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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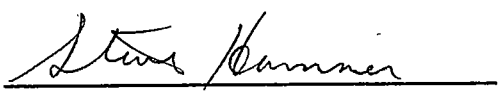
Signature 

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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

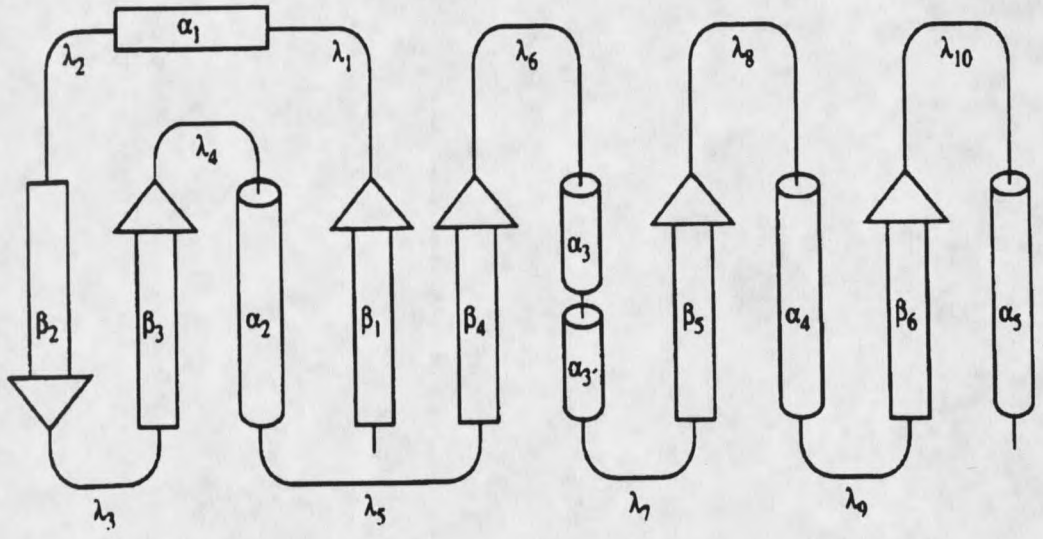

Figure 1. Schematic representation of the secondary structure of $p21^{c\text{-}H\text{-}ras}$ (residues 1-166). α-helices are represented as cylinders and are labeled α_1 through α_5. β-sheets are represented as arrows labeled β_1 through β_6. Connecting loops are labeled λ_1 through λ_{10}. Assignments 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).

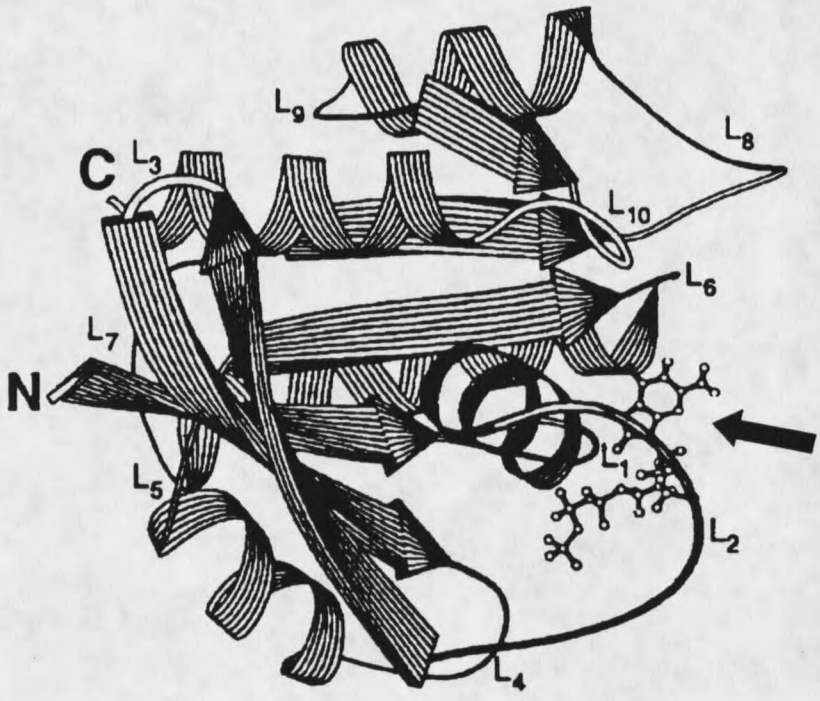

Figure 2. Schematic representation of the three-dimensional structure of $p21^{ras}$. The position of the guanine nucleotide is indicated by the solid arrow. Loops connecting α-helices and β-sheets are labeled L_1 through L_{10}. The figure is taken from Krengel et al. (1990).

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

these structures are easily crystallized. A full length protein has more recently been studied (Milburn et al, 1990), although the crystal structure of the carboxyl terminus remains somewhat disordered. The overall topological structures of these GDP-bound $p21^{ras}$ proteins are very similar. When compared to the normal protein, however, an oncogenic form of the protein containing a valine at position 12 does appear to have an enlarged loop structure in the area of the substituted amino acid (Tong et al., 1989a). This loop is involved in binding to the β-phosphate of the guanine nucleotide. A change in the conformation of this loop structure by substitutions at position 12 might account for the reduction in GTPase activity noted for the oncogenic protein.

Studies of the "active" conformation of $p21^{ras}$ when bound to GTP have made use of nonhydrolyzable or slowly hydrolyzable GTP analogues. Pai et al. (1989) elucidated the structure of $p21^{ras}$ (residues 1-166) bound to GDP-NP (guanosine 5'-(β,γ-imido)-triphosphate). A comparison of this structure with that of GDP-bound $p21^{ras}$ as described by Tong et al. (1989b) was published by Jurnak et al. (1990). Using interactive computer graphics technology, this group deduced the occurrence of conformation changes in the area where $p21^{ras}$ binds to the γ-phosphate of the GTP analog.

GTP-induced conformational changes in $p21^{ras}$ have also been noted by Kim and coworkers (Milburn et al., 1990; Brunger et al., 1990) in their comparisons of GDP- and GTP-complexed $p21^{ras}$. This group has localized the changes in conformation between the two $p21^{ras}$-guanine nucleotide complexes to two molecular regions, amino acid residues 30-38 and residues 60-76. These regions lie contiguous to each other on the surface of the $p21^{ras}$ molecule and are likely to be conformationally linked. These

regions are also candidates as recognition sites for an upstream regulatory molecule or downstream regulator such as GAP. Mutation analysis has localized GAP interaction with $p21^{ras}$ to these same regions and has demonstrated that residues 32-40 making up the effector region of $p21^{ras}$ are necessary for GAP stimulation of GTPase activity and for $p21^{ras}$ biological activity. Although GAP binds to both normal and transforming $p21^{ras}$ bound to GTP, GTPase activity is only promoted in the normal protein. Transforming mutants lack GTPase activity even in the presence of GAP interaction. The second conformation switch region made up of residues 60-76 also overlaps the epitope for antibody Y13-259. This antibody binds to $p21^{ras}$ in the region of residues 63-73. Bound antibody inhibits the exchange of GDP/GTP and neutralizes the activity of *ras* proteins. Binding of free $p21^{ras}$ to guanine nucleotides or GTP hydrolysis is not affected by antibody binding, however. It has been proposed that conformational changes are necessary during the release of GDP and exchange for GTP, and that Y13-259 binding might prevent such changes from occurring (Milburn et al., 1990). A scenario is also proposed whereby this region serves as the recognition site for an upstream regulator in the signaling pathway of which $p21^{ras}$ is a part. This region is conformationally linked to the effector region so that Y13-259 freezing of its epitope's conformation renders the effector region unable to interact with the effector molecule. This would explain the ability of Y13-259 to neutralize the transforming effects of activated $p21^{ras}$.

A time-course study of $p21^{ras}$ conformational changes accompanying the hydrolysis of bound GTP has recently been reported (Schlichting et al., 1990). This study made use of a GTP analog which only undergoes hydrolysis after photolytic dissociation of a

protecting moiety.. Similar to the results reported by Kim and coworkers described above, this group also noted major conformational changes occurring in regions of $p21^{ras}$ which interact with GAP.

Mutations which confer transforming potential on $p21^{ras}$ can now begin to be correlated with known structural features and conformation states of the $p21^{ras}$ molecule. Point mutations detected in naturally occurring *ras* oncogenes are localized to codons 12, 13, 59, and 61 (Barbacid, 1987). Substitution of valine or other amino acids seen in $p21^{ras}$ transforming mutants for the glycine normally at position 12 appears to change the orientation of the γ-phosphate of bound GTP and may block the entry of a nucleophilic attacking group into the guanine nucleotide binding pocket (Milburn et al., 1990). The result is a curtailment of GTP hydrolytic activity. Oncogenic substitution for the glycine at position 13 probably distorts the loop structure involved in binding to the β-phosphate to also result in a catalytically unfavorable conformation for GTP hydrolysis. Transforming mutations at positions 59 or 61 undoubtedly affect the loop structure of the second conformation switch region which is involved in interactions with the guanine nucleotide phosphate groups.

The structural studies described above have focused on the conserved catalytic domain of the $p21^{ras}$ protein. While not directly involved in the biochemical functioning of the protein, the carboxyl terminal 25 amino acids of $p21^{ras}$ make up a flexible region which undergoes post-translational modifications important for localization of the *ras* protein to the cytoplasmic membrane. Membrane localization is necessary for normal and oncogenic *ras* proteins to be biologically active (Willumsen et al., 1984). Thus, study

of the post-translational modifications which lead to $p21^{ras}$ association with the plasma membrane may be valuable in the development of treatments for human cancers in which oncogenic *ras* expression is involved. The carboxyl terminal four amino acid sequence found in $p21^{ras}$, CAAX (where C is cysteine, A is any aliphatic amino acid, and X is any amino acid), is highly conserved among all *ras* and *ras*-related gene sequences, and is also seen in the carboxyl termini of the α and γ subunits of certain G proteins, nuclear lamins, and yeast α mating factor. The CAAX box serves as a general signal for trafficking, modification, and targeting of $p21^{ras}$ (Santos and Nebreda, 1989). Post-translational processing of $p21^{ras}$ involves proteolytic removal of the three terminal amino acids, AAX, to place the cysteine residue at the carboxyl terminal position. This cysteine residue undergoes carboxyl-methylation and polyisoprenylation (Gutierrez et al., 1989; Hancock et al., 1989). Recent work suggests that the temporal order of these three processing steps appears to be prenylation, proteolysis, and carboxy-methylation for yeast α-mating factor and perhaps $p21^{ras}$ (Schafer et al., 1990). Polyisoprenylation increases the hydrophobicity of $p21^{ras}$ and consequently promotes the protein's association with the cell membrane. Further processing of H-*ras* and N-*ras* p21 molecules is necessary for high avidity binding of these proteins to the plasma membrane. Acylation of Cys^{181} and Cys^{184} of $p21^{H\text{-}ras}$ and Cys^{181} of $p21^{N\text{-}ras}$ with palmitic acid increases membrane binding avidity of these molecules. $p21^{v\text{-}K\text{-}ras}$ also contains a cysteine at position 180 due to expression of exon IVA of the K-*ras*-2 gene. This cysteine residue may also undergo palmitoylation (Hancock et al., 1989). $p21^{K\text{-}ras(B)}$ is not palmitoylated, however, since it lacks a cysteine residue immediately upstream of Cys^{186}. The cellular K-*ras* protein

does contain a region of basic amino acid residues (positions 175-182) including six consecutive lysine residues at positions 175-180 which is essential for membrane localization (Hancock et al., 1990). Like the palmitoylation of the other $p21^{ras}$ proteins, this polybasic domain serves as a necessary signal for targeting $p21^{K\text{-}ras(B)}$ to the plasma membrane. The polybasic domain of $p21^{K\text{-}ras(B)}$ appears to promote membrane binding through ionic interactions with the negatively charged head groups of membrane lipids. The identification of two different membrane targeting signals for the different $p21^{ras}$ proteins suggests that the different $p21^{ras}$ molecules may interact with different membrane proteins.

$p21^{ras}$ and G proteins

The ability of $p21^{ras}$ to bind guanine nucleotides and hydrolyze GTP is shared by the G proteins known to be involved in transducing membrane receptor signals. G protein mediation of signal transduction is a key component in the regulation of important cellular functions. $p21^{ras}$ can be considered to be a member of a group of small molecular weight GTP binding proteins (molecular weight range 20-25 Kd) which differ somewhat from the classical G proteins (reviewed by Freissmuth et al., 1989; Hall, 1990b; Downward, 1990). *Ras*-related gene products such as *rho* and yeast YPT-1 are also included in the small G protein group. The small molecular weight G proteins together with classical G proteins and another group of GTP binding proteins involved in protein synthesis (exemplified by bacterial elongation factor EF-Tu) make up a larger, growing family of GTP binding proteins.

Comparison of $p21^{ras}$ biochemistry with that of classical G proteins reveals both similarities and differences. Both $p21^{ras}$ and classical G proteins perform their biological activities in association with the cell membrane. Yeast $p21^{ras}$ and specific mammalian non-$p21^{ras}$ G proteins have been shown to directly regulate adenylate cyclase in their respective systems. Classical G proteins are heterotrimers consisting of α, β, and γ subunits. The functional specificity of G proteins is believed to reside in the α subunit itself. Individual G protein heterotrimers are accordingly classified on the basis of the associated α subunit. To date, at least twelve different α subunit polypeptides have been identified. Amino acid sequence comparisons reveal that $p21^{ras}$ proteins and the G protein α subunits share a conserved guanine nucleotide binding domain. Also like $p21^{ras}$, all of the α subunits exhibit GTPase activity. Unlike the α subunits, $p21^{ras}$ does not appear to interact with the equivalent of a $\beta\gamma$ subunit complex.

Classical G proteins function as signal transducers that link membrane-bound receptors to intracellular membrane-associated effectors (Freissmuth et al., 1989). G proteins were first discovered during studies of the β-adrenergic receptor system. In this system, hormone receptor binding is coupled to adenylate cyclase and the production of cyclic AMP by the related G proteins G_s and G_i which stimulate and inhibit adenylate cyclase, respectively. The α subunits of these two G protein oligomers, α_s and α_i, interact with common $\beta\gamma$ subunits, but associate selectively with different receptors.

Other G proteins include G_o and G_t which may interact with rhodopsin, muscarinic cholinergic, and α_2-adrenergic receptor systems. On the basis of molecular biological and pharmacological criteria, there appear to be at least 86 distinct receptors which are

coupled to their effectors via G proteins (Birnbaumer, 1990). These receptors interact with a large variety of hormones, neurotransmitters, and autocrine and paracrine factors to control essential cellular functions. G proteins coupled to these receptors regulate not only cyclic AMP formation, but also such activities as the mobilization of intracellular calcium, release of arachidonic acid, and control of membrane permeability and potential. Biochemical studies indicate that individual G protein species can couple receptor messages to more than one effector system, thus allowing a single hormone or neurotransmitter to modulate a variety of cellular functions at the same time. A variety of receptor types may also interact with the same class of G protein molecules so that different regulatory factors can act together to control a given cellular activity.

When linked with the $\beta\gamma$ subunit complex, the α subunit is in a GDP-bound, functionally inactive state. The $\beta\gamma$ complex serves to localize the α subunit to the plasma membrane where it can interact with the appropriate upstream regulator. Agonist binding to a receptor molecule stimulates interaction with the appropriate G protein oligomer which results in the formation of an intermediate ternary complex of agonist-receptor-G protein. Formation of this complex in turn promotes dissociation of GDP. Subsequent binding of GTP to the α subunit causes dissociation of the ternary complex. Released receptor can then recycle to activate additional G proteins. The newly activated GTP-bound α subunit separates from the $\beta\gamma$ complex and is free to interact with the appropriate effector. The activated α subunit monomer may interact with the effector molecule for several seconds, a period which allows for significant signal amplification, before the intrinsic GTPase activity of the α subunit cleaves the γ phosphate of the bound

GTP to stop the signal transmission process. The GDP-bound α subunit then dissociates from the effector which is free to interact with yet another activated α monomer. The inactive α-GDP then reassociates with $\beta\gamma$ and is available for recycling.

Activities of some of the G protein α subunits can be modified by bacterial toxins, a finding which has facilitated study of G protein functioning (reviewed by Reisine, 1990). Cholera toxin catalyzes the ADP-ribosylation of α_s at a site near the GTP binding site of the subunit. The result is that the GTPase activity of α_s is inactivated so that the monomer is left in the GTP-bound active configuration. Cholera toxin thus promotes an enhanced activation of adenylate cyclase and the overproduction of cyclic AMP. Pertussis toxin does not affect α_s, but does induce ADP-ribosylation of α_i. Instead of maintaining α_i subunits in their active states to result in an enhanced inhibitory effect on adenylate cyclase, pertussis toxin inactivates α_i and so blocks hormonal repression of the enzyme. Pertussis toxin also catalyzes ADP-ribosylation of the α_o subunit of G_o which is present in high concentration in brain tissue where it regulates ionic conductance channels. G_o is weakened in its functioning by pertussis toxin, a property which suggests that pertussis toxin acts on G_i and G_o in a similar manner. It should be mentioned that toxin effects on certain G protein-coupled effector systems are dependent on the cell type being studied.

Given the wide diversity of cellular activities for which G proteins play an intermediate regulatory role, it is not surprising that signal transduction by G proteins has been implicated in the regulation of cytoskeleton structure, cellular motility, and tumor invasion and metastasis. Treatment of malignant lymphosarcoma and T-cell hydridoma

cell lines with pertussis toxin has been shown to greatly reduce invasion in vitro as well as metastasis formation (Roos and Van de Pavert, 1987). In this work, tumor cell viability and proliferation rates were unaffected by treatment with pertussis toxin. Cholera toxin was also reported to have no effect on the tumor cell lines' invasive capacities. A highly metastatic melanoma cell line, A2058, which is known to produce and respond to an autocrine motility factor (AMF), has also been shown to display greatly decreased motility in response to the AMF after treatment with pertussis toxin (Stracke et al., 1987). Treatment of this cell line with cholera toxin or other agents which regulate the adenylate cyclase system had no effect on motility. The effect of pertussis toxin treatment on the A2058 cell line's motility responses to extracellular matrix (ECM) components in solution (chemotaxis) and bound to substrate (haptotaxis) has also been examined (Aznavoorian et al., 1990). A2058 haptotactic responses to type IV collagen, fibronectin, and laminin were unaffected by pertussis toxin treatment. Pertussis toxin treatment significantly decreased the chemotactic response to type IV collagen, however, and slightly decreased the chemotactic response to laminin. Chemotactic response to fibronectin was unaffected by the toxin. These results imply that different components of ECM induce tumor cell motility by different signal transduction pathways. For fibronectin, pertussis toxin-sensitive G proteins do not appear to be involved. All three ECM components are large molecules likely to have several different domains which interact with cells via different receptors. Signal transduction pathways invoked and cell responses seen will accordingly depend on the receptor type bound by a given domain of an ECM molecule. For both type IV collagen and laminin, haptotactic and chemotactic

responses appear to be mediated by different signal transduction pathways since they are differentiated by pertussis toxin.

Highly metastatic B16 mouse melanoma cells have been shown to express increased amounts of G_{i2} proteins compared to poorly metastatic counterpart cells (Lester et al., 1989). Treatment with pertussis toxin markedly decreased the highly metastatic cells' migration through a Matrigel matrix as well as motility on fibronectin, laminin and type IV collagen substrates. Pertussis toxin had no noticeable effect on the low in vitro invasive capacity or mobility of the poorly metastatic B16 clone. Signal transduction by G_{i2} thus appears to contribute to the motility and metastatic capacity of B16 cells.

Cellular motility is dependent on a dynamic rearrangement of cytoskeletal elements, a process which involves an orderly polymerization and depolymerization of filamentous actin. A study of actin polymerization in human neutrophils using pertussis toxin as well as agents which directly activate G proteins suggests that G proteins are involved in the process of actin polymerization (Bengtsson et al., 1990). In this experimental system, neutrophil chemotactic response to the peptide formylmethionylleucylphenylalanine is suppressed by pertussis toxin. Pretreatment of neutrophils with pertussis toxin also abrogates actin polymerization normally induced by this chemotactic peptide. When AlF_4^- is introduced into these cells, it forms a stable complex with GDP that mimics the properties of GTP. Use of this reagent or a non-hydrolyzable GTP analog leads to the perpetual activation of G protein α subunits and associated responses such as an increase in free cytosolic Ca^{2+}, activation of protein kinase C and phospholipase C, and release of arachidonic acid. Initiation of actin polymerization is also induced by these agents.

Although there remains much to be learned about G protein function and the role of G proteins in regulating cellular activity, it would appear that G proteins are intimately involved in a variety of processes directly related to the process of tumor metastasis. G proteins themselves may be subject to activating mutations which render them oncogenic (Lyons et al., 1990). Growth hormone-secreting pituitary tumors have an abnormally elevated adenyl cyclase activity and enhanced rate of cell proliferation. A mutation in the α subunit of the G_s proteins of these cells was found to have abrogated the GTPase activity of the α subunit. The α subunit thus remains in the active GTP-bound state and so continuously stimulates the adenyl cyclase pathway. A similar mutation in the α subunit of G_{i2}, the G protein implicated in motility and metastatic capability of B16 cells, has also been detected in tumors of the adrenal cortex and in endocrine tumors of the ovary.

Through studies involving G protein-coupled muscarinic receptors of atrial cells, a link between G proteins and the $p21^{ras}$ system already strongly implicated in metastasis has recently been established (Yatani et al, 1990). The G protein G_K links agonist-bound muscarinic receptors to the opening of K^+ ionic channels in atrial cells. The $p21^{ras}$ protein in association with GAP promotes the deactivation or closing of these channels. Pertussis toxin has the same effect of blocking channel opening but does so by inhibiting G_K activity. Inactivation by $p21^{ras}$-GAP may be the result of $p21^{ras}$-GAP being able to hinder the coupling of receptor to G_K. It will be of interest to determine whether the $p21^{ras}$-GAP complex interaction with G protein signal transduction is a general phenomenon which might underlie the general functioning of *ras*.

p21^{ras}, GAP and signal transduction

Whereas the intrinsic GTPase activity of G protein α subunits is sufficient to readily hydrolyze and convert bound GTP to GDP under physiological conditions, such does not appear to be the case for p21^{ras} protein. Identification of the GTPase activating protein, or GAP, which promotes the GTPase activity of p21^{ras} provided not only an explanation of how GTP-bound p21^{ras} is normally inactivated but also the first example of another protein with which p21^{ras} interacts (Trahey and McCormick, 1987). GAP now appears to be not only a modulator of p21^{ras} activity, able to inactivate p21^{ras} by stimulating GTPase activity, but also an effector necessary for p21^{ras} to interact with a target signal transduction system, the muscarinic receptor/G_K system discussed above.

