Expression of ras and metastatic behavior in panel of cell lines derived from infection of NIH 3T3 cells with Kirsten murine sarcoma virus
by Steve Hamner

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
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Abstract:
Ras genes are highly conserved throughout eukaryotic evolution and are likely to play a central role in basic cellular activity. p21ras proteins bind guanine nucleotides, have GTPase activity, and are located on the inner face of the cytoplasmic membrane. These properties suggest that p21ras may function in mediating signal transduction across the cell membrane. The ability of ras oncogenes to transform cells and render them tumorigenic, and the identification of ras oncogenes in a wide variety of human cancers suggest that ras genes may play a role in human tumor development. Understanding how ras oncogenes contribute to transformation and malignancy is likely to contribute to improvements in cancer diagnosis and treatment.

The goal of this project was to examine the role of the Kirsten murine sarcoma virus ras gene in contributing to the malignant behaviors expressed by sister cell lines derived from infection of NIH 3T3 cells with Kirsten virus. A correlation between ras mRNA expression level and metastatic potential was established in initial experiments. Experiments aimed at explaining differences in ras RNA expression among the cell lines indicated that DNA methyladon state might be important in regulating transcription of the viral ras gene. Treatment of the nonmetastatic cell line with a DNA demethyladon agent resulted in an elevation of ras mRNA level but did not alter this line's nonmetastatic phenotype. p21ras protein levels were assessed in the cell lines to test whether p21ras levels correlate with metastatic behavior. Using fluorescence activated cell sorter analysis and an antibody reactive against Kirsten virus p21, antibody binding was shown to be highest in viable cells of the metastatic cell line. The role of p21ras in contributing to malignant behavior was more directly assessed by treating the metastatic line with an anti-p21ras function blocking antibody. Treatment with blocking antibody did not decrease the in vitro invasive potential of the cells. Treatment of the metastatic cell line with pertussis toxin also had no effect on that line's invasive capacity. These findings suggest that some mechanism apart from ras or Gi proteins contributes to the invasive behavior of the metastatic cells under study.

EXPRESSION OF *RAS* AND METASTATIC BEHAVIOR IN A PANEL OF CELL LINES DERIVED FROM INFECTION OF NIH 3T3 CELLS WITH KIRSTEN MURINE SARCOMA VIRUS

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Steve Hamner

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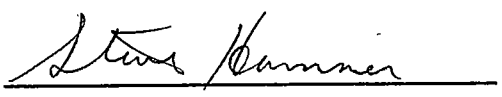
Signature 

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TABLE OF CONTENTS

TABLE OF CONTENTS-*Continued*

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Ras genes are highly conserved throughout eukaryotic evolution and are likely to play a central role in basic cellular activity. $p21^{ras}$ proteins bind guanine nucleotides, have GTPase activity, and are located on the inner face of the cytoplasmic membrane. These properties suggest that $p21^{ras}$ may function in mediating signal transduction across the cell membrane. The ability of *ras* oncogenes to transform cells and render them tumorigenic, and the identification of *ras* oncogenes in a wide variety of human cancers suggest that *ras* genes may play a role in human tumor development. Understanding how *ras* oncogenes contribute to transformation and malignancy is likely to contribute to improvements in cancer diagnosis and treatment.

The goal of this project was to examine the role of the Kirsten murine sarcoma virus *ras* gene in contributing to the malignant behaviors expressed by sister cell lines derived from infection of NIH 3T3 cells with Kirsten virus. A correlation between *ras* mRNA expression level and metastatic potential was established in initial experiments. Experiments aimed at explaining differences in *ras* RNA expression among the cell lines indicated that DNA methylation state might be important in regulating transcription of the viral *ras* gene. Treatment of the nonmetastatic cell line with a DNA demethylation agent resulted in an elevation of *ras* mRNA level but did not alter this line's nonmetastatic phenotype. $p21^{ras}$ protein levels were assessed in the cell lines to test whether $p21^{ras}$ levels correlate with metastatic behavior. Using fluorescence activated cell sorter analysis and an antibody reactive against Kirsten virus p21, antibody binding was shown to be highest in viable cells of the metastatic cell line. The role of $p21^{ras}$ in contributing to malignant behavior was more directly assessed by treating the metastatic line with an anti-$p21^{ras}$ function blocking antibody. Treatment with blocking antibody did not decrease the in vitro invasive potential of the cells. Treatment of the metastatic cell line with pertussis toxin also had no effect on that line's invasive capacity. These findings suggest that some mechanism apart from *ras* or G_i proteins contributes to the invasive behavior of the metastatic cells under study.

