysis of the non-tumorigenic parental cell strain, MSU-1.1, and its H-RAS-transformed derivative cell line expressing both dominant-negative Cdc42 and dominant-negative Rac1 mutants, under the control of a tetracycline-regulated promoter, was used to identify a subset of oncogenic H-RAS-induced genes that require intact Cdc42 and Rac1 signaling pathways. A series of such genes up-regulated or down-regulated by expression of dominant-negative Cdc42 and Rac1 mutants were identified. One such gene is EPAS1, a transcription factor specific for genes containing the hypoxia response element in the promoter region, such as that present in the vascular endothelial growth factor gene.

MATERIALS AND METHODS

Growth conditions

Unless otherwise noted, cell strains/lines were routinely grown in Eagle's minimal essential medium supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, 10% supplemented calf serum (SCS) (Hyclone Laboratories), 100 units/ml penicillin, and 100 µg/ml streptomycin (defined as growth medium) and maintained at 37°C in a humidified incubator containing 5% CO₂ in air.

Cell strains/lines

MSU-1.1, an infinite life span, near-diploid, chromosomally stable non-tumorigenic cell strain, was transfected with the H-RAS oncogene (a mutant H-RAS gene encoding constitutively active G12V-H-Ras protein), carried in a vector designed to greatly increase expression of the oncogene (Hurlin et al., 1989). Clonally-derived cells that appeared morphologically transformed were injected subcutaneously into athymic mice. PH3MT is one such MSU-1.1-derived cell line established from a tumor of an athymic mouse (Hurlin et al., 1987 and 1989).

Cell lysates and immunoblot analysis

Cell lysates were prepared with a lysis buffer consisting of 50 mM Tris-HCl, pH7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na₃VO₄, 200 mM benzamidine, 1 mM PMSF, 25 µg/ml aprotinin, and 25 µg/ml leupeptin. Total protein concentration was quantified using the Coomassie protein assay reagent (Pierce). Proteins in the lysates were denatured in 5X Laemelli sample buffer, separated by either 10% or 12.5% SDS-PAGE, and transferred to PVDF membrane. The membrane was blocked for 2 h with Tris-buffered saline containing 0.1% Tween-20

(TBST) and 5% (w/v) non-fat milk. Generally, the membrane was probed at room temperature for 2 h with the primary antibody diluted in 5% milk in TBST and then probed for 1 h with the appropriate horseradish peroxidase-linked secondary antibody (Sigma and Santa Cruz Biotechnology) diluted in 5% milk in TBST. The membrane was incubated with the Supersignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce) and then exposed to film to detect bands. The sources for the antibodies were: anti-H-Ras (Santa Cruz Biotechnology), anti-phospho-ERK1 and -ERK2 and anti-ERK1 and -ERK2 (New England Biolabs), anti-Cdc42 and anti-Rac1 (Santa Cruz Biotechnology), anti-Flag (Sigma), anti-Myc (Invitrogen), and anti-VEGF (Santa Cruz Biotechnology).

Stable transfection of dominant-negative mutant Cdc42 and Rac1

The original pTet-tTak and pTet-splice vectors were obtained from Life Technologies. This is a TET-OFF system that allows for tetracycline-regulated expression of genes in mammalian cells. To facilitate selection of cells that have stably integrated these vectors, a gene encoding a drug resistance protein was cloned into each of the original vector (histidinol resistance for pTet-tTak and puromycin resistance for pTet-splice) as previously described (Zhang et al., 1996). The Flag-N17Cdc42 and Myc-N17Rac1 cDNAs, obtained by PCR amplification using PFU polymerase, were separately subcloned into the modified pTet-splice vector. The cDNA templates used for PCR amplification were obtained from the vector constructs pRK5-Flag-N17-Cdc42 and pcDNA3-Myc-N17-Rac1, which were kindly provided by Dr. K. Gallo (Michigan State University, East Lansing, MI) and Dr. G. Bokoch (Scripps Institute, La Jolla, California), respectively. The resulting pTet-Flag-N17Cdc42 and pTet-Myc-N17Rac1 vector constructs were verified by

automated DNA sequencing (Visible Genetics). Lipofectamine was used to transfect PH3MT cells with these constructs following the general strategy described by the manufacturer (Life Technologies). Stable transfectants were selected at 1 mM histidinol (Sigma) and 0.5 $\mu\text{g/ml}$ puromycin (Sigma). Tetracycline-regulated expression of dominant-negative Flag-N17Cdc42 and/or Myc-N17Rac1 mutants in various cell strains was verified by immunoblot analysis using anti-Flag and anti-Myc antibodies, respectively. Expression of the dominant-negative cDNAs was *turned off* by including 1-2 $\mu\text{g/ml}$ of tetracycline in the growth medium and *turned on* by omitting tetracycline from the growth medium.

Growth assay

For each cell strain assayed, 1×10^4 cells were plated per 60-mm diameter dish containing growth medium with 1 $\mu\text{g/ml}$ tetracycline or lacking tetracycline (day 0). On days 1, 3, 5, and 8, cells from three replicate dishes were trypsinized and counted to determine total number of cells per dish. Cells received fresh medium on day 4. The time for a population of cells to double (h) was determined from growth curves.

Focus reconstruction assay

The focus reconstruction assay was performed essentially as described (Hurlin et al., 1989). Briefly, MSU-1.1 cells were plated at a density of 5×10^4 cells per 100-mm diameter dish containing growth medium supplemented with 0.5% SCS and 20 mM HEPES (pH 7.4) in the presence or absence of tetracycline. On the following day, the cells to be assayed for focus reconstruction were plated (100-200 cells per dish) in these same dishes. Cells received fresh medium every fourth day.

After 3 weeks of growth, the cells were fixed with neutral buffered formalin, stained with methylene blue, and then examined for quality and quantity of foci.

Tumorigenicity studies

The ability of cells to form tumors was tested in athymic mice (BALB/c). Cells were injected subcutaneously into the right and left rear flanks of mice (1×10^6 cells per site). The mice were provided with 5% sucrose drinking water containing 1 mg/ml tetracycline or lacking tetracycline. The water was replenished twice weekly. Tumor dimensions were measured with calipers every week. Tumor volume (in cm^3) was estimated from the equation used to calculate the volume of a sphere: length X width X height X 0.5236. Tumor latency is defined in these experiments as the time (in days) required for a tumor to reach a volume of 0.5 cm^3 .

Affymetrix GeneChip expression analysis

Two independent analyses were carried out using the HU95A human genome chips for each of these three cell strains: MSU-1.1 cells, PH3MT-Double-C15 cells assayed in medium supplemented with tetracycline ($1 \mu\text{g/ml}$), and PH3MT-Double-C15 cells assayed in medium lacking tetracycline. PolyA+RNA was extracted from these cells using the Micropure PolyA+RNA extraction kit following the general instructions provided by the manufacturer (Ambion). The detailed steps to synthesize cRNA products from $3 \mu\text{g}$ of polyA+RNA were followed as described in the Affymetrix expression analysis technical manual. Personnel at the on-site Genomics Technology Sequence Facility carried out the hybridization and washing steps, and the probed arrays were scanned as described in the manual.

The average difference value is reported for each gene assayed on the chip, and is directly proportional to transcript abundance. Two comparisons were made, one between MSU-1.1 cells and PH3MT-Double-C15 cells (+) tetracycline (to identify genes regulated by oncogenic H-RAS) and another between PH3MT-Double-C15 cells (+) tetracycline and (-) tetracycline (to identify genes regulated by dominant-negative Cdc42 and Rac1 mutants). For each comparison, four ratios were calculated for each gene, i.e., C1/E1, C1/E2, C2/E1, C2/E2, where C and E are two different cell strains, and 1 and 2 are two different experiments for each of the cell strains. The average and standard deviation of these ratios were calculated. Significant fold-differences, i.e., ratios significantly different from a value of 1, were assessed by Student *t*-test, $p < 0.05$. Calculation of an average and its standard deviation from two independent experiments is not a valid statistical analysis. Thus, the *t* values were not adjusted for the fact that two independent experiments gave rise to four ratios and that greater than 12,000 genes were assayed simultaneously on one chip from the same RNA sample, i.e., the analysis is not made more valid by adjusting for the experimental design. The statistical analysis was appropriately used only as a screening analysis to identify genes that warrant further verification by Northern analysis. In all comparisons, if the difference of the average of the average difference values between two samples was below an absolute value of 300, the gene was not considered further. Four gene categories resulted from such analyses as shown in Table 3.

Northern blot analysis

Total RNA was extracted from cell pellets using the RNeasy reagent as described by the manufacturer. Total RNA concentration was determined by UV

absorbance at A260. RNA samples (20 µg of total RNA or 3 µg of polyA+RNA) were fractionated on a denaturing formaldehyde agarose gel and transferred to nitrocellulose membrane. The procedures were carried out as described in *Short Protocols in Molecular Biology* (1992). The RNA was UV crosslinked to the membrane (Stratagene UV Crosslinker). Freshly denatured salmon sperm DNA was added to prehybridization and hybridization solutions immediately prior to use. In general, prehybridization was performed at 42°C for 2-4 hrs and hybridization was performed at 42°C for >12 hrs. The ³²P-labeled EPAS1 DNA probe was generated by random oligonucleotide-primed synthesis from either a 5' 1.0 kb or a 3' 1.3 kb template of EPAS1 using Klenow fragment (*Short Protocols in Molecular Biology*, 1992). The region-specific templates were obtained by SmaI digestion of pTRE-EPAS1, a generous gift from Dr. Y. Kageyama (Tokyo Medical and Dental University, Japan). The two different probes were used in separate Northern blot analyses to confirm specificity of bands. Each blot was hybridized with 20X10⁶ cpm of denatured probe. Bands were visualized and analyzed using the G5-525 phosphorimaging system (Biorad).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assays (EMSA) were carried out as described (Yoshizumi et al., 1995). Annealed double stranded (ds) oligonucleotides containing the wildtype (WT) HIF-1 binding site (5'-CCACAGTGCATACGTGGGCTCCAACAGGTCCTC TT-3') or the mutant (Gibbs et al., 1999) HIF-1 binding site (5'-CCACAGTGCATCTCGAGGCTCCAACAGGTCCTCTT-3') were used in the assays. Nuclear extracts were prepared as described (*Short Protocols in Molecular Biology*,

1992). The standard 40 μ l DNA binding reaction consisted of 10 μ g of nuclear extract, 200,000 cpm of 32 P-labeled ds oligonucleotide, 4% glycerol, 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH7.5, and 0.05 mg/ml poly(dI-dC). The DNA binding reactions were carried out at room temperature for 30 min. then incubated on ice for 30 min. In addition to the wildtype HIF-1 32 P-labeled ds oligonucleotide reaction, three control reactions were performed for each sample. Substitution of the mutant HIF-1 binding site oligonucleotide for the wildtype HIF-1 binding site oligonucleotide was the *mutant reaction*. Addition of a 10-fold molar excess (2 pmol) of unlabeled wildtype HIF-1 binding site oligonucleotide in the standard DNA binding reaction was the *competition reaction*. Addition of 4 μ l of 0.2 mg/ml goat polyclonal anti-EPAS1 antibody in the DNA binding reaction after incubation at room temperature but before incubation on ice was the *supershift reaction*. Samples were fractionated under native conditions in a 5% PAGE using 0.5X TBE buffer as described (*Short Protocols in Molecular Biology*, 1992). The G5-525 phosphorimager system (Biorad) was used to analyze the data.

VEGF secretion

Conditioned growth medium was harvested 24 or 48 h after changing the regular growth medium to low-serum (0.5% SCS) growth medium with 1 μ g/ml tetracycline or without tetracycline. The cell lysate was collected from the same dish of cells and quantified for total protein yield. The relative concentration (μ g/ml), a ratio of total protein (μ g) to total volume of the conditioned medium (ml), was used to normalize different samples. The volume of conditioned medium that is equivalent to 100 μ g of total protein was assayed for level of VEGF splice variants by immunoblot analysis.

RESULTS

PH3MT cell strains expressing dominant-negative Cdc42 and Rac1 mutants

For these studies, we used a cell line, PH3MT, derived from a malignant tumor formed in an athymic mouse following injection of MSU-1.1 cells that had been transformed by transfection of the H-RAS oncogene (Hurlin et al., 1989). Immunoblot analysis shows that PH3MT cells express H-Ras protein at a level five times higher than that of their parental MSU-1.1 cell strain (Figure 1a). To determine if there was a corresponding increase in the activation of a major Ras-mediated signaling pathway, i.e., the Ras/Raf/MEK/ERK pathway, we analyzed the level of the phosphorylated forms of ERK1 (p44) and ERK2 (p42), which are the activated states of these kinases, as well as the total amount of ERK1 and ERK2. There was no difference between MSU-1.1 and PH3MT cells in the total amount of ERK1 and ERK2, or the phosphorylated ERK1, but the level of phosphorylated ERK2 was approximately 3 times higher in the malignant cell line than that in the parent cell strain. Two other independent experiments showed similar results.

To determine if Cdc42 and Rac1 are required for H-Ras-induced malignant transformation of human fibroblasts, we first generated a clonal population of cells designated as PH3MT-C1 that expressed the tetracycline transactivator gene (tTAK). These cells were then transfected with a vector containing an epitope-tagged dominant-negative Cdc42 (Flag-N17-Cdc42) or an epitope-tagged dominant-negative Rac1 (Myc-N17-Rac1), or with both vector constructs. Transcription of genes cloned into this vector is under the regulation of the Tet promoter. A series of clonally-derived cell strains were screened for tetracycline-regulated expression of the epitope-tagged proteins. The specific cell strains chosen for use in this study

were designated as Flag-N17-Cdc42-C21 and -C35, Myc-N17-Rac1-C12 and -C22, and Double-C15. The vector-control (VC) cell strains, designated as VC-C2 and -C6, were generated using a similar strategy but the transfected vector contained no cDNA. Tetracycline-regulated expression of dominant-negative mutant(s) was observed (Figure 1b). At least three independent experiments showed similar results. A major advantage of this system is that the same clonal population of cells expressing the dominant-negative mutant(s) in the absence of tetracycline serves as the control population for the corresponding cells in medium containing tetracycline to suppress such expression.

Figure 1: PH3MT cell strains expressing dominant-negative Cdc42 and/or Rac1 mutants. a, Immunoblot analysis of H-Ras, phospho-ERK, and total ERK level in PH3MT cells compared with that in the non-tumorigenic, parental MSU-1.1 cells. **b,** Cell strains expressing either Flag-tagged dominant-negative Cdc42 or Myc-tagged dominant-negative Rac1, or both mutants (Double), under the regulation of tetracycline. Expression of the mutant(s) is suppressed in the presence of tetracycline and induced in the absence of tetracycline. Two vector-control cell strains were also generated (VC-C2 and VC-C6). Immunoblot analysis was performed with antibodies reactive to either the Flag or Myc epitope.

