IDENTIFICATION OF THE DOMAINS OF *RAS*G-G12T THAT ARE REQUIRED TO PRODUCE DEFECTS IN AGGREGATION AND CYTOSKELETAL FUNCTION IN *DICTYOSTELIUM DISCOIDEUM*

by

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ABSTRACT

Dictyostelium transformants expressing an activated *ras*G gene, *ras*G-G12T, do not aggregate when starved for nutrients. In addition, expression of the activated RasG protein results in vegetative cell populations with heterogeneous morphology. Some cells are extensively flattened and spread and exhibit lateral or dorsal membrane ruffling while others which are less flattened, exhibit prominent dorsal membrane ruffling. The expression of RasG-G12T also causes a slight increase in the average number of nuclei and results in a redistribution of F-actin to the cell periphery. These results suggest that RasG has roles in both cytoskeletal and developmental regulation in *Dictyostelium*.

To identify the functional residues required for the downstream effects of activated RasG, amino acid substitutions have been introduced into RasG-G12T within the effector domain (Tyr32-Tyr40) or within the effector distal flanking domain. Cells expressing RasG-G12T with amino acid substitutions in the effector domain (T35S or Y40C) showed normal morphology. These cells also aggregated and differentiated normally, suggesting that the defects cause by RasG-G12T required interaction of the effector domain with a downstream protein(s). In contrast an amino acid substitution in the effector distal flanking domain (T45Q) prevented the RasG-G12T induced block in aggregation, but was not able to prevent the cytoskeletal defect. This result suggests that the cytoskeletal and developmental defects induced by RasG-G12T result from the interaction of the protein with different downstream effector molecules.

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LIST OF ABBREVIATIONS

BS	Bonner's Salts buffer
cAMP	cyclic adenosine 3', 5' -monophosphate
cARs	cAMP receptors
CRAC	cytosolic regulator of adenylyl cyclase
DIF	differentiation inducing factor
ECL	enhanced chemiluminscence
EGF	epidermal growth factor
GAP	GTPase activating protein
GEF	guanine exchange factor
MAP	mitogen-activated protein
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PSF	pre-starvation factor
PKA	cAMP-dependent protein kinase A
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylene-20-sorbitan monolaurate

Single letter code for amino acids: A, Alanine; R, Arginine; N, Asparagine; D, Aspartic acid; C, Cysteine; Q, Glutamine; E, Glutamic acid; G, Glycine; H, Histidine; I, Isoleucine;L, Leucine; K, Lysine; F, Phenylalanine; P, Proline; S, Serine; T, Threonine; Y, Tyrosine; V, Valine.

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INTRODUCTION

Prologue

The primary objective of this thesis was to identify the residues of RasG-G12T that are required to produce defects in aggregation and cytoskeletal function in *Dictyostelium discoideum*. The introduction will focus on a review of the *ras* gene superfamily emphasizing primarily on the relationship between the structure and function of the Ras proteins. I will also review the literature on Ras signal transduction pathways, placing a special emphasis on the downstream effectors of Ras. I will then discuss *Dictyostelium discoideum*, and its signal transduction pathways, before summarizing what is known about the *ras* genes of *Dictyostelium*.

The Ras gene superfamily

The *ras* superfamily of genes encodes small monomeric GTPases that functionally resemble the heterotrimeric G proteins (reviewed in Zerial and Huber, 1995). The superfamily can be subdivided into three major groups (the *ras*, *rho* and *rab* sub-families), based on the degree of shared amino acid conservation and the protein function (Kahn *et al.*, 1992). More than 50 members of the mammalian *ras* superfamily have been identified to date.

The *ras* sub-family includes the three human *ras* proto-oncogenes, Ki-*ras*, H-*ras* and N-*ras* , whose encoded products share 85% amino acid identity (Bollag and McCormick, 1991); the genes encoding the closely related proteins R-Ras, TC21 and Ral, that share ~55-50% amino acid

identity with H-Ras (Cox *et al.*, 1994; Graham *et al.*, 1994), and the genes encoding the Rap proteins that share approximately 50% amino acid identity with H-Ras (Bokoch, 1993; Noda, 1993). The *ras* sub-family of genes have been highly conserved throughout evolution, since Ras proteins from organisms as diverse as human and yeast share high levels of identity.

The *rab* gene sub-family members encode proteins which share approximately 30% amino acid identity with the Ras protein (Rothman and Orci, 1992). They play a role in the regulation of vesicle trafficking between intracellular organelles (Pfeffer, 1994), a process necessary for the biogenesis of organelles and important in maintaining integrity of endomembrane compartments (Rothman, 1996). The *rho* gene sub-family encodes Rho, Rac and Cdc42 proteins which all share approximately 30% amino acid identity with Ras protein (Nobes and Hall, 1994). Rho-subfamily members regulate signal transduction from receptors in the plasma membrane, controlling cellular events related to cell shape, polarity, motility and cytoskeletal dynamics. Rho, Rac and Cdc42 induce distinct changes in actin-based cell morphology when microinjected into mammalian cells (Ridley *et al.*, 1992; Paterson *et al.*, 1990; Nobes and Hall, 1995). Specifically, Rho induces focal adhesion assembly and stress fiber formation, Rac induces lamellipodia and membrane ruffling, and Cdc42 induces formation of filopodia.

Biological roles of Ras

The *ras* genes were first identified as the transforming agents of the Harvey and Kirsten murine sarcoma viruses (reviewed by Barbacid, 1987; Lowy and Willunsen, 1993). Identification of the mammalian cellular *ras* homologues (H-*ras*, K-*ras* and N-*ras*) and the presence of mutated alleles in human tumors suggested a role for these genes in the control of cellular proliferation. High incidence of *ras* oncogenes in cancers: adenocarcinomas of the pancreas (90%), the colon (50%) and the lung (30%), thyroid tumors (50%), and myeloid leukemia (30%) indicate the importance of the genes in human(reviewed by Bos, 1989). *In vitro*, microinjection of activated Ras proteins in NIH 3T3 cells caused cell proliferation, DNA synthesis and produced a malignantly transformed cell appearance (Stacey and Kung, 1984) and the injection of antibody against Ras protein inhibited serum-stimulated growth of NIH 3T3 cells (Mulcahy *et al.*, 1985). These studies further indicated a role for Ras in cell proliferation and suggested a possible role in cytoskeletal regulation.

Ras protein also functions in differentiation and development in some cells. Activated Ras protein induce the differentiation of PC12 pheochromocytoma cells into neuronal cells (Hagag, 1986). The most clearly defined role for Ras in development, however, has been provided by a series of genetic analysis of neuronal differentiation in the R7 photoreceptor cells of the *Drosophila* eye (Wassarman *et al.*, 1995) and vulval differentiation in *C. elegans* (Kayne and Sternberg, 1995). During *Drosophila* eye development, activation of Ras disrupts normal cell fate specification in the compound eye and when microinjected into embryos, disrupts the terminal cell fates of posterior cells (Simon *et al.*, 1991; Lu *et al.*, 1993). In *C. elegans*, the *let-60 ras* gene is required for multiple

aspects of development. The vulval differentiation pathway has been the most intensively studied. Activation of Ras results in a *multivulval* phenotype while loss of Ras activity leads to a *vulvaless* phenotypes (Beitel *et al.*, 1990; Han *et al.*, 1990).

Domains of Ras proteins

The 21kD Ras protein belongs to the highly conserved GTPase superfamily which also includes the α subunits of heterotrimeric G protein and GTPases used in ribosomal protein synthesis such as bacterial elongation factor EFTu (Bourne *et al.*, 1991). These proteins all cycle between an inactive GDP-bound form and an active GTP-bound form which allows them to act as molecular switches for signal transduction pathways. The conformational state of Ras is regulated by two kinds of proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Boguski and McCormick, 1993; Wittinghofer *et al.*, 1997).

The structures of the different forms of Ras have been determined by X-ray crystallography (Fig. 1) and the functions have been studied by site-directed and random mutagenesis (De vos *et al.*, 1988, Pai *et al.*, 1989; Milburn *et al.*,1990; Nassar *et al.*, 1995). The amino acid domains required for binding and hydrolyzing GTP are located in 4 conserved sequences consisting of residues (10-17, 53-62, 112-119 and 144-146) respectively (Bourne *et al.*, 1991). The first domain (10-17) forms bonds with the α- and β-phosphates of GTP or GDP (Lowy and Willumsen, 1993). The second domain (53-62) forms a hydrogen bond with the γ-phosphate of GTP; and the third domain (112-119) forms hydrogen bonds with both the guanine ring and the first domain. The fourth domain (114-146) is

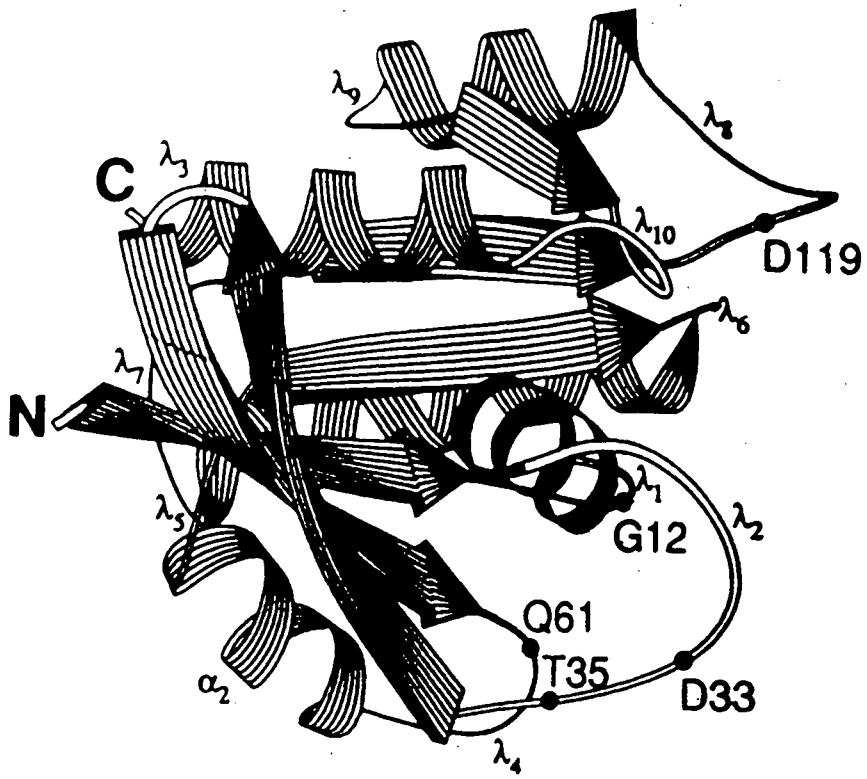


Figure1. Three dimentional structure of the nuclotide-binding domain of human c-Ha-*ras* p21.