INTRODUCTION

Cancer Metastasis

Cancer cells do not conform with the control mechanisms that govern the growth and behavior of normal cells. Abnormal cell behavior is expressed in diverse ways and can have a wide variety of effects on the host organism ranging from the benign to the lethal. A population of tumor cells which remains localized does not generally pose a serious threat to the host. Invasive neoplasms, on the other hand, can not only cause damage to the tissues surrounding the area of tumor growth but may also disseminate to distant sites in the body where secondary tumors may form. Secondary tumors are often difficult to diagnose or treat, with the result that the majority of cancer patients die due to metastatic disease.

Cancer cells able to disseminate and colonize sites away from the primary tumor must be endowed with a number of remarkable properties and must survive several types of host defenses to successfully complete the process of metastasis (Fidler and Hart, 1982). Metastasis is a complex process of sequential steps which few cells can traverse. Metastasizing cells must first invade the tissue and associated stroma surrounding the primary tumor. Invasion necessitates tumor cell attachment to components of the extracellular matrix and proteolytic breakdown of matrix to allow for tumor cell migration (Liotta, 1986). Tumor cells may produce extracellular matrix molecules to facilitate their

own attachment and movement (Varani et al., 1983). Proteases which digest extracellular matrix barriers may be produced by the tumor cells themselves or by host inflammatory cells which are responding to and interacting with the tumor cells (Liotta, 1986). Invading cells may next enter lymphatic vessels or the bloodstream through which they can spread to other sites of the body. During hematogenous dissemination, tumor cells encounter immune and nonimmune defenses of the host. Mechanical trauma during circulation in the bloodstream as well as interactions with host lymphocytes and natural killer cells destroy the majority of tumor cells which enter the circulatory system. A very few cells will reach the capillary beds of distant organs where they may attach to endothelial cells or to the exposed subendothelial basement membrane. Extravasation into the new organ site can then occur by way of the same mechanisms involved in invasion at the primary tumor site. As is true for primary tumors, unrestricted growth of secondary tumors requires induction of angiogenesis and continued resistance to host immunological responses.

Cancer Genetics

The genetic basis for tumorigenesis and progression

Cancer can be defined as a disease of the genome resulting in the uncontrolled proliferation of cells. In cancer cells the normal elements of growth control have gone awry. With some exceptions, it is generally agreed that most naturally occurring and experimentally induced cancers originate from single abnormal cells (Nowell, 1976; 1986). Once transformed, the single cell proliferates and its progeny undergo additional

genetic changes which contribute to the evolution of the tumor. Whereas normal cells are genetically stable, tumor cells are genetically unstable. Individual cells of a growing tumor will undergo genetic change independent of neighboring tumor cells so that heterogeneous subpopulations of cells displaying a range of phenotypes eventually define the tumor. Metastatic tumor cell populations that have undergone sufficient genetic alterations allowing cells to invade locally, disseminate to distant sites of the body while evading immune and non-immune responses, implant at distant sites and then establish secondary tumors represent perhaps the ultimate expression of tumor evolution and progression. Three classes of genes which can influence the initiation and evolution of tumor cell behavior are the oncogenes, tumor suppressor genes, and modulator genes (Klein, 1987).

Oncogenes

Activation and abnormal expression of specific genetic elements, the oncogenes, have been proposed to result in aberrant cell behavior and carcinogenesis (Cooper, 1982). Although oncogenes were originally defined to be the transforming genes of retroviruses, they were subsequently shown to share high degrees of homology with normal cellular genes. The normal cellular counterparts of the oncogenes, termed protooncogenes, code for proteins performing essential roles in the cell. To date, more than 40 different oncogenes have been identified. Protooncogene/oncogene proteins have been classified by biochemical function or presumed mode of action into several main groups: tyrosine kinases, GTP-binding proteins, growth factors, growth factor receptors, nuclear proteins and as yet unclassified proteins. The conversion of normal cellular protooncogene

function to that of a transforming oncogene can occur due to structural gene mutation or by overexpression of a protooncogene protein. More specific examples of oncogene function as related to cancer metastasis will be presented in greater detail in later sections of this introduction.