Figure 1a

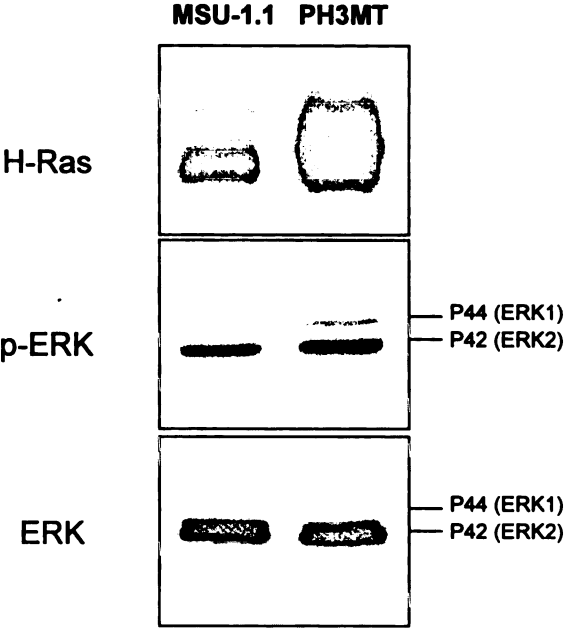
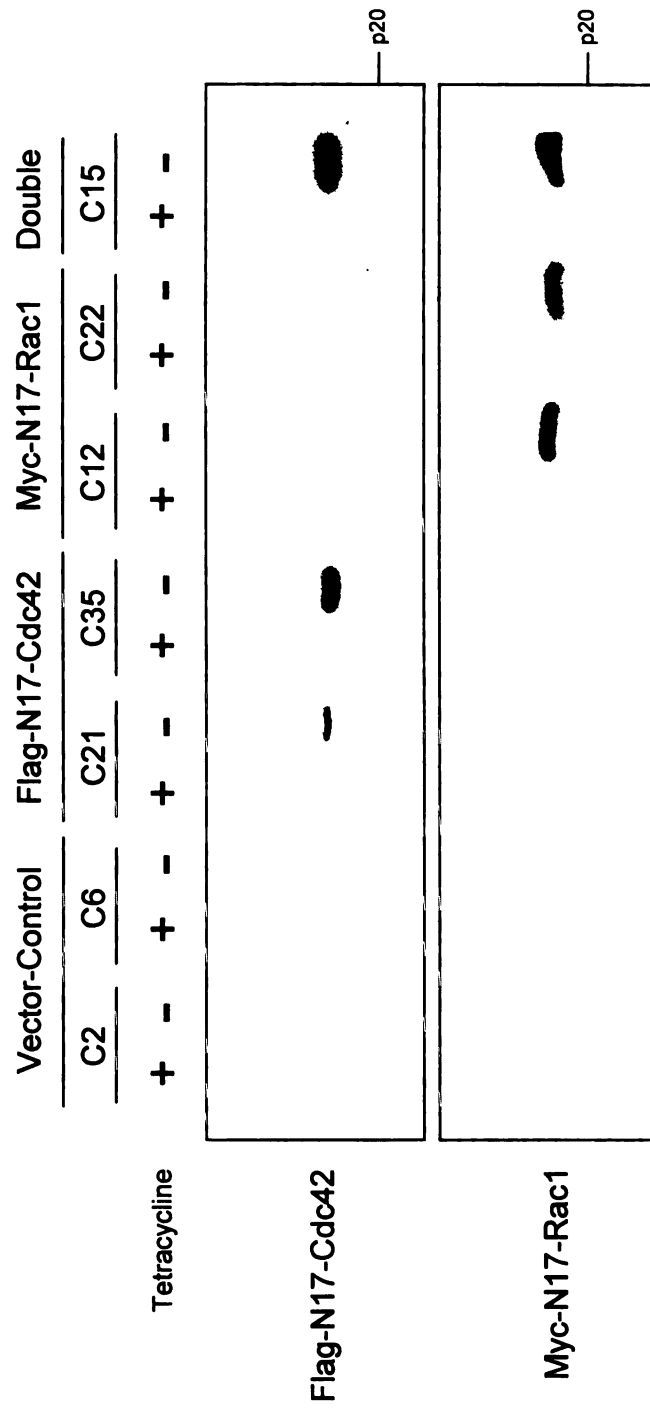


Figure 1b



Rapid proliferation of PH3MT cells requires intact Cdc42 and Rac1 activity

Oncogenic H-RAS-transformed cells display rapid cellular proliferation in the absence of extracellular mitogenic factors (Morrison et al., 1988; Gale et al., 1993). To determine the importance of Cdc42 and Rac1 activity in mediating this H-RAS-induced phenotype, we determined the doubling times of the parental cell strain MSU-1.1, its H-RAS-transformed derivative cell line, PH3MT, and the PH3MT-derived cell strains that display tetracycline-regulated expression of Flag-N17-Cdc42 and/or Myc-N17-Rac1 (Table 1). Cells were assayed in growth medium containing tetracycline or lacking tetracycline. The doubling times were determined from three or four independent experiments. As expected, PH3MT cells grew much faster than MSU-1.1 cells, i.e., the doubling times were 19.4 ± 0.7 h and 30.0 ± 0.7 h, respectively, and tetracycline had no significant effect on their growth rates. The doubling times of vector-control cell strains, VC-C2 and -C6, were comparable to that of the parental cell line PH3MT. In contrast, when tetracycline was omitted from the growth medium of the Flag-N17-Cdc42 cells (C21 and C35) and the Myc-N17-Rac1 cells (C12 and C22) to induce expression of the dominant-negative mutants, their growth rates were significantly slower than that of the same cells assayed in growth medium supplemented with tetracycline. The growth inhibitory effect of Myc-N17-Rac1 expression was slightly greater than Flag-N17-Cdc42 expression. Expression of both dominant-negative mutants (Double-C15) caused a slightly greater decrease in the growth rate than did either mutant expressed alone. These results indicate that H-Ras-induced rapid cellular proliferation is dependent on intact Cdc42 and Rac1 activity.

Table 1 • Effect of dominant-negative mutant(s) expression on growth of PH3MT cells

	Mean Doubling Time in Hours ($\pm$ s.d.) ^a	
	(+) Tet - GENE OFF	(-) Tet - GENE ON
Parental Cell Strains/Lines ^b		
MSU-1.1	30.2 ($\pm$ 1.0)	29.8 ($\pm$ 0.7)
PH3MT	19.9 ($\pm$ 0.5)	19.4 ($\pm$ 0.7)
PH3MT Cell Strains		
VC-C2 ^c	16.6 ($\pm$ 0.5)	20.4 ($\pm$ 1.2)
VC-C6 ^c	18.5 ($\pm$ 1.2)	19.4 ($\pm$ 2.2)
N17-Cdc42-C21 ^d	17.5 ($\pm$ 2.0)	28.1 ($\pm$ 2.0)
N17-Cdc42-C35 ^d	17.0 ($\pm$ 1.7)	26.6 ($\pm$ 1.2)
N17-Rac1-C12 ^d	16.3 ($\pm$ 0.2)	31.2 ($\pm$ 3.0)
N17-Rac1-C22 ^d	16.6 ($\pm$ 0.7)	31.4 ($\pm$ 1.0)
Double-C15 ^d	18.2 ($\pm$ 0.5)	35.8 ($\pm$ 1.2)

^aThe mean doubling time and standard deviation (s.d.) were determined from at least three independent experiments. Each cell strain was assayed in medium supplemented with tetracycline (expression of mutants suppressed) or in medium lacking tetracycline (expression of mutants induced).

^bAs a reference, MSU-1.1 and PH3MT cells were also assayed. The PH3MT cell line was established from a tumor of an athymic mouse following injection of a clone of MSU-1.1 cells that appeared morphologically transformed by oncogenic H-RAS (Hurlin, 1987 and 1989).

^cVC, vector-control cell strains. Some degree of cellular toxicity was associated with tTak expression alone.

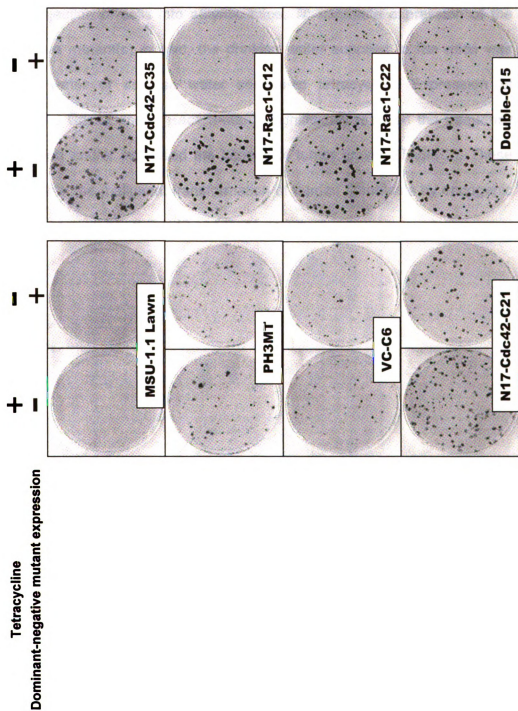
^dThere was a significant increase in the mean doubling time of cells grown in medium lacking tetracycline when compared with that of the same cells grown in medium supplemented with tetracycline (Student's *t*-test analysis, *p*<0.01).

The ability of PH3MT cells to form foci depends on Cdc42 and Rac1 activity

Human fibroblasts transformed by oncogenic H-Ras acquire the ability to form dense foci on a confluent monolayer of non-transformed fibroblasts (Hurlin et al., 1989). To determine the importance of Cdc42 and Rac1 activity as downstream mediators of H-Ras-induced focus formation, the PH3MT cell line and its derivative cell strains that display tetracycline-regulated expression of dominant-negative Cdc42 and/or Rac1 mutants were compared in focus reconstruction assays. As shown in Figure 2, MSU-1.1 cells did not form foci, whereas in the presence or absence of tetracycline, PH3MT cells formed distinct foci on a confluent monolayer of MSU-1.1 cells, as did the vector-control cell strain, VC-C6. Under conditions that suppressed mutant(s) expression, i.e., when tetracycline was included in the growth medium, the dominant-negative Cdc42 cell strains, C21 and C35, the dominant-negative Rac1 cell strains, C12 and C22, and the double mutant cell strain, C15, formed foci that were similar to those of the parental PH3MT and the VC-C6 cells. In contrast, under conditions that induced mutant(s) expression, i.e., when tetracycline was omitted from the medium, the mutant cell strains showed a reduced ability to form dense foci. Note that the cells expressing the dominant-negative Rac1 mutant as well as the cells expressing both dominant-negative mutants formed foci that were smaller and denser compared with that formed by the cells expressing the dominant-negative Cdc42 mutant. Similar results were obtained in three independent experiments.

Figure 2: Expression of dominant-negative Cdc42 and/or Rac1 mutants in PH3MT cells inhibited focus formation. The cells being tested were plated in 100-mm diameter dishes that contained 5×10^4 MSU-1.1 cells. Tetracycline was included in the growth medium to suppress dominant-negative mutant(s) expression or tetracycline was omitted from the growth medium to induce dominant-negative mutant(s) expression. Focus formation on a confluent monolayer of background cells is characteristic of transformed fibroblasts. As expected, background foci were not observed in dishes that contained only MSU-1.1 cells. In contrast, the parental PH3MT cells formed distinct foci after 3 weeks, as did the vector-control cell strain (VC-C6). The ability to form foci was significantly inhibited when the cells were assayed under growth conditions that induced mutant(s) expression compared with that of the same cells assayed under growth conditions that suppressed mutant(s) expression. The inhibition caused by Myc-N17-Rac1 expression was more striking.

Figure 2



Expression of dominant-negative mutant(s) in PH3MT cells inhibited tumor formation

The effect of inhibiting Cdc42 activity, Rac1 activity, or the activity of both small G-proteins, on the ability of PH3MT cells to form tumors was tested by subcutaneous injection of cells into athymic mice. To modulate expression of the dominant-negative mutant(s) *in vivo*, the drinking water supplied to the mice was either sucrose water or sucrose water containing tetracycline. Expression of dominant-negative Cdc42 and/or Rac1 mutants significantly reduced the frequency of tumor formation and/or increased the latency of tumor growth (Table 2). These results indicate that the malignant transformation of human fibroblasts by oncogenic H-RAS relies, in part, on intact Cdc42 and Rac1 activity.

Table 2 • Effect of dominant-negative mutant(s) expression on tumor formation by PH3MT cells				
	(+) Tet - GENE OFF		(-) Tet - GENE ON	
	# Tumors	Latency ^a	# Tumors	Latency ^a
Parental -PH3MT-C1	ND	ND	2/2	60
PH3MT-FlagN17Cdc42-C21	3/6	115	1/6	140
PH3MT-FlagN17Cdc42-C35	3/6	85	1/6	80
PH3MT-MycN17Rac1-C12	3/6	100	0/6	NA
PH3MT-MycN17Rac1-C22	4/6	110	2/6	180
PH3MT-Double-C15	4/6 ^b	80	1/6	120

^aTime in days required for the tumor to reach 0.5 cm³ in volume. When more than one tumor formed, the latency is an average of multiple tumors. ^bLatency was determined from two tumors because the other two formed in the genitalia of the mice. NA, not applicable.

Identification of H-Ras targets dependent on Cdc42 and Rac1 activity

Affymetrix GeneChip analysis was carried out to identify H-Ras targets that are dependent on intact Cdc42 and Rac1 activity. Two independent analyses were performed on two independent polyA+RNA samples for each of the following: 1) MSU-1.1 cells, 2) PH3MT-Double-C15 cells grown in the presence of tetracycline (expression of dominant-negative mutants suppressed) and 3) PH3MT-Double-C15 cells grown in the absence of tetracycline (expression of dominant-negative mutants induced). The fold-difference for each gene assayed on the HU95A chip was determined for two comparisons. First, we identified *those genes that are regulated by oncogenic H-RAS expression* by comparing the data for the MSU-1.1 cells with that obtained for the PH3MT-Double-C15 cells grown in the presence of tetracycline. Second, we identified *those genes that are regulated by dominant-negative Cdc42 and dominant-negative Rac1 expression* by comparing the data for the PH3MT-Double-C15 cells grown in the presence of tetracycline with that obtained for the same cells grown in the absence of tetracycline.

Twenty-eight genes displayed transcriptional regulation by *both* oncogenic H-RAS and dominant-negative Cdc42 and Rac1 mutants. In table 3, these genes are organized into four categories: 3A, the 9 genes that were up-regulated by H-RAS and required Cdc42 and Rac1 activity for this up-regulation, 3C, the 14 genes that were down-regulated by H-RAS and required Cdc42 and Rac1 activity for this down-regulation, 3B, the genes that were up-regulated by H-RAS and down-regulated by Cdc42 and Rac1 (none of the genes belonged in this category), and 3D, the 5 genes that were down-regulated by H-RAS and up-regulated by Cdc42 and Rac1. Thus, ~80% of these genes, 23 of 28, had expression patterns that indicate cooperative

regulation between H-Ras pathways and Cdc42 and Rac1 pathways. Also listed on this table are functional categories (FC column) of the proteins encoded by the 28 genes, and fold up-regulation (positive value) or down-regulation (negative value) of the genes resulting from expression of oncogenic H-Ras (H-RAS column) and from expression of dominant-negative Cdc42 and Rac1 mutants (C-R column). These data provide another line of evidence that H-Ras acts as an upstream regulator of Cdc42 and Rac1 pathways, especially in the pathways that lead to transcriptional modulation of gene expression.

Table 3: Proteins encoded by genes up-regulated or down-regulated by H-RAS that showed dependence on Cdc42 and Rac1 activity. Four categories resulted from analyzing the microarray data from the following two comparisons, 1) MSU-1.1 versus PH3MT-Double-C15, (+) tetracycline and 2) PH3MT-Double-C15, (+) tetracycline versus PH3MT-Double-C15, (-) tetracycline. We identified 28 genes that were regulated by both oncogenic H-RAS and dominant-negative Cdc42 and Rac1 mutants. Categories **3A** and **3C** include those genes that displayed cooperative regulation and categories **3B** and **3D** include those genes that displayed contradictory regulation. A majority of these genes showed cooperative regulation by H-Ras pathways and Cdc42 and Rac1 pathways. **H-RAS column**, fold up-regulated (+) or down-regulated (-) by oncogenic H-RAS expression. **C-R column**, fold up-regulated (+) or down-regulated (-) by dominant-negative Cdc42 and dominant-negative Rac1 expression. For example, EPAS1 (in category 3A) was up-regulated by oncogenic H-RAS and down-regulated by the dominant-negative mutants. Thus, H-RAS-induced up-regulation of EPAS1 is dependent on intact Cdc42 and Rac1 activity. **FC**, functional category: **C**, cytoskeletal and structural proteins, cell motility and adhesion; **D**, DNA replication, recombination, repair; **E**, metabolic enzymes, channels and transporters, lipid metabolism, nucleotide synthesis and modification; **I**, immunoglobulins, MHC, immune surface antigen, complement proteins; **M**, mRNA processing and translation; **R**, DNA structure, degradation, and processing; **S**, growth factors, receptors, kinases and phosphatases, G-proteins; **T**, transcription factors and regulators; **U**, unknown function, ESTs, and hypothetical proteins.

Table 3 • Proteins encoded by genes up-regulated or down-regulated by H-RAS that showed dependence on Cdc42 and Rac1 activity

Genes up-regulated by oncogenic H-RAS					Genes down-regulated by oncogenic H-RAS				
3A Down-regulated by Cdc42 and Rac1 mutants	FC	H-RAS	C-R		3C Up-regulated by Cdc42 and Rac1 mutants	FC	H-RAS	C-R	
N-methyl-D-aspartate receptor modulatory subunit 2A (hNR2A)	S	2.4	-2.0		myristoylated alanine-rich protein kinase C substrate	C	-3.6	1.7	
serine/threonine kinase	S	4.4	-1.6		collagen binding protein 2	C	-4.5	1.5	
chorionic gonadotropin beta subunit	S	1.8	-1.6		purine-rich element binding protein	D	-2.3	1.7	
sprouty 2 (SPRY2)	S	4.1	-1.5		putative diacyl-CoA isomerase (ECH1)	E	-1.6	1.6	
zinc finger DNA-binding protein (PRDM2)	T	6.9	-3.4		MHC class I HLA-J	I	-1.6	1.6	
endothelial PAS domain protein 1 (EPAS1)	T	2.4	-2.3		splicing factor, proline/glutamine rich	M	-3.1	2.5	
unknown (AB020659)	U	2.7	-3.2		histone H1x	R	-1.6	1.5	
unknown (D29810)	U	8.3	-2.4		guanine nucleotide-binding regulatory protein (G-y-alpha)	S	-2.4	4.0	
unknown (AL080218)	U	2.4	-1.8		guanine nucleotide-binding regulatory protein (G-y-alpha)	S	-2.1	2.3	
					stimulatory (GS) regulatory component of adenylyl cyclase	S	-1.5	1.8	
					copine III calcium-dependent membrane binding protein (CPNE3)	S	-2.2	1.7	
					core binding factor a1 subunit	T	-5.2	3.9	
					mSin3A associated polypeptide p30	T	-1.8	3.8	
					unknown (U79259)	U	-2.1	1.6	
3B Up-regulated by Cdc42 and Rac1 mutants					3D Down-regulated by Cdc42 and Rac1 mutants				
None					cytochrome c-2 (hCox-2)	E	-4.9	-1.6	
					insulin-like growth factor-binding protein-3	S	-3.4	-1.6	
					urokinase (uPA)	S	-2.2	-1.6	
					basic fibroblast growth factor (bFGF)	S	-1.9	-2.6	
					transcription factor LZIP	T	-1.6	-1.8	

EPAS1 is a Ras target dependent on Cdc42 and Rac1 activity for up-regulation

Genes that are up-regulated by oncogenic H-RAS expression and require intact Cdc42 or Rac1 activity for this up-regulation may play a causal role in the malignant transformation of cells. We identified endothelial PAS domain protein 1 (EPAS1), a hypoxia inducible transcription factor, as one such candidate gene. The mRNA level of EPAS1 was 2.4-fold higher in the PH3MT-Double-C15 cells grown in medium containing tetracycline, which suppressed expression of the dominant-negative Cdc42 and Rac1 mutants, than in the parental MSU-1.1 cells. The up-regulation of EPAS1 by oncogenic H-RAS, however, was reversed upon induced expression of the dominant-negative Cdc42 and Rac1 mutants by omitting tetracycline from the growth medium. This was verified by Northern blot analysis, which showed that EPAS1 mRNA abundance in the PH3MT-Double-C15 cells assayed in the presence of tetracycline was approximately 2-fold higher than that in the same cells assayed in the absence of tetracycline (Figure 3a). Two independent RNA samples were probed in several Northern blots with either a 5' probe or 3' probe to EPAS1 mRNA.