This figure is copied from (Pai *et al.*, 1989). All loops and a few important amino-acid residues are labelled for orientation.

somewhat variable and indirectly interacts with the guanine nucleotide by stabilizing the third domain.

In the Ras protein, single amino acid substitutions within the first domain (amino acid 12 or 13), significantly decrease GTPase activity. These mutations are termed activating mutations, since they confer resistance to the action of the GAP, rendering the protein constitutively GTP-bound. The activating effects of position 12 (or 13) mutations in Ras proteins have been demonstrated in mammalian cells (Barbacid, 1987; Lowy and Willumsen, 1993), *Drosophila* (Fortini *et al.*, 1992), *C. elegans* (Han and Sternbeerg, 1990), *S. cerevisiae* (Crechet *et al.*, 1990) and *Dictyostelium* (Reymond *et al.*, 1986; Khosla *et al.*, 1996). In mammalian cells these substitutions have often been identified in naturally occurring human tumors (Barbacid, 1987). Single amino acid substitution S17N strongly inhibits proliferation of NIH 3T3 cells (Feig and Cooper, 1988) and neuronal differentiation of PC12 cells (Szeberenyi *et al.*, 1990). This mutant protein is thought to bind exchange protein normally, thus promoting GDP release, but has a reduced affinity for GTP, and, as a result, remains bound to the exchange protein. This prevents activation of normal ras proteins, and thus inhibits signal transmission (McCormick, 1994).

The three-dimensional structure of the Ras protein changes upon GDP/GTP exchange. In particular, the conformations of the Asp^{30}-Asp^{38} and Gly^{60}-Glu^{76} regions change significantly, and these regions are called "switch I" and "switch II" respectively (Milburn *et al.*, 1990). The switch I region is located in the second loop and essentially overlaps with the conserved effector region (Tyr32-Tyr40; Sigal *et al.*, 1986; Willumsen *et al.*, 1986). Mutations that alter amino acid within this effector domain (32-40; Satoh *et al.*, 1987) do not influence the guanine

nucleotide-binding activity or the GTPase activity, but the biological effects such as morphological transformation of cultured fibroblasts or stimulation of the yeast adenylate cyclase are impaired. The effector domain is therefore believed to interact with the downstream target effector (Marshall, 1993). However, Rap1A is a nontransforming protein (Kitayama *et al.*, 1989), although Rap1A residues 32-44 are identical to Ras, suggesting that the transforming activity of Ras requires additional domains. Extensive genetic analysis suggested that region 26-48 should be considered to be an extended effector region. The amino acids most critical to biological function are Glu31, Pro34, Thr35, Asp38, Tyr40, Val45 and Gly48 (reviewed by Marshall, 1993). The X-ray crystal structure of the complex between the Ras-related protein Rap1A in the GTP form and the Ras effector C-Raf1 structurally confirmed the interaction is mediated by main-chain and side-chain interaction of the effector residues in the switch I region of Rap1A (Nassar *et al.*, 1995).

An additional small domain is present at the carboxyl terminal of all Ras-related proteins. This domain is subject to post translational processing in Ras, resulting in addition of a farnesyl isoprenyl group, followed by removal of the terminal 3 amino acids and then carboxymethylation of the resulting terminal cysteine (Hancock *et al.*, 1989; Clarke, 1992). This domain with its subsequent modification is required for the attachment of Ras to the inner leaflet of the plasma membrane since replacement of the cysteine with other amino acids prevents the association of the protein with the membrane and blocks ability of Ras to transform cells (Guierrez *et al.*, 1989; Willumsen *et al.*, 1984).

Regulators and effector of Ras

The binding and hydrolysis of GTP by Ras is regulated by other protein components. The GEFs function immediately upstream of Ras to stimulate the dissociation of GDP from Ras (reviewed in Boguski and McCormick, 1993). Several GEFs that interact with the Ras proteins have been identified (Feig, 1994) including the *Drosophila* SOS (*son-of sevenless*) (Simon *et al.*, 1991), the mammalian SOS homolog (Bowtell *et al.*, 1992) and *S. cerevisiae* CDC25 (Broek *et al.*, 1987). During R7 photoreceptor differentiation, the *Drosophila* SOS is linked to *Drosophila* homolog of the EGF-receptor *sevenless* via the adaptor protein DrK (*downstream of receptor kinase*). The Sev/Drk/Sos complex activates Ras1 (reviewed in Wassarman *et al.*, 1995). Ras signaling terminates when GTP is hydrolyzed to GDP. GAPs inactivate Ras by accelerating the slow intrinsic rate of GTP hydrolysis by several orders of magnitude (Fig. 2). Five mammalian GAPs for Ras have been described (Boguski and McCormick, 1993; Wittinghofer *et al.*, 1995), including $p120^{GAP}$, the prototype for this class of proteins (Trahey and McCormick, 1987; Trahey *et al.*, 1988 and Vogel *et al.*, 1988), and neurofibromin, the product of the type I neurofibromatosis (NF1) gene (Xu *et al.*, 1990; Martin *et al.*, 1990; and Ballester *et al.*, 1990).

The molecules that interact with GTP-bound Ras and transmit the signal are generally referred to as 'Ras effectors'. At present, there are several proteins have been shown to interact with Ras in a GTP-dependent manner (reviewed by Katz and McCormick, 1997).

The most studied Ras effector is serine/threonine kinase Raf. Biochemical and genetic studies identified the protein kinase Raf as involved in Ras-dependent signaling in mammalian cells, *C. elegans* and *D.*

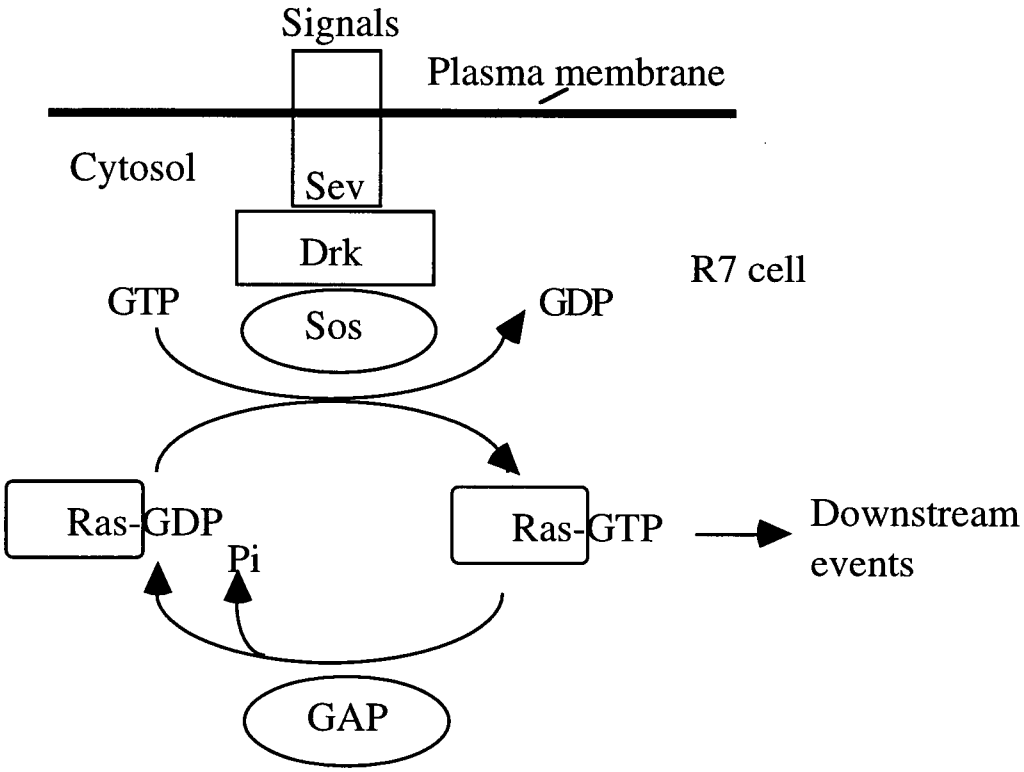


Figure 2. Ras upstream signaling events in R7 development.