Tumor suppressor genes

In addition to the oncogenes, the activation of which contributes to abnormal cell behavior, a second class of cancer-related genes, termed tumor suppressor genes (Murphree and Benedict, 1984) or antioncogenes (Knudson, 1985), has also been identified. Tumor suppressor genes are believed to play important regulatory roles in promoting normal cell growth and differentiation and may serve to prevent protooncogenes from being oncogenic (Anderson and Spandidos, 1988). Loss of, or inactivation of, tumor suppressor genes can thus contribute to oncogenesis.

The tumor suppressor gene most extensively characterized in man is the retinoblastoma (Rb) gene located on human chromosome 13. Loss of, or inactivation of, both alleles at the Rb locus results in retinoblastoma in children. Introduction of the cloned Rb gene or a normal chromosome 13 into Rb-negative retinoblastoma cell lines has been shown to result in production of normal Rb protein, reverse the transformed phenotype, and slow growth rate (Huang et al., 1988; Benedict et al., 1990). In contrast to the parental Rb-negative cell lines, cell lines expressing the introduced Rb gene have been demonstrated to be nontumorigenic when tested in nude mice. Although the precise role and function of the Rb protein is not yet fully understood, several properties of the protein suggest possible roles in control of the cell cycle and cellular senescence (Sager,

1989). The Rb protein is a nuclear phosphoprotein capable of binding DNA in vitro. When studied by gel electrophoresis, it ranges in size from 110 kD (kilodalton) to 114 kD depending upon the extent of phosphorylation it has undergone (Lee et al., 1987; DeCaprio et al., 1989). The phosphorylation state of the Rb protein varies according to position in the cell cycle with the unphosphorylated forms of the protein being seen in resting (G_0) cells (Buchkovich et al., 1989; DeCaprio et al., 1989). The more highly phosphorylated forms of the Rb protein are observed as cells near the G_1/S boundary of the cell cycle, however, and remain as cells proceed through the S and G_2 phases (Chen et al., 1989). Upon stimulation with serum treatment, the Rb protein of quiescent human diploid fibroblasts is phosphorylated and the cells enter S phase (Stein et al., 1990). Senescent fibroblasts, on the other hand, do not phosphorylate Rb protein upon treatment with serum and do not enter S phase. It has recently been demonstrated that addition of transforming growth factor β (TGF-β) to lung epithelial cells during the mid to late G_1 phase prevents phosphorylation of the Rb protein and results in cell growth arrest in late G_1 (Laiho et al., 1990). These findings strongly suggest a growth suppressive function for the Rb protein in its unphosphorylated form.

Rb protein has been shown to form complexes with the transforming proteins of several DNA tumor viruses, including SV40 large T antigen, adenovirus type 5 E1A protein, and human papilloma virus E7 protein (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989). Mutations in any of the viral sequences which block the binding of viral proteins to Rb protein also decrease or inhibit the transforming potential of the virus (Cooper and Whyte, 1989). It has thus been proposed that these DNA virus

proteins may transform and immortalize cells by binding to Rb protein and thereby blocking its associated function. Sequestering Rb protein in complexes with viral transforming proteins may allow cells to enter the cell cycle or to escape differentiation. Loss of Rb function by genetic deletion might, in an analogous fashion, lead to initiation events important in the genesis of retinoblastoma or might contribute to development of adult tumors. Rb gene involvement in adult tumors is suggested by the identification of Rb gene mutations in several common human cancers. Rb gene mutations have been noted in primary small cell lung carcinoma (SCLC) tissue and SCLC cell lines (Harbour et al., 1988), in primary breast tumors and breast cancer cell lines (T'ang et al., 1988), and in bladder cancer cell lines (Horowitz et al., 1989).

In addition to the Rb gene, one other tumor suppressor gene, p53, has been cloned and studied in some detail. The p53 gene is located on human chromosome 17 and codes for a protein of 53 kD which is found in low levels in almost all cells of the body (reviewed by Levine, 1990). Like the Rb protein, p53 is a nuclear phosphoprotein capable of binding DNA in vitro. No DNA nucleotide sequences which specifically bind to either p53 or Rb protein have yet been identified, however (Levine, 1990). Also like the Rb protein, p53 is observed to form oligomeric protein complexes with DNA tumor virus proteins in infected cells. Specifically, p53 binds to SV40 large T antigen and adenovirus type 5 E1b protein (Sarnow et al., 1982).