In response to hypoxic conditions, EPAS1 induces transcription of genes that contain the hypoxia response element in their promoter region such as that present in the VEGF gene (Tian et al., 1997; Xia et al., 2001). To determine if there was a corresponding increase in DNA binding activity of EPAS1 to the hypoxia response element, electrophoretic mobility shift assays were performed. The results (Figure 3b) show that expression of dominant-negative Cdc42 and Rac1 mutants (lanes 9 and 11) decreased DNA binding activity in these cells compared with that in the same cells containing intact Cdc42 and Rac1 activity (lanes 3 and 5). The fold-

difference is approximately 1.4 to 1.6-fold. Addition of polyclonal EPAS1 antibody in the supershift reaction decreased the band intensity but did not shift the band probably because the antibody disrupted EPAS1 binding to the labeled oligonucleotide (lanes 4, 6, 10, and 12). The amount of VEGF splice variants secreted into conditioned growth medium was assayed by immunoblot analysis. The secreted level of two splice variants of VEGF, 121- and 189-amino acid forms, was higher in the PH3MT-Double-C15 cells assayed under growth conditions that suppressed expression of the dominant-negative Cdc42 and Rac1 mutants than in the parental cell strain MSU-1.1 (Figure 3c, lanes 4 and 6 versus lane 2). Expression of oncogenic H-RAS did not increase the abundance of the 165-amino acid form. When the expression of the dominant-negative Cdc42 and Rac1 mutants was induced in these same cells, by omitting tetracycline from the growth medium, there was a marked reduction of all three splice variants of VEGF in the conditioned medium (Figure 3c, lanes 4 and 6 versus lanes 5 and 7). This experiment was performed three times.

Figure 3: EPAS1 as a Ras target dependent on Cdc42 and Rac1 activity for up-regulation. a, Northern analysis of EPAS1 mRNA level in PH3MT-Double-C15 cells grown in the presence (expression of mutants suppressed) or absence of tetracycline (expression of mutants induced). Shown here is the result of probing total RNA with a 1.0 kb probe to the 5' region of EPAS1. Three bands were noted, however, only the lowest band (arrow) was present in an independent probing of polyA+RNA with a 1.3 kb probe to the 3' region of EPAS1. The size of this band is approximately 4.0 kb, which is the expected size of EPAS1 mRNA. GAPDH was probed as the loading control. The level of EPAS1 mRNA was indeed higher in cells containing intact Cdc42 and Rac1 activity than in cells with disrupted Cdc42 and Rac1 activity. b, Electrophoretic mobility shift assay to determine EPAS1 DNA binding activity. ³²P-labeled ds oligonucleotides containing a wildtype or mutant HIF-1 binding site were used in these assays. EPAS1 DNA binding activity was higher in PH3MT-Double-C15 cells grown in the presence of tetracycline than in the same cells grown in the absence of tetracycline. c, Comparing amount of VEGF splice variants secreted into conditioned growth medium by immunoblot analysis. Conditioned medium was collected at two time points for the PH3MT-Double-C15 cells (24 and 48 h). There was a marked decrease in secretion of all three VEGF splice variants when the dominant-negative Cdc42 and Rac1 mutants were expressed (tetracycline absent). M, medium; 1.1, MSU-1.1; 6A, tumorigenic cell line transformed by BPDE carcinogen. d, Oncogenic H-Ras (*) induces VEGF secretion in a pathway requiring Cdc42- and Rac1-induced up-regulation of EPAS1. Heterodimerization of EPAS1 and aryl hydrocarbon receptor nuclear translocator (ARNT) results in a complex that activates transcription of the VEGF gene.

Figure 3a

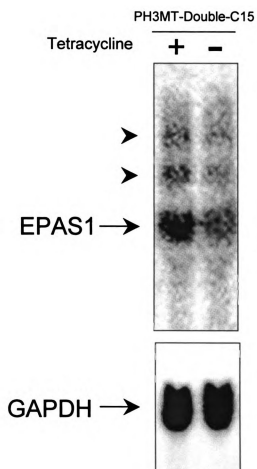


Figure 3b

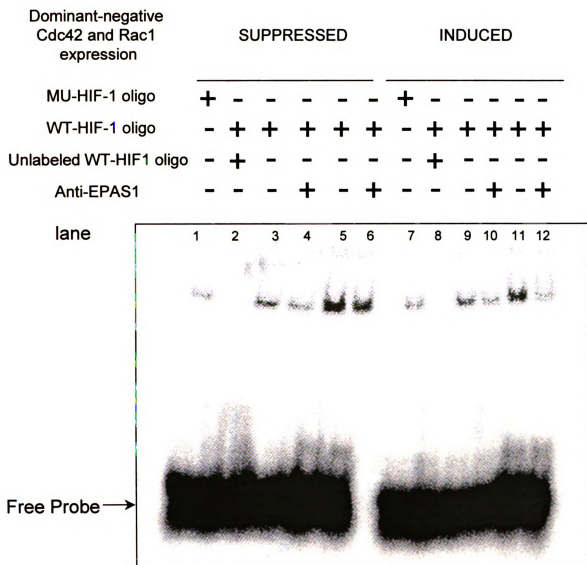


Figure 3c

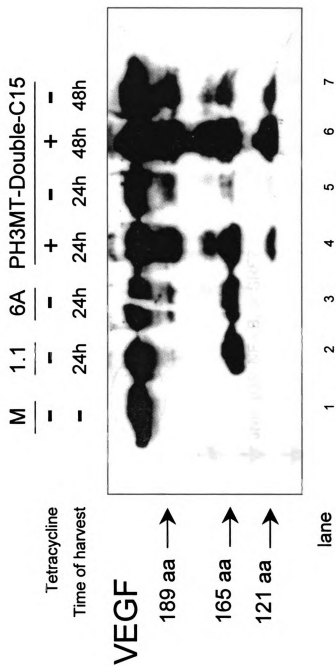
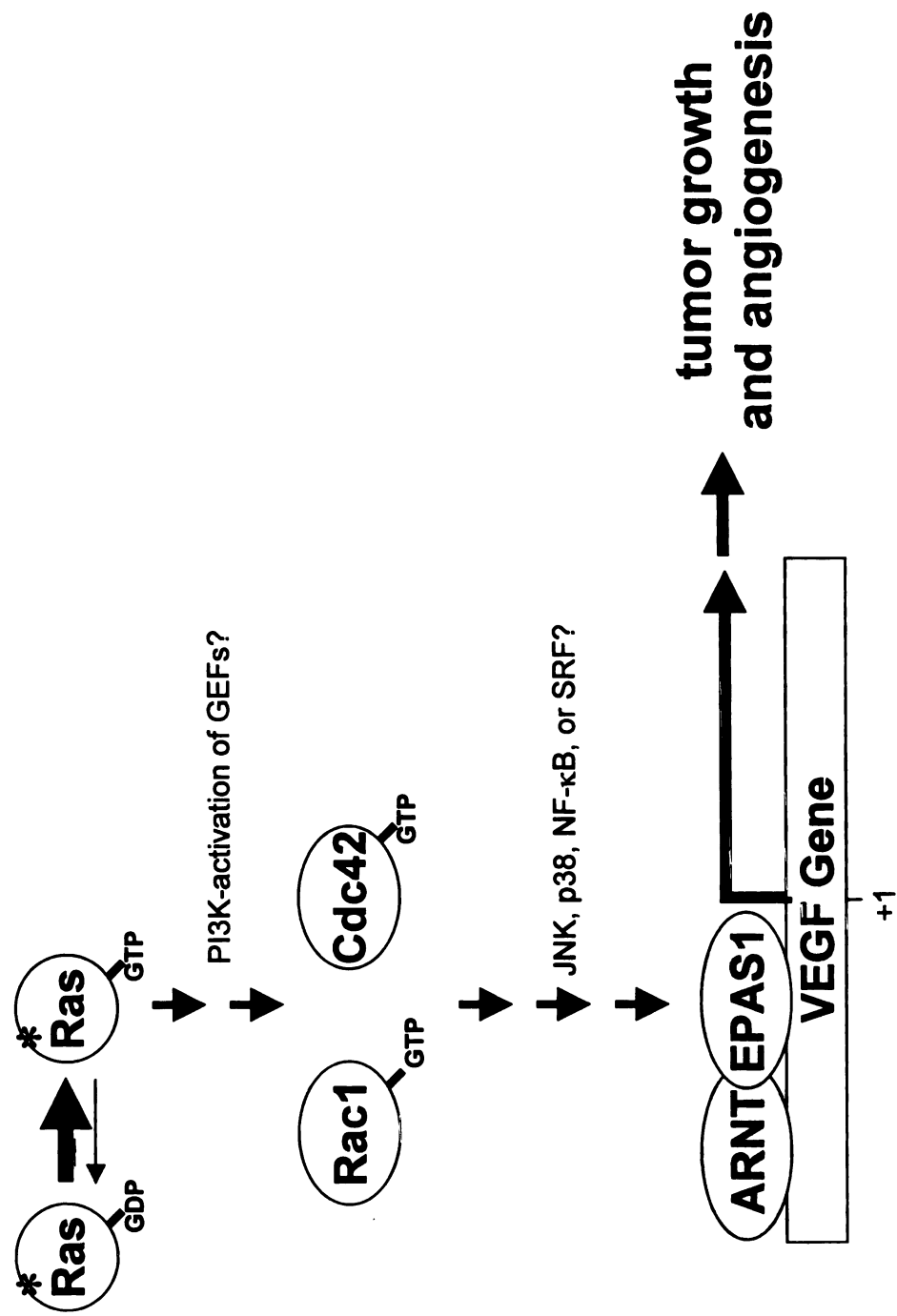


Figure 3d



DISCUSSION

Expression of RAS oncogenes in mammalian cells is known to cause critical changes that are important for tumor formation and tumor progression (Hernandez Alcoceba et al., 2000). The results of our study indicate that Cdc42 and Rac1 activity are required for oncogenic H-RAS malignant transformation of human fibroblasts. This conclusion is supported by the results of our studies of the effect of inhibiting such activity, using dominant negative mutants, on the various characteristics of such malignantly transformed cells, including their ability to form malignant tumors in athymic mice (Table 2). This is the first report of such studies involving H-RAS oncogene-transformed human fibroblasts. In our study, inhibition of Cdc42 and/or Rac1 activity by the dominant-negative mutant Cdc42 or dominant negative mutant Rac1, or both, resulted in cells that had a substantially reduced rate of proliferation (Table 1), a markedly reduced ability to form foci (Figure 2), and significantly reduced tumorigenicity (a lower frequency of tumor formation and a longer latency of tumor growth) (Table 2). Thus, loss of Cdc42 and Rac1 activity negatively affected each of the characteristics originally shown to be caused in these cells by overexpression of the H-RAS oncogene (Hurlin et al., 1989).

Dominant-negative mutant studies, carried out with an infinite life span rodent fibroblast cell line, Rat1, malignantly transformed by oncogenic H-RAS, also showed that Cdc42 and Rac1 act as essential downstream mediators of oncogenic H-RAS transformation (Prendergast et al., 1995; Qiu et al., 1997). Qiu et al. (1997) showed that expression of dominant-negative mutant Cdc42 greatly reduced the frequency of focus formation and anchorage independence, i.e., the ability of the cells to form large colonies in semi-solid medium. It also reverted the transformed morphology of these cells. However, the ability of the cells expressing the dominant-negative

mutant Cdc42 to form tumors was not investigated. Expression of dominant-negative mutant Rac1 in these H-RAS-transformed Rat1 cells also significantly reduced the frequency of focus formation and anchorage independence, but unlike dominant-negative mutant Cdc42, it failed to revert the transformed morphology of the cells. Again, the effect of dominant-negative mutant Rac1 on the cells' ability to form tumors was not studied.

Expression of the dominant-negative Cdc42 or dominant-negative Rac1 significantly inhibited the transformed characteristics of our oncogenic H-RAS-transformed human fibroblast cell line, but did not completely eliminate them. This could indicate that the level of expression of each dominant-negative mutant protein was not high enough to block all the activity of the specific endogenous protein(s), or that the expression was high enough, but the mutant form of the protein was simply not fully effective in inhibiting its target protein. Mackay and Hall (1998), studying signaling pathways, suggested that Cdc42 and Rac1 activate similar signal transduction pathways to activate transcription of genes involved in their cellular function. If these pathways are completely identical, and if dominant-negative mutant Cdc42 or Rac1 acting alone are not expressed at a high enough level or are not efficient enough to shut down the pathway completely, then use of the double dominant-negative ought to have exhibited an additive effect. This was not observed in our studies. The additive effect of expressing both dominant-negative mutants on oncogenic H-RAS transformation of human fibroblasts was minimal. Our results show that inhibition of focus formation by the dominant-negative Rac1 mutant was much more significant than that by the dominant-negative Cdc42 mutant (Figure 2) and that the results of the double mutant were the same as those seen with the dominant-negative Rac1 mutant, indicating that the dominant-negative Rac1 effect

predominates. These data support the hypothesis that there are one or more rate-limiting steps downstream of Cdc42 and Rac1.

Oncogenic RAS plays a major role in human cells becoming malignantly transformed. Because Ras is a central signaling protein from which several downstream signaling pathways diverge, any number of Ras mediators could potentially serve as targets for *selective* inhibition of Ras function (Bishop and Hall, 2000). Our study indicates that the pathway(s) controlled by Cdc42 and Rac1 and their downstream effector(s) play a critical role in the downstream pathways of Ras. Such insight may allow one to develop ways to specifically inhibit pathways that are most important for Ras-induced malignant transformation.

Investigators have characterized protein and gene changes induced by specific Ras pathways in order to identify pathway-specific targets of oncogenic RAS. The majority of these studies emphasized the contribution of the RAF/MEK/ERK pathway. For example Lewis et al. (2000) used two-dimensional gel electrophoresis to identify protein changes resulting from inhibition of MEK, and Zuber et al. (2000) performed subtractive suppression hybridization to identify transcriptional changes induced by this same pathway. To determine the global gene expression changes induced by H-Ras signaling in a pathway-specific manner, we used Affymetrix GeneChip analysis to identify the transcriptional changes dependent on Cdc42 and Rac1 activity. We compared cells expressing or not expressing oncogenic H-RAS and cells expressing oncogenic H-RAS that also expressed or did not express both dominant-negative Cdc42 and Rac1 mutants. We chose to use the double mutant with the expectation that it would exhibit a greater inhibition of the transcriptional pathways they control. Expression of genes regulated uniquely by Cdc42 or Rac1, if they exist, can be determined by Northern Blot analysis of the

RNA of the single mutants generated in our study. A total of 28 genes showed an expression pattern that indicates they are regulated by the activity of the Ras protein and the Cdc42 and/or Rac1 proteins. The relative distribution of these genes in each of the four categories shown in Table 3 suggests that for the most part, the proteins act in a cooperative manner to regulate gene transcription.