This figure is modified from (Alberts *et al.*, 1994). Activated receptor tyrosine kinase Sev activates the guanine nucleotide releasing protein Sos by the small SH adaptor protein Drk. Sos stimulates the inactive Ras-GDP to active Ras-GTP, which activates Ras to relay the signal downstream. (Although not shown here, Ras is bound to the cytosolic face of the plasma membrane.) GAP counteracts the function of Sos by stimulating Ras to hydrolyze its bound GTP and become inactive.

melanogaster (reviewed in Dickson *et al.*, 1994). Yeast two hybrid and GST-fusion protein binding studies indicate Raf is a direct target of Ras (Vojtek *et al.*, 1993; Aelst *et al.*, 1993; Zhang *et al.*, 1993; Warne *et al.*, 1993). Raf plays an essential role downstream of Ras in tyrosine kinase-stimulated signaling pathways promoting cell growth and differentiation (Marshall, 1996). The stimulation of a receptor tyrosine kinase (RTK) results in the recruitment of the guanine nucleotide-releasing factor Sos (Schlessinger, 1994) to the plasma membrane through the SH2 domain of an adaptor protein Grb2 (Clark *et al.*, 1992; Lowenstein *et al.*, 1992). Sos activates Ras and promotes its direct binding to Raf and translocates Raf to the plasma membrane (Moodie *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). Activated Raf is the member of a MAP kinase cascade that results in the activation of ERK1 and /or ERK2 which, in turn, phosphorylate and activate transcriptional factors that induce transcription of genes required for entry into S phase of the cell cycle (reviewed in Marshall, 1995; McCormck, 1995; Marshall, 1996; Katz and McCormick, 1997).

Ral-GDS (Ral guanine nucleotide dissociation stimulator), an exchange factor for the Ras-related protein Ral was also identified to interact with Ras through the effector loop in a GTP-dependent manner *in vitro* in insect cells and in the two hybrid systems (Hofer *et al.*, 1994; Spaargaren and Bischof, 1994; Kikuchi *et al.*, 1994), making it a candidate for a biological effector of Ras action. It was discovered recently (Urano *et al.*, 1996) that Ras can stimulate exchange activity of RalGDS in cells. RalGDS may mediate its effects via Ral, although this issue is still controversial (Katz and McCormick, 1997). At present, the function of Ral is unknown.

Identification the proteins in cell extracts that bind to p21Ras in a GTP-dependent manner has provided direct biochemical evidence for the interaction between phosphoinositide 3-OH kinase (PI 3-kinase) and *ras* (Rodriguez-Viciana *et al.*, 1994). PI 3-kinase interacts with Ras-GTP but not with Ras-GDP and is activated both *in vitro* and *in vivo* as a result of this interaction (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1996). PI 3-kinase has been implicated in the regulation of the actin cytoskeleton by growth factors such as PDGF and insulin (Kotani *et al.*, 1994; Wennstrom *et al.*, 1994; Nobes *et al.*, 1995). Furthermore, PI 3-kinase may provide a link between Ras and the Rho GTPases and appears to function upstream of Rac, since generating 3' phosphorylated phosphoinositides activate Rac guanine nucleotide exchange factors (Hawkins *et al.*, 1995), mediating Ras control of the cytoskeleton (Fig. 3).

Other putative mammalian Ras effectors (Katz and McCormick, 1997) include the family of Ras GTPase-activating proteins, among them p120GAP, neurofibromin, and Gap1 (Marshall, 1996), but it is still open question whether GAPs and neurofibromin are both regulators and effectors of Ras. Two serine/threonine kinases other than Raf: MEK kinase 1 (MEKK1) and protein kniase Cζ (PKCζ) bind Ras, although it is not known whether MEKK1 or PKCζ are activated directly by Ras or not. Two structurally related proteins AF-6 and Canoe have been found recently to bind GTP-Ras (Kuriyama *et al.*, 1996), but the function of these proteins are not known. Finally, kinase suppressor of Ras (KSR-1) has been shown to be downstream of *ras* genetically, but there is no biochemical evidence for interaction between Ras and KSR-1.

Recent evidence has indicated that Ras proteins can regulate more than one signal transduction pathway. Raf-dependent pathway is

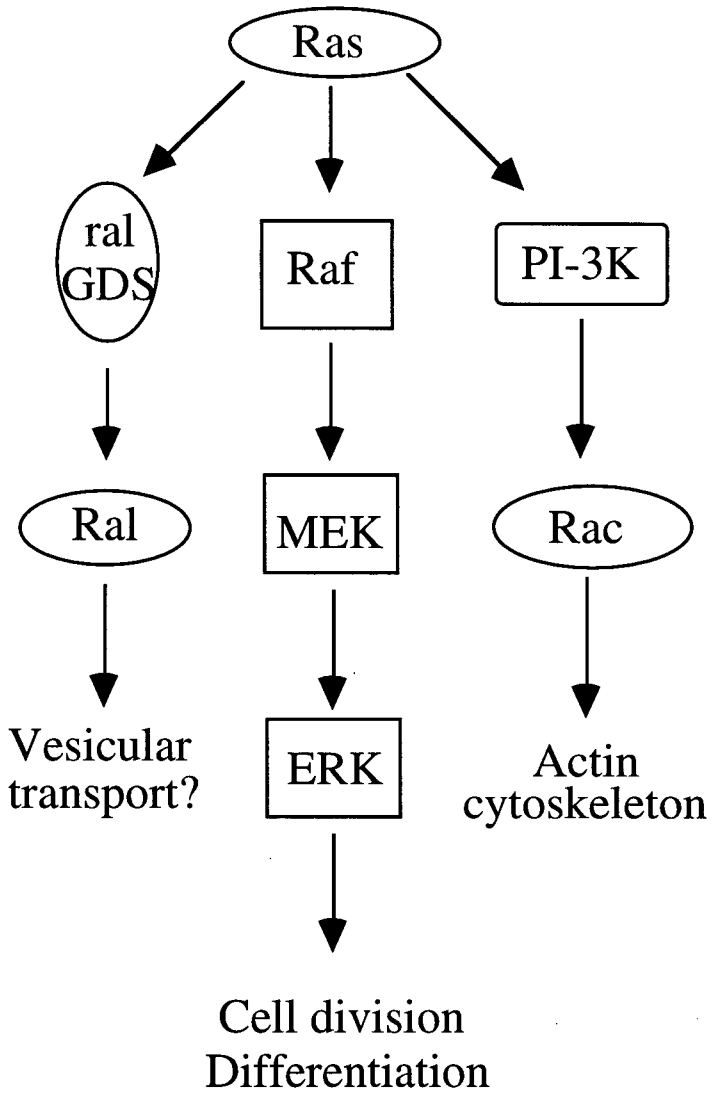


Figure 3. Vertebrate Ras downstream signaling.

This figure is modified from (Marshall, 1996). The strongest candidates for physiological effectors of vertebrate Ras are shown along with the pathways they are proposed to control.

essential for fibroblast proliferation but not needed for Ras to induce membrane ruffling in fibroblasts (Joneson *et al.*, 1996). Further analysis indicates PI 3-kinase is the effector by which Ras induces membrane ruffling, acting through Rac (Ridley, 1994; Rodriguez-Viciana *et al.*, 1997). Efficient neoplastic transformation by Ras requires activation of PI 3-kinase in addition to Raf. (Rodriguez-Viciana *et al.*, 1994; Klinghofer *et al.*, 1996; Marshall, 1996; Rodriguez-Viciana *et al.*, 1997). In fact, in fission yeast *S. pombe*, Ras has been shown to directly regulate two effectors. One is a MAP Kinase kinase kinase, Byr2, and the other is Scd1, a guanine nucleotide exchange factor for the Rho family protein Cdc 42, that is involved in regulation of cell morphology (Chang *et al.*, 1994).

Dictyostelium discoideum **Life cycle**

D. discoideum is a simple eukaryote that has a life cycle that renders it attractive for studies on both proliferation and differentiation since the processes are largely distinct (Loomis, 1982; Firtel *et al.*, 1989; Mann *et al.*, 1991). Vegetative *D. discoideum* cells exist as individual amoebae and use phagocytosis to ingest bacteria and pinocytosis ingest liquid media. Upon nutrient deprivation, the cells initiate an interactive developmental program. Approximately 3 h after starvation, cells within the population initiate a cAMP-mediated response/relay cascade by secreting pulses of cAMP into its surroundings. Nearby cells respond by moving towards the stimulus and by producing cAMP pulses themselves. This chemotaxis and signal relay results in the formation of an aggregate of cells. The aggregation process begins at 5 h and typically requires 2 h for completion. By 12 h after starvation, a single tip, which functions as an organizing center for

morphogenesis, can clearly be distinguished on the mound. At the same time, the initial spatial pattern of two functionally distinct cell types (prestalk and prespore) is established. The tipped mound gradually extends vertically and then falls to the substratum forming a migrating slug by 16 h of development. The anterior 20% of the slug is composed of prestalk cells and the posterior 80% is composed of predominantly prespore cells. Under appropriate conditions, the migrating slug culminates with the formation of a mature fruiting body containing 80% spore cells and 20% vacuolated dead stalk cells rising from a basal disk. The process is complete by 25 h after starvation (Fig. 4)

Signal transduction events during the life cycle of *D. discoideum*

Vegetative growth and aggregation

Vegetative *D. discoideum* cells prey on bacteria by detecting and chemotaxing toward folate and pterin, two bacterial metabolites (Pan *et al.*, 1972; Pan *et al.*, 1975), The transition from single cells to multicellularity is mediated by a variety of signaling molecules. Growing amoebae secrete an autocrine factor, prestarvation factor or PSF, which accumulates in proportion to cell density, serving as a sensor for the availability of nutrients (Clarke *et al.*, 1987, Rathi *et al.*, 1991; Rathi and Clarke, 1992). At a high PSF/bacteria ratios, a prestarvation response is initiated and the expression of several genes involved in early aggregation is increased. (Burdine and Clarke, 1995). Another factor,