GAP is a cytosolic protein with a molecular weight of about 120 kD. Although GAP activity is found primarily in the cytosol, the amino terminal domain of the protein is very hydrophobic and may facilitate association with the plasma membrane. p21^{ras} GTPase-stimulating activity resides in the carboxy-terminal third of the GAP molecule (Marshall et al., 1989). The amino terminal domain is dispensable with regard to the stimulation of p21^{ras} GTPase activity (McCormick, 1989), but may be important in the putative effector functioning of GAP. Compared to the intact GAP protein, a truncated form containing only the carboxy-terminal 334 amino acids shows greatly reduced ability to block muscarinic receptor/G_K opening of ionic channels (Yatani et al., 1990).

Despite its obvious role in down-regulating p21^{ras} by stimulating GTP hydrolysis, GAP exhibits a number of other properties which suggest a more complex relationship

with $p21^{ras}$. GAP is present at higher levels in the cell than is $p21^{ras}$, a characteristic which is unexpected if GAP's function is simply to down-regulate $p21^{ras}$-GTP (Hall, 1990a). To allow $p21^{ras}$-GTP an opportunity to interact with effector, GAP GTPase stimulation may itself need to be regulated. In fact, there is evidence that during the cellular response to growth factors, certain phospholipids such as phosphatidylinositol phosphate and products of their metabolic pathways such as arachidonic acid do block GAP promotion of $p21^{ras}$ GTPase activity (Tsai et al., 1989). Sequence analysis has revealed homology between two amino terminal regions of GAP and segments of the *src* oncogene protein (Vogel et al., 1988; Trahey et al., 1988). Putative regulatory regions of some nonreceptor tyrosine kinases also share homology with these GAP/*src* protein regions. This homology suggests a possible link between tyrosine kinase oncogenes and $p21^{ras}$.

Before the recent association of $p21^{ras}$-GAP with the muscarinic receptor-G_K system, there was already the suspicion that GAP might be a target of $p21^{ras}$-GTP. The portion of $p21^{ras}$ which is necessary for GAP binding and stimulation of GTPase activity, residues 32-40, is also absolutely required for the biological activity of *ras*. GAP binds to activated, oncogenic *ras* proteins although it is unable to promote GTPase function in these mutants. Mutations involving residues 32-40 of $p21^{ras}$ which suppress the biological activity of oncogenic *ras* proteins also destroy in wild type proteins the ability of GAP to bind to $p21^{ras}$ and hence the ability of GAP to stimulate GTPase activity. These findings lend strong support for GAP being a target effector protein for $p21^{ras}$.

When the GAP gene is transfected into NIH 3T3 cells also expressing an activated H-*ras* gene, the transforming capability of the *ras* oncogene remains unaffected by the overexpressed GAP (Zhang et al., 1990). Transformation of the NIH 3T3 cells by overexpression of normal *ras* is greatly diminished by overexpression of cotransfected GAP. The enhanced level of GAP activity is presumably able to thwart the transforming potential of overexpressed normal $p21^{ras}$ by reducing the level of activated, GTP-bound $p21^{ras}$ protein.

GAP has recently been identified as one of four substrates which bind to ligand-activated platelet derived growth factor (PDGF) β-receptor (Kaplan et al., 1990). GAP and the other three proteins which form a complex with the receptor, phospholipase c-γ, phosphatidylinositol kinase, and the c-raf protooncogene-encoded serine/threonine kinase, are also all tyrosine phosphorylated by the activated PDGF receptor. These three other proteins have previously been implicated in mediating cellular response to PDGF as well as in the control of cell proliferation. Several observations suggest that the multiprotein complex may serve an important role in normal signal transduction. In cells transformed by activated *ras*, PDGF receptors are unable to stimulate phospholipase activities. In *ras*-transformed cells, GAP also fails to associate with activated PDGF receptor. Thus, receptor-GAP association may be necessary for receptor activation of phospholipase C. Although the precise relationship of GAP to the PDGF system is presently unclear, the findings do suggest a GAP-mediated linkage of growth factor receptors and *ras*.

Activation of another receptor system, the T lymphocyte antigen receptor, has recently been shown to result in the rapid activation of $p21^{ras}$ by a mechanism which may

involve down-regulation of GAP activity (Downward et al., 1990). Stimulation of the T-cell receptor was seen to result in a tenfold increase in the level of $p21^{ras}$ bound to GTP. During this increase in the active form of the *ras* protein, no change in the rate of nucleotide exchange was detected. A decrease in GAP activity responsible for catalyzing GTP hydrolysis was noted however. Stimulation of the T-cell antigen receptor is known to activate certain signal transduction pathways including the protein kinase C system. Treatment of T cells with a protein kinase C-activating phorbol ester also resulted in significant activation of $p21^{ras}$ and an accompanying inhibition of GAP activity. Although the exact mechanism of regulation is as yet unknown, it appears that protein kinase C is an important mediator of $p21^{ras}$ activation.

In addition to GAP, another cytosolic protein has recently been discovered to interact with $p21^{ras}$. This protein has been termed *ras*-guanine nucleotide-releasing factor (*ras*-GRF) since it appears to catalyze the release of GDP bound to $p21^{ras}$ (Wolfman and Macara, 1990). Under physiological conditions, GDP does not readily dissociate from $p21^{ras}$, so it seemed apparent even before the discovery of *ras*-GRF that some factor should interact with $p21^{ras}$-GDP to promote nucleotide exchange and activation of normal cellular $p21^{ras}$. Further investigation of *ras*-GRF should reveal how guanine nucleotide exchange and activation of *ras* is regulated, and how $p21^{ras}$ may be coupled to growth factor responses and signal transduction pathways.

Although it is not yet well characterized, the protein coded for by the neurofibromatosis type 1 (NF1) gene has recently been described as having a domain which shares about 30% sequence homology with the catalytic domains of mammalian

GAP and the yeast IRA (inhibitory regulators of the *ras*-cAMP pathway) proteins (Xu et al., 1990a). Subsequent to this report of sequence homology between the NF1 gene and IRA/GAP, several researchers have expressed the homology domain of NF1 in vitro and found that the truncated protein interacts with normal $p21^{N\text{-}ras}$ and $p21^{H\text{-}ras}$ to stimulate *ras* GTPase activity (Xu et al., 1990b; Martin et al., 1990; Ballester et al., 1990). The NF1 protein also complements yeast mutants which have a loss of IRA function. Further studies are required to fully elucidate the function of the NF1 protein and how it might be involved with p21 and GAP.

Regulation and activation of *ras* genes

Ras genes can become oncogenic when overexpressed above normal levels or, as discussed in the earlier section on $p21^{ras}$ structure, when activated by point mutations affecting domains of the $p21^{ras}$ protein involved in guanine nucleotide binding. Mutations which result in an increase in GDP/GTP nucleotide exchange rate or a decrease in GTPase activity will accordingly accelerate the formation or favor the maintenance of the biologically active $p21^{ras}$-GTP complex (Feig and Cooper, 1988). *Ras* proteins maintained in the active state are presumed to send a continuous flow of growth-promoting messages along a signal transduction pathway or pathways and thus promote malignant transformation of cells (Barbacid, 1987). Overexpression of normal *ras* proteins appears to result in the formation of a higher-than-normal number of $p21^{ras}$ molecules bound to GTP which are then able to induce transformation. In general, transformation induced by increased expression of normal *ras* is not as efficient or extensive as that achieved by activating mutations, however.

Ras genes exhibit a number of properties characteristic of "housekeeping genes" which are required for routine cellular functioning and growth control. Like other housekeeping genes, *ras* genes are expressed in most, if not all, cell types at low levels (Barbacid, 1987). During proliferation, many cells show increased expression of *ras* genes. In certain tissues such as brain or heart, however, high *ras* expression is associated not with active proliferation but with differentiated states. *Ras* genes and many housekeeping genes have 5'-region promoter and regulatory regions that are rich in CG (cytosine, guanine) clusters. For most housekeeping genes, there is strong evidence that the methylation state of specific cytosine residues within these CG clusters is related to the control of gene expression (reviewed by Jones, 1986). For many genes, the presence of 5-methyl-deoxycytidine appears to be able to abrogate protein DNA interactions in the CG-rich regulatory regions (reviewed by Doerfler et al., 1990). In general, genes associated with hypermethylated CG regions are transcriptionally silent, while undermethylated CG clusters are identified with transcriptionally active genes. There are of course exceptions to this rule in that expression of certain genes may be dependent on DNA methylation (Doerfler et al., 1990).

Several studies have suggested that the control of *ras* gene expression and oncogenic activation of *ras* genes may be influenced by methylation status of associated CG-rich regions. In the mouse, tissue types of epithelial origin show greater incidences of expressing an activated H-*ras* oncogene and exhibiting associated neoplastic changes and development than do other tissue types (Ramsden et al., 1985). These same epithelial tissues have c-H-*ras* genes which are hypomethylated relative to the other tissue types.

A related finding is that in fibroblast cells including the NIH 3T3 line, c-H-*ras* genes are hypermethylated compared to normal mouse cells of epithelial lineage. When NIH 3T3 cells are transformed by chemical treatment, activation of K-*ras* rather than the methylated H-*ras* gene results. Thus, there appears to be a correlation between the methylation state of c-H-*ras* genes and their susceptibility to activation. The H-*ras* genes of epithelial cell types are hypomethylated compared to H-*ras* genes of other tissues and, as a possible consequence, are more prone to becoming activated (Ramsden et al., 1985).

To examine the relationship between methylation state and the transforming capability of an H-*ras* gene, one group introduced methylated or unmethylated H-*ras* oncogenes into NIH 3T3 cells (Borrello et al., 1987). In this study, transfection of cells with the unmethylated gene gave rise to many transformed foci, while transfection with the methylated gene decreased transformation efficiency by some 80%. Subsequent treatment of non-transformed cells harboring the methylated H-*ras* oncogene with the demethylating agent 5-azacytidine resulted in demethylation of the introduced H-*ras* gene and induction of morphological transformation.

Compared to normal control tissues, the H-*ras* gene isolated from some mouse and human tumor tissues is hypomethylated (Ramsden et al., 1985; Vousden and Marshall, 1984; Feinberg and Vogelstein, 1983). A recent study found that H-*ras* sequences from breast carcinoma tissues and normal breast epithelial tissues from the same patients showed apparently identical methylation patterns (Barbieri et al., 1989). In both normal and disease tissues, the H-*ras* gene was unmethylated at the 5' region. This finding lends support for the idea that hypomethylation of the H-*ras* gene in an epithelial tissue may

precede the onset of transformation. Analysis of mRNA levels showed the tumor tissue to express high levels of H-*ras* message compared to normal tissue. Accordingly, it appears that although DNA methylation may influence the expression of certain genes including members of the *ras* family, transcriptional activity of *ras* genes is not strictly dependent on methylation state. Other factors in addition to DNA methylation must be acting to regulate gene expression.

The promoter regions of many housekeeping genes and members of the *ras* gene family contain one or more copies of the DNA sequence GGGCGG and its inverted complement CCGCCC (termed a GC box). This sequence serves as the recognition site for the transcription factor Sp1. The promoters of many other eukaryotic genes contain a CCAAT or TATA box which is also recognized by transcription factors (reviewed by Saltzman and Weinmann, 1989). For genes with these types of promoters, binding of the transcription factor TFIID to the TATA box is believed to lead to the recruitment of other transcription factors as well as RNA polymerase into a transcription complex. Additional factors such as Sp1, through binding to their recognition sites, also promote transcriptional activation. Sp1 may also serve to recruit additional transcription factors or to sequester the transcription complex during the activation of TATA-less promoters.

As studied by one group, the promoter region of the human c-H-*ras* gene has been shown to initiate transcription at four different sites and contain eight copies of the GC box recognized by Sp1 (Ishii et al., 1985 and 1986). This promoter was localized to a region between two Nae1 restriction sites lying 1553 and 1004 bases upstream of the first coding ATG site. While no TATA box is present in this region, one CAAT box is found

some 170 bases upstream of the 5'-most transcription start site. Sp1 was shown to bind to six of the eight GC boxes identified, although only the three SP1 recognition sites closest to the transcription start sites appear to be essential for transcription. Through a competition assay using non-*ras* DNA sequences containing GC boxes, transcription from the c-H-*ras* sequence was shown to be reduced as Sp1 was competitively bound by the non-*ras* GC boxes. Sp1 thus appears to be a regulator required for transcription for the c-H-*ras* gene. Another group studying the promoter region of an activated c-H-*ras* gene has made use of a focus formation assay to test for expression and transforming activity of the H-*ras* oncogene (Honkawa et al., 1987). In this study, an essential promoter region located between positions 1418 and 1368 upstream of the first ATG codon was identified. This region lacks TATA and CCAAT sequences, is CG rich and contains a GC box at its 3' end. Additional transcription start sites not recognized by Ishii et al. (1985) were also detected in this study.

The promoter regions of the mouse c-K-*ras* and guinea pig N-*ras* genes have also been characterized in detail (Hoffman et al., 1987; Doniger and DiPaolo, 1988). Both of these *ras* promoter regions lack TATA or CCAAT boxes. Like the promoters of the H-*ras* gene and many other housekeeping genes, these promoters are rich in CG residues, have several GC boxes, and exhibit multiple transcription start sites. Comparison of human and guinea pig intron sequences has also revealed at least a 65% homology (Doniger and DiPaolo, 1988). Comparison of the non-coding exons of human and guinea pig N-*ras* genes (Doniger and DiPaolo, 1988) as well as of the non-coding exons of human and mouse c-K-*ras* genes (Hoffman et al., 1987) show similarly high degrees of

homology. These high degrees of conservation between the intron sequences and between the non-coding exon sequences suggest possible roles in regulation of expression of these *ras* genes.

Further investigation of the c-K-*ras* promoter has led to the identification of a 29 base pair homopurine/homopyrimidine-rich domain which appears to be important in control of transcription (Hoffman et al., 1990). This domain is so-named because one of its complementary strands is comprised mainly of purine nucleotides while pyrimidine residues predominate in the other complementary strand. This purine/pyrimidine (pur/pyr) motif has also been identified in the promoter regions of other housekeeping genes including the epidermal growth factor receptor (EGF-R) and insulin receptor (I-R) genes. Gel mobility-shift and DNase protection assays revealed that a 166 base pair length of double stranded c-K-*ras* promoter DNA containing this pur/pyr region bind several nuclear proteins in vitro. One of these proteins is probably Sp1. The pur/pyr region itself binds at least one nuclear protein in vitro. A nuclear factor bound by the c-K-*ras* pur/pyr segment is also competitively bound by the same sequence present in the EGF-R and I-R promoters. Deletion of the pur/pyr sequence from the c-K-*ras* promoter markedly decreases transcription. These findings suggest an important role for the pur/pyr element in regulating transcription of the c-K-*ras* gene and perhaps other housekeeping genes.

Transformation by *ras*: experimental induction of tumor metastasis and associated phenotypic changes

In some experimental tumor systems, metastatic variants arising from tumorigenic but nonmetastatic cell populations have been shown to express activated *ras* genes not detectable in the parent cell population. In comparing metastatic variant cells with their parental nonmetastatic T-lymphoma cell line, Vousden and Marshall (1984) found that only the metastatic variant cells possessed an activated c-K-*ras* gene. Although activation of cellular *ras* genes may contribute to the natural progression of some tumor cell types, a simple correlation between expression of activated *ras* and promotion of metastasis has not been established for tumor cells in general, however. Studies of *ras* expression and metastatic phenotype in various naturally occurring human tumors have failed to provide direct evidence that the activity of *ras* is necessary for inducing or maintaining metastatic behavior in spontaneous tumors (reviewed by Collard et al., 1988). In certain human and animal tumors, metastatic lesions have been identified which express higher or lower levels of *ras* compared to the original primary tumor (reviewed by Nicolson, 1987). Studies of certain experimental tumor lines such as the mouse melanoma B16 line have revealed that variant low-metastatic and high-metastatic sublines express similar amounts of K-*ras* oncogene mRNA and similar amounts of the activated K-*ras* protein (Kris et al., 1985). The only safe conclusion to be drawn from these studies is that expression of activated *ras* genes cannot be a sole determinant of metastatic behavior in spontaneous tumors.

To more directly study the possible influence of *ras* genes on promoting metastasis, *ras* genes have been introduced into a wide variety of cell types. In several of these studies, tissue culture cells have been shown to be readily transformed through the introduction of activated *ras* genes or by overexpression of normal *ras* genes. In certain cell types, transformation by *ras* has been shown to confer tumorigenic and even metastatic potential on the cells. Thorgeirsson et al. (1985) were the first to show that NIH 3T3 cells transfected with an activated c-H-*ras* gene acquired the ability to metastasize in nude mice. Transformed cells also expressed elevated levels of type IV collagenase activity and could invade human amnion basement membrane. Several other investigators have also confirmed that introduction of an activated *ras* gene into NIH 3T3 cells promotes metastatic ability (Bernstein and Weinberg, 1985; Bondy et al., 1985; Bradley et al., 1986; Egan et al., 1987a, 1989; Greig et al., 1985; Muschel et al., 1985). The ability of transfected *ras* sequences to induce metastasis in freshly isolated, normal cell types has also been tested. NIH 3T3 cells represent an aneuploid cell line which has become adapted to long term propagation in culture. When NIH 3T3 cells are allowed to grow to confluence in culture, spontaneous transformants arise which can undergo density-independent growth. Thus, the NIH 3T3 line may be considered to be already partially transformed and may be predisposed to becoming metastatic. Despite these caveats regarding NIH 3T3 cells, the finding that *ras* can induce metastatic behavior has been reproduced in work with other cell types. Early passage rat diploid fibroblasts transfected with activated *ras* readily form tumors and metastasize in nude mice (Muschel et al., 1985). In this same study, a contact inhibited mouse mammary cell line

transfected with the same activated *ras* gene became tumorigenic but remained nonmetastatic. In in vitro studies, a secondary factor or factors in the genetic background of the recipient cell type appear to be important in addition to the transfected *ras* gene in regulating acquisition of metastatic potential.

Transfection of activated *ras* alone or together with the immortalizing oncogene polyoma large T has also been shown to induce invasive and metastatic behavior in other rat fibroblast cell types (Van Roy et al., 1986). Pozzatti et al. (1986) found that co-transfection of activated H-*ras* with adenovirus type 2 E1a gave rise to a higher frequency of transformation of primary rat embryo fibroblasts than was the case with *ras* transfection alone. Transformed cells expressing both oncogenes were tumorigenic but nonmetastatic, however. Further investigation of the inhibitory effect of E1a on metastasis in this system revealed that cells transfected with *ras* alone showed elevated production of type IV collagenase, while cells cotransfected with *ras* and E1a failed to increase production of type IV collagenase (Garbisa et al., 1987).

Certain tumorigenic cell lines that are not metastatic or have low metastatic potential are also induced to become metastatic after introduction of activated *ras*. Noninvasive and nonmetastatic T-lymphoma cells have been shown to exhibit invasive properties in vitro and metastasis in vivo upon transfection with activated H-*ras* (Collard et al, 1987). The induced invasive and metastatic potentials of the transfected cells correlated with the expression level of the introduced *ras* gene. In this study, levels of mRNA expression were compared for the transformed cells before injection into animals and for the experimental metastatic lesions. Isolated metastases showed similar, increased or

decreased levels of *ras* transcription compared to the cells before injection. Vousden et al. (1986) have reported that mouse mammary carcinoma cells with low metastatic ability exhibited enhanced metastatic capability after transfection with an activated H-*ras* gene. Similar to the finding with *ras*-transfected lymphoma cells, individual metastases arising after inoculation of mice with the transfected carcinoma cells also expressed varying levels of the *ras* transcript and p21. These observations suggest that while high levels of *ras* expression may be necessary for metastasizing cells to grow and escape from the primary tumor site, growth of disseminated cells at secondary, metastatic tumor sites is less dependent on continued high *ras* expression. A rat mammary tumor cell line with low metastatic ability has also been shown to acquire higher metastatic ability after transfection with the v-H-*ras* oncogene (Kyprianou and Isaacs, 1990). In the oncogene transformed cells, any level of $p21^{v\text{-}H\text{-}ras}$ expression was sufficient for the development of metastatic potential. In contrast to findings from other researchers, this study suggests that, at least for some cell types, there is no simple dose-response relationship between the level of *ras* expression and metastatic ability.

A three-step hypothesis has been formulated which describes the events which tumor cells undergo during invasion of extracellular matrix (Liotta, 1986). Malignant cells first attach to matrix components such as laminin or fibronectin via specific cell surface receptors. Once bound to the matrix, tumor cells may secrete hydrolytic enzymes which degrade structural components of the matrix as well as the attachment elements themselves. Proteolytic enzymes present as proenzymes in the matrix may also be activated by the tumor cells. Additionally, normal cells of the host can be induced by

tumor cells to release proteases. The third step of the invasion process entails tumor cell movement into and through the area of the matrix broken down by proteolysis. In addition to the more general studies regarding induction of transformation and metastasis, introduction of *ras* genes into cells has also been shown to result in specific phenotypic changes relating to the three steps of cellular adhesion, motility, and expression of proteolytic activity. Appropriate modulation of such properties could of course contribute to invasive and metastatic ability of cells.