Mutant p53 genes are capable of transforming cells when introduced by transfection (Hinds et al., 1989). Co-transfection of mutant and wild type p53 genes leads to a lower frequency of transformation, however. Introduction of wild type p53 into transformed

cells also leads to reversion of transformation. Since p53 protein molecules exist as dimers in the cell, mutant p53 protein may inactivate wild type protein function by the formation of heterodimeric complexes (Levine, 1990). Such complexes are readily detected in transformed cells. Similarly, viral transforming proteins may alter or inactivate wild type p53 through the formation of oligomeric protein complexes.

Tumor modulator genes

Whereas the balanced expression of oncogenes and tumor suppressor genes is responsible for normal cell behavior, disruption of such a balance can ultimately result in cells becoming cancerous. Most studies of the genetic determinants of cancer have focused on perturbations of oncogene and, more recently, tumor suppressor gene expression and how these changes affect fundamental aspects of growth control. Not all cancer cells are capable of invasion and metastasis, however. This is because the metastatic phenotype is determined independently at the genetic level. It is the altered expression of what Klein (1987) terms modulator genes which differentiates the metastatic cell from the cancer cell which is not yet malignant. The so-called modulator genes determine whether cancer cells are invasive, motile, and capable of forming metastases (Klein, 1987). Invasiveness is determined by genes which govern the cell's interaction with extracellular matrix (Sobel, 1990). The ability of a cell to modify the extracellular matrix by production of its own extracellular matrix components to facilitate attachment or by production of proteases capable of degrading matrix components is determined by specific genes. Motility is influenced by genes which affect such properties as cytoskeletal structure, membrane fluidity, and the ability of cells to sense and respond to

extracellular signals. As will be discussed in detail, studies involving the aberrant expression of *ras* oncogenes have provided a powerful model system for the induction and examination of those modulator genes whose expression contributes to the metastatic phenotype. Also included under the category of modulator genes are the genes which determine whether metastasizing cells generate immune responses during their dissemination. As an example, the association of tumor cell surface molecules with specific antigens coded for by the major histocompatibility complex appears to be an important determinant of immunogenicity and whether tumor cells otherwise capable of metastasizing are recognized and destroyed by immune system effector cells such as cytotoxic T lymphocytes (reviewed by Feldman et al., 1988).

The multigene nature of cancer

Cancer is the result of an accumulation of genetic changes occurring over time (Klein and Klein, 1985; 1986). While childhood cancers such as retinoblastoma may appear to be the result of only one or a few genetic events happening soon after birth, pedigree analyses and molecular genetic studies indicate the existence of predisposing genotypes with specific genetic lesions which contribute to development of cancer in the young. Similarly, cytogenetic studies and statistical analyses of age-incidence curves of cancer data give strong support for the development of adult cancer being a multistep process.

Historically and early in the course of discovering and understanding oncogenes, "one oncogene" and "two oncogene" models of carcinogenesis were proposed (reviewed by Duesberg, 1985; Klein and Klein, 1985; Sager, 1986). Introduction of a human tumor-derived H-*ras* (Harvey-*ras*) oncogene was shown to transform certain aneuploid mouse

cells. Since the cells used in these experiments had already undergone numerous chromosomal rearrangements, they could already be considered partially transformed. The only other example of a single oncogene being able to transform cells is that of retrovirally-transduced oncogenes such as v-*src*. Retroviral infection of cells introduces not only the transduced oncogene but additional genetic elements such as LTRs (long terminal repeats) which are strong promoters probably capable of exerting unspecified effects on neighboring genes surrounding the retrovirus integration site. Thus, the mechanism of retroviral transformation is likely to be somewhat different from that involved in spontaneous tumor development.