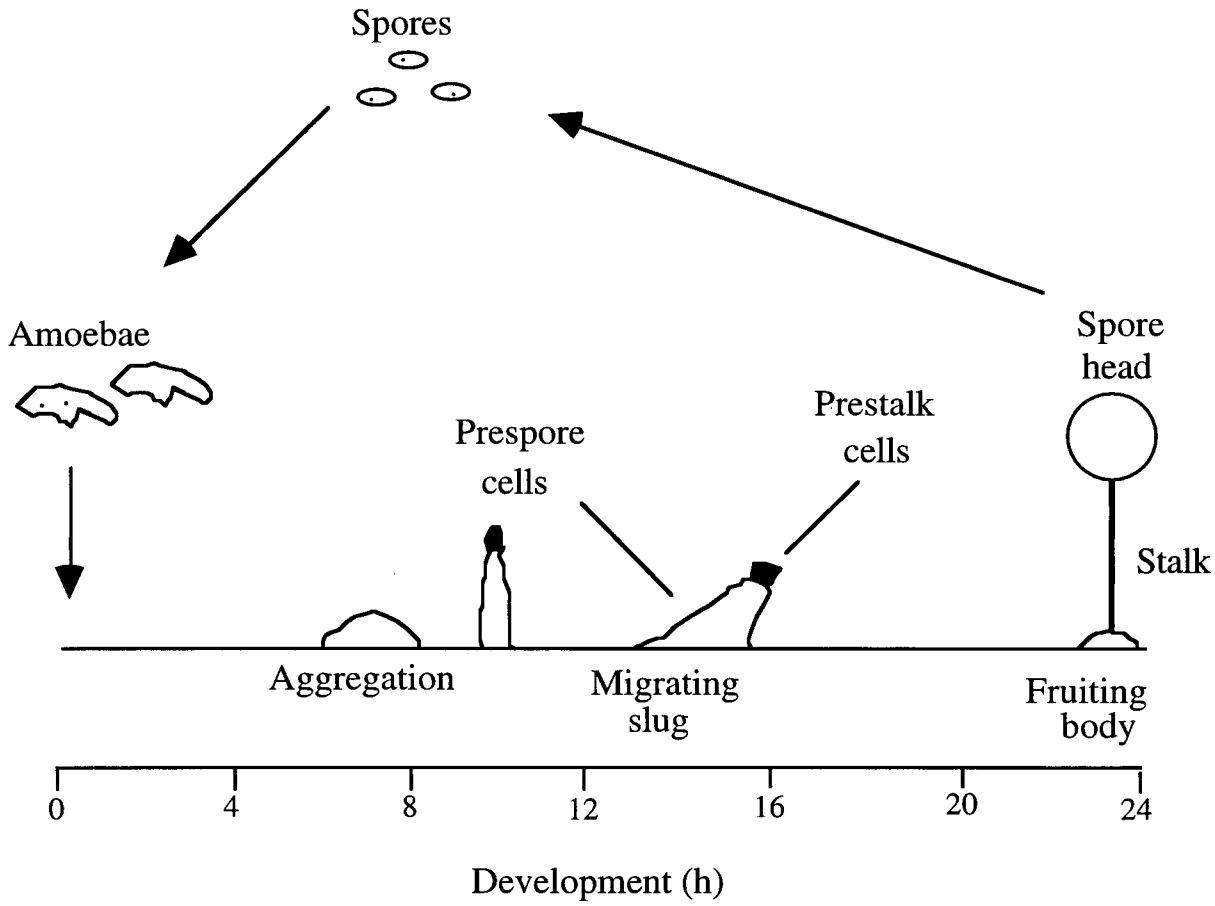


Figure 4. The life cycle of *D. discoideum*

Black areas represent prestalk and stalk cells while clear areas represent prespore and spore cells. The time in development is shown below in hours.

conditioned medium factor (CMF) provides a similar density sensing function which is involved in regulating cAMP signaling after starvation has initiated (Gomer *et al.*, 1991; Yuen *et al.*, 1991).

Aggregation in *Dictyostelium* is mediated by chemotactic responses to pulsatile extracellular cAMP that binds to cell surface receptors and activates intracellular signaling pathways including the activation of adenylyl cyclase, leading to the secretion of cAMP and the relay of the chemotactic signal; the activation of guanylyl cyclase, which is important for chemotaxis; and the expression of genes (including the cAMP receptor cAR1 and the coupled Gα2 subunit), whose encoded products are essential for the aggregation process. These pathways are regulated by oscillatory pulses of cAMP, which control the sequential activation and adaptation of these pathways (Van Haastert 1991; Devereotes 1994; Firtel 1995; Firtel 1996). During aggregation, cAMP, acting through cAMP receptor (cAR1), stimulates adenylyl cyclase producing cAMP, which is then relayed outward from aggregation center to stimulate additional cells. Following stimulation, there is a subsequent adaptation response leading to an inactivation of adenylate cyclase. Extracellular phosphodiesterase (PDE) hydrolyzes the excess extracellular cAMP, thus preparing the cell for response to the next pulse. The roles of many of the components in aggregation have been defined by biochemical, physiological and genetic studies (reviewed in Firtel, 1995; Firtel, 1996; Parent and Devreotes, 1996). Activation of adenylyl cyclases requires the G protein containing the Gα2 subunit coupling to cAR1 and cAR3 (Kumagai *et al.*, 1991; Kesbeke *et al.*, 1988; Insall *et al.*, 1994a) and Gβ subunit regulates adenylyl cyclase directly. Cytosolic regulator of adenylyl cyclase (CRAC) is also required for AC activation (Lilly and Deverotes 1994) possibly by interacting with Gβ subunit (Insall

et al., 1994b; Touhara *et al.*, 1994). cAMP-dependent protein kinase (PKA), presumably activated by increased intracellular cAMP, has also been shown to be required for AC activation.

A possible role for Ras in aggregation has been indicated by the finding that ERK2 and a GEF are essential for aggregation (Segall *et al.*, 1995; Insall *et al.*, 1996). However, cells expressing an activated form of *ras*G fail to aggregate, suggesting that RasG-GTP could be negative regulator of the signal transduction pathway that leads from cAMP receptor to adenylate cyclase activation.

Cell differentiation

Upon the formation of the mound, rising levels of cAMP activate a second signaling pathway that results in the repression of aggregation-stage gene expression and the induction of postaggregative gene expression and the regulatory cascade that leads to the subsequent expression of prestalk- and prespore-specific genes, cell-type differentiation, morphogenesis (Abe and Yanagisawa 1983; Schnitzler *et al.*, 1994, 1995; Firtel 1995). In contrast to the cAMP-mediated responses during aggregation, post-aggregate gene expression and cell-type differentiation require a high, continuous level of cAMP. The expression and activation of the transcription factor GBF is essential for prestalk and prespore gene expression.

The differentiation of cells into prestalk and prespore cells within the multicellular aggregate is regulated by a number of factors including cAMP (reviewed in Gross, 1994; Firtel, 1995; Firtel, 1996). cAMP initially promotes the development of both prestalk and prespore cells but at later stages acts to promote spore development and inhibit stalk

development. Although both prestalk and prespore gene expression requires GBF function, they are regulated by additional distinct mechanisms. Protein kinase A (PKA), is required for normal development and has been shown to be a positive regulator of prespore gene expression and spore maturation, presumably functioning to mediate the effects of intracellular cAMP (Simon *et al.*, 1989; Firtel and Ahapman, 1990; Mann *et al.*, 1994; Hopper *et al.*, 1995). Protein kinase A also appears to inhibit prestalk development but plays a role in subsequent stalk formation (Mann and Firtel, 1993; Harwood *et al.*, 1992b). Glycogen synthase kinase 3 (GSK-3) and ERK2 are also essential for prespore gene expression (Harwood *et al.*, 1995, Gaskins *et al.*, 1996), but it is unknown whether GSK3, PKA and ERK2 function in common or parallel pathways. The morphogen DIF (differentiation-inducing factor) is required for the prestalk pathway and inhibits the prespore pathway (Kopachik *et al.*, 1983; Williams *et al.*, 1987). DIF activates a STAT (Signal Transducers and Activators of Transcription) protein which induces prestalk cell differentiation and represses stalk cell differentiation (Kawata *et al.*, 1997). The low affinity cAMP receptor cAR4 is also required for prestalk cell differentiation (Louis *et al.*, 1994).

Ras and *ras* related genes in *D. discoideum*

In *Dictyostelium*, five *ras* genes (*ras*D, *ras*G, *ras*B, *ras*S, and *ras*C) and one *rap* gene (*rap1*) have been reported, each having a specific expression pattern (Reymond *et al.*, 1984; Robbins *et al.*, 1989; Daniel *et al.*, 1993; Daniel *et al.*, 1994) and recently two additional genes have been identified (Wilkins and Insall, unpublished). The first five *ras* genes share the four well-conserved GTP-binding domains as well as the C-

terminal CAAX box. *ras*G encodes a protein which shares 69% overall amino acid identity with the human H-*ras* gene product, and the gene is expressed during growth and early development with expression declining markedly during aggregation (Robbins *et al.*, 1989). *ras*D, whose gene product share 65% identity with H-*ras*, is expressed only during late aggregation and slug formation (12-16 hours) (Reymond *et al.*, 1984). The RasG and RasD proteins are 82% identical to each other and they have identical effector and effector-proximal domains. The binding site for the Ras-specific monoclonal antibody, Y13-259, is also totally conserved in RasD and RasG. Four other *ras* sub-family genes (*ras*B, *ras*C, *ras*S, and *rap1*) encode products more distantly related to Ha-*ras*. The RasB, RasC and RasS gene products share 59%, 56% and 54% amino acid identity respectively with H-*ras*. *ras*B is maximally expressed during vegetative growth and early development but expression remains relatively high during the remainder of development (Daniel *et al.*, 1994). *ras*C is expressed maximally during aggregation and slug migration but significant expression is detected during vegetative growth throughout the remainder of development (Daniel *et al.*, 1993). *ras*S is expressed only during aggregation (4-8 hours).