NIH 3T3 cells transformed by various *ras* and non-*ras* oncogenes show a high chemotactic response to laminin compared to control cells (Melchiori et al., 1990). Laminin is a predominant glycoprotein found in basement membranes and plays an important function in the adhesion and interaction of normal and malignant cells with the basement membrane (reviewed by Liotta, 1986; Sobel, 1990). Laminin not only regulates cell adhesion to the basement membrane, but also influences cell growth, morphology, and migration. Both normal and tumor cells bind to laminin via high affinity cell surface receptors specific for laminin. Certain tumor cell types show an increased number of laminin receptors on their surfaces compared to normal cells. Once malignant cells are anchored to the basement membrane via binding to laminin, laminin binding to the cells may then serve to increase the release of type IV collagenase activity from the cells (Turpeenniemi-Hujanen et al., 1986). By treating cells in tissue culture with laminin or laminin fragments, it was found that the stimulation of laminin receptor by ligand binding results in induction of type IV collagenase release from the cells. Pretreatment of cells with an anti-receptor antibody which blocks laminin binding to the receptor blocked the

collagenase-inducing effect of laminin. Type IV collagenase itself specifically digests type IV collagen which is one of the important structural elements of the basement membrane. Thus, completion of the first step of tumor cell invasion of extracellular matrix, that of adhesion to the matrix, appears to directly trigger the release of a protease which can degrade matrix in the second step of the invasion process.

Subsequent to finding that *ras* transfection induced type IV collagenase activity and metastatic ability in NIH 3T3 cells (Thorgeirsson et al., 1985) and in rat embryo fibroblasts (Garbisa et al., 1987), followup studies with the same and additional *ras*-transfected cell types have revealed that the major collagenolytic activity induced by *ras* is that of a 92 kD metalloproteinase (Ballin et al., 1988; Bernhard et al., 1990). This enzyme degrades not only type IV collagen but also gelatin. A 92 kD gelatinase activity has also been identified in motile, normal cell types such as neutrophils and capillary endothelial cells. Type IV collagenase/gelatinase activity appears to contribute directly to the invasive and metastatic abilities of the cells which express it. This has been demonstrated in experiments in which malignant cells are treated with inhibitors of type IV collagenase activity or antibodies specific for the enzyme (Reich et al., 1988; Hoyhtya et al., 1990). Treated cells are inhibited in their abilities to invade basement membrane matrices in vitro and to metastasize in vivo.

NIH 3T3 cells transformed by either cellular or viral activated H-*ras* oncogenes have been shown to be not only metastatic when tested in vivo but also more motile in vitro when compared to control cells (Varani et al., 1986). In this study, both control and *ras*-transformed cells were stimulated by treatment with laminin or fibronectin to attach to

various substrates. Only the transformed cells were stimulated to become motile in response to laminin or fibronectin, however. In another study, v-H-*ras* transfection of a rat prostatic tumor cell line was also reported to induce both metastatic behavior and increased cell motility (Partin et al., 1988). Cell motility was graded by in vitro characterization of membrane ruffling patterns, pseudopodal extension, and cell translocation. Membrane ruffling is defined as high frequency, low amplitude changes in well-defined areas of the cell membrane, while pseudopodal extension is described as low frequency, high amplitude modulation of larger regions of the cell membrane. Highly metastatic tumor cells resulting from *ras* transformation and nontransformed control cells grown in tissue culture were observed microscopically and cell images were recorded using a high resolution video camera. When the recorded images were visually graded by investigators, the highly metastatic cells were seen to exhibit greater degrees of ruffling, extension of pseudopodia and translocation relative to control cells. Both membrane ruffling and pseudopodial extension are believed to play important roles in cell spreading and motility (reviewed by Sawa et al., 1989). The phenomena of ruffling is poorly understood, while pseudopodial extension is thought to be induced by membrane receptor binding to subtratum-linked ligands. In v-K-*ras* transformed cells, $p21^{v\text{-}K\text{-}ras}$ has been found to be concentrated in ruffling areas of the cell membrane (Myrdal and Auersperg, 1985). Signal transduction by p21 may promote modulation of cytoskeletal or membrane structure. Dynamic rearrangements in these elements might then result in cell motility and the appearance of associated phenomena such as ruffling.

An autocrine motility factor initially isolated from the human melanoma cell line A2058 is also produced by NIH 3T3 cells transfected with activated *ras* (Liotta et al., 1986b). Autocrine motility factor is not detected in the conditioned medium of untransfected 3T3 cells. The factor induces random motility and also serves as a chemoattractant for responsive cells. Thus, autocrine motility factor may serve to promote the invasive behavior of individual cells as well as regulate and coordinate the migration of groups of tumor cells.

In addition to eliciting specific phenotypic changes associated with transformation and metastasis, activated *ras* may also exert a more general transforming effect on cells through the induction of genetic instability (reviewed by Nicolson, 1987). A recent study which addressed this hypothesis found that an activated *ras* gene may indeed promote genetic instability in recipient cells (Ichikawa et al., 1990). In this study, nine clonal transfectants were isolated and characterized after a rat mammary tumor cell line was transfected with a plasmid carrying both a neomycin resistance gene and a v-H-*ras* oncogene. Of these nine clones, four were highly metastatic, four displayed low metastatic ability, and all nine exhibited numerous structural chromosomal anomalies and/or changes in chromosome number. All four of the highly metastatic clones showed loss of part of chromosome 1 and a gain in chromosome 4 number. One of the four clones with low metastatic ability showed a similar pattern of chromosome 1 loss and chromosome 4 gain. In contrast, five clonal transfectants isolated and studied after transfection of the same parent cell line with a plasmid containing only the neomycin resistance gene were all nonmetastatic. Only one of these clones had a single structural

chromosomal aberration, and only one clone showed a change in number of a single chromosome type. The loss of portions of chromosome 1 and the gain in chromosome 4 in several of the metastatic clones may reflect a loss of a gene or genes which have metastasis suppressor function and the gain of an oncogene or genes which promote metastasis. Similar alterations in these two chromosomes have been reported for other rat tumors also. The difference in the number of chromosomal alterations seen in the *ras*/neomycin transfectants compared to the number observed for the neomycin-only transfectants is statistically significant and suggests that activated *ras* may promote genetic instability and chromosomal abnormalities.

Ras oncogenes are not the only genes able to induce metastatic behavior. NIH 3T3 cells have been shown to acquire metastatic potential when transfected with the protein kinase oncogenes *mos*, *raf*, *src*, *fes* and *fms* (Egan et al., 1987b). NIH 3T3 cells transformed with either of the two nuclear oncogenes *myc* or mutant p53 were tumorigenic but not metastatic. Microinjection of anti-p21 antibody into cells has been shown to block transformation by *src*, *fes* or *fms* (Smith et al., 1986). This finding suggests that these oncogenes may exert their transforming effects by way of a common *ras*-mediated pathway.

Suppression of *ras* transformation

Work with phenotypic revertants of v-K-*ras* transformed NIH 3T3 cells has led to the identification of a gene which suppresses the transforming properties of activated *ras*. After finding that phenotypic revertants could be induced in a population of Kirsten sarcoma virus transformed 3T3 cells through treatment with a mutagen (Noda et al.,

1983), the virus transformed cells were transfected with a cDNA expression library prepared from normal human fibroblasts (Noda et al., 1989). Recombinant plasmids harboring a putative *ras*-suppressor cDNA were then isolated from the phenotypic revertant lines obtained after transfection with the human DNA fragments. A 1.8 kb cDNA clone termed K*rev*-1 with *ras*-suppressor activity has been isolated by using this strategy (Kitayama et al., 1989). In revertant cell lines or *ras* transformed cell lines transfected with K*rev*, the level of expression of K*rev* transcript has been found to correlate well with the extent of suppression of the *ras*-induced transformation. The K*rev* cDNA codes for a protein of 21 kD which shares some 50% homology with $p21^{K\text{-}ras}$. K*rev* homologs have also been detected in chicken, rat and mouse DNA. K*rev* gene transcripts have been detected in all organs of the rat tested. Because of the high degree of homology between the K*rev* protein and p21, and because the amino acids at positions 32 to 44, the region implicated in the effector function of p21, were noted to be identical between the two proteins, it was hypothesized that the K*rev* protein binds to GAP (Frech et al., 1990). In testing this hypothesis, the K*rev* protein was indeed found to be able to bind to GAP and thereby inhibit the GAP-mediated GTPase activity of p21 (Frech et al., 1990). K*rev* binding to GAP was found to be strictly dependent on the K*rev* protein being bound to GTP. Since normal tissues and cells express the K*rev* gene, K*rev* may function as an antioncogene and serve to prevent transformation of cells by activated *ras* genes. By competitively binding to GAP, K*rev* proteins may modulate normal *ras* function also.

Other studies suggest that there may be additional genes which can suppress transformation by *ras* oncogenes. Samid et al. (1984, 1985) found that long term interferon treatment of NIH 3T3 cells transformed by an activated H-*ras* gene gave rise to persistent phenotypic revertants. These revertants continued to display a nontransformed phenotype even after cessation of treatment with interferon, yet still expressed pre-reversion levels of activated *ras*-encoded p21. Revertants also were resistant to retransformation when infected with various other retroviruses carrying other *ras* oncogenes or the *abl* or *fes* oncogenes. Contente et al. (1990) have recently obtained from one of these persistent revertants a cDNA clone for a gene the expression of which is down-regulated in cells transformed by activated *ras* genes. Northern blot analysis revealed that parental NIH 3T3 cells as well as the persistent revertant lines express the gene, termed *rrg* for "*ras* recision gene", at significant levels. Expression of the gene is markedly decreased in *ras* transformed NIH 3T3 cells as well as in cells transformed by v-*fes* or v-*raf*, however. To further study the properties of the *rrg* gene, persistent revertant cells were transfected with *rrg* linked to an expression vector suitable for the production of antisense mRNA. The antisense colonies obtained from this procedure exhibited a transformed phenotype and were tumorigenic when tested in nude mice. These findings suggest that *rrg* expression may need to be down-regulated in order for activated *ras* to exert a transforming effect on cells.

Metastasis suppressor genes

In addition to the tumor suppressor genes already identified which are able to reverse or inhibit the transformed phenotype, there is also evidence for the existence of metastasis

suppressor genes which can obstruct the development of metastasis (reviewed by Steeg, 1989; Sobel, 1990). When various metastatic cell lines have been fused to nonmetastatic cell types, the resulting hybrid cells often maintain a tumorigenic phenotype but are no longer metastatic (Ramshaw et al., 1983; Layton and Franks, 1986). Metastatic behavior may reappear in these hybrids as chromosomes segregate and are lost during cell division, however. In studies involving fusion of tumorigenic and normal cells, hybrid cells are similarly found to be nontumorigencic but may regain a tumorigenic phenotype when specific chromosomes contributed by the normal parent cells are lost (reviewed by Harris, 1990). By cytological examination of hybrid cells during passage in tissue culture, loss of specific chromosomes can be correlated with reappearance of tumorigenic or metastatic behavior. Chromosomes whose loss corresponds with reexpression of tumorigenicity or metastatic behavior are likely to contain genes which suppress tumorigenic behavior or the metastatic phenotype specifically.

In a cell fusion study where type IV collagenase activity was also looked at, the fusion of metastatic mouse melanoma cells expressing a relatively high collagenase activity with normal mouse fibroblasts gave rise to hybrid cells which were nonmetastatic and exhibited greatly reduced collagenase activity relative to the metastatic parent cell line (Turpeenniemi-Hujanen et al., 1985). A total of six parental cell lines expressing various degrees of metastatic potential and type IV collagenase activity were used to generate eight hydrid clones for further study. In hybrids, the levels of collagenase activity expressed paralleled the metastatic capacities displayed, lending further support for a linkage between type IV collagenase activity and the metastatic phenotype. If the

collagenase gene is closely involved in promoting metastasis, then genes able to inhibit the expression or function of a gene product (i.e., collagenase) which is involved in promoting metastasis would certainly qualify as metastasis suppressor genes. Accordingly, the adenovirus E1a gene able to suppress both collagenase secretion and metastatic behavior in *ras*-transfected rat embryo fibroblast cells (Pozzatti et al., 1986; Garbisa et al., 1987) may be termed a metastasis suppressor gene. The metastasis-suppressing properties of E1a may well be dependent on the genetic background of the cell type being studied, however. E1a transforms a number of cell types, but did not transform rat embryo fibroblasts when introduced into the cells without activated *ras*.

Tissue inhibitor of metalloproteinases (TIMP) glycoproteins are secreted by several cell types and inactivate metalloproteinases such as type IV collagenase by forming complexes with the enzymes. Treatment of mouse melanoma cells with recombinant TIMP has been shown to inhibit cell invasion in vitro and decrease the number of metastases formed in vivo (Schultz et al. 1988). A rat embryo cell line rendered metastatic after transfection with H-*ras* has also been shown to be inhibited in its secretion of collagenase and expression of metastatic potential when treated with recombinant TIMP (Alvarez et al., 1990). TIMP genes may therefore also be considered to be metastasis suppressor genes.

Differential or subtractive hybridization techniques offer a powerful strategy for the identification of candidate metastasis suppressor genes which are downregulated in metastatic cells (Steeg, 1989). Dear et al. (1988) used a subtractive hybridization protocol in the isolation of a cDNA for a previously unidentified gene, WDNM1, which

is expressed at a greater level in nonmetastatic rat mammary adenocarcinoma cells relative to sister metastatic clones. Using a differential hybridization test, Schalken et al. (1988) have found the fibronectin gene to be expressed to a greater degree in nonmetastatic cells derived from the Dunning R-3327 rat prostatic tumor than in metastasizing sublines.

Another gene, NM23, which was originally isolated from the K-1735 murine melanoma cell line may also be associated with regulation of metastasis (Steeg et al., 1988a). In screening several sublines derived from both the K-1735 melanoma and a rat mammary carcinoma, it was found that NM23 RNA levels were high in poorly metastatic cells and decreased in cells with greater metastatic potential. Rat embryo fibroblasts cotransfected with *ras* and E1a or transfected with *ras* alone as described by Pozzatti et al. (1986) have also been tested for expression of NM23 (Steeg et al., 1988b). NM23 RNA levels were 2- to 8-fold higher in the nonmetastatic, *ras*/E1a cotransfected cell lines than in the highly metastatic lines expressing only *ras*. NM23 codes for a 17 kD protein found both in the nucleus and cytoplasm (Rosengard et al., 1989), and shares a 78% homology with the predicted product of the abnormal wing disc gene (*awd*) of *Drosophila melanogaster*. Wallet et al. (1990) have recently reported that two nucleoside diphosphate (NDP) kinases expressed in the slime mold *Dictyostelium discoideum* share a 70-80% homology with the NM23 and *awd* proteins. NDP kinases are important in the generation of nucleoside triphosphates such as GTP, and have been found to be associated with G proteins as well as with microtubules (reviewed by Liotta and Steeg, 1990). NDP kinases might generate the GTP required during G protein signal transduction and during the process of microtubule assembly. If the NM23 protein is indeed a NDP kinase, it

may serve a regulatory function during the activation of G proteins and $p21^{ras}$ (Liotta and Steeg, 1990). Aberrant expression of NM23 might thus be relevant to the function of *ras* in contributing to tumorigenicity and metastasis. Reduced expression of NM23 NDP kinase activity or expression of a mutant NM23 gene might also lead to abnormal microtubule assembly and disassembly which in turn could result in chaotic mitosis and induction of chromosomal abnormalities associated with tumor cells. Further research focusing on NM23 and NDP kinase activity may provide valuable insights regarding the roles of G protein signal transduction and $p21^{ras}$ in the processes of tumor development and metastasis.

Preview to the Experiments

In general terms, the aim of the investigation was to examine the relationship between viral Kirsten *ras* gene expression and the absence or appearance of metastatic behavior in a panel of related cell lines infected with the Kirsten murine sarcoma virus. The opportunity to study these cell lines was presented in 1985 by members of Dr. Roger Avery's research group who were working with Kirsten virus infected derivatives of NIH 3T3 cells. Among the lines derived after Kirsten virus infection of parental NIH 3T3 4E cells were two designated CC1 and R2-1. Dr. Avery's group had characterized the CC1 line as being a relatively high expressor of viral Kirsten *ras* mRNA compared to the R2-1 line and had determined that only the CC1 line was tumorigenic when tested in immunocompetent mice. Further details regarding the derivation and characteristics of these lines are given in the "Materials and Methods" section of this paper. Given the

concurrent reports from other investigators (detailed in a previous section of this introduction) that transfection of activated *ras* sequences into certain nonmetastatic cell types was capable of conferring on these cells a metastatic phenotype when tested in immunocompromised mice, we decided to test Dr. Avery's lines for tumorigenic and metastatic behavior both in immunocompetent and nude mice. Initial testing indicated that CC1 cells were highly metastatic in nude mice while R2-1 cells were essentially nonmetastatic. Furthermore, the metastatic phenotype appeared to correlate with a relatively high level of expression of *ras* mRNA in CC1 cells compared to the other nonmetastatic cells. These findings prompted a more detailed examination of how the viral *ras* gene may be differentially regulated in the metastatic vs. nonmetastatic cells. While a study of *ras* gene regulation at the level of RNA transcription did not prove extremely informative, experimental results were obtained suggesting differential *ras* protein expression among the metastatic and nonmetastatic cell lines studied. Additionally, experimental findings are presented which provide evidence for a mechanism or pathway operating in CC1 cells which may not be directly coupled to the *ras* pathway but which is important in defining the invasive phenotype of these cells.

MATERIALS AND METHODS

Materials

Plastic tissue culture ware was obtained from Costar (Cambridge, MA) and from Nunclon (Denmark). Transwell cell culture chamber inserts were also obtained from Costar. Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Penicillin, streptomycin, trypsin and G418 antibiotic were from GIBCO (Grand Island, NY). Guanidine thiocyanate was from Kodak (Rochester, NY). Oligo(dT)-cellulose and Matrigel were from Collaborative Research (Bedford, MA). Nitrocellulose membrane was from Schleicher and Schuell (Keene, NH). Agarose and restriction endonucleases were from Bethesda Research Laboratories (BRL, Gaithersburg, MD). Formamide, redistilled phenol, chloroform, and Pristane (2, 6, 10, 14-tetramethylpentadecane) were from Aldrich (Milwaukee, WI). Tris (tris(hydroxymethyl)aminomethane), Tween-20 detergent (polyoxyethylene sorbitan monolaurate), Zeta-Probe membrane, horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) antibody, and horseradish peroxidase color development reagent (4-chloro-1-naphthol) were from Bio-Rad (Richmond, CA). Sodium dodecyl sulfate (SDS), sulfo-m-maleidobenzoyl (sulfo-MBS), and Protein G-agarose were from Pierce (Rockford, IL). Pertussis toxin, insulin, and HEPES were from Calbiochem (LaJolla, CA). Anti-pan-*ras* antibody and radionucleotide for nick-translation were from DuPont/New England Nuclear (Boston, MA). Anti-*ras* function

blocking antibody (Y13-259) was from Oncogene Science (Manhasset, NY). Reagents for peptide sythesis were from MilliGen/Biosearch (Burlington, MA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, 5-azacytidine, keyhole limpet hemocyanin (KLH) and all other reagents were from Sigma (St. Louis, MO).

Cell Lines

All tissue culture procedures were conducted using aseptic technique and filter sterilized tissue culture media and solutions. Isolation of transformed and revertant derivatives of a clone of NIH 3T3 cells (designated 4E) infected with Kirsten murine sarcoma/leukemia virus complex (K-MSV/MLV) has been previously described (Morris et al., 1980). Briefly, the morphologically transformed CC1 line was cloned from NIH 3T3 4E cells after low multiplicity of infection with K-MSV/MLV complex containing excess MSV. The phenotypic revertant R2-1 line was selected from CC1 cells which had been mutagenized with N'-methyl-N-nitroso guanidine followed by killing with 5-bromodeoxyuridine/irradiation to eliminate phenotypically transformed cells either able to grow at low serum concentration (1%) or at high cell density. R2-1 cells are phenotypically flat when grown on a plastic substrate and have a morphology similar to the nontransformed parent line NIH 3T3 4E. Upon subcutaneous injection of 10^6 cells into the scapular region of two day old inbred NIH mice, the transformed CC1 cells were originally found to be highly tumorigenic, while the revertant R2-1 line, as well as the parent NIH 3T3 4E line, were nontumorigenic (Morris et al., 1980). The CC1 and R2-1 lines are nonvirus producers, but both have rescuable sarcoma virus upon superinfection

with MLV. Cells were routinely grown in DMEM supplemented with 10% FBS, 100 ug/ml penicillin and 100 units/ml streptomycin. Cells were routinely incubated at 37°C in a 5% CO_2 humidified atmosphere. Cells were removed from tissue culture dishes by brief treatment with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Tyrode's calcium and magnesium-free saline solution (Tyrode's CMF: 8 gm NaCl, 0.2 gm KCl, 0.18 gm NaH_2PO_4, 0.19 gm Na_2HPO_4, 0.5 gm $NaHCO_3$, 1 gm glucose and 2 ml 0.5% phenol red stock solution dissolved in 1 liter sterile distilled water; pH 7.5). Upon release from the plastic substrate, cells were immediately transferred to a sterile capped tube and centrifuged. The supernatant medium was discarded and the cell pellet resuspended in serum-containing medium to inactivate any residual trypsin. Cells were counted using a hemocytometer and viability determined through the use of Trypan blue stain exclusion. Where indicated, R2-1 cells were treated with 3 uM 5-azacytidine in culture medium for seven days, passaged into new tissue culture dishes and grown up in culture medium lacking 5-azacytidine. R2-1 cells treated with 5-azacytidine were designated R2-1(aza-Cyd). NIH 3T3 4E cells were freshly infected in our laboratory using a K-MSV-containing tissue culture cell supernatant medium kindly provided by Dr. R. Avery. NIH 3T3 4E cells were incubated overnight with this supernatant medium, after which time the cells were grown to confluency in regular culture medium and passaged. Cells began to exhibit density-independent growth three passages after treatment with the K-MSV supernatant medium. The freshly infected cells were designated K-NIH-R. NIH 3T3 4E and R2-1 cells were transfected with the pSV2neo plasmid (Southern and Berg, 1982) or with pSV2neo and pEJ plasmid (containing the

Harvey-*ras* gene cloned from a bladder carcinoma line by Shih and Weinberg, 1982) using the technique of electroporation as described by Chu et al. (1987). For this procedure, cells in log (logarithmic) phase of growth were harvested and resuspended in electroporation buffer at a concentration of 3 x 10^6 cells per ml. Electroporation buffer was an aqueous solution of 20 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose. The electroporation buffer also contained pSV2neo plasmid DNA at a concentration of 10 ug/ml, or 10 ug/ml pSV2neo and 10 ug/ml pEJ. In a control preparation, plasmid DNA was omitted from the buffer solution. Aliquots of 0.8 ml of cells in suspension were dispensed into sterile plastic cuvettes and the electroporation electrode fitted into the cuvette. Cells were exposed at room temperature to a voltage pulse of 275 volts using an electroporation device built to the specifications described by Chu et al. (1987). Cells were left in the electroporation buffer for 10 minutes and then plated with the addition of 10 ml of serum-containing medium into tissue culture dishes. Cells were allowed to grow in serum-containing DMEM for three days, after which time the transfected cells were selected for by growth in medium containing 400 ug/ml G418.