The "two oncogene" model of carcinogenesis arising from the experiments of Land, Parada, and Weinberg (1983 a, b) provided for the functional classification of oncogenes into two groupings. Members of one group, which initially included the various *ras* genes and polyoma middle T, are seen as being competent to confer a transformed phenotype upon cells. *Ras* transformed cells, unlike normal counterparts, are thus able to undergo anchorage-independent growth and grow in a density-independent manner. Members of the other group, including myc, polyoma large T, and adenovirus E1a, code for nuclear proteins and are able to immortalize or confer an extended lifespan on cells. (It is noteworthy that the DNA tumor virus proteins referred to earlier which all bind to Rb protein serve immortalizing functions in cells transformed by the respective viruses. It appears that the viruses have all converged on a common cellular target to bring about a change in cellular growth state conducive to the activities of the viruses.) Freshly isolated rodent fibroblasts, unlike established cell lines, have been shown to require the

contribution of an oncogene from each of the two groups if full transformation and tumorigenic potential are to be attained. Earlier studies did not include a cytogenetic examination of cells after they were coinfected with members of each oncogene group, and so the occurrence and contribution to transformation of additional post-transfection genetic changes was not evaluated. In cases where cytogenetic examination has been carried out after oncogene transfection, chromosomal changes have been consistently noted (Sager, 1986). These gross alterations in the genetic material suggest that genetic events in addition to or apart from the introduction of oncogenes contribute to transformation. In fact, transformation and tumorigenicity have been induced in certain cells such as Chinese hamster embryo fibroblasts (CHEF) by transfection with antibiotic resistance gene-carrying plasmids where stable insertion of a cotransfected oncogene was not observed (Lau et al., 1985). In these transformants, gross chromosomal changes were also noted. An important note of caution resulting from these and related experiments is that any manipulation or treatment of cells may upset genetic stability and result in secondary genetic changes distinct from the introduction of an oncogene being studied. The process of treating certain nonmetastatic cells with calcium phosphate alone, a reagent commonly employed in gene transfection procedures, has been shown to result in induction of metastatic behavior in a subset of the treated cells (Kerbel et al., 1987). Such a change in phenotype is presumed to result from changes in gene expression caused by the experimental procedures and/or reagents themselves. Such possible changes must always be considered when gauging the effects of an introduced gene on cell behavior. Cytogenetic data such as those just mentioned, as well as the time-course of development

of naturally occurring tumors, suggest that numerous mutation-like events or genetic changes must occur to impart the tumorigenic phenotype. It is thus highly unlikely that activation of a single cellular oncogene or introduction of an exogenous oncogene can by itself ever cause a normal diploid cell to become tumorigenic.

Ras

Reasons for studying *ras* genes

Ras genes have been the subject of intense research activity in recent years for several reasons (reviewed by Barbacid, 1987). Of the known oncogenes, *ras* genes are the ones most frequently encountered in studies of carcinogen-induced animal tumors and naturally occurring human tumors. *Ras* oncogenes are seen in a wide variety of human carcinomas as well as in leukemias, lymphomas, fibrosarcomas, rhabdomyosarcomas, neuroblastomas, melanomas and gliomas. *Ras* gene alterations have been identified in some 10-15% of all human tumors examined, with the incidence being lower or higher depending on the type of cancer. The highest frequency of mutated *ras* genes has been noted in pancreatic adenocarcinomas (about 90% of cases tested) and in colon carcinomas (about 50% of cases examined) (Bos, 1989). As detection assay methods and sensitivities are improved, these incidence figures will undoubtedly increase. Increased expression of *ras* transcripts and $p21^{ras}$ protein in human tumors relative to normal control tissue has also been found in about 50% of the cases examined. Such incidence data suggest a role for *ras* genes in human neoplasia.

Mutated *ras* genes isolated from human tumors as well as retrovirally-transduced *ras* genes are capable of transforming a variety of cell types in vitro. Additionally, *ras* oncogenes can confer metastatic capability on certain cell types such as NIH 3T3 cells and rat embryo fibroblasts. These observations have made possible a wide variety of in vitro studies which are contributing to our understanding of the many genes and their products involved in tumorigenicity in general and the metastatic process specifically.

Ras genes are also of interest from the standpoint of furthering our understanding of basic cell biology. *Ras* genes are found in all eukaryotes and are highly conserved to the point that appropriately activated yeast *ras* genes can transform mammalian cells in vitro and mammalian *ras* genes can replace mutant yeast *ras* genes to support yeast growth (reviewed by Santos and Nebreda, 1989). $p21^{ras}$ proteins are located on the inner face of the plasma membrane. They share sequence homology with G proteins and, like G proteins, bind guanine nucleotides and exhibit GTPase activity. All of these properties together suggest that $p21^{ras}$ proteins participate in essential cellular processes such as that of signal transduction.