Our data indicate that EPAS1, a newly identified member of the PAS superfamily of basic helix-loop-helix proteins (Tian et al., 1997), is a Ras target dependent on functional Cdc42 and Rac1 for up-regulation (Table 3). The activity of EPAS1 was also partially dependent on Cdc42 and Rac1 activity (Figure 3b). Up-regulation of EPAS1 by itself is unlikely to result in an equal increase in EPAS1 DNA binding activity to HIF-1 binding sites because EPAS1 is regulated by at least two mechanisms such as transcription and phosphorylation. This prompted us to investigate whether the secretion of vascular endothelial growth factor, a gene that is known to be up-regulated by EPAS1 and HIF-1 α , is also affected by inhibiting the activity of Cdc42 and Rac1 in cells malignantly transformed by oncogenic H-RAS. Cdc42 and Rac1 activity may affect the amount of VEGF secreted by altering the level of transcription of VEGF through EPAS1 or regulating the organization of the actin cytoskeleton in secretory processes. It is well established that oncogenic RAS induces VEGF expression and secretion (Rak et al., 1995; Feldkamp et al., 1999b). Consistent with this is the finding that farnesyltransferase inhibitors block Ras-induced up-regulation of VEGF (Charvat et al., 1999; Feldkamp et al., 1999a; Gu et al., 1999). The results of our study show clearly that H-Ras-induced VEGF secretion into the medium is highly dependent on the intact activity of Cdc42 and Rac1 (Figure 3c). The successful *in vivo* establishment of tumors is highly dependent on the induction of angiogenesis (Bouck et al., 1996; Hanahan and Folkman, 1996;

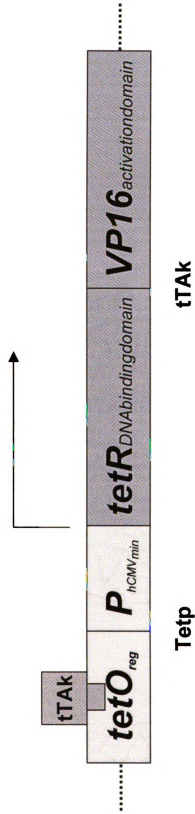
Folkman, 1997), and VEGF is known to play a major role in this process. Inhibition of VEGF activity in tumorigenic cells, by microinjection of anti-VEGF antibodies or expression of dominant-negative mutants, significantly impairs tumor growth (Kim et al., 1993; Millauer et al., 1996; Folkman, 1997). In summary, investigating the downstream effector pathways of oncogenic H-RAS, such as those pathways involved in VEGF up-regulation, may uncover pathway-specific targets for selective inhibition of Ras function.

APPENDICES

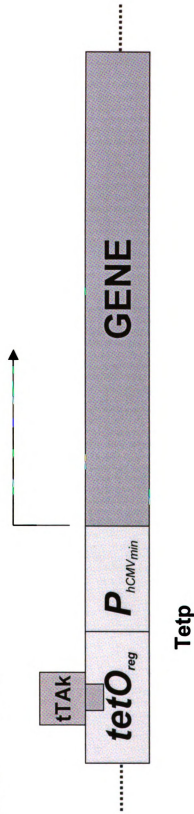
Appendix 1: Tet-OFF mammalian expression system. This is a two-vector system that allows for tetracycline-regulated expression of target genes in mammalian cells (Life Technologies). Drug resistance genes were cloned into the original vectors to facilitate selection of stable transfectants as described (Zhang et al., 1996). In this system, both the tetracycline-regulated transactivator kozak sequence (tTAk) and the target gene are under the regulation of the Tet promoter, which consists of the regulatory sequences of the Tet operator (TetO) and the human minimal CMV promoter sequences (hCMVmin). tTAK is a fusion protein consisting of the DNA binding domains of the Tet repressor (TetR) and the VP16 activation domain. **a**, In the **absence of tetracycline**, tTAk binds to the Tet promoter and activates transcription of itself and of the target gene. **b**, In the **presence of tetracycline**, tTAK is bound to tetracycline, which prevents tTAk from binding to the Tet promoter, and the expression of tTAK and the target gene is suppressed.

Appendix 1a

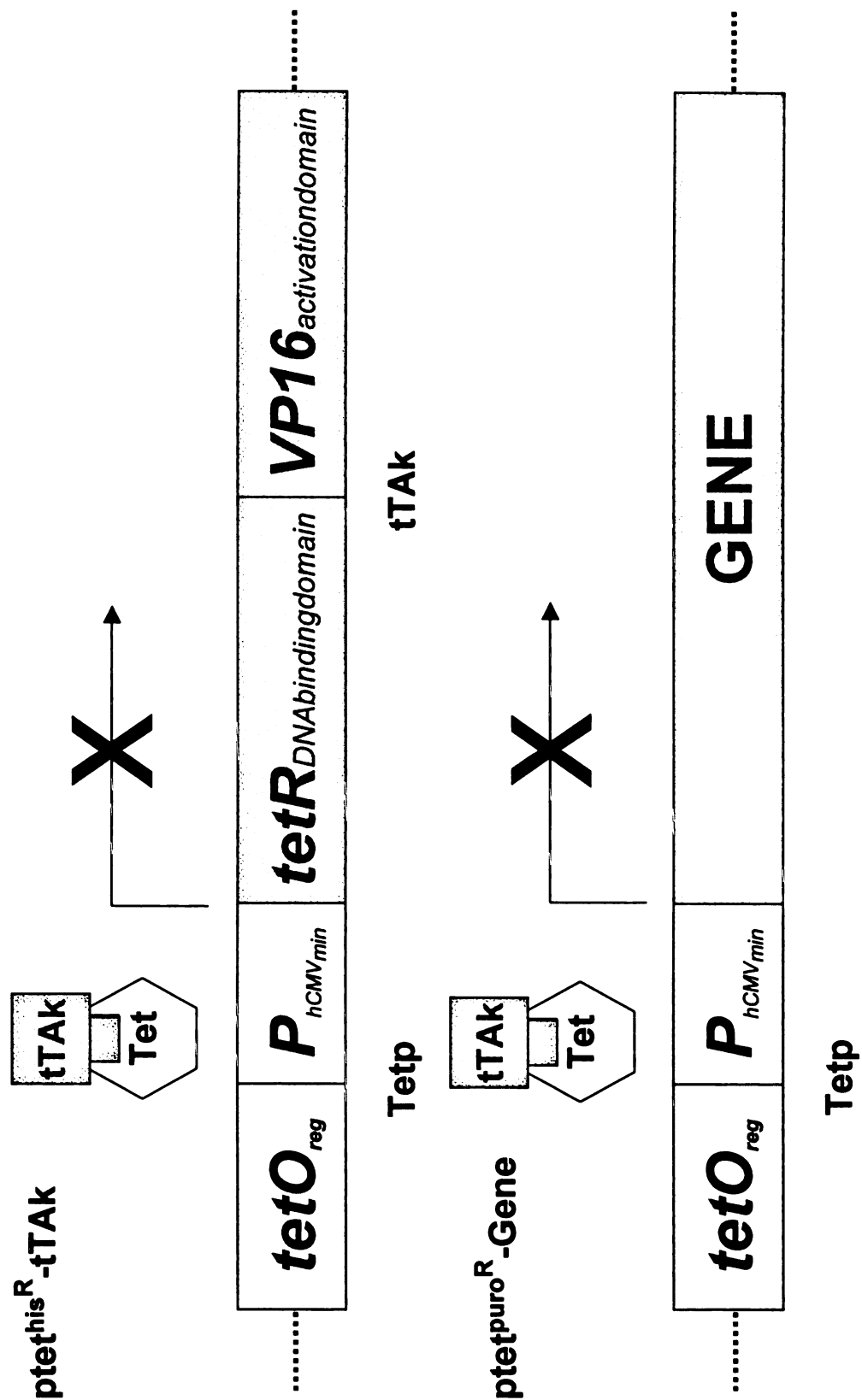
ptet^{his^R}-tTAK



ptetpuro^R-Gene

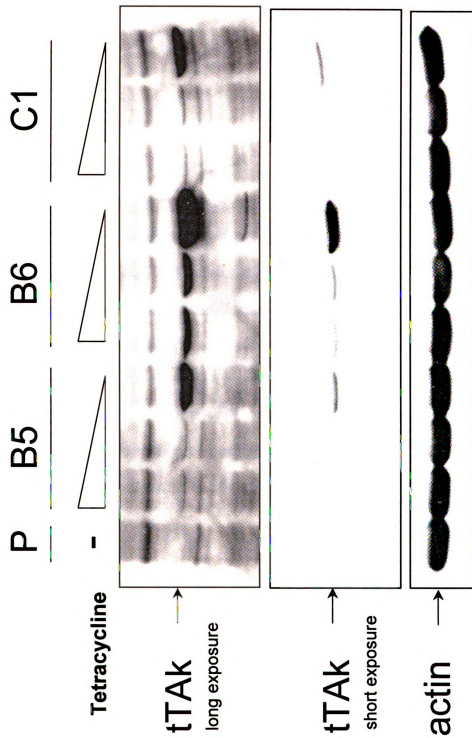


Appendix 1b



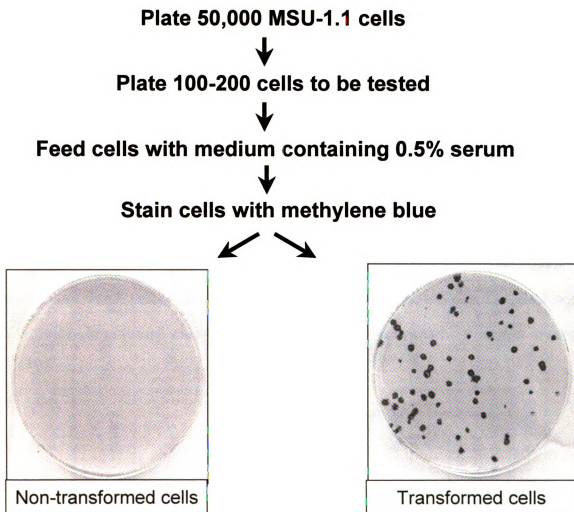
Appendix 2: PH3MT cell strains with tetracycline-regulated expression of tTAk protein. The first vector of the Tet-OFF system, pTet-tTAk, was transfected into PH3MT cells. Histidinol-resistant cell strains were then screened for tetracycline-regulated expression of tTAk by immunoblot analysis using an anti-VP16 polyclonal antibody (Invitrogen). Cell lysates were extracted from cells grown in medium containing 0, 0.5, or 1.0 $\mu\text{g/ml}$ tetracycline. P, parental cell line PH3MT. Actin was probed as the loading control. The C1 cell strain was used for subsequent transfection of the dominant-negative Cdc42 and/or Rac1 mutants.

Appendix 2



Appendix 3: Focus reconstruction assay. Transformed cells have the capacity to grow on a confluent monolayer of non-transformed cells. The focus reconstruction assay involves plating non-transformed cells, such as MSU-1.1 cells, at a cell density of 50,000 cells per 100-mm diameter dish. The next day, 100-200 cells that are being tested for focus reconstruction are plated into these same dishes. The growth medium is supplemented with 0.5% SCS and changed every week. After 2 or 3 weeks, foci are examined after fixing the cells with neutral buffered formalin and staining the cells with methylene blue. In control dishes, i.e., those dishes plated with only MSU-1.1 cells, there should be no or low background foci as shown here.

Appendix 3



Appendix 4: Summary of role of Cdc42 and Rac1 in oncogenic H-RAS-transformed rodent and human fibroblasts. Several studies established Cdc42 and Rac1 as downstream mediators of oncogenic H-RAS transformation of rodent fibroblasts (Prendergast et al., 1995; Qiu et al., 1997). In my studies, such a role for Cdc42 and Rac1 was investigated in a human fibrosarcoma cell line, PH3MT, which was established from a tumor that formed in an athymic mouse following the injection of oncogenic-H-RAS-transformed MSU-1.1 cells. The table summarizes the effect of expressing the dominant-negative Cdc42 and/or Rac1 mutants on growth properties characteristic of H-RAS-transformed cells (Hurlin et al., 1989).

**Appendix 4 • Summary of role of Cdc42 and Rac1 in
oncogenic H-RAS-transformed rodent and human fibroblasts**

Cells ¹	N17 Mutant ¹	Growth ¹	Focus Formation ¹	Anchorage Independence ¹	Tumorigenicity ¹	Reference
NIH3T3	Rac1	ND	↓↓	ND	ND	Qiu et al. 1995
Rat1	Cdc42	↔ ²	↓↓	↓↓	ND	Qiu et al. 1997
	Rac1	↓↓ ²	ND	↓↓	ND	
MSU-1.1	Cdc42	↓↓ ³	↓ ⁴	ND	↓	my work
	Rac1	↓↓ ³	↓↓ ⁴	ND	↓	my work
	Cdc42 and Rac1	↓↓ ³	↓↓ ⁴	ND	↓	my work

¹NIH3T3, mouse fibroblasts; Rat1, rat fibroblasts; MSU-1.1, human fibroblasts; N17, dominant-negative mutant; ND, not determined; ↔, no change; ↓, inhibition, ↓↓, significant inhibition.