Assays for Tumorigenicity and Metastatic Potential

Tumorigenicity and metastatic potential of the cell lines were assayed in mice bred and maintained in the Montana State University Animal Resource Center. To test for tumor incidence and spontaneous metastatic ability, nude BALB/c mice as well as immunocompetent BALB/c mice 9-15 weeks of age were each inoculated with 5 x 10^5 -

1×10^6 cells suspended in 0.2 ml of growth medium. Mice were sacrificed and autopsied four-six weeks later or when moribund. Lung sets were fixed in Bouin's fixative and metastatic colonies counted using a dissecting microscope. Suspect tumor lesions were confirmed by histopathology. Experimental metastatic ability of the cell lines was tested by injecting nude BALB/c mice 9-15 weeks of age with 5×10^4 cells per mouse via the lateral tail vein. Mice were sacrificed after four weeks and lung metastases enumerated as before.

Dot Blot Hybridization

Tissue culture cells, primary tumor tissue and metastatic lung nodules were processed in guanidine thiocyanate solution according to the method of Chirgwin et al. (1979). Cells, fragments of tumor tissue pooled from three mice, or metastatic lung nodules pooled from three mice were homogenized in an aqueous solution of 4 M guanidine thiocyanate containing 25 mM sodium citrate (pH 7.0), 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol, and 0.1% Sigma Antifoam A. One gram of cesium chloride (CsCl) was added for each 2.5 ml of the homogenate. The homogenate was then transferred into Beckman SW50.1 polyallomer centrifuge tubes already containing a 1.2 ml cushion of an aqueous solution of 5.7 M CsCl and 0.1 M EDTA (pH 7.5). The loaded tubes were centrifuged in a Beckman SW50.1 rotor at 36,000 rpm for 12 hours at 20°C. Supernatant fluids were discarded and the RNA pellets each dissolved in 0.5 ml of an aqueous solution of 10 mM Tris (pH 7.4), 5 mM EDTA, and 1% SDS. The dissolved RNA solutions were extracted once with an equal volume of a 4:1 mixture of chloroform

and 1-butanol. RNA was precipitated from solution by addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes 95% ethanol and cooling for two hours at -20°C. RNA was then pelleted by centrifugation. RNA pellets were dissolved in 1 ml of sterile distilled water prior to selection of polyadenylated RNA. Polyadenylated RNA was isolated using an oligo(dT)-cellulose column (Maniatis et al., 1982). Oligo(dT)-cellulose was equilibrated in a sterile aqueous loading buffer of 20 mM Tris (pH 7.6), 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS. A 1 ml column of oligo(dT)-cellulose was poured into a disposable plastic column (Bio-Rad) and the column washed successively with 5 ml sterile distilled water, 5 ml aqueous solution of 0.1 M NaOH and 5 mM EDTA (pH 7.5), 5 ml sterile distilled water, and 5 ml sterile loading buffer. RNA dissolved in sterile distilled water was heated at 65°C for five minutes, mixed with an equal volume of 2 x sterile loading buffer, cooled to room temperature, and applied to the column. Flow-through from the column was collected, heated at 65°C for five minutes, cooled to room temperature, and reapplied to the column. The column was washed with 10 ml loading buffer and then with 5 ml loading buffer containing 0.1 M NaCl. 1 ml fractions of flow-through were collected and the OD_{260} monitored to insure that the later fractions contained no OD_{260} absorbing material. Polyadenylated RNA was eluted from the column by washing with 3 ml of a sterile aqueous solution of 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.05% SDS. The RNA was then precipitated as described above. RNA pellets were dissolved in sterile distilled water and the concentration determined and purity verified by measurements of OD_{260} and OD_{280} (for RNA, an OD_{260} of 1 corresponds approximately to a concentration of 40 ug/ml; a pure preparation of RNA

has an OD_{260}/OD_{280} ratio of about 2.0). Equal quantities of polyadenylated RNA samples were spotted onto nitrocellulose sheets (pretreated with 20 x SSC, standard sodium citrate buffer: 1 x SSC = 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0) using a Bio-Dot microfiltration apparatus (Bio-Rad). Nitrocellulose blots were baked under vacuum for two hours at 80°C and then hybridized to nick-translated probes according to the method of Thomas (1980). K-*ras*, c-myc and v-fes fragments were obtained from restriction enzyme digestion of plasmids KCC7 (Norton and Avery, 1982), c-myc/pBR322 (kindly provided by Michael Cole, Dept. Biochemistry, St. Louis University School of Medicine), and pST4/S_r (American Type Culture Collection), respectively. Plasmids were cut with the appropriate restriction enzymes using buffers and standard protocols provided by the supplier of the restriction enzymes (BRL). Probes were labeled with ^{32}P using BRL's nick translation kit following the manufacturer's instructions. Hybridization was carried out in sealed plastic bags at 42°C for 20 hours with the probes in an aqueous buffer of 50% (volume/volume) formamide, 5 x SSC, 50 mM Na phosphate (pH = 6.5), 250 ug/ml sheared denatured salmon sperm DNA, and 5 x Denhardt's solution (0.02% each bovine serum albumin, Ficoll, and polyvinyl pyrrolidone). After washing, blots were exposed to Kodak X-Omat AR X-ray film at -70°C.

Southern Analysis of Methylation State

High molecular weight DNA was isolated from tissue culture cells using a standard protocol (Maniatis et al., 1982). Cells in log phase of growth were harvested, pelleted by centrifugation, resuspended in TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA) at

a concentration of 10^8 cells per ml, and then lysed by addition of ten volumes of lysis buffer (aqueous solution of 0.5 M EDTA (pH 8.0), 100 ug/ml proteinase K, and 0.5% Sarcosyl). Cells in lysis buffer were incubated in a 50°C water bath for three hours during which time the solutions were mixed gently by inversion every 30 minutes. Samples were deproteinized by repeated extraction three times with an equal volume of buffered phenol (prepared by mixing 80 ml redistilled phenol with 30 ml 50 mM Tris (pH 8.0)/10 mM EDTA and 0.11 gm hydroxyquinoline) followed by repeated extraction three times with chloroform/isoamyl alcohol (mixed 24:1, volume:volume). DNA was precipitated by addition of 0.1 volume 2 M sodium acetate (pH 5.5) and 2.2 volumes 95% ethanol. Precipitated DNA present at the aqueous phase/ethanol interface was spooled onto a sterile glass rod, rinsed with 70% ethanol, and then dissolved overnight in 5 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNase A was added to the DNA solutions to give a final concentration of 10 ug/ml and the solutions incubated at 37°C for one hour. SDS was added to 0.5% and proteinase K to 100 ug/ml. The DNA solutions were incubated at 50°C for one hour and then deproteinized again by repeated extraction with phenol and chloroform/isoamyl alcohol. The DNA was again precipitated and allowed to solubilize in TE buffer at 4°C for two days. DNA concentration was determined by measurement of OD_{260} (for DNA, an OD_{260} of 1 corresponds approximately to a concentration of 50 ug/ml). 20 ug samples of DNA were digested overnight with either HpaII or MspI restriction endonuclease at a concentration of five units of enzyme per ug of DNA. A second overnight treatment with the same concentration of fresh enzyme was then carried out to insure complete digestion of the

DNA samples. Restriction enzymes from different commercial sources were also compared to insure that samples were completely digested. Digested samples were electrophoresed in 1% agarose gels and blotted onto Zeta-Probe membrane by capillary transfer using 0.4 M NaOH. The membrane was hybridized to a 6 kb v-K-*ras* probe from plasmid KCC7 (Norton and Avery, 1982). The probe was labeled with ^{32}P using BRL's nick translation kit. Prehybridization and hybridization buffer consisted of 0.36 M NaCl, 20 mM Na-phosphate (pH 7.7), 2 mM EDTA, 1% SDS, and 0.5% nonfat dried milk. After hybridization at 65°C overnight, the membrane was washed with agitation at room temperature in 2 x SSC/0.1% SDS for 15 minutes, and then washed at 65°C for 15 minutes in 0.5 x SSC/7.5 mM trisodium citrate/1% SDS. After washing, the membrane was exposed to Kodak X-Omat AR X-ray film at -70°C.

Northern Analysis

Total RNA was isolated from tumor tissues using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (1987). Briefly, tumor tissue fragments or lung nodules pooled from three mice were homogenized in 1 ml of denaturing solution (aqueous solution of 4 M quanidine thiocyanate, 25 mM sodium citrate (pH 7), 0.5% Sarcosyl and 0.1 M 2-mercaptoethanol) and then transferred to a sterile 15 ml centrifuge tube. 0.1 ml of 2 M sodium acetate (pH 4), 1 ml of buffered phenol, and 0.4 ml of chloroform/isoamyl alcohol (49:1 mixture) were sequentially added to the denaturing solution homogenate with the solution being vortexed briefly as each reagent was added. The mixture was finally vortexed for 15

seconds and then allowed to cool on ice for 15 minutes. Samples were then centrifuged for 20 minutes at 10,000 x g at 4°C. The aqueous phase containing RNA was transferred to a clean tube in which it was mixed with 1 ml of isopropanol. The tube was incubated at -20°C for two hours to allow the RNA to precipitate. RNA was pelleted by centrifugation at 10,000 x g for 20 minutes and the pellet dissolved in 0.3 ml of denaturing solution. The RNA in solution was transferred to a 1.5 ml microfuge tube and reprecipitated by addition of 0.3 ml isopropanol and cooling for one hour at -20°C. RNA was pelleted by centrifugation in an Eppendorf microcentrifuge for ten minutes, the pellet washed with 75% ethanol, and the RNA then dissolved in 50 ul of an aqueous solution of 0.5% SDS by heating at 65°C for ten minutes. The concentrations of RNA samples were determined by measurement of OD_{260}. 20 ug samples of total cellular RNA were electrophoresed in 1% agarose/formaldehyde gels (Maniatis et al., 1982). Capillary transfer of RNA onto a Zeta-Probe membrane was accomplished using 10 x SSC. Hybridization with a ^{32}P-labeled 1 kb v-K-*ras* probe obtained from plasmid pHiHi-3 (Ellis et al., 1981) was performed at 50°C. Prehybridization/hybridization buffer consisted of an aqueous solution of 60% formamide (volume/volume), 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), 1 mM EDTA, 0.5% nonfat dried milk, and 1% SDS. After hybridization, the membrane was washed and exposed to X-ray film as described for the Southern hybridized membrane.

Assay of Anchorage-Independent Growth

A 5% aqueous solution of agarose was autoclaved and diluted to a 0.5% solution using DMEM containing 10% FBS. A base layer of 9 ml of this 0.5% solution was plated in a 25 cm^2 plastic tissue culture flask. After setting of the base layer, a covering layer of 6 mls of 0.33% agarose containing 1 x 10^5 cells was added. Three flasks were plated for each of the R2-1, R2-1(aza-Cyd), and CC1 cell lines. After equilibrating the flask at 37°C in a 5% CO_2 humidified atmosphere for two hours, the flasks were sealed and incubated for ten days at 37°C. Colonies containing ten or more cells were counted using a microscope and the numbers expressed as the percentage of cells plated which formed colonies. Ten areas of each flask were examined in the counting procedure.

Synthesis of *Ras* Peptide Fragments and Production of Antibody

Three synthetic peptides corresponding to amino acid positions 5 through 17 of $p21^{ras}$ protein species were made with a MilliGen 9020 Peptide Synthesizer (MilliGen/Biosearch). This instrument applies a continuous flow method to the standard solid phase peptide synthesis chemistry originally introduced by Merrifield (1963). Fmoc (7-fluorenylmethoxycarbonyl) amino acid PFP (penta-fluorophenyl) esters were used for synthesis of the peptides. These amino acids have an Fmoc α-amino protecting group and a penta-fluorophenol group esterified at the the α-carboxy position. The support resin used in the reaction column was a polyacrylamide-kieselguhr composite resin precoupled

to the protected carboxy-terminal amino acid of the peptides (Fmoc-Ser-PepSyn-KA, MilliGen/Biosearch).

There are four basic steps making up a peptide synthesis cycle using the Milligen synthesizer. 1) A solution of 20% piperidine in dimethylformamide (DMF) is pumped through the reaction column. Piperidine removes the Fmoc protecting group of the amino-terminal amino acid of the nascent peptide chain. 2) A wash with DMF follows to remove piperidine from the reaction column. 3) A solution of Fmoc amino acid PFP ester and 1-hydroxybenzotriazole (HOBT) dissolved in DMF is introduced into the synthesizer system by injection. This amino acid solution is recirculated through the column. HOBT serves as a catalyst to activate the incoming amino acid for reaction with the amino-terminus of the peptide chain. 4) The column is again washed with DMF to remove any unreacted amino acid. A new synthesis cycle is then initiated by deprotection of the new amino-terminal amino acid.

Assembled peptides were cleaved from the support resin and the side chain protecting groups removed by treatment with 95% trifluoroacetic acid/5% phenol. Free peptide was separated from resin by filtration through a sintered glass funnel. Resin trapped by the filter was washed out 4 x with trifluoroacetic acid. The peptide was concentrated by rotary evaporation and then precipitated by trituration with cold ether. Precipitated peptide was washed 5 x with ether to remove residual trifluoroacetic acid and phenol. The peptide was then dried in a vacuum dessicator.

Homogeneity of the synthesized peptides was confirmed by reverse-phase liquid chromatography (HPLC). The HPLC apparatus consisted of a Beckman model 420

microprocessor, Beckman model 110A pumps, an Altech C18 column, a Hitachi model 100-10 spectrophotometer, and a Hewlett-Packard model 3390A integrator. Wavelength was monitored at 220 nm. The mobile phase solvents used were: buffer A) 0.1% trifluoroacetic acid in water (pH 2.2), and buffer B) 0.1% trifluoroacetic acid, 95% acetonitrile, 5% water (pH 2.2). 1 mg of synthetic peptide was dissolved in 1 ml of buffer A. A 20 ul aliquot of peptide in solution was introduced into the HPLC system by injection. The reverse-phase HPLC program employed a flow rate of 1 ml/minute and a linear gradient running from 6% to 62% solvent B over 30 minutes.

Amino acid ratios for the synthetic peptides were confirmed by amino acid analysis using reverse-phase HPLC (Heinrikson and Meredith, 1984). A standard mixture of amino acids (Pierce Chemical) in 0.1 N HCl was used. The 17 amino acids included in the standard are alanine, aspartic acid, arginine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. A 20 ul sample of the standard containing 50 nanomoles of each amino acid was dried and then dissolved in 100 ul of coupling buffer (coupling buffer: prepared by mixing 1.8 ml acetonitrile, 1.5 ml pyridine, 0.6 ml triethylamine, and 2.1 ml water). This solution was dried in a vacuum dessicator to remove residual HCl which might interfere with the subsequent derivatization with phenylisothiocyanate (PITC). The amino acid residue was again dissolved in 100 ul of coupling buffer. 5 ul of PITC was added to the amino acid solution. After reaction at room temperature for 5 minutes, the resulting phenylthiocarbamyl(PTH)-amino acids were dried using a vacuum dessicator. The dried PTC-amino acid standards were dissolved in 125 ul of 10%

acetonitrile in water. 20 ul samples of the standard solution were analyzed by reverse-phase HPLC. For analysis of synthetic peptides, 1 mg of peptide was hydrolyzed in 6 N HCl *in vacuo* overnight at 110°C. Acid was removed by rotary evaporation. The sample was washed and coupled with PITC as described above for the amino acid standards. The derivitized peptide residue was then dissolved in 250 ul of 10% acetonitrile in water. 5 ul samples of the synthetic peptides were injected for reverse-phase HPLC analysis. For amino acid analysis, the C18 column used was maintained at 52°C by a circulating water bath to enhance resolution of individual amino acids. Wavelength was monitored at 254 nm. The mobile phase solvents used included: buffer A) 0.05 M ammonium acetate in water (pH 6.8), and buffer B) 0.05 M ammonium acetate (pH 6.8), 44% acetonitrile, 10% methanol, and 46% water. The HPLC program directed a flow rate of 1 ml/minute and step-wise gradient shifts of 0% to 15% buffer B over 15 minutes, 15% to 50% buffer B over 15 minutes, and 50% to 100% buffer B over one minute. Comparision of absorbance profiles for the hydrolyzed peptides with those of the amino acid standards and integration of absorbance peaks allowed for determination of amino acid ratios for the synthetic peptides.

Production of the three peptides and a related anti-*ras* antibody followed the strategy used by Clark et al. (1985). As outlined in Figure 3, these peptides differ from each other by the amino acid substituted at position 12. A cysteine residue was incorporated into the peptides between amino acids 16 and 17 to allow for coupling of the peptide to a carrier protein for immunization and raising of antibody. The K-*ras* synthetic peptide was conjugated to KLH (keyhole limpet hemocyanin) by means of a sulfo-MBS (sulfo-m-

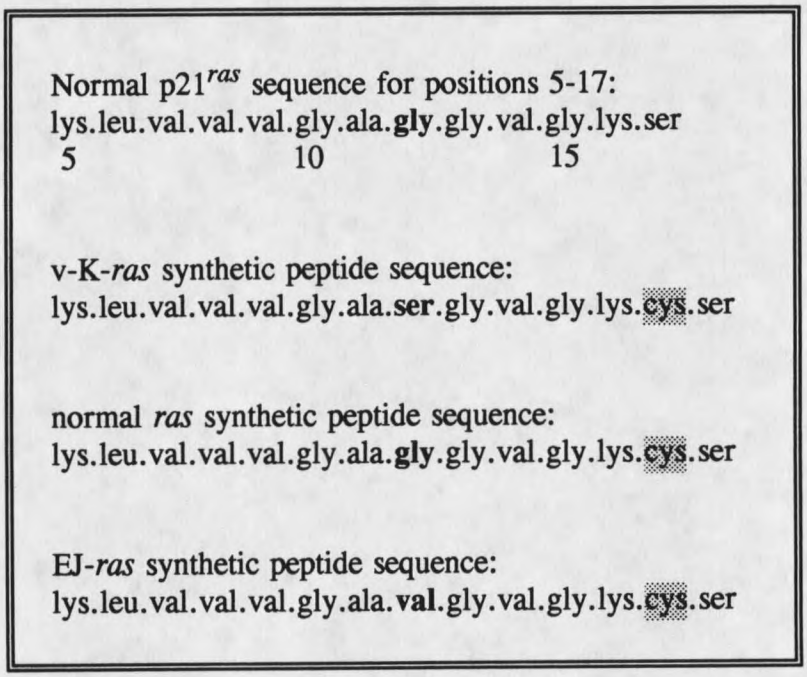

Figure 3. Amino acid sequences of the synthetic peptides. The amino acid sequence of positions 5-17 common to all normal K-*ras*, H-*ras*, and N-*ras* p21 proteins is given at the top of the figure. Substitutions at position 12 of amino acids characteristic of the v-K-*ras* protein and the EJ-*ras* protein compared to the normal $p21^{ras}$ protein are highlighted in boldface type. A cysteine residue (highlighted by shading) was included in the synthetic peptides between amino acids 16 and 17 of the $p21^{ras}$ sequence to allow for coupling of the peptide to a carrier protein for immunization. The figure is modified from Clark et al. (1985).

maleidobenzoyl) linker following the protocol of Lerner et al. (1981). 5 mg of KLH were dissolved in 0.25 ml aqueous solution of 0.05 M inorganic phosphate (pH 8.0). 1 mg of sulfo-MBS linker was added to the solution and the mixture incubated with agitation at room temperature for 30 minutes. The coupled KLH/linker solution was dialyzed against several changes of an aqueous solution of 0.05 M inorganic phosphate (pH 7.25) over three days to remove unreacted sulfo-MBS. The KLH/linker solution (now at pH 7.25) was then reacted with 5 mg of synthetic peptide at room temperature for three hours with agitation. For immunization, the KLH carrier protein/linker/peptide conjugate was diluted in phosphate buffered saline (PBS: aqueous solution of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4; pH 7.4). 50 ug of the conjugate in a volume of 100 ul PBS was mixed with an equal volume of Freund's complete adjuvant and injected intraperitoneally (I.P.) into each of 3 BALB/c mice. Three weeks later, the mice were anesthetized and the spleens injected directly with 20 ug of conjugated peptide in 100 ul PBS (Spitz et al., 1984). Spleens were harvested and processed four days later.

Just prior to fusion of immune spleen cells with myeloma cells, feeder cells were prepared from the thymus gland of one mouse. After removal from the mouse, the thymus gland was dispersed into single cells by passing the gland through a stainless steel wire strainer (70 mesh) using the rubber end of a syringe plunger. Cells were collected in HAT (hypoxanthine/aminopterin/thymidine) medium (DMEM containing 10% FBS, 5 ug/ml insulin, 100 uM hypoxanthine, 0.4 uM aminopterin, and 16 uM thymidine) and the cells pelleted by centrifugation. Supernatant medium was discarded and the cells

resuspended in 10 ml of HAT medium. Cell number and viability were determined using a hemocytometer and Trypan blue stain exclusion. The cell suspension was then diluted with HAT medium to give 1 x 10^5 cells/ml. 100 ul aliquots of the thymus feeder cell suspension were dispensed into the wells of three 96-well plates.