Aside from their patterns of expression, little is known about the functions of *Dictyostelium* Ras protein. Overexpression of an activated form of RasD (G12T) gene during development resulted in formation of aggregates with multitips, instead of the normal single tips, and a block in further development (Reymond *et al.*, 1986). Further analysis has indicated that this development defect is accompanied by a profound change in the fate decision; prestalk cell gene expression is markedly enhanced and prespore gene expression is almost totally inhibited (Louis *et al.*, 1997), suggesting that RasD has a role in regulating cell type

determination during differentiation. Surprisingly, *ras*D null mutant show temporally and morphologically normal development although there is a significant increase in spore cell formation. (Khosla and Wilkins, unpublished data).

Overexpression of *ras*G containing an activating mutation (RasG-G12T) during growth caused a block in aggregation (Khosla *et al.*, 1996). The defect in aggregation was rescued by pulsing cells with cAMP and by mixing with wild type cells, suggesting that the mutant cells are able to receive cAMP signals but not able to generate them. In contrast, cells overexpressing wild type *ras*G gene or a presumptive dominant negative *ras*G-S17N differentiated normally, suggesting that the inhibition of the initiation of aggregation is due to enhanced levels of RasG-GTP. *ras*G-G12T expression also caused marked cytoskeletal changes and wild type *ras*G produced less pronounced morphological changes. *ras*G-S17N produced a different cell shape change, and the *ras*G null cells have a defect in the cytoskeleton, which results in abnormal cytokinesis when cells are grown in suspension (Tuxworth *et al.*, 1997). These results are all consistent with RasG having a role in regulating the cytoskeleton. Finally transformants overexpressing *ras*G or *ras*G-G12T also exhibit a slight defect in cytokinesis, they have on average 1.7 nuclei per cell under conditions where wild type cells have a single nucleus.

MATERIALS AND METHODS

Materials

The following reagents were purchased from suppliers indicated in brackets. Cyclic adenosine monophosphate (cAMP), streptomycin, ampicillin, and folate (Sigma, St. Louis, USA); X-ray film (Kodak, Canada); Radiolabelled [α^{35}S] dATP (Dupont NEN Canada Inc.); filters (Millipore, USA); the enhanced chemiluminescence kit for western blot analysis (Amersham, Canada); NuclebondR Ax-20 kit for isolating plasmid DNA was (Macherey-Nagel, Germany); Bacteriological peptone and yeast extract for the *D. discoideum* growth media (Oxoid, UK) and for bacterial growth media (Canadian Life Technologies GIBCO BRL); G418 (Geneticin), restriction endonucleases and modifying enzymes (GIBCO BRL); Sequenase (United States Biochemical). All other chemicals were purchased from Fisher Scientific Co. (Vancouver, Canada) or BDH (Vancouver, Canada). Hoechst 33258 dye was a gift from Dr. R. E. W. Hancock's laboratory (UBC). Oligonucleotides were synthesized by the NAPS facility (UBC).

The preparation and use of the RasG-GST-fusion protein antibody for western blot analysis has been described previously (Khosla *et al.*, 1994) Goat anti-rabbit antibody, conjugated to horseradish peroxidase was purchased from Amersham (USA).

The *E. coli* strains DH5αF', XL-1 and RZ1032 were used for the various cloning manipulations. The genotype of DH5αF' is: F'/*endA1 hsdR17*($r_K^- m_K^+$) *supE44 thi-1 recA1gyrA*(Nal^r) *relA1 Δ(lacZYA-argF)U169 deoR (Ø80dlacΔ(lacZ)M15)* (Raleigh *et al.*, 1989). The genotype of XL1MRF' is: *endA1*, *hsdR17* (r_K-, m_K+), *supE44*, *thi-1* , l-, *recA1*, *gyrA96, relA1,* (*lac*-), F'[proAB, *lacI*^{q}ZΔM15, *Tn10* (tetR)] (Stratagene). The genotype of

RZ1032 is: *HfrKL16PO/45[]ys*A (61-62)*] dut1, ung1, thi1, relA1Zbd-279:: Tn10supE44* (Kunkel *et al.*, 1987). The pVEII vector was donated by Wolfgang Nellen and modified to remove the discoidin ATG translation site by Meenal Khosla (Rebstein *et al.*, 1993). The pTZ19R-*ras*G-G12T vector was constructed by Meenal Khosla (Khosla *et al.*, 1996).

D. discoideum **growth and differentiation**

The parental axenic line Ax-2 strain of *D. discoideum* that was used in all experiments was grown axenically in HL5 medium (Watts and Ashworth, 1970) (14.3 g neutralized bacteriological peptone, 7.15 g yeast extract, 0.96 g Na_2HPO_4 and 0.486 g KH_2PO_4 per liter of water) with gyratory shaking at 175 rpm at 22°C or on SM nutrient agar (10 g glucose, 10 g neutralized bacteriological peptone, 1g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 1.55 g $NH_2PO_4.H_2O$, 1 g $KHPO_4$ and 20 g bacto-agar per liter of water) plates in association with *E. aerogenes*. Cell numbers were determined in duplicate with a hemacytometer. The transformed Ax-2 strains were maintained in HL5 medium in the presence of 10 μg/ml G418 (Geneticin) and 1 mM folate in rotatory agitated suspension (175 rpm) at 22°C.

D. discoideum development on filters was initiated as previously described (Khosla *et al.*, 1990). Exponentially growing vegetative cells at a density of between 1×10^6 and 2×10^6 cells per ml were harvested by centrifugation at 700 × g for 2 min and then washed twice in Bonner's salt (10 mM NaCl, 10 mM KCl and 2 mM $CaCl_2$), (Bonner, 1947). For development, 2.5×10^7 washed cells were plated on a 4.0 cm diameter nitrocellulose filter (pore size = 0.45 μm), resting on a Bonner's salt (BS) saturated pad in a 60 mm petri dish. The filters were incubated at 22°C

in a moist chamber. To observe *D. discoideum* development following growth on bacteria was accomplished by pipetting 1- 5 µl of 2×10^6 cells/ml *Dictyostelium* cells in HL5 medium onto a freshly inoculated lawn of *Enterobacter aerogenes* on an SM nutrient agar plate. Plates were incubated at 22°C and after the *D. discoideum* cells had consumed the bacteria (usually 4 days), development could be observed in the zone depleted of bacteria.

Induction of the discoidin promoter

To maintain strains containing genes under the control of the discoidin *dis* I γ gene promoter in a suppressed state, 1 mM folate was added to the HL5 medium. To maximally induce expression from the discoidin promoter, cells were incubated with conditioned HL5 medium, since conditioned medium contains a pre-starvation factor (PSF) (Clarke *et al.*, 1987; Clarke *et al.*, 1988), that induces expression from the discoidin promoter (Rathi *et al.*, 1991). Conditioned HL5 medium was prepared by growing Ax-2 cells to a density of approximately 2×10^6 cells/ml, removing the cells by centrifugation and filtering the medium through a 0.2 µm pore size nitrocellulose filter. In some experiments, induction was achieved by growing cells to $1\text{-}2 \times 10^6$ cells/ml in the absence of folate.

Transformation of *D. discoideum*

D. discoideum Ax-2 cells were transformed by the calcium phosphate precipitation technique (Nellen *et al.*, 1984) in Bis-Tris HL5 (Egelhoff *et al.*, 1989). A total of 8×10^6 exponential-phase Ax-2 cells

were incubated with 10 μg of vector DNA in the form of calcium phosphate DNA precipitate for 4 hours, the cells were given a 2 minute osmotic shock with 15% glycerol as previously described (Early and Williams, 1987). Transformants were selected in HL5 medium containing 30 μg/ml G418, 50 μg/ml stretomycin, 50 μg/ml ampicillin, and 1mM folate and colonies were visible after approximately 14 days. Individual clones were picked using a pipette and transferred initially to 24 well plates, and then to 100 mm plates. Once cell growth was well established, stable transformants were maintained in shake suspension in HL5 media containing 10 μg/ml G418, 50 μg/ml streptomycin, 50 μg/ml ampicillin, and 1 mM folate.

Nuclear staining and analysis of cell morphology

To examine cell morphology, cells were plated 3×10^3 cells/cm^2 on a glass coverslip in a 60 mm petri dish and incubated for 24 hours either in the presence of 5 ml of conditioned HL5 medium or in HL5 medium containing 1mM folate to induce or repress the *discoidin* promoter, respectively. To determine nuclear number, the adherent cells were washed, fixed with 3.7% formaldehyde in BS for 10 minutes and then washed 3 times with PBS (8 g NaCl, 0.2 g KCl, 1.44 g $NaHPO_4$, 0.24 g KH_2PO_4 in one liter of distilled water). Cells were then permealibilized for 5 minutes in -20°C actone, rehydrated in PBS, overlaid with 0.0005% Hoechst dye #33258 for 5minutes and then washed with PBS (Harlow and Lane, 1988). The glass coverslips were briefly dried before mounting on glass slides using 50% glycerol as mounting solution and viewed with a Zeiss Axiophot microscope equipped with epifluorescence. Cell morphology of the same cells were then observed using phase contrast

microscopy. All photographs were taken using Kodak TMAX 400 black and white film.