Mammalian *ras* genes

Ras genes were first identified as the transforming elements present in the Harvey and Kirsten strains of rat sarcoma virus (Harvey, 1964; Kirsten and Mayer, 1967). The viral gene sequences thus represent the transduced and mutated versions of the rat Harvey-*ras*-1 (H-*ras*-1) and Kirsten-*ras*-2 (K-*ras*-2) cellular protooncogenes. In addition to the H-*ras*-1 and K-*ras*-2 genes, mammalian cells also have a third *ras* protooncogene, N-*ras*, which was originally identified in neuroblastoma cells (Shimizu et al., 1983b). Human and rat

cells contain two noncoding pseudogenes, H-*ras*-2 and K-*ras*-1. Additional *ras* pseudogenes exist in mouse and other mammalian species. In the human, the N-*ras*, H-*ras*-1 and K-*ras*-2 genes are located on chromosomes 1p, 11p and 12p, respectively.

All three *ras* genes code for proteins of molecular weight 21 kD. Each gene contains four exons coding for the 189 amino acids comprising the $p21^{ras}$ protein. The K-*ras*-2 gene also has an alternative coding exon for the fourth exon (Shimizu et al., 1983a). Thus, two isomorphic K-*ras* proteins of 188 or 189 amino acids result from the expression of exon IVA in the v-K-*ras* oncogene or exon IVB of the mammalian protooncogene, respectively (Shimizu et al., 1983a). There is significant variation in the intron structure among the *ras* genes. The overall size of the H-*ras*-1 gene is 4.5 kbp (kilobase pairs), while the K-*ras*-2 gene is 50 kbp in length. The mammalian *ras* genes also possess a 5' noncoding exon downstream from promoter sequences. These noncoding exons are rich in CG (cytosine, guanine) clusters and have been shown to bind to the transcription factor Sp1. C residue methylation state may also be important in regulation of *ras* gene expression (Ramsden et al., 1985).

The *ras* gene family

Ras genes coding for p21-type proteins have been identified throughout the eukaryotic kingdom (Barbacid, 1987). In addition to mammalian species, highly conserved *ras* genes are present in chickens, amphibians, fruit flies, mollusks, slime molds, plants, and yeasts. Striking examples of the high degree of evolutionary conservation are provided by studies in which mammalian and yeast *ras* genes have been shown to be functionally interchangeable. In yeast, native *ras* proteins serve to activate adenylate cyclase (Toda

et al., 1985). Both yeast and mammalian *ras* proteins have been shown to efficiently activate the adenylate cyclase present in crude yeast membrane fractions (Broek et al., 1985). Mammalian *ras* genes also support the growth of yeast cells containing inactivated yeast *ras* sequences. Yeast *ras* genes which contain amino acid substitutions similar to those which activate mammalian *ras* genes are also capable of transforming NIH 3T3 cells (DeFeo-Jones et al., 1985).

In addition to the eukaryotic *ras* genes which code for p21, there is also a growing number of genes being identified which share sequence homology with the *ras* genes. Together with the *ras* genes, these *ras*-related genes are considered to be members of a super gene family which may have evolved from common ancestral genes. The *rho* gene products identified in snails, humans and yeasts share some 30-40% sequence homology with $p21^{ras}$ protein. An 85% sequence homology exists between the *rho* genes of snails and humans, making them evolutionarily highly conserved like *ras* genes. Other *ras*-related genes include R-*ras* identified in humans and rodents, *ral* genes found in primates and humans, D-*ras* in fruit flies, yeast YPT, rat brain *rab*, and human *rap*. The yeast YPT1 gene and an equivalent gene found in mammalian cells are involved in cellular secretion processes (Segev et al., 1988). The human *rap*1 gene, also known as K*rev*-1, has the interesting property of counteracting the transforming effects of activated *ras* genes (Kitayama et al., 1989).