P3X63-Ag8.653 myeloma cells (Kearney et al., 1979), harvested in the log phase of growth and determined to be greater than 95% viable by Trypan blue exclusion, were used for hybridoma production. Spleens were removed from immunized mice and gently passed through a stainless steel wire strainer (70 mesh) using the rubber end of a syringe plunger. Cells were collected in DMEM containing 10% FBS and pelleted by centrifugation. After discarding the supernatant medium, 1 ml of sterile distilled water was added to the pellet and the pellet rapidly dispersed with the aid of a pipette to lyze red blood cells. 10 ml of DMEM/10% FBS was rapidly added to the dispersed cells. After determining the number of viable spleen cells, spleen cells and myeloma cells were mixed at a ratio of 2.5:1 (spleen cells:myeloma cells). Cells were pelleted by centrifugation and the supernatant medium removed. 1 ml of sterile polyethylene glycol (PEG) solution (50% PEG, 45% serum-free DMEM, and 5% dimethylsulfoxide) was slowly added to the mixed cell pellet over a three minute period with the cell pellet being gently dispersed with a pipette tip. 10 ml of serum-containing HAT medium was then slowly added over a 10 minute period. An additional 10 ml of medium was then added over five minutes. The volume was brought to 30 ml with HAT medium. 100 ul of the cell suspension was aliquoted to each well of the three 96-well plates containing thymus feeder cells. Fused cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

On each day of culture, 100 ul of spent medium was removed from each well and replaced with 100 ul of fresh HAT medium. After ten days of growth, the culture medium was changed to HT (hypoxanthine/thymidine) medium (identical to HAT medium, but without aminopterin).

Wells containing actively growing hybridomas were tested for the production of K-*ras* synthetic peptide-reactive antibody. Nitrocellulose membrane was fitted into a Bio-Dot 96 well microfiltration apparatus (Bio-Rad) and wetted with Tris-buffered saline (TBS: 0.02 M Tris, 0.5 M NaCl, pH 7.5). 10 ul aliquots of K-*ras* synthetic peptide/carrier protein (10 ug synthetic peptide/10 ug carrier protein per 10 ul PBS) or 10 ul aliquots of carrier protein alone (10 ug carrier protein per 10 ul of solution) were placed in each well and vacuum applied to allow for binding of protein to the membrane. Each well was washed with 100 ul TBS. To block any membrane sites not containing bound antigen, each well was treated for two hours with 100 ul of 1% nonfat dried milk dissolved in TBS. Blocking solution was removed and each well rinsed 3 x with 100 ul TBS prior to application of hybridoma supernatant fluids to the microfiltration apparatus. The membrane was allowed to incubate with 50 ul aliquots of undiluted hybridoma supernatant fluids for two hours. Supernatant fluids were discarded and each well rinsed 5 x with 100 ul aliquots of TBS over a 30 minute period. 50 ul aliquots of secondary antibody solution (horseradish peroxidase-conjugated goat anti-mouse IgG(H+L), diluted 1:100 in PBS from the commercial stock solution) were then applied to the microfiltration apparatus and left to incubate for one hour. Secondary antibody solution was removed and each well washed once with 100 ul TBS containing 0.05% Tween-20 detergent for

5 minutes and then 4 x with 100 ul aliquots of TBS over a 20 minute period. The membrane was removed from the microfiltration apparatus and placed in a staining tray. Horseradish peroxidase (HRP) color development solution was then applied for visualization of hybridoma antibody-antigen binding results. This solution was prepared by dissolving 60 mg of HRP color development reagent (4 chloro-1-naphthol) in 20 ml ice cold methanol (a), adding 60 ul of ice cold 30% stock H_2O_2 to 100 ml TBS (b), and then mixing (a) and (b) together. Hybridomas producing antibody binding to K-*ras* synthetic peptide/carrier protein but not to carrier protein alone were expanded in culture after cloning by limiting dilution (Galfre and Milstein, 1981). In this procedure, all hybridoma cells of a given well shown to contain synthetic peptide-reactive antibody were transferred to a sterile tube containing 1 ml HT medium. A viable cell count was made using the Trypan blue stain exclusion method and the cells diluted with HT medium to a concentration of 8 cells per ml. 100 ul aliquots of this dilution of cells were then plated in the wells of 96-well plates. By plating an average of 0.8 cells per well, Poisson statistics gives a probability that 36% of the wells will contain one cell per well. Hybridoma cells were then incubated as described previously until wells showing active growth were about 50% confluent. Hybridoma supernatant fluids were again tested for production of synthetic peptide-specific antibody. Another cycle of cloning and testing was performed for antibody-producing subclones to yield stable hybridoma lines.

Production of anti-K-*ras* synthetic peptide monoclonal antibody was accomplished by induction of ascites tumors. BALB/c mice were primed by I.P. injection of 0.5 ml Pristane. Ten days after Pristane injection, mice were injected I.P. with 5 x 10^6

hybridoma cells in a volume of 0.5 ml PBS. Three mice were injected for each of three hybridoma lines used. At about 10 to 14 days after injection of hybridoma cells, or when mice were displaying swollen abdomens and were moribund, mice were sacrificed and ascites fluids collected. Fluids were incubated for two hours at 4°C to allow clot formation and then centrifuged at 5,000 x g for 15 minutes. Supernatant fluids were collected and stored at -20°C.

Monoclonal antibodies were purified from ascites supernatant fluid through the use of a Protein G-agarose affinity column. A 2 ml column of protein G-agarose was poured into a disposable plastic column and the column equilibrated with 10 ml of PBS (pH 7.4). Ascites fluid was diluted with 3 volumes of PBS and applied to the Protein G-agarose column. Unbound material was washed from the column with 30 ml of PBS. Monoclonal antibody was eluted from the column using a low pH glycine buffer (aqueous solution of 0.05 M glycine and 0.15 M NaCl, titrated to pH 2.3 with HCl). 1 ml fractions were collected and OD_{280} monitored using a UV flow-through detector. Fractions containing antibody were pooled and the pH neutralized by addition of an aqueous solution of 0.5 M inorganic phosphate (pH 7.7). Eluted antibody fractions were further tested for antigen binding specificity as outlined above, except that testing against normal *ras* synthetic peptide and EJ *ras* synthetic peptide was also performed.

$p21^{ras}$ Profiles of the Cell Lines

Anti-Kirsten *ras* synthetic peptide antibody described above, as well as anti-pan-*ras* antibody (Dupont NEI-703) and *ras* function blocking antibody Y13-259, obtained from

commercial suppliers, were used to examine $p21^{ras}$ protein expression levels and function in the cell lines under study. To allow monitoring of cell labeling using a Becton Dickinson FACS 440 fluorescence activated cell sorter, antibodies were first conjugated with fluorescein isothiocyanate (FITC) (Mishell and Shiigi, 1980). In this procedure, a 1 ml aliquot of antibody solution was dialyzed against 0.15 M NaCl at 4°C for two days. Dialysis solution was changed three times during this period. Antibody was next dialyzed against 0.05 M bicarbonate-buffered saline, pH 8.5 (prepared by mixing 10 ml of 0.5 M $NaHCO_3$ in 0.15 M NaCl with 5.8 ml of 0.5 M Na_2CO_3 in 0.15 M NaCl, adding 6 M HCl to adjust the pH, and then diluting the solution 1:10 with 0.15 M NaCl), for five hours, and then against bicarbonate-buffered saline, pH 9.2, for two hours. FITC was dissolved in 20 ml of bicarbonate-buffered saline (pH 9.2) at a concentration of 100 ug/ml, and the antibody solution dialyzed against the FITC/bicarbonate solution for 16 hours at 4°C. The reaction was stopped by changing the dialysis buffer to 0.2 M PBS (pH 7). Antibody solution was dialyzed against this solution for three hours at 4°C. FITC-conjugated antibodies were introduced into live cells by electroporation following the DNA transfection protocol of Chu et al. (1987) as modified by Berglund and Starkey (1989). In the modified procedure, cells were resuspended in an electroporation buffer of Hank's balanced saline solution (0.14 gm $CaCl_2$, 0.4 gm KCl, 0.06 gm KH_2PO_4, 0.1 gm $MgCl_2$-$6H_2O$, 0.1 gm $MgSO_4$-$7H_2O$, 8 gm NaCl, 0.09 gm Na_2HPO_4-$7H_2O$, 0.35 gm $NaHCO_3$, 1 gm glucose, and 2 ml 0.5% phenol red stock solution dissolved in 1 liter sterile distilled water; pH 7.5) at a concentration not exceeding 3×10^6 cells per ml. 0.7 ml of cells in suspension were transferred to a plastic cuvette and from 10-100 ul of

FITC-conjugated antibody solution added and mixed in with the cells in the cuvette. Saturating antibody concentrations were used in order to yield maximal cell fluorescent signals when monitored with the FACS. The electrode of the electroporation apparatus was fitted onto the cuvette and a voltage pulse of 275 volts applied to the solution. Electroporated cells were left to incubate at room temperature for ten minutes. Electroporated cells were then transferred to a centrifuge tube containing 2 ml DMEM/10% FBS and pelleted by centrifugation at 1,000 rpm for five minutes. Cells were resuspended in DMEM prior to analysis with FACS. In experiments testing for the presence of stable subpopulations of cells within a given cell line, subpopulations of a cell line were sorted using the FACS on the basis of low and high fluorescence levels measured after electroporation with anti-*ras* antibody. These "bright" and "dim" subpopulations, so named on the basis of FACS-measured fluorescence, were then grown up in tissue culture and the expanded populations retested for anti-*ras* antibody binding using the FACS. In testing for antibody binding capacity of fixed cells, freshly harvested cells pelleted by centrifugation were resuspended in 70% ethanol and incubated at room temperature for 30 minutes. Fixed cells were then pelleted by centrifugation and resuspended in Hank's balanced saline solution at 3×10^6 cells per ml. Antibody at saturating concentration was added to the fixed cells in suspension and the cells incubated for ten minutes. Cells were pelleted by centrifugation, the supernatant fluid discarded, and cells resuspended in DMEM prior to analysis by FACS.

In Vitro Assay of Tumor Cell Invasion

Transwell cell culture chamber inserts (Costar) resting in 24 well cluster dishes (Nunc) were used to evaluate tumor cell line invasion in vitro. Transwell inserts with polycarbonate membranes having 8 um pores were used. In an initial experiment, a commercial preparation of extracellular basement membrane matrix (Matrigel) was diluted 1:20 with serum-free DMEM. In subsequent experiments, the stock solution of Matrigel was diluted 1:20 with a 1 mg/ml solution of type IV collagen isolated from human placenta. The polycarbonate membranes of each Transwell chamber were coated with extracellular matrix by dispensing 100 ul aliquots of the diluted Matrigel solution into the chambers and then allowing the solution to dry by evaporation overnight in a laminar flow hood. Just prior to use, the extracellular matrix was rehydrated by addition of 100 ul of serum-free DMEM to the Transwell chamber insert. Rehydration was allowed to proceed for a minimum of two hours before the Transwell chamber was used. For each experiment, 5×10^4 cells of a given cell line or treatment group were plated in each of four Transwell chambers. Cells were plated in a volume of 200 ul of DMEM containing 10% FBS per Transwell chamber. Each of the cluster plate wells holding the individual Transwell chamber inserts contained 800 ul of growth medium. Medium in both the Transwell chamber inserts as well as in the cluster dish wells was changed daily. Dishes were examined microscopically on a daily basis to allow enumeration of cells that had invaded through the extracellular matrix/polycarbonate membrane layer of the Transwell insert and come to attach to and grow on the cluster dish well bottoms. In the experiment

examining the effect of the function blocking antibody Y13-259 on cell invasion, CC1 cells were first electroporated in the presence or absence of the antibody. Electroporated cells were washed in serum-containing DMEM to remove unbound antibody. Washed cells were then plated in the Transwell chamber inserts. In the experiment examining the effect of pertussis toxin on cell invasion, CC1 or B16BL6 cells in log growth phase were removed from plastic substrate by treatment with trypsin and EDTA and replated at a density of 10^6 cells per ml in serum-containing medium. After a recovery period of one hour, cells were then preincubated for four hours in growth medium containing 1 ug/ml pertussis toxin. Cells were removed from the plastic substrate and washed with fresh growth medium to remove excess pertussis toxin. Washed cells were then plated in the Transwell chamber inserts.

RESULTS

Tumorigenicity and Metastatic Behavior of the Cell Lines

NIH 3T3 4E cells were nontumorigenic and nonmetastatic when tested in either immunocompetent or nude mice (Table 1). Infection of the NIH 3T3 4E cell line with K-MSV in our laboratory gave rise to cells (designated K-NIH-R) which were highly tumorigenic in both normal and nude mice. While not metastatic in normal mice, K-NIH-R cells did display spontaneous metastatic ability in nude mice. The CC1 line was highly tumorigenic in both normal and nude mice. CC1 cells did not metastasize when tested in immunocompetent mice, but did give rise to spontaneous lung metastases in 100% of the nude mice tested. In an assay for experimental metastatic ability in which cells were injected intravenously into nude mice, the CC1 line gave rise to lung metastases in only three of ten mice tested. The revertant R2-1 line was nontumorigenic when tested in normal immunocompetent mice. R2-1 cells were highly tumorigenic but essentially nonmetastatic in nude mice. R2-1 cells gave rise to experimental lung metastases in only one of ten mice tested. R2-1(aza-Cyd) cells gave rise to a primary tumor in only one of five normal mice tested. No spontaneous metastatic ability was noted for these cells when tested in normal mice. However, since only one tumor-bearing mouse was obtained, the possibility of a low spontaneous metastatic potential could not be ruled out. R2-1(aza-Cyd) cells were highly tumorigenic but nonmetastatic when introduced into nude mice.

Table 1. Tumorigenicity and metastatic behavior of the cell lines

Cell line	Tumor incidence in normal BALB/c mice	Tumor incidence in nude BALB/c mice	Spontaneous metastases in nude BALB/c mice	Experimental metastases in nude BALB/c mice
NIH 3T3 4E	0/5	0/5	-	-
CC1	8/8	9/9	9/9	3/10
R2-1	0/5	8/10	-	1/10
R2-1 (aza-Cyd)	1/5	5/5	-	NT
K-NIH-R	3/4	4/4	3/4	NT

Note: No spontaneous metastases were detected in any of the normal, immunocompetent mice tested.

For tests of tumorigenicity and spontaneous metastasis, 5×10^5 - 1×10^6 cells were injected subcutaneously into the flanks of mice. Mice were sacrificed 4-6 weeks later when moribund, and lung sets were examined for metastases. For testing of experimental metastasis, 5×10^4 cells were introduced into mice via lateral tail vein injection.
Explanation of symbols:
"-" denotes absence of metastases in all animals tested;
"NT" denotes not tested.

Dot Blot Hybridization Analysis of Oncogene Expression

Polyadenylated RNA samples isolated from tissue culture cells and from tumor tissues were subjected to a dot blot hybridization analysis using cDNA probes specific for the v-K-*ras*, *myc*, and *fes* oncogenes. As can be seen in Table 2, where the results of this hybridization analysis are presented, there appeared to be no correlation between *fes* or *myc* mRNA expression levels and malignant behavior of the cell lines. A relatively high level of v-K-*ras* mRNA expression was noted in primary tumor tissue resulting from subcutaneous inoculation of CC1 cells. Lower relative levels of *ras* mRNA were detected in CC1 cells grown in tissue culture and in R2-1 subcutaneous tumor tissue. No *ras* mRNA expression was detected in CC1 metastases isolated from lung tissue or in tissue culture cells of the NIH 3T3 4E and R2-1 cell lines. These results suggested a positive correlation between the level of *ras* mRNA expression and metastatic potential since the highest level of *ras* expression seen occurred in the CC1 primary tumor tissue which gave rise to metastases. Although high *ras* expression may contribute to a cell population's ability to traverse the early stages of metastasis, continued high expression of *ras* does not appear to be necessary for growth of CC1 cells once they are established at the secondary tumor site.

Table 2. Oncogene expression in the cell lines and tumor tissues

	Cell line and RNA source					
	NIH 3T3 4E	CC1			R2-1	
Oncogene probe	tissue culture cells	tissue culture cells	subcuta-neous tumor	lung metastases	tissue culture cells	subcuta-neous tumor
v-*fes*	+++	++	+	+	0	0
c-*myc*	+	+	+	0	0	0
v-K-*ras*	0	++	+++	0	0	+

Polyadenylated RNA bound to nitrocellulose sheets was hybridized to ^{32}P nick-translated oncogene probes as described in "Methods". Relative levels are only comparable within data for each separate probe.
"0" denotes no hybridization detected under the conditions used.

Methylation State of the v-K-*ras* Sequences

DNAs isolated from CC1 and R2-1 cells were digested with the restriction enzymes HpaII and MspI and subjected to Southern blot probing with v-K-*ras* cDNA following an experimental design widely used to compare methylation states of DNA from different cell types. Results of this analysis (Figure 4) indicated that for at least one HpaII/MspI restriction site contained within the v-K-*ras* sequences of the two cell lines, the CC1 v-K-*ras* sequence is hypomethylated relative to the R2-1 sequence. The isoschizomeric enzymes HpaII and MspI recognize the double-stranded, palindromic DNA sequence CCGG and will cleave both strands of the DNA molecule at this site between the two C residues. HpaII will not digest DNA at this site if the internal C residue is methylated, however. Similarly, MspI will not cleave DNA if the external C residue is methylated. In Figure 4, there exist bands of approximately 0.47 kbp length for MspI digestion of R2-1 DNA, and HpaII or MspI digestion of CC1 DNA. The absence of a band of this size for HpaII digested R2-1 DNA indicates that one HpaII/MspI recognition site within the v-K-*ras* sequence of R2-1 cells has its internal C residue methylated. The corresponding site within the CC1 v-K-*ras* sequence must be unmethylated since it is cleaved by HpaII treatment to give rise to the 0.47 kbp band. Such demethylation may be related to the higher *ras* transcript level seen in CC1 cells (Table 2). The presence of a 0.68 kbp band for both R2-1 and CC1 DNAs digested with MspI, but the absence of such a band for both DNAs treated with HpaII argues for another HpaII/MspI site being methylated at the internal C residue in both cell lines.

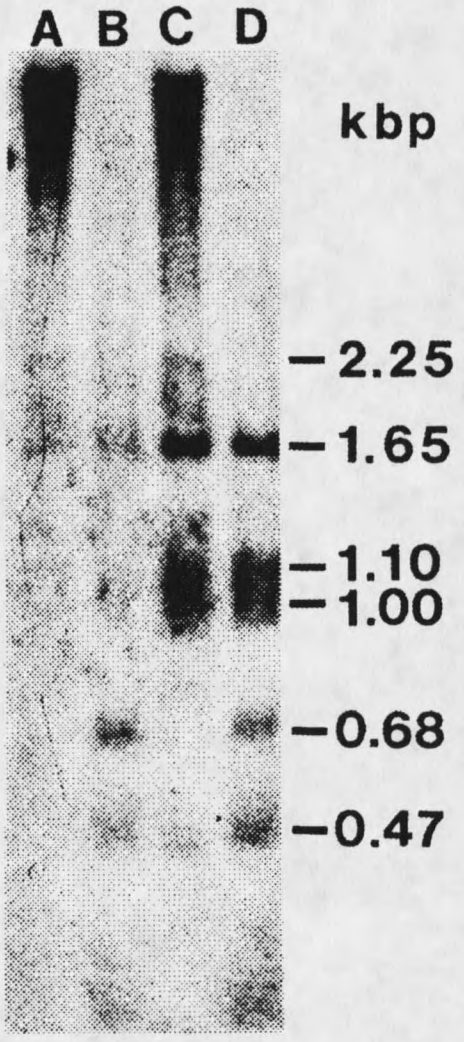

Figure 4. Southern hybridization analysis of tissue culture DNAs. 20 ug samples of DNA were digested with HpaII or MspI restriction endonuclease, electrophoresed in an agarose gel, blotted onto Zeta-Probe membrane, and subjected to Southern hybridization analysis using the KCC7 v-K-*ras* cDNA probe.
Lane A: R2-1 DNA digested with HpaII
Lane B: R2-1 DNA digested with MspI
Lane C: CC1 DNA digested with HpaII
Lane D: CC1 DNA digested with MspI

R2-1 cells were treated with the demethylating agent 5-azacytidine to see whether increased *ras* transcription would result. Compared to untreated cells, 5-azacytidine-treated R2-1 cells [designated R2-1(aza-Cyd)] showed a more rounded and density-independent phenotype as they grew on plastic substrate. R2-1(aza-Cyd) cells also showed a greatly increased ability to proliferate and form colonies when embedded in agarose (Table 3). Both of these observations are indicative of a shift towards a more transformed and potentially malignant phenotype. The R2-1(aza-Cyd) cells were tested for tumorigenic and metastatic potential in both immunocompetent and nude mice. Although the R2-1(aza-Cyd) cells formed primary tumors in one of five immunocompetent mice and five of five nude mice tested, metastatic behavior was not induced (Table 1). RNAs were isolated from the primary tumor tissue resulting from injection of R2-1(aza-Cyd) cells as well as from R2-1 primary tumor tissue, CC1 primary tumor tissue, and CC1 metastatic tissue. The results of these tumor tissue RNAs being tested by Northern blot analysis using a v-K-*ras* cDNA probe are shown in Figure 5. R2-1(aza-Cyd) tumor tissue displayed a marked increase in *ras* mRNA expression compared to that seen for the R2-1 tumor tissue. The level of expression of *ras* mRNA in R2-1(aza-Cyd) tumor tissue was comparable to the level observed for CC1 primary tumor tissue. The increase in *ras* message expression was not accompanied by an induction of metastatic behavior in R2-1 (aza-Cyd) cells.

Table 3. Anchorage-independent growth of the cell lines

Cell line	Colony formation in soft agar (± S.D.)
CC1	52.6% (± 1.8)
R2-1	18.2% (± 1.1)
R2-1(aza-Cyd)	64.0% (± 1.2)

Tumor cells were plated in agarose suspension and incubated at 37°C for ten days. Three flasks were plated for each cell line. After ten days of incubation, cultures were examined under a microscope and anchorage-independent growth expressed in terms of the percentage of cells initially plated which formed colonies.
"S.D." denotes standard deviation.