Scanning electron microscopy

A total 4×10^3 cells were plated on 1/2 inch glass coverslip in a 60mm petri dish and incubated for 24 hours in the presence of 5 ml of conditioned HL5 medium to induce the *discoidin* promoter. The coverslips were removed and the cells were fixed in 2.5% gluteraldehyde buffered with 100mM sodium cacodylate, pH 7.4. The cells were then treated successfully with 1% OsO_4, 2% Tannic acid and 1% OsO_4 again. The attached cells were dehydrated in a graded ethanol series and critical point dried with liquid carbon dioxide. The coverslips with adhering dried cells were mounted on specimen stubs, sputter coated with gold and viewed with an Cambridge 250T Scanning Electron Microscope.

Plasmid DNA preparation

Plasmid DNA was isolated from DH5αF' *E. coli* cells using alkaline lysis miniprep procedure (Maniatis *et al.*, 1989) or a PEG-precipitation large sale procedure (Maniatis *et al.*, 1989). Competent DH5αF' *E. coli* cells were prepared using the rubidium chloride technique and transformation of competent cells was done as described (Maniatis *et al.*, 1989). Transformed DH5αF' *E. coli* cells were selected on LB ampicillin plates (10 g Bacto-tryptone, 5 g Bacto yeast extract, 10 g NaCl, 15 g Bacto-agar pH 7.0, with 60 μg/ml ampicillin).

Sequencing

Single stranded DNA was generated following infection of DH5αF' with K07M13 helper phage, by PEG precipitation. The precipitated DNA was isolated on glass filters and then eluted into TE (Maniatis *et al.*, 1989). Double stranded DNA was isolated using the NucleobondR Ax-20 kit Macherey-Nagel (Duren, Germany). DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) with modified T7 DNA polymerase. Sequencing reactions were performed according to the manufacturer's (United States Biochemical) protocol except that the Sequenase reaction buffer was added after DNA denaturation.

Site directed mutagenesis and vector constructions

Missense mutations of *ras*G-G12T gene were created as follows. The pTZ19R-*ras*G-G12T vector (Khosla *et al.*, 1996) was transformed into *Escherichia coli* strain RZ1032 (Kunkel *et al.*, 1987). Uracil containing single-stranded phagemid DNA was isolated after infection with the helper phage, M13K07 (Vireira *et al.*, 1987) and was used as a template for oligonucleotide-directed mutagenesis reaction (Kunkel *et al.*, 1987). To create the mutations, the mutagenic oligonucleotides had the following sequences:

5'-TACGATCCAT<u>T</u>CTATCGAAG-3' (T35S)

5'-CTATCGAAGATTCAT<u>GT</u>AGAAAACAAGTTAC-3' (Y40C)

5'-CATACAGAAAACAAGTT<u>CAA</u>ATTGATGAAGAAACTTG-3' (T45Q)

In each case the substituted bases are underlined. The mutated genes were transformed into *Escherichia coli* strain DH5αF' (Stratagene). To

obtain the pVEII-*ras*G construct, the pVEII vector was digested by *Kpn*I, and treated with T4 DNA polymerase to generate blunt ends. The *ras*G inserts were isolated by an *Bgl*II/*Eco*RI digestion, treated with Klenow and dNTPs to generate blunt ends and then ligated into blunt ended pVEII vector. The *ras*G fragment was ligated into the pVEII vector in the sense orientation downstream of the discoidin promoter and created the 3' flanking *Eco*RI site (Fig. 5). The junctions of the constructs were confirmed by *Eco*RI digestion and by double stranded DNA sequencing reactions. pVEII vector also contains the actin 15-Tn903 resistance cassette (Blusch *et al.*, 1992) as a G418 selectable marker. The constructs were transformed into XL-1.

Electrophoresis and immunoblotting

SDS-PAGE and immunoblotting techniques were performed as described (Robbins, 1991). For western blot analysis of transformants expressing genes under the control of the discoidin promoter, cells were inoculated at a density of 5×10^5 cells/ml and grown for 24 hours in shake suspension in either conditioned medium or HL5 containing 1mM folate. Cells were lysed in 1% SDS. Protein concentration was estimated by UV Absorbance (Harlow and Lane, 1988). About 20 μg of protein from each transformant was mixed with an equal volume of 2 × loading dye (20% glycerol, 10% β-mercaptoethanol, 4.6% SDS, 125mM Tris-HCl, pH 6.8), boiled for 5 min, then were electrophoresed on a 12% SDS-ploy acrylamide gel.

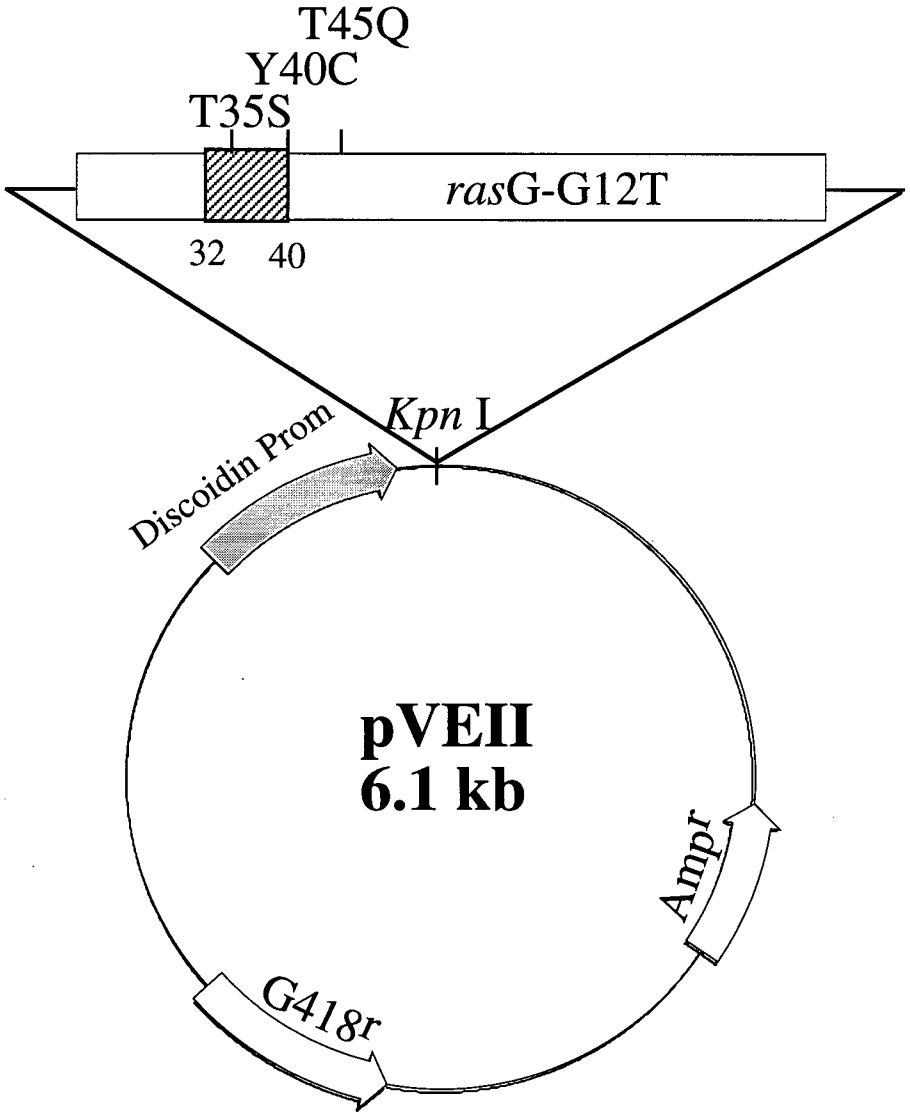


Figure 5. Amino acid substitutions of RasG-G12T and vector constructions

The positions of the substituted amino acid are indicated, the hatched box indicate the proposed effector domain. The mutated *ras*G-G12T genes were cloned under the control of dicoidin promoter in the pVEII vector.

After electrophoresis, the protein were transferred to a nitrocellulose filter for 1 hour at 90V (Towbin *et al.*, 1979). Prestained molecular weight markers (BioRad) were used to estimate protein sizes. The nitrocellulose blots were stained with Ponceau S (Harlow and Lane, 1988) to confirm that equal amounts of protein had been loaded and transferred in all lanes. The membrane was blocked at room temperature with 1 × TBS (8 g NaCl, 0.2 g KCl, 3 g Tris-HCl in 1 liter H_2O, pH 7.4) 5% skim milk and o.1% Tween-20 at least for 1 h.and were then washed twice for 5 min. with TBS and 1% Tween-20. The RasG protein was detected with a specific anti-RasG-GST protein antibody at a 1: 500 dilution containing 0.5% powered milk, 0.1% Tween-20 and incubated with nitrocellulose membranes at room temperature for overnight. The membranes were then washed four times (for 5 min. each) in TBS-Tween. And the binding of the anti-RasG antibody was detected using a secondary goat anti-rabbit antibody by ECL which was recorded on X-ray film.

Chemotaxis and motility assays

Chemotaxis assays were performed as previously described (Khosla *et al.*, 1996). Chemotaxis to folate was assessed by plating approximately 10^6 cells in 1µl on 2% agar plates containing PDF/MES (20 mM KCl, 1.2 mM $MgSO_4$, 7.5 mM morpholinethanesulfonic acid [MES; pH 6.5]) and 40 µM folate. The average distance migrated by the halo of cells that escape from the cell mass was determined after 24 h. Chemotaxis to cAMP was determined in an identical fashion except that the 2% agar plates contained PDF/MES and 10 uM cAMP, and the cells were shaken at 150 rpm for 4 h in PDF/MES at 5 × 10^6 cells per ml prior to plating.