$p21^{ras}$ structure and biochemistry

A comparison of the amino acid sequences for $p21^{ras}$ proteins has led to the recognition of four molecular domains (Barbacid, 1987; Santos and Nebreda, 1989).

Using mammalian p21^{ras} as a model, the first domain encompassing amino acids 1-86 is identical in amino acid sequence for human H-*ras*-1, K-*ras*-2, and N-*ras*, mouse K-*ras*-2 and N-*ras*, and rat H-*ras*-1. This region is also very highly conserved throughout the eukaryotic kingdom. The second domain covering amino acid positions 87 through 164 is somewhat less conserved than the first domain. Between any pair of human *ras* genes, there is at least 85% homology for this amino acid sequence. For any pair of vertebrate *ras* genes, the homology is in the range of 70-80%. The remaining portion of the protein except for the last four carboxyl terminal amino acids make up the third domain. This region is poorly conserved with the sequence being unique to the *ras* protein being examined. In all *ras* proteins, the carboxyl terminal domain includes a cysteine residue at position 186, two aliphatic amino acids at the next two positions and any amino acid at the terminal position. This terminal domain CAAX sequence is a motif found not only in *ras* and *ras*-related proteins, but also in the carboxyl ends of several unrelated proteins. Post-translational removal of the AAX terminal amino acids and biochemical modification of the C residue appear to be important in the targeting and association of p21 with the cell membrane (Santos and Nebreda, 1989).

The domain structure of p21^{ras} appears to be related to the general biochemical function and the specific target localization of the proteins. As indicated in Figures 1 and 2, study of the secondary structure of p21 proteins has led to the identification of 5 α-helices, 6 β-sheets and 10 connecting loops within the p21 molecule (Pai et al., 1989). All *ras* proteins are able to bind guanine nucleotides and exhibit GTPase activity. X-ray crystallography studies and mutation analysis have made possible the identification of

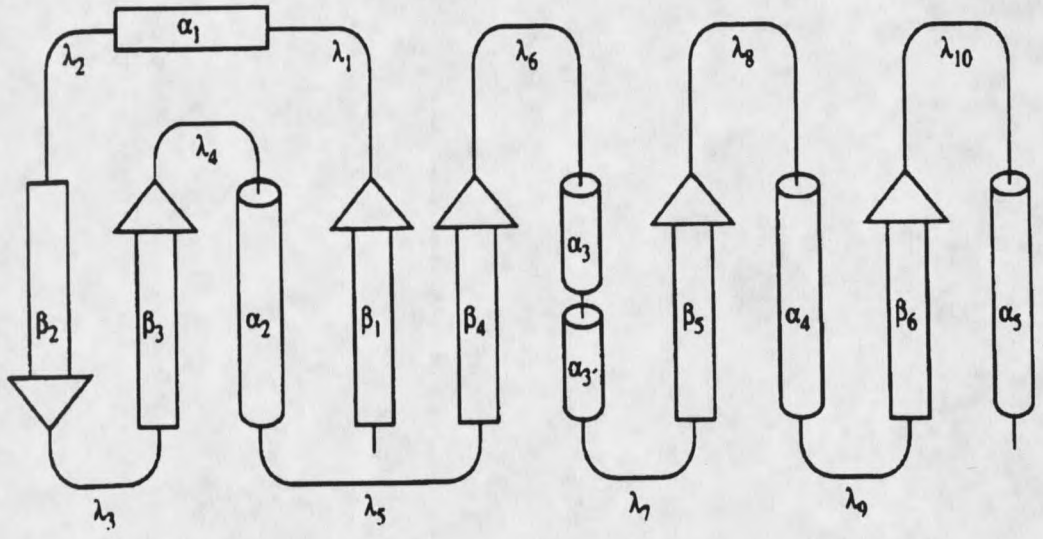

Figure 1. Schematic representation of the secondary structure of $p21^{c\text{-}H\text{-}ras}$ (residues 1-166). α-helices are represented as cylinders and are labeled α_1 through α_5. β-sheets are represented as arrows labeled β_1 through β_6. Connecting loops are labeled λ_1 through λ_{10}. Assignments for the beginning and ending residues for each secondary structure elements are as follows: α_1(16-25), α_2(66-74), α_3(87-103), α_4(127-136), α_5(152-165), β_1(2-9), β_2(37-46), β_3(49-58), β_4(77-83), β_5(111-116), β_6(141-143), λ_1(10-15), λ_2(26-36), λ_3(47-48), λ_4(59-65), λ_5(75-76), λ_6(84-86), λ_7(104-110), λ_8(117-126), λ_9(137-140), and λ_{10}(144-151). The figure and secondary structure assignments are taken from Pai et al. (1989).