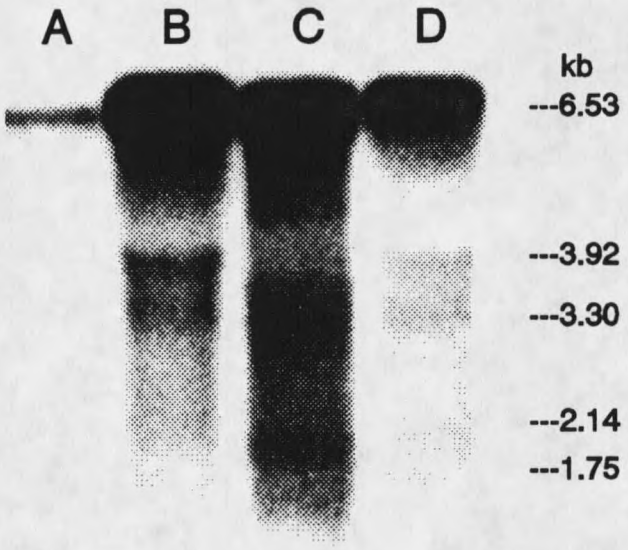

Figure 5. Northern analysis of tumor tissue RNAs. 20 ug samples of total cellular RNA were electrophoresed in an agarose/formaldehyde gel, blotted onto Zeta-Probe membrane, and subjected to Northern hybridization analysis using the pHiHi3 v-K-*ras* cDNA probe.
Lane A: R2-1 cell line, primary tumor tissue
Lane B: R2-1 (aza-Cyd) cell line, primary tumor tissue
Lane C: CC1 cell line, primary tumor tissue
Lane D: CC1 cell line, metastatic tissue from lung

Introduction of an Activated Cellular *Ras* Gene into NIH 3T3 4E and R2-1 Cells

The plasmid pEJ containing an activated cellular H-*ras* gene was introduced into R2-1 cells via the technique of electroporation to test whether the combined expression of two oncogenic *ras* genes (i.e., the endogenous v-K-*ras* gene plus the newly introduced EJ *ras* gene) would induce metastatic behavior in R2-1 cells. pEJ was also introduced into NIH 3T3 4E cells as a control. As discussed earlier, NIH 3T3 cells have been previously shown by other researchers to become metastatic when activated *ras* genes are introduced into them by the calcium phosphate transfection protocol. To allow for G418 antibiotic selection of pEJ-electroporated cells, pSV2neo plasmid was introduced into cells along with the pEJ plasmid. Transfection with the EJ *ras* gene had no noticeable effect on the tumor forming capability or nonmetastatic phenotype of R2-1 cells (Table 4). NIH 3T3 4E cells transfected with the neo plasmid alone or with neo and EJ plasmids and then introduced into nude mice eventually formed tumors after a four month latency period. NIH 3T3 4E cells treated with both the neo and EJ plasmids, but not cells treated with neo plasmid alone, acquired a more rounded shape and were more density-independent in their growth pattern in vitro (Figure 6). Thus, it appeared that the EJ *ras* gene did transform NIH 3T3 4E cells to some degree although a highly tumorigenic phenotype or metastatic phenotype was not induced.

Table 4. Tumorigenicity of NIH 3T3 4E and R2-1 cell lines transfected with pSV2neo or pSV2neo and pEJ

Cell line	Tumorigenicity in nude BALB/c mice	Average latency period (days) $\pm$ S.D.	Tumorigenicity in immunocompetent BALB/c mice
NIH 3T3 4E(neo)	5/9	134.0 $\pm$ 7.6	NT
NIH 3T3 4E (neo/EJ)	9/9	110.8 $\pm$ 19.1	NT
R2-1(neo)	8/8	21.0 $\pm$ 3.2	6/10 *
R2-1(neo/EJ)	8/8	24.3 $\pm$ 3.8	6/10 **

Note: No metastases were detected in any of the mice tested.

Mice were injected subcutaneously in the flank with 5 x 10^5 cells. Mice were sacrificed when moribund, and lung sets examined for metastases.
Explanation of symbols:
"neo" denotes transfection with pSV2neo plasmid.
"neo/EJ" denotes transfection with both pSV2neo and pEJ plasmids.
"S.D." denotes standard deviation.
"NT" denotes not tested.
"*": 6/10 mice tested developed small tumors no greater than 1 cm^3 in volume within two weeks after inoculation with R2-1(neo) cells. Tumors in all six of these mice regressed to the point of not being palpable within six weeks after inoculation.
"**": 6/10 mice tested developed small tumors no greater than 1 cm^3 in volume within two weeks after inoculation with R2-1(neo/EJ) cells. Tumors in all six of these mice regressed to the point of not being palpable within six weeks after inoculation.

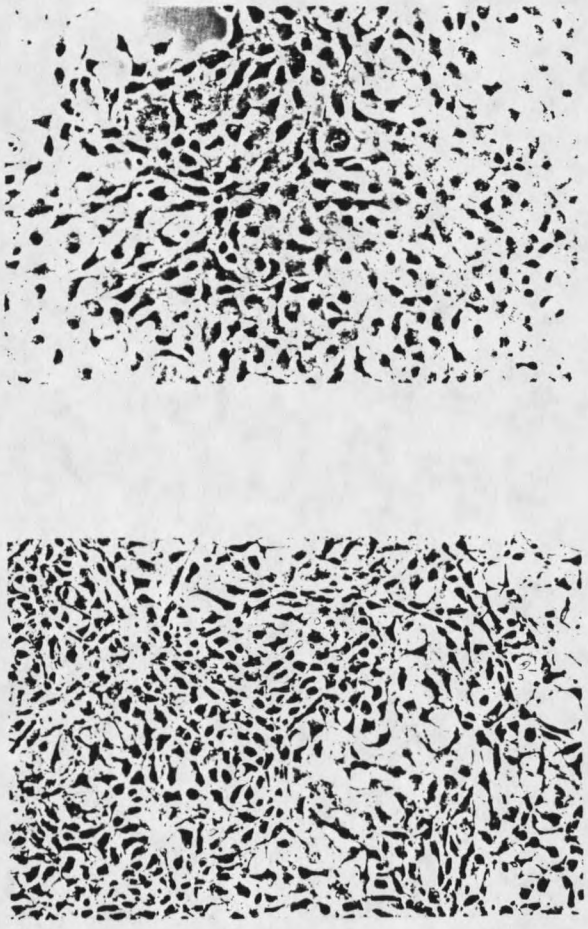

Figure 6. NIH 3T3 4E cells electroporated with pSV2neo plasmid alone, or with both pSV2neo and pEJ plasmids. Cells transfected with pSV2neo alone (photomicrograph, top) show a flattened morphology on plastic substrate similar to that of untransfected NIH 3T3 4E cells. Cells transfected with pEJ (photomicrograph, bottom) show a more rounded morphology and are more density-independent in their growth than are untransfected cells or cells transfected with pSV2neo alone. Magnification of both photomicrographs is 150x.

p21 Profiles of the Cell Lines Determined Using Anti-*ras* Antibodies and FACS

Cell lines were electroporated in the presence of *ras* protein-specific antibody tagged with a fluorescent label, and the cells analyzed using the FACS. The histograms obtained from a set of experiments employing FACS and anti-pan *ras* antibody labeling (Figure 7) indicated bimodal patterns for anti-*ras* antibody binding capacity for the cell lines under study. These data suggested that the cell lines might contain distinct subpopulations of cells expressing different levels of p21 protein able to interact with the antibody. Accordingly, subpopulations of R2-1 as well as R2-1(aza-Cyd) cells were sorted on the basis of high and low fluorescence levels after electroporation with anti-*ras* antibody. These subpopulations, designated "bright" and "dim" on the basis of FACS-measured fluorescence, were then grown up for one passage in tissue culture and the expanded populations retested for anti-*ras* antibody binding using the FACS. In two trials of this experiment, the expanded "bright" and "dim" subpopulations of either cell line showed similar unimodal patterns of antibody binding (Figure 8). This finding indicated that subpopulations of cells sorted on the basis of anti-*ras* antibody binding were not stable with regard to maintenance of a low or high antibody binding capability. In expanding each of the "bright" and "dim" subpopulations during one passage in tissue culture, low and high anti-*ras* antibody binding subpopulations were rapidly regenerated. For either cell line, the fluorescence profiles of the populations resulting from expansion of "bright" and "dim" subpopulations were indistinguishable from each other. Sorting, expansion, and retesting of "bright" and "dim" subpopulations of the CC1 line were performed with

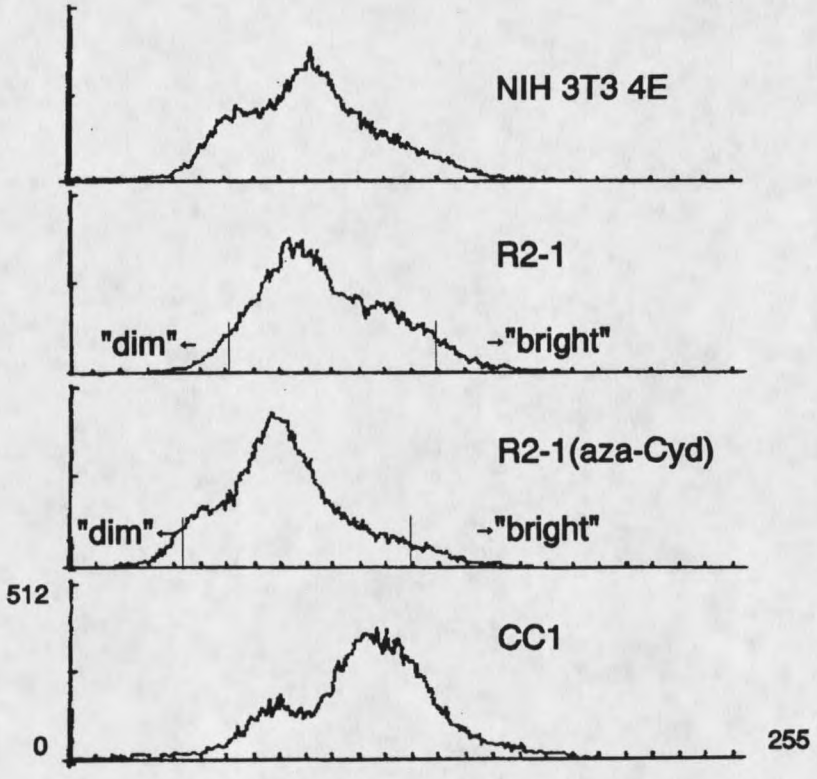

Figure 7. FACS analysis of p21 content of the cell lines. FITC-conjugated anti-pan-*ras* antibody was introduced into cells by electroporation. Labeled cells were then subjected to FACS analysis. Brightly fluorescent and dimly fluorescent subpopulations of the R2-1 and R2-1 (aza-Cyd) cell lines were sorted and expanded in culture for further analysis (see Figure 8). The horizontal axis denotes log of fluorescence intensity. The vertical axis represents number of events, or cell number.

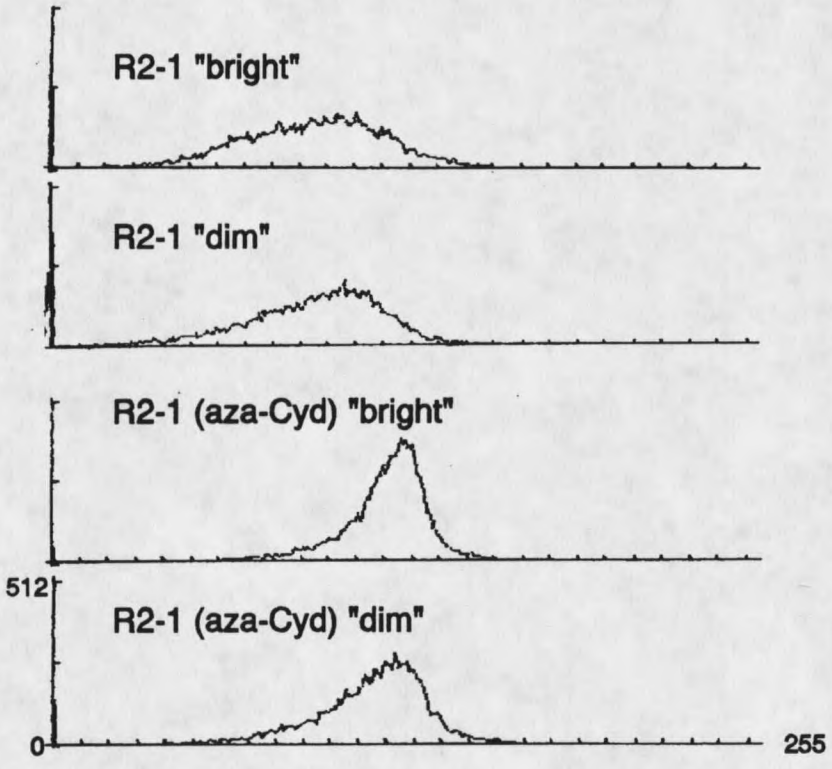

Figure 8. FACS analysis of brightly fluorescent and dimly fluorescent subpopulations of the R2-1 and R2-1(aza-Cyd) cell lines after expansion in tissue culture. The cell lines were first labeled with FITC-conjugated anti-pan-*ras* antibody using electroporation and then sorted using FACS into "bright" and "dim" subpopulations on the basis of fluorescence intensity (see Figure 7). The subpopulations were then expanded by one passage in tissue culture, labeled with conjugated antibody, and retested for fluorescence profiles. The horizontal axis denotes log of fluorescence intensity. The vertical axis represents number of events, or cell number.

similar results. The instability of "bright" and "dim" subpopulations sorted from these cell lines contrasts with the findings obtained from similar experiments performed using the B16BL6 mouse melanoma cell line. B16BL6 cells grown to a post-confluent state on a plastic substrate and then sorted into "bright" and "dim" subpopulations based on FACS monitoring of anti-pan-*ras* antibody binding yield subpopulations of cells which are stable with regard to high and low antibody binding capacity for at least six passsages in culture (Berglund and Starkey, 1989). Stable subpoplations of "bright" and "dim" cells could not be obtained when B16BL6 cells were used after harvest in a pre-confluent growth state since these cells showed a unimodal staining pattern with anti-*ras* antibodies. R2-1, R2-1 (aza-Cyd) and CC1 cells showed bimodal staining patterns and did not yield stable "bright" and "dim" subpopulations regardless of whether they were harvested in pre-confluent, just confluent, or post-confluent growth states.

To more specifically examine the role of the activated viral Kirsten *ras* protein in the malignant behavior of the cell lines, a synthetic peptide fragment corresponding to amino acid positions 5 through 17 of the viral Kirsten *ras* p21 protein was made and used to generate a mouse monoclonal antibody. This domain of the p21 molecule is involved in GDP/GTP binding. The oncogenic Kirsten virus p21 protein differs from the normal *ras* p21 by the substitution of a serine residue for Gly^{12}. The Kirsten virus p21 fragment-specific antibody was tagged with FITC and introduced into the cell lines by electroporation. Histograms obtained from FACS analysis of the cell populations labeled with the Kirsten *ras*-specific antibody are presented in Figure 9. CC1 cells exhibited greater fluorescence than both the R2-1 and R2-1(aza-Cyd) cell lines, which in turn

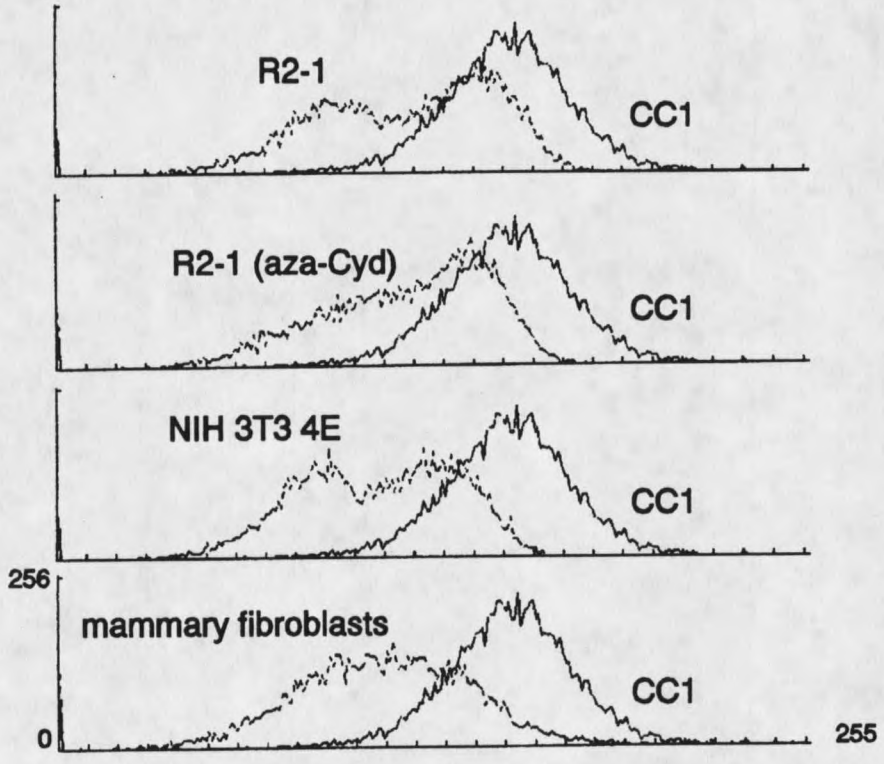

Figure 9. FACS analysis of p21 content of the cell lines. FITC-conjugated anti-K-*ras* antibody was introduced into cells by electroporation. Labeled cells were subjected to FACS analysis. The horizontal axis denotes log of fluorescence intensity. The vertical axis represents number of events, or cell number.

displayed greater fluorescence than either NIH 3T3 4E cells or mouse mammary fibroblasts. In prescreening of the monoclonal antibody, it was found that although the antibody bound avidly to the Kirsten *ras* synthetic peptide fragment immobilized on nitrocellulose paper, the antibody also displayed a much weaker non-specific binding to equivalent peptide fragments containing either a glycine or a valine residue at position 12. Thus, it is to be expected that the antibody might show some non-specific binding of non-Kirsten *ras* p21s when the NIH 3T3 4E cells or other cell types also lacking the Kirsten virus *ras* gene were subjected to FACS analysis.

Cells were fixed with 70% ethanol and then labeled with either the anti-pan-*ras* antibody or the anti-Kirsten-*ras* antibody. Unimodal FACS fluorescence profiles were obtained for these fixed preparations of the cell lines regardless of the antibody used (Figure 10). This indicated single populations of cells not having readily distinguishable "bright" and "dim" subpopulations. The mean fluorescence levels for these fixed populations were higher than those of the viable cell populations, indicating that fixed cells had a greater number of p21 molecules available for binding to antibody than did live cells in which a certain proportion of the p21 molecules were unavailable for binding to antibody. The fixed cells of all the cell lines studied also showed similar levels of fluorescence, suggesting similar levels of p21 molecules available for binding to the anti-Kirsten-*ras* antibody. Similar results were obtained when "bright" and "dim" subpopulations of B16BL6 cells were fixed in 70% ethanol prior to anti-pan-*ras* labeling and FACS analysis (Berglund and Starkey, 1989).

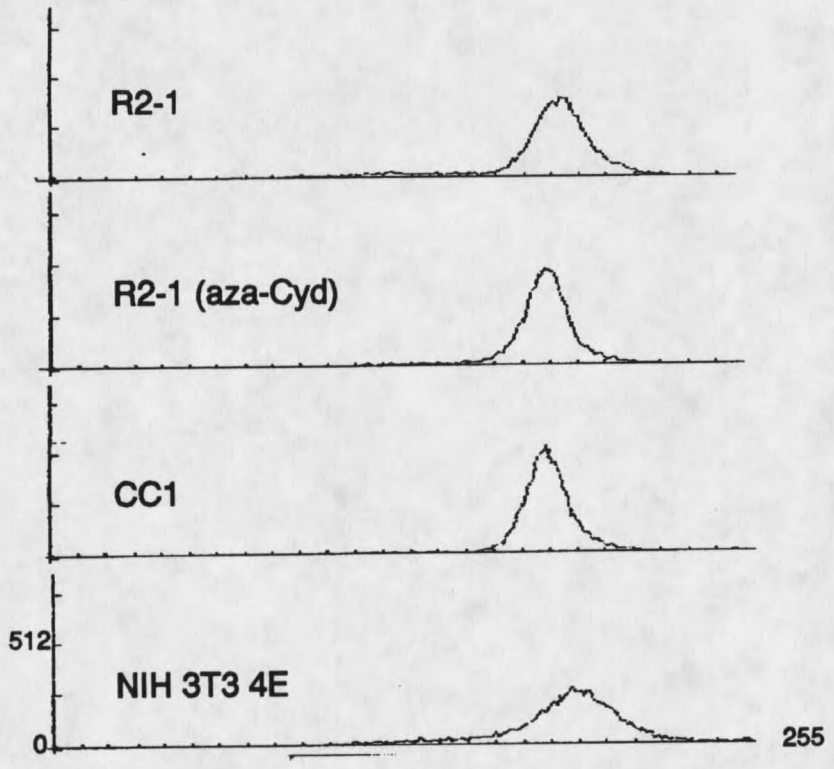

Figure 10. FACS analysis of p21 content of the cell lines after cells were fixed in ethanol. Cells were fixed in 70% ethanol prior to labeling with FITC-conjugated anti-K-*ras* antibody. Labeled cells were subjected to FACS analysis. Ethanol-fixed cells of all cell lines exhibited a greater level of antibody binding/fluorescence intensity than did unfixed cells. Similar results were obtained when fixed cells were labeled with the anti-pan-*ras* antibody. The horizontal axis denotes log of fluorescence intensity. The vertical axis represents number of events, or cell number.