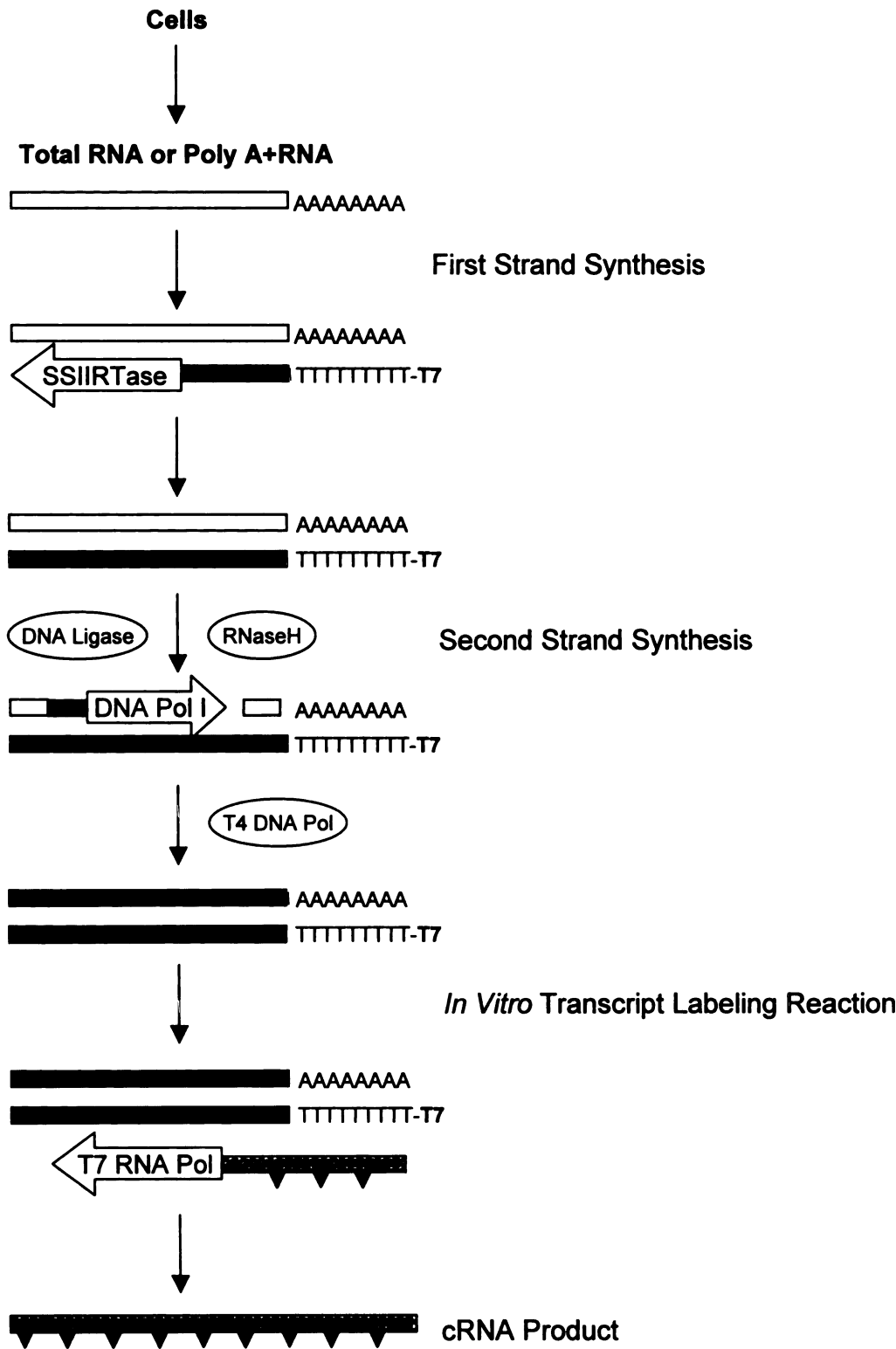
²Medium supplemented with 0.5% serum

³Medium supplemented with 10% serum

⁴Focus reconstruction assays

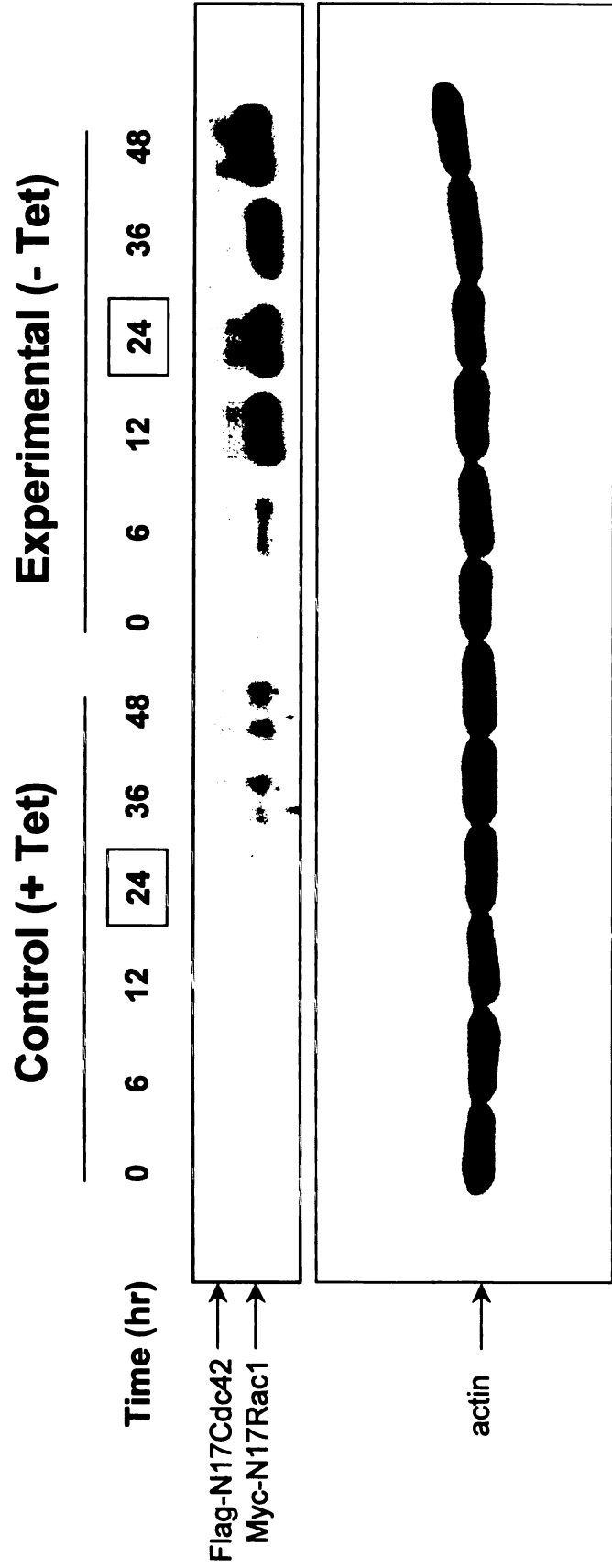
Appendix 5: Preparation of cRNA for Affymetrix GeneChip expression analysis. These are the enzymatic steps involved in converting mRNA, from either total RNA or polyA+RNA, into cRNA products for use as probes on the HU95A chip. The steps were performed as recommended by the manufacturer. Incorporation of oligo-dT(24)-T7 in the first strand synthesis reaction is required to generate biotinylated cRNA products from double stranded cDNA using T7 RNA polymerase. The cRNA products were chemically fragmented and heat denatured before use as probes. SSIIRTase, superscript II reverse transcriptase.

Appendix 5



Appendix 6: Optimal time point for polyA+RNA extraction from PH3MT-Double-C15 cells in the presence or absence of tetracycline. Cells were plated in growth medium supplemented with tetracycline. After 24 hr, the cells were washed once with PBS and the medium was changed with fresh growth medium containing tetracycline or lacking tetracycline (0 hr). Cell lysates were extracted at the indicated times, and 50 µg of total protein was subjected to immunoblot analysis using anti-Flag and anti-Myc antibodies. Actin was probed as the loading control. The 24 hr time point (boxed) was determined to be the optimal time point to extract polyA+RNA because there was significant induced-expression of the mutants (tetracycline absent) without loss of regulation in the control cells (tetracycline present) or cell death due to near-confluent growth of cells.

Appendix 6



Appendix 7: Expression of oncogenic RAS isoforms induces VEGF secretion into the growth medium. Six RAS-transformed human fibrosarcoma cell lines, each expressing the mutant V12 oncoprotein of one of the RAS isoforms, were compared with their parental cell strain MSU-1.1 for amount of VEGF secretion into the conditioned growth medium by immunoblot analysis using an anti-VEGF antibody. The cell lines tested included: H-RAS-2MT and -3MT (referred in paper as MSU-1.1-T24 cell strain 2 and 3) (Hurlin et al., 1989), K-RAS-2DT and -3DT (referred in paper as cell strain 1 and 2) (Fry et al., 1990), and N-RAS-3T and -8T (referred in paper as cell strain 1 and 2) (Wilson et al., 1989 and 1990). Alternative splicing of the VEGF gene resulted in at least three splice-variants that were detected in conditioned growth medium: 121-, 165-, and 189-amino acid forms (Poltorak et al., 2000). All of the oncogenic RAS-transformed cell lines secreted a much higher level of VEGF splice variants than the parental MSU-1.1 cell strain.

Appendix 7

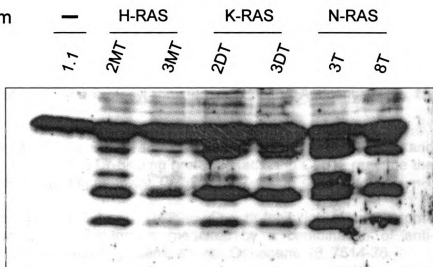
V12-RAS isoform

VEGF

189 aa →

165 aa →

121 aa →



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**APPENDIX A: INVESTIGATING THE CONTRIBUTION OF ENDOGENOUS,
HYPERACTIVE CDC42 AND RAC1 TO THE MALIGNANT TRANSFORMATION
OF HUMAN FIBROBLASTS**

ABSTRACT

The activity of the Rho GTPases is required for malignant transformation of cells by oncogenic mutants of RAS and oncogenic derivatives of guanine nucleotide exchange factors. However, the role of endogenous, hyperactive Cdc42 and Rac1 activity in the maintenance of growth properties characteristic of human fibrosarcomas has not been investigated. To determine this role, dominant-negative Cdc42 and Rac1 mutants were expressed in L210-6A/SB1 cells, a cell line derived from the tumor of an athymic mouse following subcutaneous injection of MSU-1.1 cells that displayed morphological transformation by BPDE carcinogen treatment. This cell line was chosen because it has an elevated level of GTP-bound Cdc42 and Rac1 as determined by an affinity-precipitation assay. The L210-6A/SB1 cells were infected with retroviruses carrying the cDNA(s) encoding either β -galactosidase, dominant-negative Cdc42, dominant-negative Rac1, or both mutants, followed by selection with puromycin, the selectable marker. Three cell populations were generated for each of these protein(s), and the cells have been injected subcutaneously into athymic mice to investigate the effect of dominant-negative mutant(s) expression on the ability of L210-6A/SB1 cells lacking hyperactive Cdc42 and/or Rac1 to form tumors in athymic mice. Clonally-derived populations expressing either a low or high level of these protein(s) were also generated to test directly the effect of dominant-negative mutant(s) expression.

INTRODUCTION

The Rho subfamily belongs to the Ras superfamily of small guanine-nucleotide binding proteins (Hall, 1990 and 1992b). RhoA, Cdc42, and Rac1 are three main members of this subfamily, and they each have multiple isoforms that differ in their tissue-specific expression pattern. They were originally identified by their biochemical and structural similarities to the prototype Ras protein. However, the Rho GTPases regulate diverse cellular processes, including actin cytoskeleton reorganization, transcriptional activation pathways, cell cycle progression, cell differentiation, survival and apoptosis pathways, and membrane trafficking (Van Aelst and D'Souza Schorey, 1997; Takai et al., 2001). Cdc42 and Rac1 act in parallel in several pathways, such as the p38, c-jun N-terminal kinase (Coso et al., 1995; Minden et al., 1995), nuclear factor κ B (Sulciner et al., 1996; Perona et al., 1997), and serum response factor pathways (Hill et al., 1995), to regulate transcription of genes.

Like all guanine-nucleotide binding proteins, Cdc42 and Rac1 cycle between active, GTP-bound states and inactive, GDP-bound states. In their active GTP-bound states, Cdc42 and Rac1 activate effector proteins that are involved in carrying out their cellular functions. GTPase-activating proteins stimulate Cdc42 and Rac1 conversion from their active states to their inactive states by enhancing the intrinsic GTP hydrolysis rates. The guanine-nucleotide dissociation inhibitors and the guanine-nucleotide exchange factors are two other families of proteins that regulate the activity of Cdc42 and Rac1 by increasing and decreasing the affinity for GDP, respectively.

Several lines of evidence suggest the involvement of Cdc42 and Rac1 in the malignant transformation of cells. First, although expression of constitutively active Cdc42 or Rac1 mutants in rodent fibroblasts only weakly transforms these cells (Hall, 1990 and 1992b), the guanine-nucleotide exchange factors, the positive regulators of Cdc42 and Rac1, are fully oncogenic when expressed as truncated derivatives in certain cell types (Cerione and Zheng, 1996). Second, several studies show that the Rho GTPases are required for malignant transformation of rodent fibroblasts by oncogenic H-RAS (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997). For example, when dominant-negative Rho GTPases were expressed in rodent fibroblasts malignantly transformed by oncogenic H-RAS, investigators found impaired ability of the cells to exhibit growth factor independence, anchorage independence, focus formation, and tumorigenicity in athymic mice. These studies indicate that Cdc42 and Rac1 act downstream of H-RAS. Third, Mira et al. (2000) show that highly proliferative breast carcinoma cell lines have endogenous, hyperactive Rac3 protein. They also show that this phenotype is dependent on activation of p21-activated kinase. The role of Cdc42 and Rac1 in maintenance of transformed characteristics of human fibrosarcomas has not been investigated. L210-6A/SB1 is a human fibrosarcoma cell line that has hyperactive Cdc42 and Rac1 proteins. These cells were infected with retroviruses carrying cDNA(s) encoding the dominant-negative Cdc42 and/or dominant-negative Rac1 to inhibit endogenous activity of target protein(s). The effect of inhibiting the protein(s) on the ability of L210-6A/SB1 cells to form tumors in athymic mice is being examined.

MATERIALS AND METHODS

Growth conditions

Unless otherwise noted, cell strains/lines were routinely grown in Eagle's minimal essential medium (Life Technologies), supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, 10% supplemented calf serum (SCS) (Hyclone Laboratories), 100 units/ml penicillin (Sigma), and 100 µg/ml streptomycin (sigma) (growth medium) and maintained at 37°C in a humidified incubator containing 5% CO₂ in air. The growth medium was supplemented with 0.5 µg/ml puromycin (Sigma) when cells were selected for the resistance marker.

Cell strains/lines

MSU-1.1, an infinite life span, near-diploid, chromosomally stable non-tumorigenic human fibroblast cell strain (Morgan et al., 1991), was treated with a reactive derivative of benzo[a]pyrene. Cells that formed foci were isolated and injected subcutaneously into athymic mice. L210-6A/SB1 is one such MSU-1.1-derived cell line established from a tumor of an athymic mouse (L. Milam, unpublished data). Three other MSU-1.1-derived fibrosarcoma cell lines used in this study were similarly established from tumors formed in athymic mice but were transformed by treatment with ethylnitrosourea (MSU-1.1-E32, unpublished studies), irradiation with cobalt-60 (MSU-1.1-γ3-A2) (O'Reilly et al., 1998), or expression of H-RAS (PH3MT) (Hurlin et al., 1989).

Cell lysates and immunoblot analysis

Cell lysates were prepared with 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na₃VO₄, 200 mM benzamidine, 1 mM

phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, and 25 µg/ml leupeptin. Total protein concentration was quantified using the Coomassie protein assay reagent (Pierce). Proteins in the lysates were denatured in 5X Laemelli sample buffer at 95°C for 5 min., separated by either 10% or 12.5% SDS-PAGE, and transferred to Immobilon-P membrane (Millipore). The membrane was blocked for 2 h with Tris-buffered saline containing 0.1% Tween-20 and 5% (w/v) non-fat milk. The membrane was probed at room temperature for 2 h with the primary antibody diluted in 5% milk in Tris-buffered saline containing 0.1% Tween-20 then probed for 1 h with the appropriate horseradish peroxidase-linked secondary antibody (Sigma and Santa Cruz Biotechnology) diluted in 5% milk in Tris-buffered saline containing 0.1% Tween-20. The membrane was incubated with the Supersignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce) and exposed to film to detect bands. The sources for the antibodies used in these studies were: anti-H-Ras (Santa Cruz Biotechnology), anti-phospho-ERK1 and -ERK2 and anti-ERK1 and -ERK2 (New England Biolabs), anti-Cdc42 and anti-Rac1 (Santa Cruz Biotechnology), anti-Flag (Sigma), anti-Myc (Invitrogen), and anti-V5 (Invitrogen).

Glutathione-S-transferase-p21-binding domain pull-down assay

The use of the glutathione-S-transferase-p21-binding domain (GST-PBD) as a strategy for affinity-precipitation of GTP-bound forms of Cdc42 and Rac1 was recently described (Benard et al., 1999). The cDNA sequence encoding amino acids 67-150 of the p21-activated kinase 1 was obtained by RT-PCR amplification, using primers that annealed to gene-specific sequences and contained convenient restriction enzyme sites for cloning into pGEX-KG (Pharmacia). The sequence integrity of the vector construct was verified by automated DNA sequencing (Visible

Genetics). The pGEX-KG-PBD construct was transformed into *Escherichia coli* BL21 (DE3) cells (Promega). Large cultures of bacteria were grown at 37°C with shaking to a cell density of approximately O.D. 600nm of 0.8. Cultures were then transferred to a shaker at room temperature and isopropyl-1-thio- β -D-galactopyranoside (5 mM final concentration) was added to induce GST-PBD expression, which is under the regulation of a LACZ promoter. GST-PBD fusion protein was batch-purified from bacteria cells using glutathione-agarose (Sigma). The GST-PBD assay conditions were carried out as described (Benard et al., 1999). Two reactions were carried out for each cell strain/line: the unloaded reaction was assayed for level of GTP-bound Cdc42 and Rac1 and the loaded reaction was performed as a control after artificially stimulating exchange with 10 mM EDTA in the presence of 100 μ M GTP γ S. For each cell strain/line, 250 μ g of total protein was assayed in a standard reaction, and for protein amounts greater than 250 μ g, the standard reaction was scaled-up accordingly.

Retroviral infection of cells with dominant-negative Cdc42 and Rac1 cDNAs

The cDNA of V5-epitope-tagged β -galactosidase, β -galactosidase-V5, Flag-epitope-tagged dominant-negative Cdc42 mutant, Flag-N17-Cdc42, and Myc-epitope-tagged dominant-negative Rac1 mutant, Myc-N17-Rac1, were separately cloned into the pBABE-puro vector. The cDNA encoding a puromycin resistance protein was cloned into the original pBABE vector as a selectable marker for stable transfectants (H. Zhang, unpublished results). Lipofectamine was used to transfect these vector constructs into Phoenix-ampho cells using the general strategy provided by the manufacturer (Life Technologies). Virus-containing medium was harvested 72 hr post-transfection. After spinning cellular debris down at 1500 rpm

for 5 min., the viral supernatant was transferred to a fresh conical tube and sterilized by 0.2 μ m filtration. Polybrene was added to an aliquoted amount of viral supernatant to a final concentration of 4 μ g/ml. This mixture was then added to dishes containing cells that were plated the day before. For every dish from which the viral supernatant was obtained, three equivalent dishes of cells plated at 40-60% confluence were infected. After 48 hr incubation with viral supernatant, cells were selected with puromycin or lysed for whole cell lysate.

Tumorigenicity studies

The ability of cells to form tumors was tested in athymic mice (BALB/c). Cells were injected subcutaneously into the right and left rear flanks of mice (1×10^6 cells per site). Tumor dimensions were measured with calipers every week. The tumor volume (in cm^3) was estimated from the equation used to calculate the volume of a sphere: length X width X height X 0.5236. Tumor latency is defined in these experiments as the time in days required for a tumor to reach a volume of 0.5 cm^3 .

RESULTS

Identifying fibrosarcoma cell lines that exhibit increased Cdc42 and Rac1 activity

A series of cell strains/lines of the MSU1 cell lineage were assayed for their level of GTP-bound Cdc42 and Rac1 proteins using a GST-PBD pull-down assay (Figure 1). The loaded reaction (+) served as a control to determine if the proteins could be detected if exchange was stimulated by the addition of EDTA in the presence of GTP γ S, a non-hydrolyzable GTP analog. The unloaded reaction (-) assayed endogenous GTP-bound level of Cdc42 and Rac1. The results are shown in Figure 2. GTP γ S-Cdc42 was detected in all cell lines tested whereas GTP γ S-Rac1 was detected only in the L210-6A/SB1 cell line (6A). LG1, a normal human fibroblast, and its two non-tumorigenic, derivative cell strains, MSU-1.0 and MSU-1.1, exhibited a very low level of both GTP-bound Cdc42 and Rac1 in the unloaded reaction, as did three of the four MSU-1.1-derived fibrosarcoma cell lines tested (E32, 3A2, and 3MT). The strategy that was used to establish these fibrosarcoma cell lines is described in the materials and methods section. The L210-6A/SB1 cell line displayed a very high level of GTP-bound Cdc42 and Rac1. To quantify this result, the L210-6A/SB1 cell line and its parental cell strain MSU-1.1 were assayed in at least three independent experiments. The results were similar to that shown in Figure 3. The level of GTP-bound Cdc42 was on average 5-fold higher in L210-6A/SB1 cells compared with that in MSU-1.1 cells. The fold-increase in the level of GTP-bound Rac1 could not be determined because the reference MSU-1.1 cells did not have a detectable level of GTP-bound Rac1.