RESULTS

Identification of the effector residues of RasG-G12T that are required for phenotypes caused by activated RasG In *Dictyostelium discoideum*

Introduction

Recent studies have provided overwhelming evidence that Ras regulates at least two distinct signal transduction pathways in mammalian cells; one involves the Raf-dependent activation of ERK1 and ERK2, resulting in transcription of genes that regulate growth and differentiation; the other pathway is Raf-independent and involves activation of Rho family GTPases which play a role in actin cytoskeletal reorganization (Katz and McCormick, 1997). Partial loss of function mutants of Ras have been used help to define its downstream effector pathways (White *et al.*, 1995; Joneson *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997). For example in quiescent fibroblasts, ectopic expression of activated H-RAS (H-RASV12) induces membrane ruffling, MAP kinase activation and stimulation of DNA synthesis. When secondary mutations (T35S or Y40C) were introduced into the activated H-RAS effector domain (32-40), either MAP kinase activity (Y40C) or the membrane ruffling (T35S) effects were not induced (Joneson *et al.*, 1996). It has been suggested that the effector mutations S35 and C40 in a V12 background correlate with either of two distinct Ras effector pathways to induce transformation in mammalian cells (Rodriguez-Viciana *et al.*, 1997).

Expression of activated RasG (G12T) protein in vegetative *D. discoideum* cells resulted in cytoskeletal and cytokinesis defects and

cells that did not aggregate when starved for nutrients (Khosla *et al.*, 1996). To identify the effector resides of the RasG that block the aggregation and/or produce the cytoskeletal and cytokinesis defects in *Dictyostelium* and to determine if there are multiple downstream effectors involved, secondary mutations (T35S, Y40C) were introduced into *ras*G-G12T within the effector domain (32-40) and a mutation (T45Q) was introduced within one of the effector flanking regions (Fig. 6). Residue 45 is not conserved in most of the *Dictyostelium* Ras subfamily proteins and the amino acid in RasG is different from that found in mammalian Ras (Fig. 6). In mammalian Ras, V45 has been found to be one of the most critical amino acids required for Ras function (reviewed by Marshall, 1993). A V45E mutation prevented an activated *ras* gene from producing foci formation in NIH 3T3 fibroblast cell and from promoting neurite outgrowth of PC12 cells (Marshall *et al.*, 1991; Fujita-Yoshigaki *et al.*, 1991).

Overexpression of the mutated *ras*G-G12T genes

Mutated *ras*G genes T35S (ACT→TCT), Y40C (TAC→TGT), or T45Q (ACC→CAA) were cloned downstream of the folate-repressible discoidin (*dis*1γ) promoter in the vector pVEII (Fig. 5), and the vector was introduced into *D. discoideum* by transformation using the calcium phosphate precipitate procedure. Transformants were selected in the presence of 30 μg G418 to select for the uptake of the vector and 1mM folate to repress the *discoidin* promoter.

For each transformation twelve G418 resistant clones were isolated and RasG protein levels under inducing conditions were determined by Western blot analysis using a RasG-specific antibody. The western blot

```
          26                *         *         *     48
H-Ras     N H F V D E Y D P T I E D S Y R K Q V V I D G
RasG      - - - I - - - - - - - - - - - - - - T - - E
RasD      - - - I - - - - - - - - - - - - - - S - - D
RasB      - - - I E - - - - - - - - - - - R - C Q V - E
RasC      - - - I A - - - - - - - N - - - - - N - - E
RasS      - - - - - - - - - - L - - - - - - T T V - -
```

Figure 6. Alignment of the human H-Ras extended effector sequence with the *Dictyostelium* Ras subfamily protein sequences.

Numbers indicate amino acid positions in each protein (aa 26-48). Stars indicates amino acid positions of mutants (35, 40 or 45). Dashes indicate amino acids identical to those in H-Ras. The effector domain residues (Sigal *et al.*, 1986) are underlined.

data for six of the isolates are shown in Figure 7. Most of the levels of RasG were considerably higher than that found in the parental Ax-2 cells (Fig. 7). For each double mutant construct, a transformant clone that expressed a level of RasG protein equal to or higher than that of original pVEII-*ras*G-G12T transformant was selected for further analysis (Fig. 7).

The effect of mutated *ras*G-G12T genes on cell morphology

To determine the effects of high levels of the mutated RasG protein on cell morphology in vegetative *Dictyostelium*, transformants were plated on glass coverslips and incubated for 24 hours in conditioned medium. Conditioned medium contains a pre-starvation factor (PSF) (Clarke *et al.*, 1987; Clarke *et al.*, 1988), which enhances expression from the discoidin promoter (Rathi *et al.*, 1991). The cells were fixed with formaldehyde and then observed by phase contrast microscopy. The transformant clones expressing *ras*G-G12T/T35S (Fig. 8I) or *ras*G-G12T/Y40C (Fig. 8J) had a morphology that resembled the parental Ax-2 cells (Fig. 8K). In contrast, many of the cells in a population expressing high levels of *ras*G-G12T/T45Q exhibited a flattened and spread out morphology (Fig. 8 panel E, F, G, H) that was similar to that of cells expressing activated *ras*G-G12T (Fig 8 panel A, B, C, D). To examine if there were subtle differences in the morphology of the transformants expressing *ras*G-G12T and transformants expressing *ras*G-G12T/T45Q that were not readily apparent by phase contrast microscopy, vegetative cells were observed by scanning electron microscopy (SEM) and their appearance is shown in Figure 9. The transformant expressing *ras*G-G12T/T45Q (Fig. 9C and 9D) resembled the *ras*G-G12T transformant (Fig. 9A and 9B). Both populations exhibited heterogeneity in their morphology.

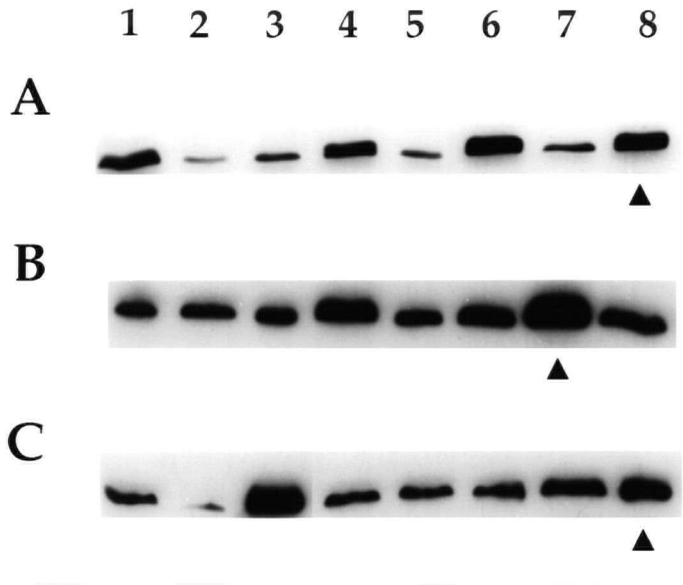
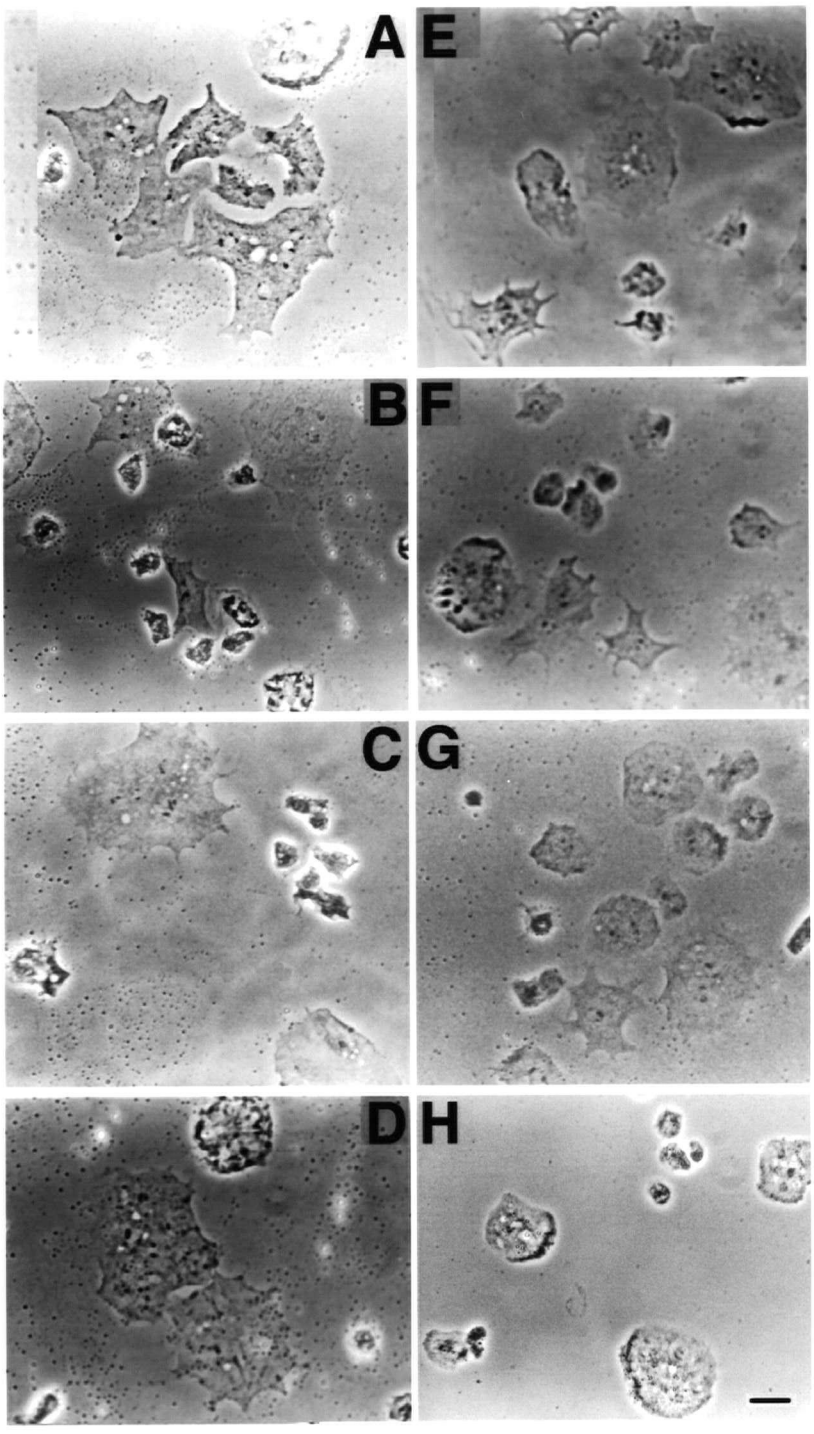