In Vitro Assay of Tumor Cell Invasion

In an initial experiment in which only EHS tumor extracellular matrix (Matrigel) was coated onto the Transwell insert membranes, the NIH 3T3 4E, R2-1, R2-1(aza-Cyd), and CC1 cell lines all displayed rapid invasion through the matrix barrier within one or two days after plating such that differences among the cell lines could not be distinguished. Because the Matrigel extracellular basement membrane matrix used is a processed extract of the EHS tumor matrix, it is likely to be somewhat deficient in type IV collagen. This is because type IV collagen serves as a "scaffold" within the matrix structure (Liotta et al., 1986b) and remains highly insoluble during chemical extraction procedures. Since type IV collagen degradation appears to be closely linked to matrix invasion and to expression of *ras* oncogenes, it was reasoned that addition of type IV collagen to the EHS matrix solution would probably retard the invasion of the cell lines under study to varying degrees. This in fact was found to be the case. NIH 3T3 4E cells did not exhibit invasion through the collagen-fortified EHS matrix until six days after plating (Figure 11). Cells of the R2-1, R2-1(aza-Cyd), and CC1 lines were only slightly affected in that they were able to pass through the EHS-collagen IV matrix as soon as three days after plating (Figure 11). Greater numbers of metastatic CC1 cells invaded through the Transwell matrix than were observed for the non-metastatic R2-1 or R2-1(aza-Cyd) lines even though all three lines displayed invasion beginning three days after plating. This greater rate of invasion observed in vitro for the CC1 cells compared to the R2-1 or R2-1(aza-Cyd) lines may be a factor which contributes to the metastatic phenotype of CC1 cells.

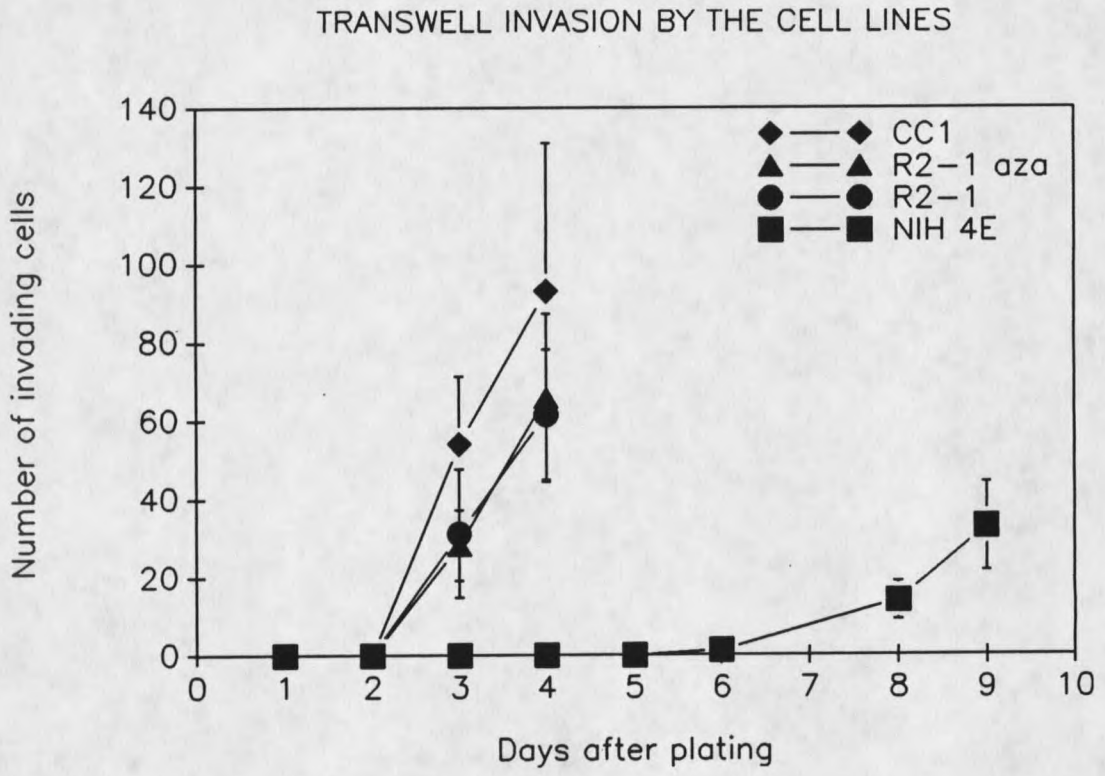

Figure 11. Transwell invasion by the cell lines. The polycarbonate membranes of Transwell inserts were coated with a mixture of Matrigel extracellular matrix and type IV collagen. 5 x 10^4 cells in a volume of 0.2 ml culture medium were plated per Transwell insert. Each cell line was plated in quadruplicate. The number of cells invading through each membrane barrier and attaching to the bottom of the tissue culture dish well was evaluated daily. Data points plotted are the means of quadruplicate values. Error bars represent standard deviations.

To study the role of *ras* p21 expression in contributing to invasive behavior observed in vitro, CC1 cells were electroporated in the presence of a *ras* p21-specific antibody known to block p21 function. Introduction of the blocking antibody had no detectable effect on the in vitro invasive capacity of CC1 cells (Figure 12). In contrast, the in vitro invasive ability of B16BL6 cells is significantly blocked when these cells are treated with the same blocking antibody (Starkey and Berglund, manuscript submitted for publication).

The possible involvement of G proteins in contributing to in vitro invasion was investigated by treating cells with pertussis toxin prior to plating in the Transwell chambers. Because the in vitro invasive capacities of the CC1 and B16BL6 cell lines were differentially affected by treatment with the anti-*ras* function blocking antibody, the effects of pertussis toxin on the two cell lines' invasive behaviors were compared. As shown in Figure 13, Transwell invasion by CC1 cells was unaffected by treatment with pertussis toxin. On the other hand, pretreatment of B16BL6 cells with pertussis toxin significantly hindered this cell line's ability to invade in vitro (Figure 14). The decrease in in vitro invasive capacity observed for B16BL6 cells after treatment with anti-*ras* function blocking antibody or pertussis toxin suggests that *ras* p21 and a G protein-mediated signal transduction pathway both contribute to the invasive behavior of this cell type. The finding that the invasive behavior of CC1 cells is unaffected by treatment with either the anti-*ras* function blocking antibody or pertussis toxin suggests that yet another biochemical pathway may be responsible for driving the invasion process in these cells. A role for *ras* p21 or G proteins in contributing in some more subtle fashion to CC1 invasive behavior is not discounted by these findings, however.

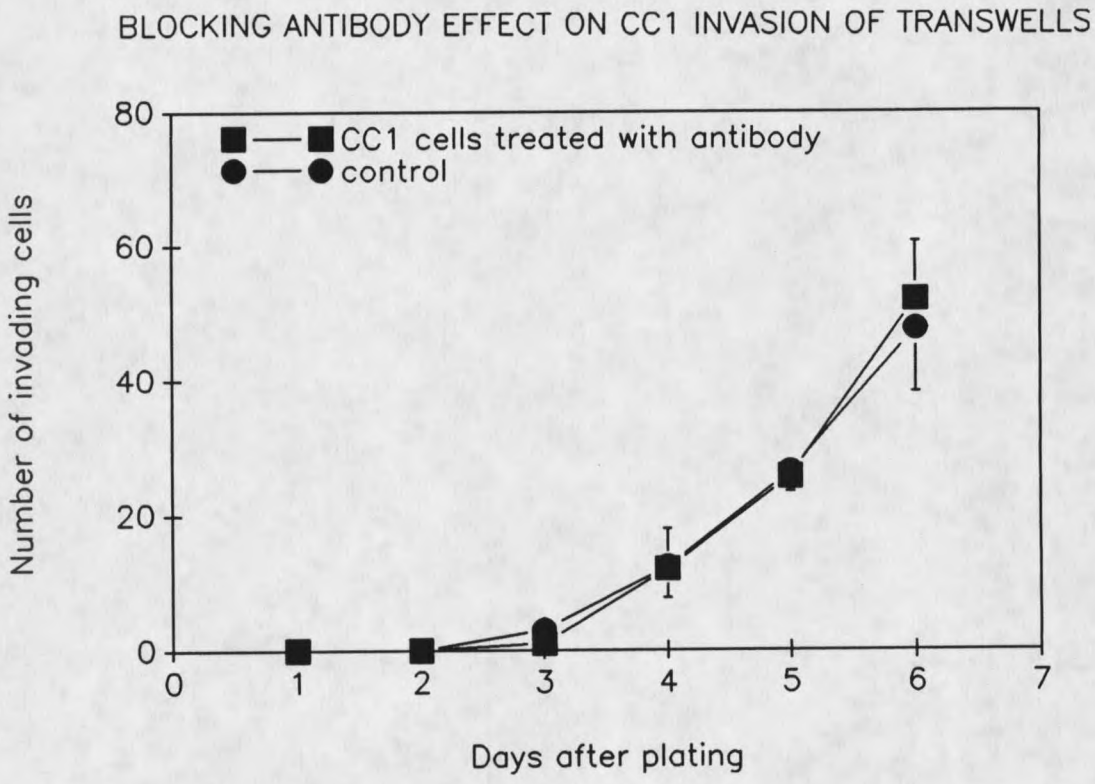

Figure 12. Anti-*ras* function-inhibiting antibody effect on CC1 cell line invasion of Transwells. The anti-*ras* function inhibiting antibody Y13-259 was introduced into CC1 cells by electroporation. Antibody-treated cells were then plated into Transwell chambers. The polycarbonate membranes of the Transwell inserts were precoated with a mixture of Matrigel extracellular matrix and type IV collagen. 5×10^4 cells in a volume of 0.2 ml culture medium were plated per Transwell insert. Treated and control cells were each plated in quadruplicate. The number of cells invading through each membrane barrier and attaching to the bottom of the tissue culture dish well was evaluated daily. Data points plotted are the means of quadruplicate values. Error bars represent standard deviations.

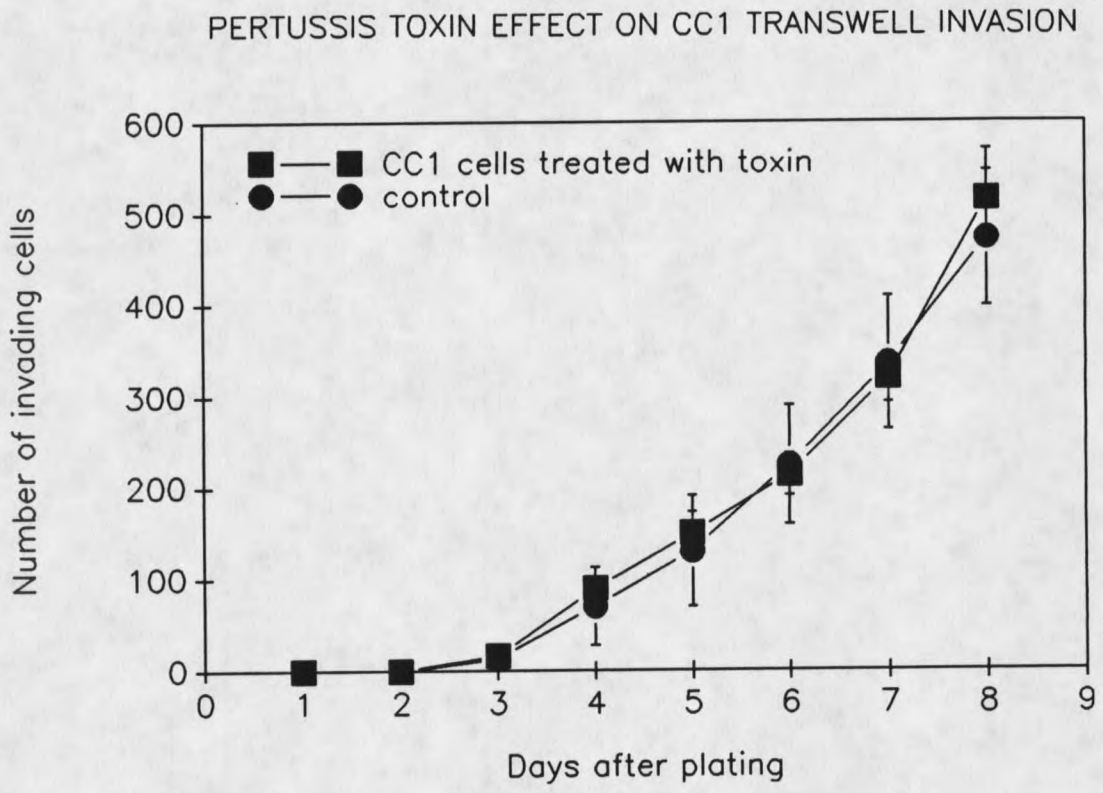

Figure 13. Effect of pertussis toxin on CC1 cell line invasion of Transwells. Cells were treated with culture medium containing 1 ug/ml pertussis toxin for four hours prior to plating in Transwell chambers. The polycarbonate membranes of the Transwell inserts were precoated with a mixture of Matrigel extracellular matrix and type IV collagen. 5 x 10^4 cells in a volume of 0.2 ml culture medium were plated per Transwell insert. Treated and control cells were each plated in quadruplicate. The number of cells invading through each membrane barrier and attaching to the bottom of the tissue culture dish well was evaluated daily. Data points plotted are the means of quadruplicate values. Error bars represent standard deviations.

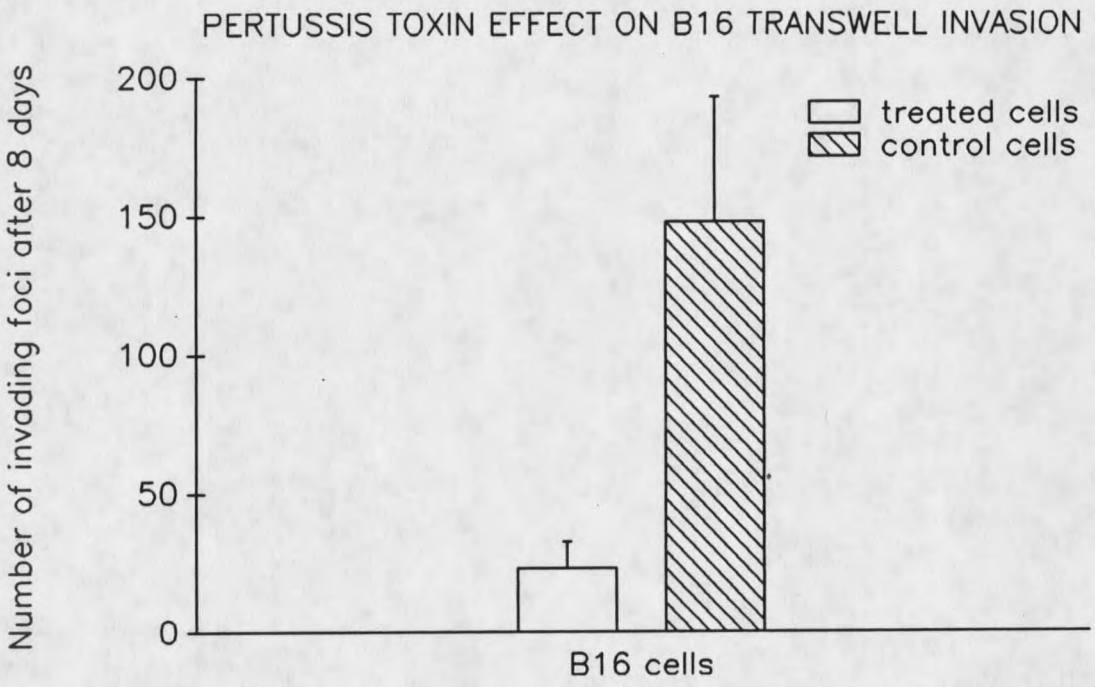

Figure 14. Effect of pertussis toxin on B16BL6 cell line invasion of Transwells. Cells were treated with culture medium containing 1 ug/ml pertussis toxin for four hours prior to plating in Transwell chambers. The polycarbonate membranes of the Transwell inserts were precoated with a mixture of Matrigel extracellular matrix and type IV collagen. 5 x 10^4 cells in a volume of 0.2 ml culture medium were plated per Transwell insert. Treated and control cells were each plated in quadruplicate. The number of colonies growing on the bottom of the tissue culture dish wells was counted eight days after plating. Data points plotted are the means of quadruplicate values. Error bars represent standard deviations.

DISCUSSION

Analysis of Viral Ras Gene Expression and Metastatic Potential

Thorgeirsson et al. (1985) were the first to report that nonmetastatic mouse cells transfected with a *ras* oncogene were able to metastasize when tested in athymic nude mice. Because the technique of transfection itself may perturb the genetic stability and phenotype of treated cells, it was desirable to confirm this finding using an alternate means of introducing a *ras* oncogene into cells. Two cell lines, CC1 and R2-1, derived from NIH 3T3 mouse fibroblasts being transformed with a Kirsten *ras* oncogene by way of retroviral infection were thus evaluated with respect to metastatic potential. The CC1 cell line was found to be highly metastatic when tested in nude mice. CC1 cells readily metastasized to the lungs from a primary tumor formed after subcutaneous injection of cells into the flanks of test animals. CC1 cells also formed metastatic colonies in the lungs after being introduced directly into the bloodstream by tail vein injection. The phenotypic revertant R2-1 cell line showed only a very limited experimental metastatic capability and no spontaneous metastatic capability when tested in nude mice. Both lines readily formed tumors in nude mice upon subcutaneous injection of cells into the animals, but only CC1 cells were capable of forming progressively growing tumors in young adult immunocompetent mice. A third cell line, K-NIH-R, derived in our laboratory by infection of NIH 3T3 cells with Kirsten murine sarcoma virus, was also shown to be

highly metastatic in nude mice. Like CC1 cells, K-NIH-R cells were tumorigenic but nonmetastatic in normal mice.

The R2-1 cell line was originally derived by mutagenic treatment of CC1 cells and subsequent selection for cells unable to grow under conditions in which transformed CC1 cells readily proliferate. Because the original transforming virus is defective with respect to replicative ability, both cell lines are virus nonproducers (Morris et al., 1980). K-MSV particles can be rescued from either cell line by superinfection with K-MLV. Furthermore, the rescued virus particles from either cell line are indistinguishable from the original transforming K-MSV, suggesting that phenotypic reversion in R2-1 cells is caused by an alteration in cellular rather than viral genetic material. It is thus possible to speculate that mutagenic treatment may have inactivated a gene in R2-1 cells which cooperates with *ras* in CC1 cells to confer on CC1 cells a fully transformed and malignant phenotype. Alternatively, mutagenesis may have activated a gene in R2-1 cells which is inactive in CC1 cells and which exerts some transformation suppressor activity to render the R2-1 cells more phenotypically normal and nonmetastatic. As a test of these hypotheses, applying a strategy such as that of subtractive hybridization might reveal RNA populations which are differentially expressed between the two cell lines. This type of approach has been used successfully in identifying putative metastasis suppressor genes, the expression of which is decreased in metastatic lines relative to nonmetastatic sister cell types (Dear et al., 1988; Schalken et al., 1989; Steeg et al., 1989). Identification and characterization of such RNAs will undoubtedly prove valuable in

extending our understanding of tumor progression and the genetic elements important in control of metastasis.

Another strategy that might be used in the identification of possible tumor suppressor genes in phenotypic revertant lines such as R2-1 is the approach used by Noda and coworkers in their identification of the K*rev* gene having *ras* suppressor activity (Noda et al., 1983; Kitayama et al., 1989). Their initial work with phenotypic "flat" revertants obtained after mutagenic treatment of NIH 3T3 cells transformed by the viral Kirsten *ras* gene led to experiments involving transfection of the parental transformed cell line with a cDNA expression library from normal human cells. The K*rev* gene was then isolated in the form of recombinant plasmids from phenotypic revertant cells obtained after transfection with the human DNA.

RNA dot blot analysis using cDNA probes specific for the three oncogenes v-*fes*, c-*myc*, and v-K-*ras* indicated a selective correlation between *ras* mRNA expression and the metastatic phenotype of CC1 cells. RNAs from CC1 tissue culture cells and CC1 primary tumor tissue both gave strong hybridization signals with the v-K-*ras* probe. No detectable v-K-*ras* hybridization signals were seen for RNAs of CC1 metastatic tissue, NIH 3T3 4E tissue culture cells, or R2-1 tissue culture cells. This trend was confirmed by Northern blot analysis where tumor tissue RNAs were fractionated by gel electrophoresis before blotting onto membranes for hybridization analysis. In this experiment, *ras* mRNA levels were highest for the CC1 primary tumor tissue which gave rise to metastases. Compared to CC1 primary tumor tissue, CC1 metastatic tissue showed decreased expression of *ras* mRNA. These findings lend support for the idea that

a high level of *ras* expression is important for cells to successfully complete the early stages of metastasis but is not critical for growth of tumor cells at secondary sites. Vousden et al. (1986) noted that transfection with *ras* increased the spontaneous metastatic ability of mouse adenocarcinoma cells but did not influence the lung colonization potential of cells injected intravenously into test animals. An individual metastasis was identified in which the activated *ras* sequence introduced by transfection was lost. When retested, cells of this metastasis showed an incidence of spontaneous metastasis identical to that displayed by the original untransfected adenocarcinoma cell line. Accordingly, Vousden et al. (1986) proposed that while expression of an activated *ras* gene may be important in allowing metastasizing cells to escape from the primary tumor site, continued expression may be less important for the cells' survival during dissemination in the circulatory system and for the cells to be able to initiate secondary tumors. Other workers have presented evidence suggesting that *ras* oncogenes may play an important role during the early stages of tumor progression. Brown et al. (1986) found that *ras* sequences of the Harvey and BALB murine sarcoma viruses can serve as initiators in a two stage induction of mouse skin carcinogenesis. The H-*ras* gene has been shown to be mutationally activated when mouse skin is treated with a chemical carcinogen; upon activation, the *ras* gene can then serve as an initiator for benign papilloma development upon subsequent application of a tumor promoter (Balmain et al., 1984).

RNA dot blot analysis and Northern gel blot analysis both revealed that v-K-*ras* RNA transcript levels were higher in the CC1 cell line than in the R2-1 line. As detailed in the introduction of this thesis, a number of studies have implicated changes in DNA

methylation in the possible regulation of *ras* gene expression. Accordingly, the methylation states of the v-K-*ras* sequences of CC1 and R2-1 cells were examined to seek a possible explanation for the difference in *ras* transcript levels observed between the two cell lines. Southern analysis indicated a relative hypermethylation for one HpaII/MspI restriction site within the v-K-*ras* sequence of R2-1 cells compared to the CC1 sequence. With the discovery of an apparent difference between the two cell lines in v-K-*ras* methylation, R2-1 cells were treated with the DNA demethylating agent 5-azacytidine to test whether these cells could be rendered metastatic by the resulting perturbation in DNA methylation. R2-1 cells treated with 5-azacytidine acquired a more transformed phenotype but remained nonmetastatic. It was of interest to determine whether R2-1 v-K-*ras* DNA itself was specifically demethylated by 5-azacytidine treatment. Unfortunately, technical difficulties with followup Southern analyses of R2-1(aza-Cyd) DNA prevented this question from being answered. Northern blot analysis of tumor tissue RNAs indicated that DNA demethylation treatment did enhance *ras* gene expression in R2-1 (aza-Cyd) cells. R2-1 primary tumor tissue showed only a modest level of *ras* transcripts compared to the high levels observed for R2-1(aza-Cyd) and CC1 cells growing at primary tumor sites.