Figure 1: GST-PBD pull-down assay of GTP-bound Cdc42 and Rac1 proteins.

Glutathione-S-transferase-p21-binding domain (GST-PBD) is a fusion protein that was purified from bacteria cells transformed with pGEX-KG-GST-PBD. PBD is a domain in p21-activated kinase that is necessary and sufficient for binding to active, GTP-bound forms of Cdc42 and Rac1. The activity level of Cdc42 and Rac1 in cells is assayed by incubating cell lysates with GST-PBD and glutathione agarose, followed by several washes and immunoblot analysis (using anti-Cdc42 and anti-Rac1 antibodies) (Benard et al., 1999).

Figure 1

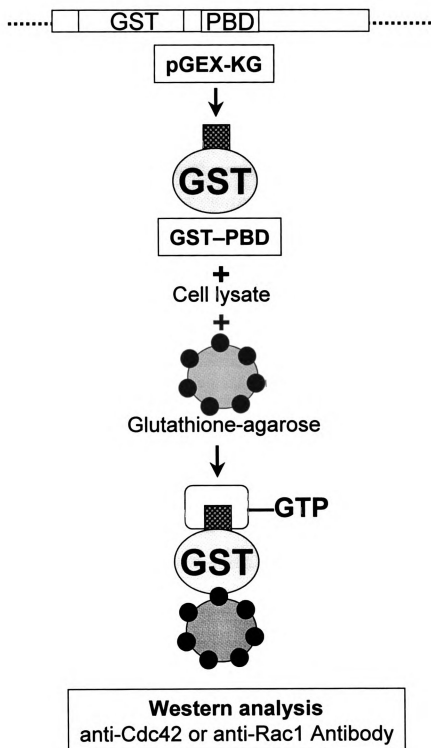


Figure 2: Level of GTP-bound Cdc42 and Rac1 proteins in cell strains/lines of the MSU1 cell lineage. The GST-PBD pull-down assay was carried out with cell lysates from LG1, a normal human fibroblast cell line, its non-tumorigenic derivative cell strains, MSU-1.0 and MSU-1.1, and four MSU-1.1-derived fibrosarcoma cell lines. Colleagues in this laboratory generated the fibrosarcoma cell lines by transforming MSU-1.1 cells by carcinogen treatment or oncogene expression including ENU (E32), BPDE (6A), γ -irradiation (3A2), and oncogenic H-RAS (3MT). Two reactions were set-up for each cell strain/line: a loaded reaction (EDTA and GTP γ S included in the cell lysate) and an unloaded reaction. The latter reaction assays for the endogenous level of GTP-bound Cdc42 and Rac1 proteins. As expected, there was a low level of activated Cdc42 and Rac1 proteins in all the cell strains/lines tested except in the L210-6A/SB1 (6A) cell line. This cell line had a high level of such proteins. Furthermore, the Rac1 protein was loaded in this cell line whereas it was not loaded in all the other cell strains/lines tested.

Figure 2

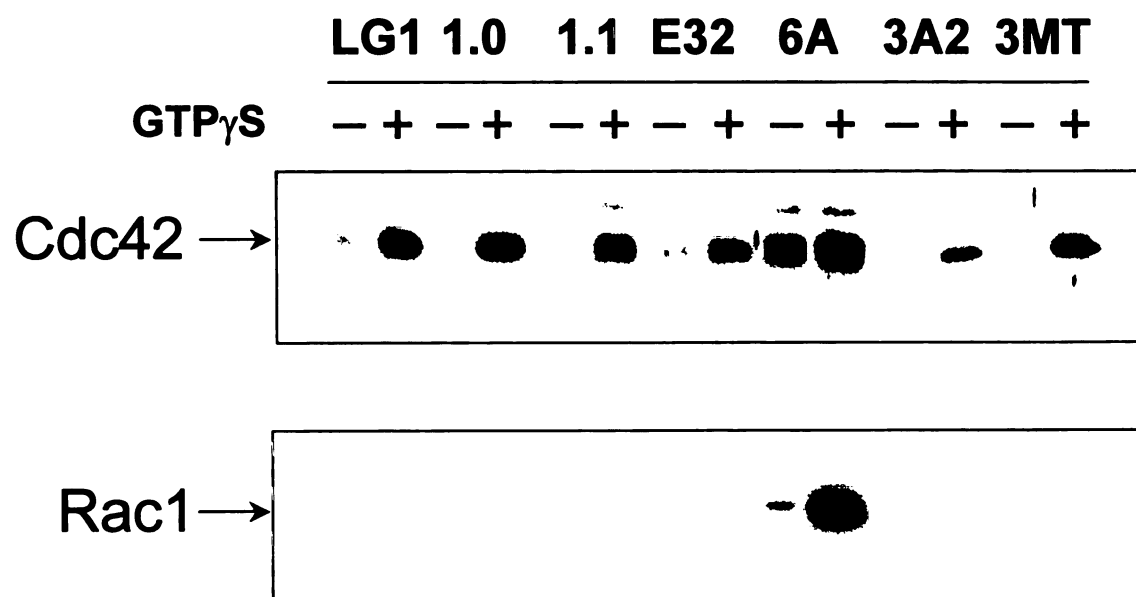
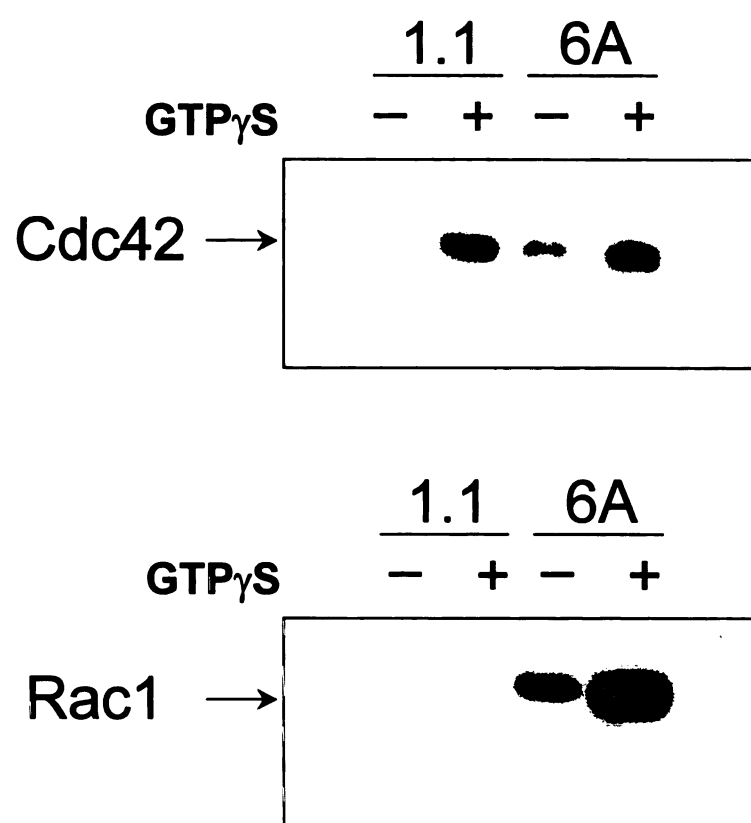


Figure 3: Endogenous, hyperactive Cdc42 and Rac1 proteins in L210-6A/SB1 cells compared with that in its parental cell strain MSU-1.1. The level of active, GTP-bound Cdc42 and Rac1 was determined by the GST-pull-down assay. L210-6A/SB1 (6A) cells have a 5-fold higher level of GTP-bound Cdc42 than that in MSU-1.1 cells (1.1). The fold-increase in the level of GTP-bound Rac1 could not be determined because this form was undetectable in MSU-1.1 cells. The experiment was repeated several times to quantify the fold-increase.

Figure 3

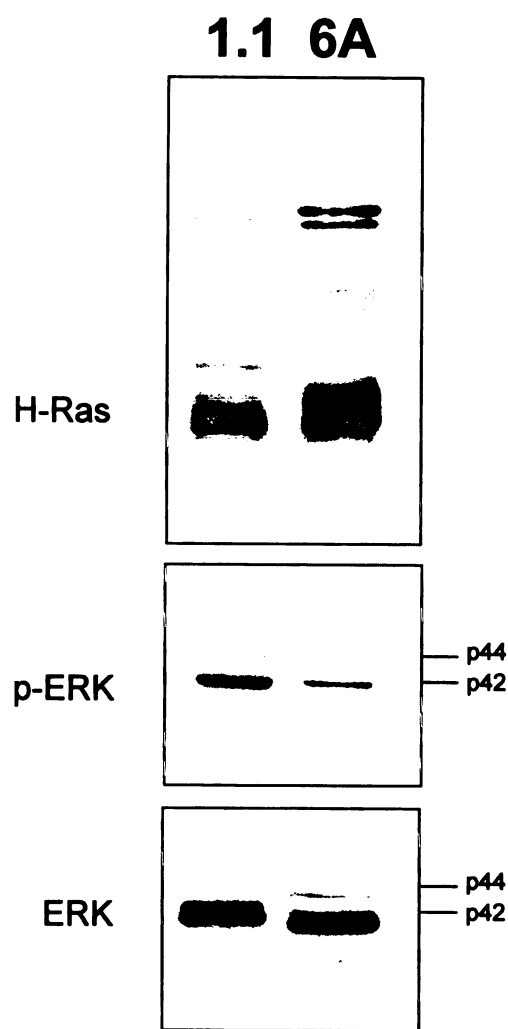


Level of H-Ras, p-ERK1/2, and ERK1/2 in MSU-1.1 and L210-6A/SB1 cells

The level of H-Ras, p-ERK, and ERK in MSU-1.1 and L210-6A/SB1 cells was determined by immunoblot analysis. The results in Figure 4 are similar to that obtained in two other experiments. An increase in H-Ras protein level could account for some of the activation of Cdc42 and Rac1 in L210-6A/SB1 cells. The level of H-Ras was 2-fold higher in L210-6A/SB1 cells than that in MSU-1.1 cells (top panel). However, there was not a corresponding increase in phospho-ERK level, which is the activated form of the kinase (middle panel). Total ERK level was similar between MSU-1.1 and L210-6A/SB1 cells, but the p42-ERK2 protein in L210-6A/SB1 cells ran slightly faster, suggesting that there was a difference in post-translational modification of the protein (bottom panel). It is not clear whether this increased level of H-Ras is sufficient to activate Cdc42 and Rac1.

Figure 4: Comparing level of H-Ras, p-ERK, and ERK in MSU-1.1 and L210-6A/SB1 cells. A 2-fold increase in H-Ras protein level was observed in L210-6A/SB1 (6A) cells compared with that in MSU-1.1 cells (1.1). Activity of the Ras/Raf/MEK/ERK pathway was also determined by immunoblot analysis. Phosphorylated ERK1 and ERK2 are activated forms of the kinases and their level in cells should reflect activity of the pathway. Two forms of ERK were detected, a 44 kD ERK1 and a 42 kD ERK2. Although L210-6A/SB1 cells had a higher level of H-Ras protein than that in MSU-1.1 cells, there was not a corresponding increase in amount of phospho-ERK1 or -ERK2 in L210-6A/SB1 cells. As expected, the total amount of ERK, i.e., phosphorylated and unphosphorylated, is the same for both these cells.

Figure 4



L210-6A/SB1 cells expressing dominant-negative Cdc42 or Rac1 mutants

To test the importance of Cdc42 and Rac1 activity in malignant transformation of L210-6A/SB1 cells, these cells were infected with retroviruses carrying the cDNA that encodes either dominant-negative Cdc42 (Flag-N17-Cdc42) or dominant-negative Rac1 (Myc-N17-Rac1) mutant. As a control population, L210-6A/SB1 cells were infected with retroviruses carrying the cDNA that encodes β -galactosidase. Immunoblot analysis of such population of cells is shown in Figure 5. Similar results were obtained in another experiment. The retroviral vector containing the cDNA has a puromycin resistance gene as the selectable marker. Puromycin-resistant cells infected with retroviruses carrying the cDNA that encodes β -galactosidase were stained for such expression to evaluate efficiency of retroviral infection and stable integration of target gene. Approximately 50% of the cells stained light to dark blue, indicating that cells in the population expressed various levels of β -galactosidase (Figure 5, inset of L2 population). Since the same retroviral vector was used for infection of dominant-negative Cdc42 and Rac1 mutants, it is assumed that expression of these proteins follows a similar pattern to that of the β -galactosidase populations. All of these cell populations have been injected subcutaneously into athymic mice. Tumors will be removed and tumor-derived cells will be tested for expression level of epitope-tagged dominant-negative mutant(s) and compared with the same population of cells that were not injected into athymic mice. If there is an *in vivo* selection against expression of dominant-negative Cdc42 and/or Rac1 mutants, then the tumor-derived cells should have a low level of mutant(s) expression. This experiment will provide evidence for or against the

hypothesis that activated Cdc42 and Rac1 proteins in L210-6A/SB1 cells play a causal role in malignant transformation of these cells.

Clonally-derived cells from the L210-6A/SB1 cell populations shown in Figure 5 were assayed for level of expression of β -galactosidase, dominant-negative Cdc42, dominant-negative Rac1, or both mutants, by immunoblot analysis (data not shown). The analysis was repeated before combining relatively low (L) or high (H) expressors from each of the cell population type generated (Figure 6). The double mutant (CR) is a single cell strain that displayed an intermediate expression level of both Flag-N17-Cdc42 and Myc-N17-Rac1. The clonal populations provide a more direct approach of investigating effect of Cdc42 and Rac1 inhibition on ability of L210-6A/SB1 cells to form tumors in athymic mice.

Figure 5: Cell populations of L210-6A/SB1 that express β -galactosidase, dominant-negative Cdc42, dominant-negative Rac1, or both mutants. Cell populations were generated by infecting L210-6A/SB1 cells with retroviruses carrying the cDNA encoding either β -galactosidase, dominant-negative Flag-N17-Cdc42, dominant-negative Myc-N17-Rac1, or both mutants, followed by selection with puromycin. Three independent cell populations were established for each of these. Within each cell population, the cells express various levels of protein(s) as demonstrated by β -galactosidase staining of cells from the L2 population. Overall expression level of these protein(s) in the cell populations was determined by immunoblot analysis using anti-Flag or anti-Myc antibody. β -galactosidase-expressing cell populations were generated as control cell populations. Actin was probed as a loading control. L, LacZ; C, Flag-N17-Cdc42, R, Myc-N17-Rac1; CR, double mutant.