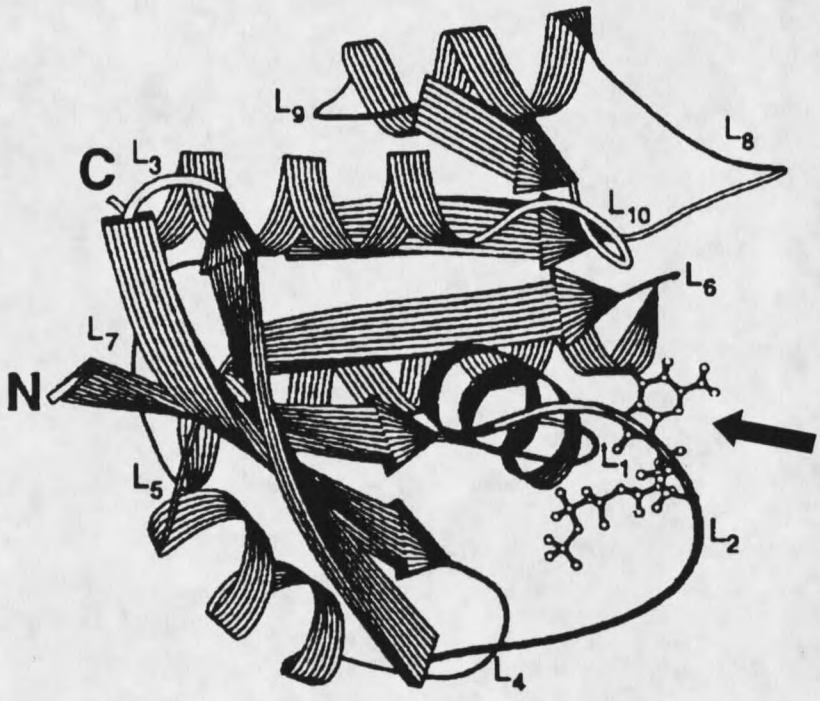

Figure 2. Schematic representation of the three-dimensional structure of $p21^{ras}$. The position of the guanine nucleotide is indicated by the solid arrow. Loops connecting α-helices and β-sheets are labeled L_1 through L_{10}. The figure is taken from Krengel et al. (1990).

regions of the p21^{ras} molecule involved in nucleotide binding. Four noncontiguous connecting loop regions including amino acid positions 10-16 (connecting loop 1, or λ1), 59-63 (λ4), 116-119 (λ8), and 143-147 (λ10) participate in binding to GTP/GDP. During binding, GTP/GDP phosphate groups appear to interact with the amino acids of positions 10-16 and 59-63, while the guanine nucleotide base is localized in a pocket formed by amino acids of positions 116-119 and 143-147. These positions are completely conserved in mammalian p21^{ras} species as might be expected for regions of the protein which interact with a common molecule having regulatory significance. Biochemical functioning of normal p21^{ras} appears to be determined by whether GTP or GDP is bound to the protein, with the GTP-bound form being in the active state. The GTP-bound, active form of p21^{ras} is normally short-lived to insure that p21^{ras} biochemical functioning is expressed in a controlled manner. Although p21^{ras} by itself has intrinsic GTPase activity, interaction with GTPase activating protein (GAP) (Trahey and McCormick, 1987) promotes GTP hydrolysis in normal cells to give rise to a functionally inactive GDP-p21^{ras} complex. Amino acid substitutions in p21^{ras} which block GTPase activity confer transforming potential on p21^{ras} molecules which are perpetually bound to GTP in a functionally active state.

Recent X-ray crystallography studies have provided information on the conformation states of GDP and GTP-bound forms of p21^{ras}. The crystal structures of GDP-bound forms of genetically engineered normal and oncogenic c-H-*ras* proteins have been determined and compared (de Vos et al., 1988; Tong et al., 1989a, b). In these studies, normal and oncogenic proteins containing amino acid residues 1-171 were used because