5-azacytidine treatment has been reported to have variable effects on the progression of tumor cell populations (reviewed by Jones, 1986). 5-azacytidine has been shown both to be able to impart metastatic behavior on tumor cells not previously metastatic as well as to render nonmetastatic cell lines which were previously fully metastatic. In R2-1 cells treated with 5-azacytidine, demethylation of DNA is likely to have deregulated the activity

of unidentified genes in addition to increasing the expression of the v-K-*ras* gene so as to impart a more transformed phenotype on R2-1(aza-Cyd) cells. Changes in DNA methylation patterns induced in vitro, and the accompanying changes in phenotype observed, appear to mimic events which occur in such naturally occurring processes as cellular senescence and tumor progression. Aging cells have been reported to show decreased levels of DNA methylation compared to younger cells having undergone fewer cycles of replication (Wilson and Jones, 1983). Hypomethylation of DNA has been noted in a wide variety of primary and metastatic tumors of animals and humans (reviewed by Jones, 1986; Hoffman, 1990). Hoffman (1990) has proposed that a generalized imbalance in the regulation of methylation and alteration of methionine metabolism may underlie the changes in methylation patterns observed during tumor development. A detailed analysis of overall differences in DNA methylation status between the tumorigenic but nonmetastatic R2-1 cell line and the metastatic CC1 line would certainly be a worthwhile project and might help to explain the phenotypic differences observed for these cell lines. Such a project was not undertaken during the course of my study, since the effort required would have diverted attention from the study of *ras* effects on metastasis specifically.

The EJ-*ras* oncogene was introduced into NIH 3T3 4E and R2-1 cell lines to test whether a second activated *ras* gene might induce metastatic ability in R2-1 cells. Cells were transfected using the technique of electroporation with the plasmids pSV2neo and pEJ. pSV2neo carries an antibiotic resistance gene which can serve as a dominant selectable marker in transfected cells. Plasmid pEJ harbors a mutationally activated H-*ras*

oncogene which has been shown to confer experimental metastatic capability on transfected NIH 3T3 cells (Bradley et al., 1986). It was reasoned that if the v-K-*ras* gene carried in R2-1 cells was somehow deficient in being able to promote metastatic behavior, a second *ras* oncogene previously shown to confer metastatic potential on NIH 3T3 cells might be able to impart a metastatic phenotype on R2-1 cells. The H-*ras* oncogene failed to induce metastatic behavior in either NIH 3T3 4E or R2-1 cells as indicated by testing in nude mice. NIH 3T3 4E cells transfected with pSV2neo alone or with a combination of pSV2neo and pEJ plasmids were observed to be tumorigenic in nude mice but only after a latency period of about four months. The *ras* transfected NIH 3T3 4E cells also acquired a more rounded, less adherent appearance compared to untransfected cells or cells transfected with pSV2neo plasmid alone. The *ras* transfected NIH 3T3 4E cells also exhibited a somewhat shorter latency period for tumor formation (average of 110 days until palpable tumors were noted) than did cells transfected with pSV2neo alone (134 day average latency period). It would thus seem that transfection with *ras* did exert a low degree of transforming activity on transfected NIH 3T3 4E cells. Transfection of R2-1 cells with the H-*ras* oncogene had no effect on that cell type's morphology, latency period for primary tumor formation, or nonmetastatic phenotype.

Although NIH 3T3 cells have generally been described as being nontumorigenic, Greig et al. (1985) reported that subcutaneous inoculation of 1×10^6 NIH 3T3 cells produced tumors of approximately 2 cm in diameter as soon as 32-50 days after injection into mice. No metastases were observed in these animals. EJ-*ras* transfected NIH 3T3 cells showed tumors of similar size as soon as 16-20 days after injection and displayed

numerous metastases to the lungs. In this same study, 0.5 x 10^6 cells of either cell type were also injected into mouse footpads where more slowly growing primary tumors formed. For the untransfected NIH 3T3 cells growing in the footpad, tumors of 1-1.5 cm in diameter were seen 84-125 days after injection, while *ras* transfected cells displayed tumors of this size 18-25 days after injection. Although a similar number of cells were injected into mice in the present study, tumor latency periods were noticeably longer than those observed in the Greig et al. (1985) study. The latency periods reported in this study are based on initial observation of palpable tumors, while Greig et al. (1985) define latency periods after tumors have achieved a rather large mass. It would thus appear that the NIH 3T3 4E cells used in this study differ somewhat in their behavior from the stock of NIH 3T3 cells employed by Greig et al. (1985). The NIH 3T3 4E line is the product of two cycles of cloning from the parental NIH 3T3 cell line (Morris et al., 1980), and may thus represent a subset of the original NIH 3T3 population which is more limited in its potential to become metastatic after transfection with a *ras* oncogene.

Analysis of $p21^{v\text{-}K\text{-}ras}$ Expression and Its Bearing on Malignant Behavior

Viable cell preparations of the cell lines under study displayed bimodal distribution patterns for anti-*ras* antibody binding capacity. Since ethanol fixed cells of all the cell lines gave unimodal antibody binding profiles with very similar mean fluorescence values, it is unlikely that subpopulations of cells exist within any given cell line which express high or low levels of p21. Within one tissue culture passage, brightly fluorescent and dimly fluorescent cells sorted from R2-1, R2-1(aza-Cyd), or CC1 cell lines generated cell

populations which were indistinguishable from each other in terms of antibody binding-associated fluorescence profiles. This finding also suggests the absence of subpopulations of cells which stably express high or low total levels of the *ras* protein. Instead, although cells of the lines studied appear to express similar total levels of p21, viable cells appear to have only a fraction of their pool of p21 molecules available for binding to anti-*ras* antibody.

Using both an anti-pan-*ras* antibody and an anti-viral Kirsten *ras* antibody, cells of the metastatic CC1 cell line consistently displayed a higher mean level of antibody binding than did cells of the tumorigenic but nonmetastatic R2-1 or R2-1(aza-Cyd) lines. If viral *ras* p21 is contributing to the metastatic capability of CC1 cells, it is tempting to speculate that p21 availability for binding to antibody may correlate with p21 molecules being in a biochemically functional state. If this is so, perhaps only the post-translationally modified p21 molecules associated with the cell membrane of viable cells are able to bind antibody. In this case, p21 molecules not fully processed and not yet associated with the cell membrane might in some manner be sequestered from binding to antibody. After fixation of cells with ethanol, however, these p21 molecules become accessible for binding to antibody. Even when processed and associated with the cell membrane, p21 interaction with as yet unidentified regulatory or effector molecules may alter p21 accessibility to antibody binding. Conformation of the p21 protein could be affected by p21 interaction with other molecules and could accordingly dictate whether or not the p21 is able to bind to antibody. Once again, the high antibody binding capacity displayed by viable cells of the metastatic CC1 cell line prompts speculation that

this line might have the highest average level of conformationally active p21 molecules on a per cell basis. There is as yet no direct evidence to support these speculations, however. The nature of factors which permit or restrict antibody binding to p21 or, for that matter, other proteins within the cell remains an intriguing question.

It is noteworthy that while viable cells of all cell lines studied reproducibly displayed bimodal distribution patterns for anti-*ras* antibody binding, sorted "bright" and "dim" subpopulations quickly gave rise to populations having more unimodal distribution patterns than were observed for the original parental cell lines. Perhaps the cells of "mature" cell populations interact with each other in such a way as to result in a distribution of cells having varying proportions of their p21 populations accessible to antibody binding. Accessibility to antibody binding may have functional significance for p21 molecules. Cells having different levels of p21 capable of binding antibody, i.e., different levels of p21 in a biochemically active state, may be related to the operation of a dynamic homeostasis for the cell population as a whole. Tumor cell populations are often heterogeneous for the expression of a wide range of phenotypic traits (Fidler and Hart, 1982; Nowell, 1976, 1986). Production of genetic and phenotypic variant cells often provides the tumor with a selective advantage during its evolution (Nowell 1976; 1986). Variation in expression of different levels of p21 able to bind antibody may be yet another manifestation of such heterogeneity. Sorted "bright" and "dim" subpopulations may first be driven to achieve a unimodal, median balance of p21 expression as defined by accessibility to antibody binding. In retrospect, it would have been interesting to carry the "bright"- and "dim"-derived populations for additional passages in culture to test

whether they would regain a bimodal distribution pattern for antibody binding characteristic of the "mature" parental cell lines.

$p21^{ras}$ molecules must be appropriately processed and targeted to the inner face of the cell membrane for them to be biochemically functional (reviewed by Barbacid, 1987). To determine whether the levels of membrane-associated p21 molecules correlate with the levels of p21 molecules able to bind with anti-*ras* antibody in a cell line-specific manner, cells of each line under study could be fractionated into cell membrane and cytosolic components. For each cell line, the cell membrane fraction would then be assayed for total p21 content. In this manner, the prediction that, among the different cell lines, CC1 cells contain the highest number of p21 molecules associated with the cell membrane could be tested. Presumably, these membrane-associated p21 molecules are competent for biochemical functioning within the cell. The higher level of membrane-associated p21 found in CC1 cells might accordingly contribute to this cell line's metastatic phenotype.

When determining the levels of total p21 found associated with the cell membrane fractions of the different cell lines, it would also be worthwhile determining what fraction of membrane-associated p21 is coded for by cellular *ras* genes compared to the fraction of membrane-associated p21 which is v-K-*ras*-encoded. Since two different membrane targeting signals are presently known to be operating for the various $p21^{ras}$ species (Hancock et al., 1990), it is conceivable that $p21^{v\text{-}K\text{-}ras}$ proteins are targeted to interact with different membrane-associated signal transduction regulatory and effector molecules than are the various p21 proteins coded for by the different cellular *ras* genes. Little is

presently known about the functional significance, if any, of there being more than one species of p21 expressed in a given cell type.

The instability of "bright" and "dim" subpopulations sorted from R2-1, R2-1(aza-Cyd), and CC1 cell lines contrasts markedly with the relative stability of "bright" and "dim" subpopulations derived from the B16BL6 line. Viable cells of the B16BL6 subpopulations maintain their respective "bright" and "dim" anti-*ras* antibody fluorescence profiles for several passages after sorting (Berglund and Starkey, 1989). Despite this stability in expression of p21 levels accessible to antibody binding, ethanol fixed B16Bl6 "bright" and "dim" subpopulations both show similar mean anti-*ras* antibody binding levels which are greater than the values seen for viable cells of the two subpopulations (Berglund and Starkey, 1989 and personal communication). Thus, similar to what was found with the R2-1, R2-1(aza-Cyd), and CC1 cell lines, "bright" and "dim" subpopulations of the B16BL6 line have only a portion of their p21 populations available for anti-*ras* antibody binding. Why the B16BL6 subpopulations should be relatively stable and the R2-1, R2-1(aza-Cyd), and CC1 subpopulations unstable with regard to the levels of p21 which are able to bind antibody is unknown.

An in vitro assay for tumor cell invasion of extracellular basement membrane matrix allowed not only a comparison of invasive potential for the cell lines under study, but also an opportunity to more directly examine the role of *ras* p21 in contributing to malignant behavior in the cell lines. Basement membrane matrix is a barrier which metastatic tumor cell types must breach early in the course of metastasizing (Liotta et al., 1986). An in vitro assay of invasion through basement membrane thus serves as a legitimate test for

partially characterizing the malignant potential of a tumor cell line. The commercially obtained basement membrane matrix used for this assay, Matrigel, contains a number of components, including laminin, type IV collagen, heparan sulfate proteoglycan, and entactin, found in naturally occurring basement membranes. It should be kept in mind when working with extracellular matrix systems in vitro that the presence and proportional amounts of specific components found in basement membrane can vary significantly in a tissue-specific manner (Yurchenco and Schittny, 1990). Use of Matrigel by itself did not allow for any comparison of the invasive potentials of the NIH 3T3 4E, R2-1, R2-1(aza-Cyd), and CC1 cell lines. Supplementing Matrigel with type IV collagen significantly impeded NIH 3T3 4E invasion through the matrix barrier while slightly slowing down invasion by the other three cell lines. Given the linkage between oncogenic *ras* expression and type IV collagenase expression established by other researchers (Thorgeirsson et al., 1985; Garbisa et al., 1987), it is quite likely that the Kirsten virus transformed R2-1, R2-1(aza-Cyd) and CC1 lines do express elevated levels of type IV collagenase activity compared to the nontransformed NIH 3T3 4E line, and are thus able to invade through the Matrigel-type IV collagen barrier more aggressively. Type IV collagenase activity is of course not the only determinant of metastatic potential in metastatic cells. B16BL6 cells invade through Matrigel not supplemented with type IV collagen more slowly than do CC1 cells through type IV collagen-supplemented Matrigel, but are nevertheless more highly metastatic in mice than are CC1 cells.

Another remarkable feature of the B16BL6-derived "bright" and "dim" subpopulations which bind high and low levels of anti-*ras* antibody, respectively, is that the "bright"

subpopulation is able to invade through the Matrigel barrier more readily than cells of the "dim" subpopulation (Starkey and Berglund, manuscript submitted for publication). "Bright" cells are also more highly metastatic in animals than are "dim" cells. Treatment of B16BL6 cells with a $p21^{ras}$-function inhibiting antibody also decreases the invasive potential of cells in vitro as well as the metastatic potential of cells introduced into mice (Starkey and Berglund, manuscript submitted for publication). These data provide substantial support for p21 playing a direct role in promoting invasive and metastatic behavior in this cell line. CC1 cells similarly treated with the p21-function inhibiting antibody did not show a reduction in in vitro invasive behavior. It was accordingly deemed not fruitful to test antibody-treated CC1 cells in mice since an alteration of metastatic behavior was unlikely. It is possible that oncogenic *ras* induction of phenotypic changes associated with metastasis may not always be reversed by short term inhibition of p21 function. For example, a *ras* oncogene may turn on or increase the expression of a gene such as type IV collagenase, which could then remain constitutively expressed in a cell type-specific manner. CC1 degradation of and invasion through extracellular matrix might also be more dependent on a signaling pathway other than *ras* and so would not be noticeably affected by inhibition of p21 function. Laminin binding to CC1 cells may serve as the signal for this cell type to express type IV collagenase activity as has been documented for other cell types (Turpeenniemi-Hujanen et al., 1986). CC1 production of type IV collagenase activity both in the absence and presence of laminin could easily be assayed for to test this possibility.

The in vitro invasion assay system was further exploited to test for the involvement of G protein signal transduction in determining the invasive behaviors of B16BL6 and CC1 cells. Cells were treated with pertussis toxin prior to plating on the extracellular matrix-coated Transwell membranes. CC1 cell invasive behavior was unaffected by treatment with toxin, while B16BL6 cell invasive potential was significantly hampered by treatment with pertussis toxin. This finding implicates the involvement of a G_i or G_o protein in promoting extracellular matrix invasion by B16BL6 cells. G protein activity has been implicated in the regulation of cytoskeleton structure, cellular motility, and tumor invasion and metastasis (Bengtsson et al., 1990; Roos and Van De Pavert, 1987; Stracke et al., 1987; Aznavoorian et al., 1990; Lester et al., 1989). The G_{i2} subset of G proteins in particular has been previously identified as being singularly abundant in a highly metastatic B16 subline compared to a sister subline having low metastatic ability (Lester et al., 1989). Pertussis toxin treatment of these highly metastatic B16 cells decreased the motility and invasion of the cells without affecting cellular cyclic AMP levels. Lester et al. (1989) suggest that G_{i2} proteins may possibly be linked to some second messenger pathway other than the adenyl cyclase system and that this secondary pathway may contribute to the metastatic potential of B16 cells. If a panel of antibodies capable of inhibiting the function of known G_i/G_o proteins were obtained, B16BL6 cells could be screened with the various G_i/G_o-specific antibodies using the same electroporation protocol used for labeling cells with the anti-*ras* antibodies. Such screening might lead to identification of specific G proteins involved in invasion. B16BL6 cells might also be treated with p21-function inhibiting antibody and pertussis

toxin concurrently to test for a synergistic effect on invasion between *ras* and the G protein signal transduction pathway. Given the association now established by Starkey and Berglund between p21 expression and the invasive/metastatic phenotype in B16BL6 cells, continued use of this system should be useful in elucidating the pathway(s) of which *ras* is a part which are involved in imparting metastatic behavior.

How viral *ras* expression may be contributing to the invasive potential of CC1 cells remains an enigma. The answer may come from further study at the level of p21 expression itself since viable CC1 cells do bind higher levels of antibody directed at p21 than do the nonmetastatic R2-1 or R2-1(aza-Cyd) lines. On the other hand, it might be worthwhile to look for possible effects that integration of the Kirsten virus *ras* gene may be having on the cell's genome. Is integration of the viral sequences promoting genetic instability within the recipient cells as has been reported in the case of *ras* transfectants (Ichikawa et al., 1990)? If this is so, how has the metastatic phenotype been reversed in R2-1 cells? As alluded to earlier, the detection of differences in gene expression between the two cell lines at the level of mRNA expression may prove to be the most useful strategy in identifying the genetic elements which contribute to or restrict expression of the metastatic phenotype in this system.

CONCLUSIONS

1) R2-1 cells may express a tumor suppressor gene able to block the metastasis-promoting effects of the viral Kirsten *ras* gene. R2-1 cells are similar to the phenotypically "flat" revertants generated by Noda et al.(1983) after mutagenic treatment of cells also transformed by Kirsten murine sarcoma virus. Work with those revertants led to identification of the K*rev* gene which exhibits *ras* oncogene suppressor activity (Kitayama et al., 1989). R2-1 cells should be screened for expression of K*rev* activity. If K*rev* activity is not detected in R2-1 cells, continued study of this cell line may well lead to identification of a novel tumor suppressor or *ras* suppressor gene. Work with the class of phenotypic revertant cell types and transformed parental cell lines exemplified by the R2-1/CC1 system is also likely to be valuable in investigating the possible interaction of *ras* with other oncogene activities. As an example, the phenotypic revertants studied by Noda et al. (1983) have been shown in cell fusion experiments to suppress transformation by *fes* and other *ras* genes, but not transformation by certain other oncogenes.

2) $p21^{ras}$ and G_i/G_o activities do not appear to be directly promoting the invasive behavior of CC1 cells. Another biochemical pathway or mechanism other than *ras* must be involved in driving invasion in the CC1 cell line. To the best of my knowledge, in all other studies of *ras* and its relationship to invasion and metastatic potential, the *ras* system appears to be a primary determinant responsible for promoting malignant

behavior. On the basis of information gained studying the expression of *ras* and metastatic behavior in CC1 cells, we are now forced to search for an alternate biochemical mechanism or pathway responsible for promoting invasion in these cells. Such a pathway is unlikely to involve G_i/G_o proteins, or more specifically the G_{i2} subset of G proteins identified by Lester et al. (1989) as possibly contributing to the malignant properties of highly metastatic B16 cells.

3) For the CC1 cell line, high *ras* mRNA expression and the relatively high level of p21 able to bind antibody specific for $p21^{v\text{-}K\text{-}ras}$ appear to correlate well with metastatic potential. These findings provide strong circumstantial evidence that *ras* is contributing in at least an indirect fashion to the promotion of metastatic behavior in CC1 cells. For example, *ras* oncogene activity in CC1 cells may be activating another secondary pathway more closely linked to cellular motility or expression of proteolytic activity directed at extracellular matrix. Aberrant increases in such activities could certainly contribute to invasion and metastasis and might not be affected by short-term inhibition of p21 function.

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APPENDIX

Abbreviations

A	adenine or adenosine
ADP	adenosine diphosphate
AMF	autocrine motility factor
bp	base pair
BSA	bovine serum albumin
C	cytosine or cytidine
CMF	calcium and magnesium-free
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
c-H-*ras*	cellular Harvey *ras*
c-K-*ras*	cellular Kirsten *ras*
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF-R	epidermal growth factor receptor

FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gravity
gm	gram
G	guanine or guanosine
G protein	guanine nucleotide binding protein
GAP	GTPase activating protein
GDP/GTP	guanosine 5'-diphosphate/triphosphate
GTPase	guanosine 5'-triphosphatase
HAT	hypoxanthine/aminopterin/thymidine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
HT	hypoxanthine/thymidine
Ig	immunoglobulin
I-R	insulin receptor
IRA	inhibitory regulators of the *ras*-cAMP pathway
kD	kilodalton
KLH	keyhole limpet hemocyanin
mM	millimolar
mRNA	messenger ribonucleic acid
M	molar

MLV	murine leukemia virus
MSV	murine sarcoma virus
NDP	nucleoside diphosphate
NF	neurofibromatosis
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEG	polyethylene glycol
ras-GRF	*ras*-guanine nucleotide releasing factor
Rb	retinoblastoma
RNA	ribonucleic acid
SCLC	small cell lung carcinoma
SDS	sodium dodecyl sulfate
SSC	standard sodium-citrate solution
T	thymine or thymidine
TBS	Tris buffered saline
TIMP	tissue inhibitor of metalloproteinase
Tris	tris(hydroxymethyl)aminomethane
ug	microgram
U	uracil or uridine
v-K-*ras*	viral Kirsten *ras*

Three-Letter Codes for Amino Acids

ala	alanine	leu	leucine
arg	arginine	lys	lysine
asn	asparagine	met	methionine
asp	aspartic acid	phe	phenylalanine
cys	cysteine	pro	proline
glu	glutamic acid	ser	serine
gln	glutamine	thr	threonine
gly	glycine	trp	tryptophan
his	histidine	tyr	tyrosine
ile	isoleucine	val	valine