Figure 5

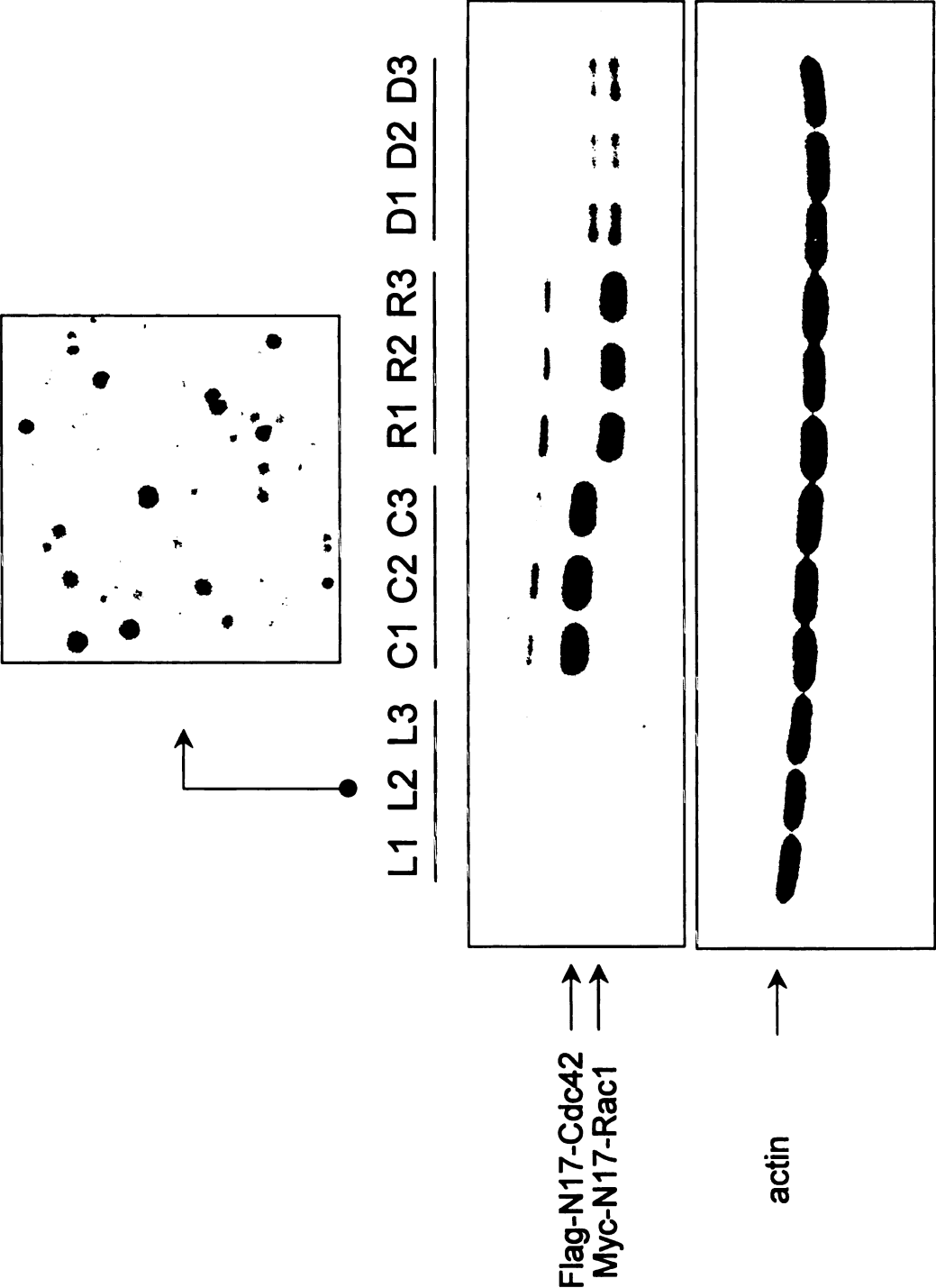
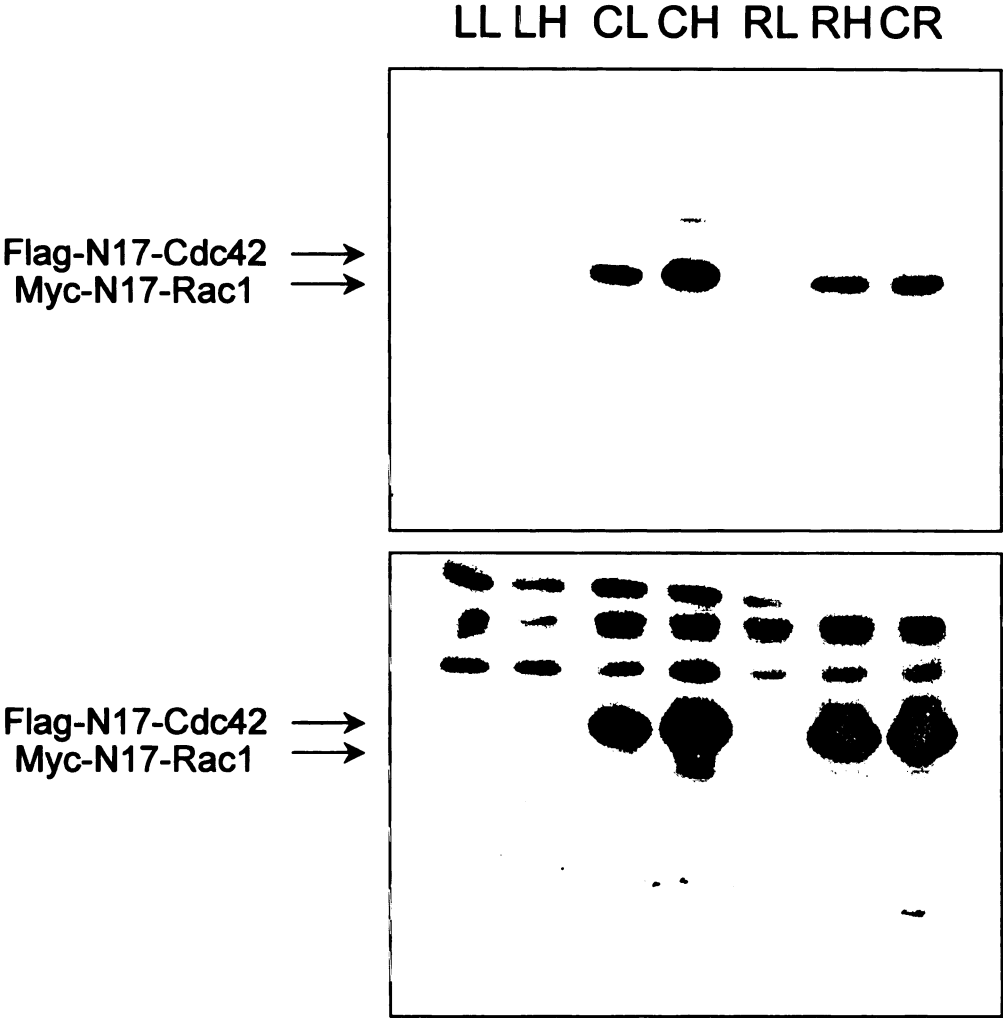


Figure 6: Clonal populations of L210-6A/SB1 that express a low or high level of β -galactosidase, dominant-negative Cdc42, dominant-negative Rac1, or both mutants. Clonally-derived cells from the cell populations shown in Figure 5 were screened by immunoblot analysis for expression level of β -galactosidase, dominant-negative Flag-N17-Cdc42, dominant-negative Myc-N17-Rac1, or both mutants, using anti-V5, anti-Flag, or anti-Myc antibody (data not shown). Individual cell strains that expressed either a low or high level of the specific protein were combined to form low- and high-expressing clonal populations except in the case of the double dominant-negative mutant. LL, β -galactosidase, low expressors; LH, β -galactosidase, high expressors; CL, Flag-N17-Cdc42, low expressors; CH, Flag-N17-Cdc42, high expressors; RL, Myc-N17-Rac1, low expressors; RH, Myc-N17-Rac1, high expressors; CR, a single clonally-derived cell strain that expressed an intermediate level of both mutants. The gel did not resolve Flag-N17-Cdc42 and Myc-N17-Rac1 proteins in this experiment. Top panel is a short exposure whereas bottom panel is a long exposure.

Figure 6



DISCUSSION

The malignant transformation of cells by expression of oncogenic mutants of RAS (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997) or oncogenic derivatives of GEFs (Cerione and Zheng, 1996) is dependent on Cdc42 and Rac1 activity. However, the contribution of endogenous, hyperactive Cdc42 and Rac1 to such transformation has not been studied. In this study, a MSU-1.1-derived fibrosarcoma cell line, L210-6A/SB1, which was transformed by a reactive derivative of benzo[a]pyrene (L. Milam, unpublished studies), was found to express a much higher level of GTP-bound Cdc42 and Rac1 than that in its parental cell strain (Figures 2 and 3). It is highly unlikely that a single treatment of MSU-1.1 cells with a chemical carcinogen for 1 hr resulted in two independent activating mutations in the CDC42 and RAC1 genes. Furthermore, a recent analysis showed that the level of GTP-bound Rac3 is also elevated in L210-6A/SB1 cells (D. Appledorn, unpublished data). Since these are three related proteins found in their hyperactive states in L210-6A/SB1 cells, their activation is likely due to an activating mutation of a common stimulator or an inactivating mutation of a common inhibitor.

In this study, it was also determined that the level of H-Ras protein is 2-fold higher in L210-6A/SB1 cells than that in its parental cell strain MSU-1.1 (Figure 4). However, it is unlikely that this caused significant activation of Cdc42 and Rac1 because previous studies in our laboratory showed that expression of *wildtype* H-RAS even at a level 5-fold higher than that in MSU-1.1 cells was not sufficient to malignantly transform MSU-1.1 cells (J. McCormick, unpublished studies). In contrast, it is well established that rodent fibroblasts are more easily transformed by carcinogen treatment or oncogene expression. Immortalized rodent fibroblasts that express constitutively active V12-H-Ras mutant display most characteristics of the

malignant phenotype. Recent studies show that intact Cdc42 and Rac1 activity are required for such malignant transformation (Prendergast et al., 1995; Qiu et al., 1995a, 1995b, and 1997). When the investigators expressed dominant-negative Cdc42 or dominant-negative Rac1 in such oncogenic H-RAS-transformed cells, certain characteristics of the malignant phenotype were suppressed.

To investigate whether hyperactive Cdc42 and Rac1 proteins are required for maintaining transformed growth characteristics of L210-6A/SB1 cells, dominant-negative Cdc42 or dominant-negative Rac1, or both mutants, was expressed in these cells. L210-6A/SB1 cells were infected with retroviruses carrying the cDNA(s) encoding β -galactosidase, Flag-N17-Cdc42, Myc-N17-Rac1, or both mutants. Three puromycin-resistant cell populations were generated for each. These cells have been subcutaneously injected into athymic mice. If tumors do form, tumor-derived cells will be tested for expression level of the respective protein(s) and compared with the same cell populations that were not injected into athymic mice. Such analysis should indicate whether there is an *in vivo selection* against cells that express dominant-negative mutant(s). If hyperactive Cdc42 and Rac1 proteins are required for the malignant phenotype of L210-6A/SB1 cells, then tumor-derived cells should express a minimal amount of dominant-negative Cdc42 and/or dominant-negative Rac1. The effect of dominant-negative mutant(s) expression on other growth properties characteristic of malignant cells may be tested if the tumor data indicate that this is of interest. Clonal populations of cells expressing a low or high level of mutant protein(s) will also be tested for their ability to form tumors in athymic mice.

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**APPENDIX B: IDENTIFYING CANDIDATE CANCER-RELATED GENES BY
CDNA SUBTRACTION ANALYSIS OF A MALIGNANT CELL LINE WITH
ELEVATED CDC42 AND RAC1 ACTIVITY AND ITS PARENTAL CELL STRAIN
MSU-1.1**

ABSTRACT

The MSU-1.1-derived, human fibrosarcoma cell line, L210-6A/SB1, was discovered to have a higher than normal level of Cdc42 and Rac1 activity (K-H. Dao, unpublished studies). This cell line was established in the Carcinogenesis Laboratory from a tumor formed in an athymic mouse following subcutaneous injection of a cell strain derived from a focus formed by MSU-1.1 cells that had been treated with a reactive derivative of benzo[a]pyrene (L. Milam, unpublished studies). As part of an ongoing study investigating the role of hyperactive Cdc42 and Rac1 proteins in maintenance of the transformed phenotype of these cells, cDNA expression differences between L210-6A/SB1 and its parental cell strain, MSU-1.1, were analyzed by PCR-Select subtractive hybridization. Sixteen genes were found differentially expressed.

INTRODUCTION

Cdc42 and Rac1 act in parallel pathways to activate transcription of genes, including the p38, c-jun N-terminal kinase (Coso et al., 1995; Minden et al., 1995), nuclear factor κ B (Sulciner et al., 1996; Perona et al., 1997), and serum response factor pathways (Hill et al., 1995). Recent studies, mainly with rodent cells, indicate that Cdc42 and Rac1 contribute to the malignant transformation of cells by regulating such pathways (Mackay and Hall, 1998; Van Aelst and D'Souza Schorey, 1997). In a comparative study it was shown that a human cell line, L210-6A/SB1, derived from a fibrosarcoma formed in an athymic mouse following subcutaneous injection of a cell strain derived from a focus formed by MSU-1.1 cells that had been treated with a reactive derivative of benzo[a]pyrene, has an elevated level of Cdc42 and Rac1 activity compared with its non-tumorigenic, parental cell strain MSU-1.1 (K-H. Dao, unpublished studies). The strategy for establishing tumor-derived, fibrosarcoma cell lines from MSU-1.1 cells (Morgan et al., 1991) following treatment with a carcinogen (O'Reilly et al., 1998; Yang et al., 1992) or expression of an oncogene has been described (Hurlin et al., 1989). The activity level of Cdc42 and Rac1 were determined using a glutathione-S-transferase-p21-binding domain pull-down assay described by Benard et al. (1999). Increased activation of Cdc42 and Rac1 may lead to transcription of genes that are important mediators of malignant transformation.

To identify genes that may play a role in the malignant transformation of MSU-1.1 cells treated with a reactive derivative of benzo[a]pyrene, PCR-Select subtractive cDNA hybridization was performed between the parental cell strain, MSU-1.1, and its malignant derivative cell line, L210-6A/SB1. The forward

subtraction (cDNA from MSU-1.1 cells subtracted by cDNA from L210-6A/SB1 cells) and the reverse subtraction (cDNA from L210-6A/SB1 cells subtracted by cDNA from MSU-1.1 cells) were performed. A total of 384 cDNA products were analyzed by dot blot analysis. Those genes that showed differential expression between MSU-1.1 and L210-6A/SB1 cells by such analysis were analyzed further by Northern blot analysis. Of the 55 genes analyzed further, 16 genes were verified to be differentially expressed by this more quantitative analysis of mRNA abundance. The 16 genes were sequenced to facilitate identification of genes that may play a causal role in malignant transformation of MSU-1.1 cells.

MATERIALS AND METHODS

PCR-Select subtractive hybridization

PCR-Select cDNA subtraction (Clontech) was performed between MSU-1.1 and L210-6A/SB1 cells (Figure 1). The detailed steps were followed according to the manufacturer's instruction. Briefly, double stranded cDNA was synthesized from MSU-1.1 (sample #1) and L210-6A/SB1 (sample #2) cells. This was followed by digestion with *RsaI* restriction enzyme to generate shorter, blunt-ended products. Each sample's digested double stranded cDNAs were ligated to two different adaptors, adaptor1 and adaptor2R. The first hybridization consisted of two separate reactions: adaptor1-ligated cDNAs or adaptor2R-ligated cDNAs of sample #1 each combined with excess non-ligated cDNAs of sample #2. The second hybridization consisted of a mixture of these two reactions and a fresh addition of excess non-ligated cDNAs of sample #2. Twice-hybridized cDNA species that were unique or were expressed at a higher level in sample #1 than in sample #2 contain adaptor1 and 2R at the ends, which facilitates exponential amplification and enrichment by PCR. This describes the forward subtraction. The subtracted cDNA products were then cloned into pCMV-SPORT (Life Technologies) and transformed into DH5 α *Escherichia coli* bacteria for isolation of individual cDNA products. Approximately 1,000 total bacteria colonies were obtained from the forward and reverse subtraction. The *forward subtraction* consisted of MSU-1.1 cDNAs subtracted by L210-6A/SB1 cDNAs, i.e., genes that are expressed at a higher level in MSU-1.1 cells compared with that in L210-6A/SB1 cells. The *reverse subtraction* consisted of L210-6A/SB1 cDNAs subtracted by MSU-1.1 cDNAs, i.e., genes that are expressed at a higher level in L210-6A/SB1 cells compared with that in MSU-1.1 cells.

Dot blot analysis

Each of the 384 bacteria colonies selected contained a single subtracted cDNA product cloned into pCMV-SPORT vector. Plasmid DNA was isolated from a small bacteria culture, chemically denatured with basic buffer, and then spotted onto nylon membrane using a dot blot manifold apparatus. Two replicate blots were spotted, one was probed with forward-subtracted cDNA products and the other was probed with reverse-subtracted cDNA products. ^{32}P -labeled cDNA probes were generated by PCR incorporation of $\alpha\text{-}^{32}\text{P}$ -dCTP, using primers that annealed to adaptor1 and 2R sequences ligated to subtracted cDNA products. Approximately 20×10^6 cpm of ^{32}P -labeled, subtracted cDNA products were denatured at 95°C for 5 min. and then added to hybridization buffer immediately prior to the hybridization step. The procedures for prehybridization, hybridization, and low- and high-stringency wash steps were carried out as described in *Short Protocols in Molecular Biology* (1992). Bands were visualized and analyzed using the G5-525 phosphorimaging system (Biorad).

Northern blot analysis

Total RNA was extracted from cells using the RNeasy reagent as described by the manufacturer (Qiagen). Total RNA concentration was determined by UV absorbance at A260. A sample of total RNA (5 μg) was fractionated on a denaturing formaldehyde agarose gel and transferred to nitrocellulose membrane. The procedures were carried out as described (*Short Protocols in Molecular Biology*, 1992). The RNA was immobilized on membrane with a Stratagene UV Crosslinker. Freshly denatured salmon sperm DNA was added to prehybridization and hybridization solutions immediately prior to use. Prehybridization was performed at

42°C for 2 to 4 hr, and hybridization was performed at 42°C for >12 hr. ³²P-labeled DNA probes were generated by PCR incorporation of α-³²P-dCTP, using the T7 and SP6 priming sites present in the pCMV-SPORT vector. Each strip, containing one lane of MSU-1.1 RNA and one lane of L210-6A/SB1 RNA, was hybridized with ~1X10⁶ cpm of denatured probe. Bands were visualized and analyzed using the G5-525 phosphorimaging system (Biorad).

RESULTS/DISCUSSION

PCR-Select cDNA subtractive hybridization

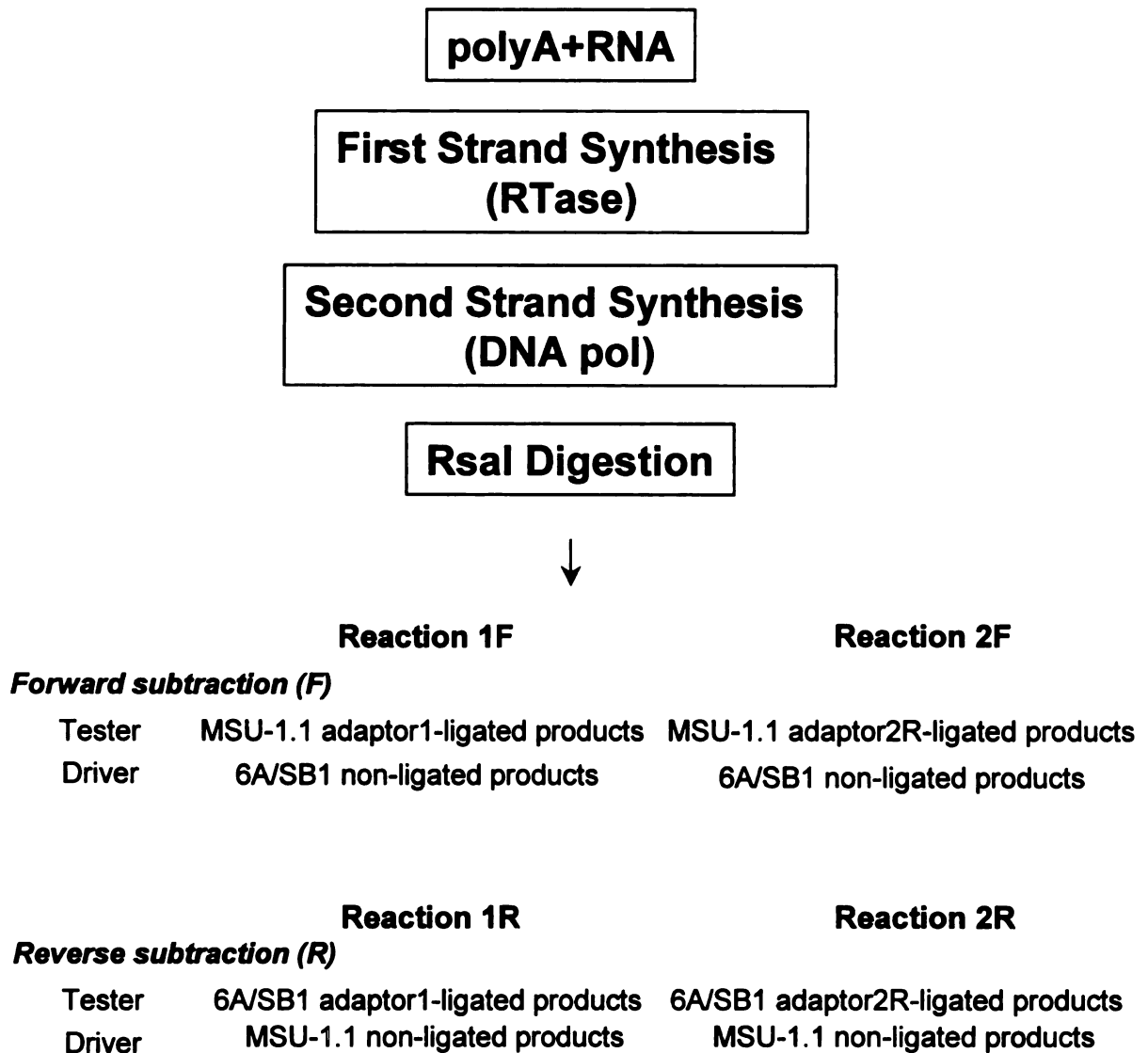
PCR-Select subtractive hybridization was performed between MSU-1.1 and L210-6A/SB1 cells to identify genes that may play a causal role in transformation of MSU-1.1 cells to a malignant derivative (Figure 1). Results of dot blot analysis of a total of 384 cDNA products from the forward and reverse subtraction are shown in Figure 2. Only one such analysis was performed since it was used as a screening strategy. The “positives” are those genes that were enriched in either direction of the subtractive hybridization and displayed the correct pattern of expression between MSU-1.1 and L210-6A/SB1 cells (Figure 2, panels A and D). Overall, the forward subtraction proved to be more stringent and specific than the reverse subtraction. This judgment is based on a comparison of signals between the top panel and the bottom panel of each blot. For example, the top panel (A) of the forward subtraction had more positive signals than the bottom panel (B), indicating that positive signals in panel A are more likely to be truly differentially expressed between MSU-1.1 and L210-6A/SB1 cells. In contrast, the same comparison for the reverse subtraction, panel D versus panel C, showed little difference. Thus, subtracted cDNAs obtained in this subtracted direction (D) were just as likely to be false positives.

Relative signal intensity was determined for each subtracted cDNA product analyzed by dot blot analysis, and a graphical presentation of these values is shown in Figure 3. A majority (84%) of the genes, 324 of 384, were expressed at a level that is approximately equal between MSU-1.1 and L210-6A/SB1 cells. Using a 2.5-fold difference as the minimum, 35 genes were expressed at a higher level in MSU-1.1 cells than in L210-6A/SB1 cells, and 20 genes were expressed at a higher level

in L210-6A/SB1 cells than in MSU-1.1 cells. To verify expression differences of these 55 genes, Northern analysis was performed using subtracted cDNA products as probes. Only a select subset of the results is shown in Figure 4 for the reverse subtraction. Those cDNAs that displayed expression differences by Northern analysis were sequenced to facilitate identification of genes (Table 1). The sixteen genes that were sequenced included nine genes expressed at a higher level in MSU-1.1 cells and seven genes expressed at a higher level in L210-6A/SB1 cells. Four of nine genes that were expressed at a higher level in MSU-1.1 cells encode ribosomal proteins, whereas four of seven genes that were expressed at a higher level in L210-6A/SB1 cells encode glyceraldehyde-3-phosphate dehydrogenase. These results are interesting, especially the higher laminin expression in L210-6A/SB1 cells than in MSU-1.1 cells, but the function of these genes in maintaining the malignant phenotype of L210-6A/SB1 cells remains to be investigated.

Figure 1: General strategy of PCR-Select cDNA subtractive hybridization. The procedures were followed as described by the manufacturer. Double stranded cDNA was synthesized from polyA+RNA extracted from each MSU-1.1 and L210-6A/SB1 cells. RsaI-digested products were ligated to adaptor1, ligated to adaptor 2R, or non-ligated to act as driver for the opposite subtraction. Two reactions were carried out separately for the forward (F) subtraction and the reverse (R) subtraction. The various hybridization steps that were performed are shown. RTase, reverse transcriptase; DNA pol, DNA polymerase I.

Figure 1



- First hybridization: reaction 1 and 2 for each of the subtracted direction are hybridized separately in the presence of excess driver
- Second hybridization: reaction 1F and 2F are mixed together and excess driver is added again; the same is done for reaction 1R and 2R
- PCR amplification using a primer that anneals to both adaptor1 and adaptor2R (all products containing any combination of these primers are amplified)
- PCR amplification using two primers, each annealing to adaptor1 or adaptor2R (only products containing adaptor1 at one end and adaptor2R at the other end are exponentially amplified)
- Digest subtracted, PCR-amplified products with suitable restriction enzymes and clone into pCMV-sport for isolation of cDNA products and dot blot analysis

Figure 2: Dot blot analysis of 384 subtracted cDNA products from the forward and reverse subtraction. PCR-amplified subtracted cDNAs were spotted onto nylon membrane and ^{32}P -labeled forward- or reverse-subtracted products were used as probes. **A**, putative cDNA products expressed at a higher level in MSU-1.1 cells than in L210-6A/SB1 cells and probed with forward-subtracted products. **D**, putative cDNA products expressed at a higher level in L210-6A/SB1 cells than in MSU-1.1 cells and probed with reverse-subtracted products. Panels **B** (vs. **A**) and **C** (vs. **D**) should have fewer signals because they were isolated from the subtraction of one direction but probed with the subtraction of the opposite direction.

Figure 2

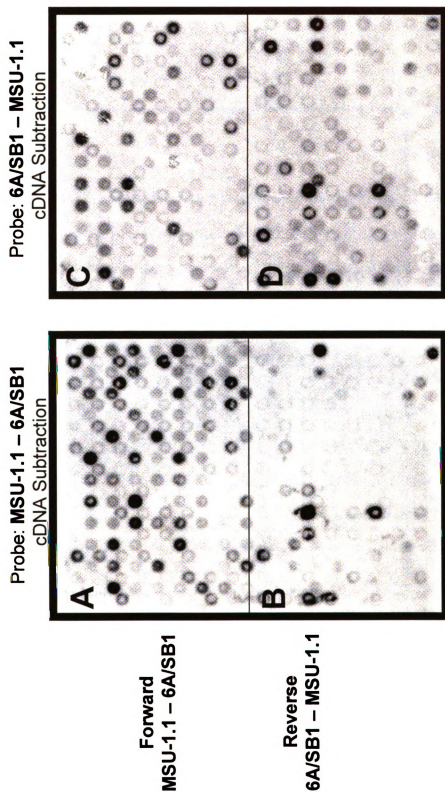


Figure 3: Relative signal intensity of each subtracted cDNA product analyzed by dot blot analysis. The relative signal, MSU-1.1 versus L210-6A/SB1, was determined for each spot using phosphorimager analysis (Biorad). A graphical presentation of these values for the 384 subtracted cDNA products analyzed is shown here. Most of the genes had a relative signal intensity between 1 and 2-fold, which indicates no significant expression difference between MSU-1.1 and L210-6A/SB1 cells. There were 55 genes that had a relative signal intensity above 2.5. Genes that were expressed at a higher level in MSU-1.1 cells have signals toward the *left*, whereas those expressed at a higher level in L210-6A/SB1 cells have signals toward the *right*.

Figure 3

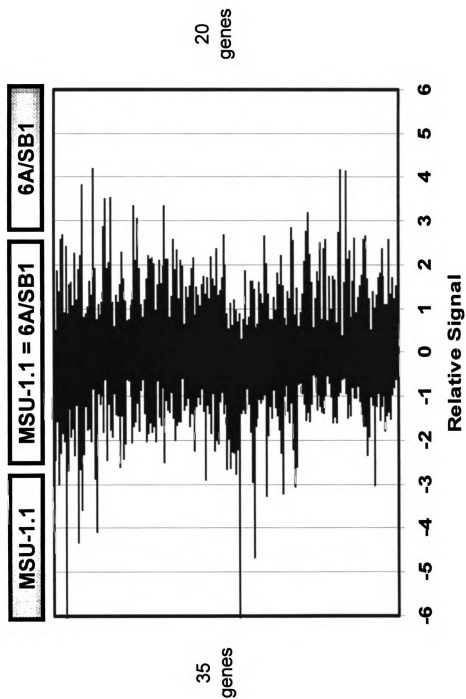


Figure 4: Northern blot analysis to verify differential expression of subtracted cDNA products. Northern blot analysis was performed to verify 55 genes that had greater than 2.5-fold expression difference as determined by dot blot analysis. A select subset is shown here. A sample of total RNA (5 µg) from each MSU-1.1 (1.1) cells and L210-6A/SB1 (6A) cells were probed with PCR-amplified, ³²P-labeled subtracted cDNA. Not all probes resulted in detectable signals. Those genes that were differentially expressed between MSU-1.1 and L210-6A/SB1 cells, as indicated by *boxes*, were sequenced to facilitate identification of the gene.

Figure 4

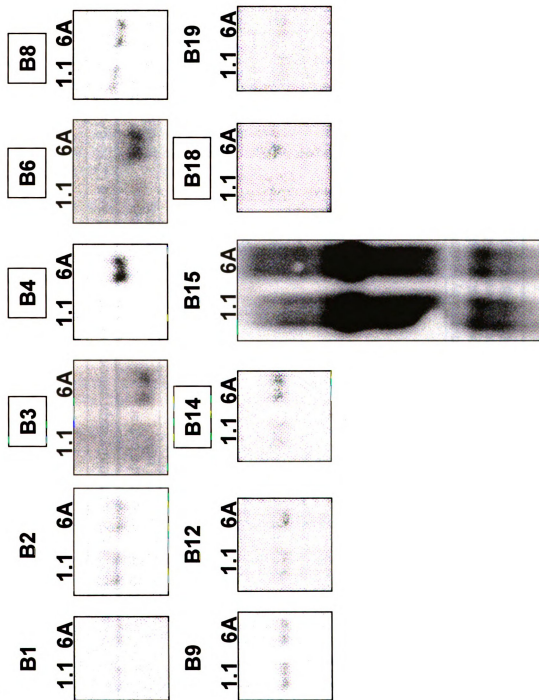


Table 1 • Genes differentially expressed between MSU-1.1 and L210-6A/SB1 cells			
Subtracted cDNA product	Genes expressed at a higher level in MSU-1.1 cells	Subtracted cDNA product	Genes expressed at a higher level in L210-6A/SB1 cells
A10	ribosomal protein S8	B3	glyceraldehyde 3-phosphate dehydrogenase
A19	translation elongation factor 1 α 1	B4	glyceraldehyde 3-phosphate dehydrogenase
A22	γ -actin	B6	glyceraldehyde 3-phosphate dehydrogenase
A26	ribosomal protein like 7a	B8	mitochondrion protein
A30	ribosomal protein like L4	B12	translation elongation factor 1 α 1
A31	translation elongation factor 1 α 1	B14	glyceraldehyde 3-phosphate dehydrogenase
A32	ribosomal protein like 21	B18	laminin receptor
A34	mitochondrion protein		
A35	translation elongation factor 1 α 1		

For each cDNA that was differentially expressed, as determined by Northern analysis, approximately 300 bases of DNA sequence were entered in a BLAST search and the gene that was identified is reported here. Listed are nine genes from the forward subtraction (genes expressed at higher level in MSU-1.1 cells) and seven genes from the reverse subtraction (genes expressed at higher level in L210-6A/SB1 cells).

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FUTURE DIRECTIONS

In light of my dissertation research, there are at least four areas of focus that are worth pursuing. First, very few studies address the effect of inhibiting endogenous activity of Cdc42 or Rac1, by dominant-negative mutant expression or by other strategies such as antisense expression or mutant effector protein expression, on transformed growth characteristics of malignant cell lines. In studies that have been published, the investigators' use of rodent fibroblasts should encourage caution when extrapolating data to apply to human fibroblasts. Alternatively, similar studies could be investigated in human fibroblasts. My studies showed that Cdc42 and Rac1 are required for malignant transformation of human fibroblasts by oncogenic H-RAS. These results are consistent with the results obtained in similar studies with rodent fibroblasts. It would be important to determine whether such a role for Cdc42 and Rac1 exists in more common types of human cancers such as lung, colon, breast, and prostate carcinomas. Second, my finding that EPAS1 is a RAS target dependent on Cdc42 and Rac1 for up-regulation suggests a possible pathway that oncogenic RAS isoforms induce VEGF secretion, which is known to promote tumor growth and angiogenesis. The role of EPAS1 in the malignant transformation of human fibroblasts or other human cell types has not been investigated. It would be worthwhile to determine the effect of selective inhibition of EPAS1 in human cancer cell lines on the ability of such cells to form tumors in athymic mice. Since EPAS1 is a hypoxia-inducible factor, it is highly likely that members of this family of transcription factors have a critical role in establishment of solid human tumors. Third, in my studies I identified H-RAS-induced gene changes that are dependent on Cdc42 and Rac1 activity to investigate

the pathway-specific contribution of oncogenic H-RAS to the malignant transformation of human fibroblasts. As previously reported, this approach should facilitate identification of potential targets for selective inhibition of RAS function. Inhibition of only those RAS mediators that contribute to the malignant transformation of cells is likely to be less cytotoxic than inhibiting Ras directly because Ras is a central signaling protein that is required for normal cellular processes. I followed-up on EPAS1 but the other genes listed in Table 3, Chapter II should also be investigated further. Finally, the reports in the literature suggest that there are at least two ways that increase Cdc42 and Rac1 activity. In the first scenario, there is an increase in exchange activity, for example, due to G12V or Q61L mutations or oncogenic GEF activation. In the second scenario, there is a concomitant increase in GTPase activity with the increase in exchange activity, for example, due to F28L mutation or ABR or RAS activation. I described these two scenarios in the literature review (Chapter I) as static versus dynamic mechanisms, respectively, of increasing Cdc42 and Rac1 activity. The mechanism of activating Cdc42 and Rac1 is likely to modulate their specific contribution to the malignant transformation of cells. However, this remains to be investigated.

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