Figure 7. Expression of RasG proteins containing G12T/T35S, G12T/Y40C and G12T/T45Q substitutions.

Cells were incubated in conditioned medium for 24 hours and then lysed in 1% SDS, 20 μg of total protein was separated by SDS-PAGE, transferred to nitrocellulose and probed with an anti-RasG antibody. Lane 1 is the pVEII-*ras*G-G12T transformant. Lane 2 is the parental Ax-2. Lane 3-8 represent independent transformants expressing the doubly mutated RasG proteins RasG-G12T/T35S (panel A), RasG-G12T/Y40C (panel B) or RasG-G12T/T45Q (panel C). The arrows indicate the transformants selected for further detailed analysis.


A
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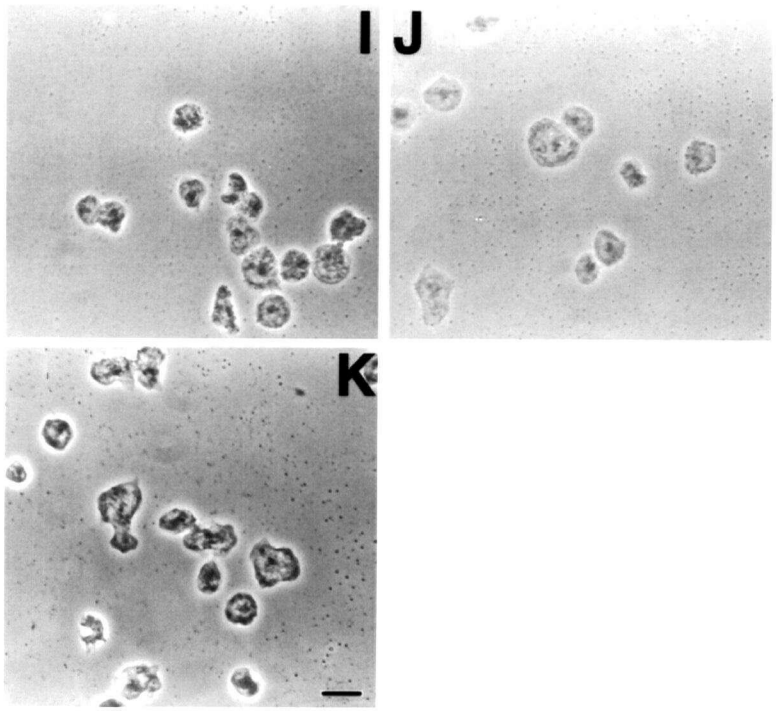

Figure 8. Morphology of vegetative cells

pVEII-*ras*G-G12T (A, B, C, D), pVEII-*ras*G-G12T/T45Q (E, F, G, H), pVEII-*ras*G-G12T/T35S (I), pVEII-*ras*G-G12T/Y40C (J) and Ax-2 (K), cells were incubated with conditioned media for 24 hours and fixed with formaldehyde and then photographed with phase contrast optics. The cell morphology of Ax-2 and *ras*G-G12T cells shows no significant change before and after fixation with formaldehyde (data not shown). The bar is 10 μm.

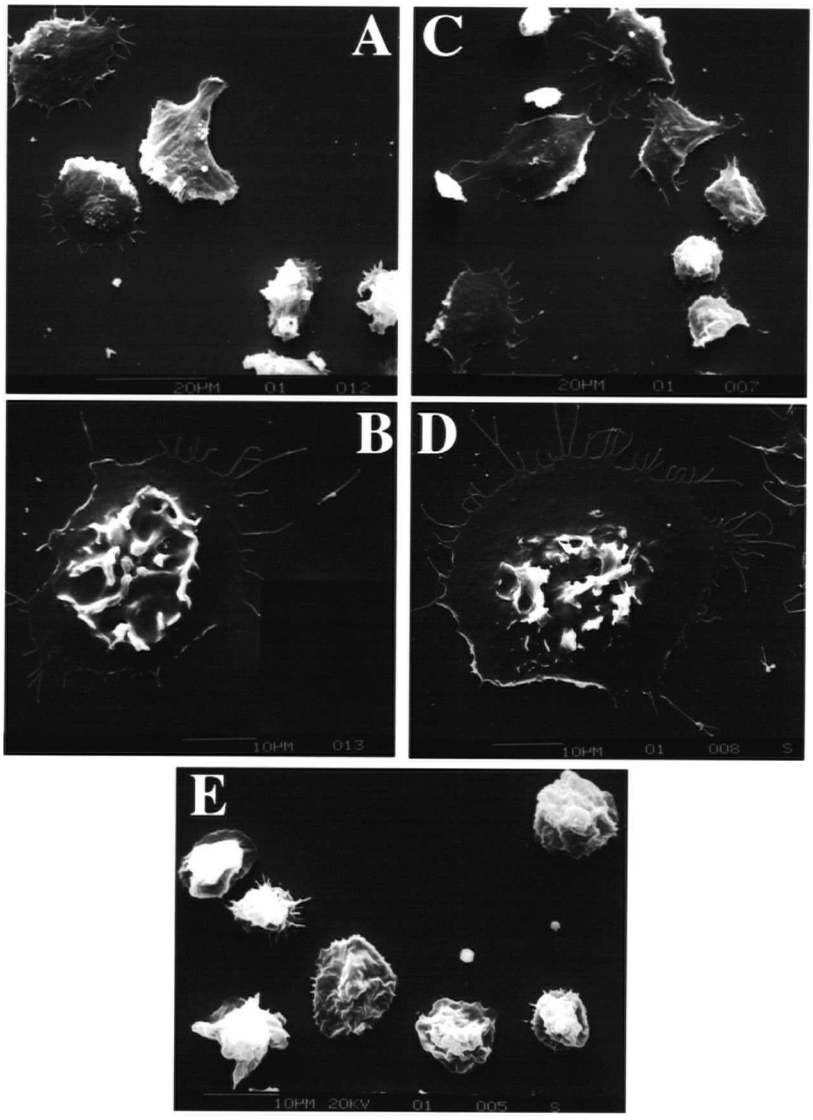

Figure 9. Scanning Electron Microscopy of vegetative cells pVEII-*ras*G-G12T (A and B); pVEII-*ras*G-G12T/T45Q (C and D) and Ax-2 (E) cells were grown in conditioned medium for 24 hours, fixed, dehydrated, critical point dried, coated with gold and then photographed using scanning electron microscope as described in the Materials and Methods.

Some cells were extensively flattened and spread and exhibited considerable dorsal membrane ruffling with many fine elongated filopodia (Fig. 9B and 9D), whereas some cells were flattened and spread but exhibited lateral membrane ruffling with few elongated filopodia (Fig. 9A and 9C). Other cells in the two populations had an appearance more characteristic of wild type Ax-2 cells (Fig. 9E).

Determination of the number of nuclei in transformed cells

There was an increase in the number of multinucleate cells in populations of cells expressing *ras*G-G12T, suggesting a defect in cytokinesis (Rebstein, 1996). When cells were induced with conditioned medium during growth on a surface, multinucleate cells were frequently observed in transformants expressing *ras*G-G12T/T45Q (Fig. 10C). In contrast, most of the cells expressing *ras*G-G12T/T35S (Fig. 10A) or *ras*G-G12T/Y40C (Fig. 10B) had single nuclei like Ax-2 (Fig. 10E). The number of nuclei in the cells transformed with pVEII-*ras*G-G12T/T35S, pVEII-*ras*G-G12T/Y40C and pVEII-*ras*G-G12T/T45Q was determined and compared to the number in Ax-2 and *ras*G-G12T cells (Table 1). Transformants expressing *ras*G-G12T/T45Q had an average of 2.0 nuclei/cell, which was significantly different from the value (1.2 nuclei/cell) for Ax-2 ($P<.05$), but was not significantly different from the value (1.7 nuclei/cell) for pVEII-*ras*G-G12T transformant ($P>.05$). Folate repression of the discoidin promoter, which drives expression of the introduced *ras* gene, abolished the multinucleate phenotype of cells transformed with *ras*G-G12T or *ras*G-G12T/T45Q (Table 1). To test if growth conditions affected cytokinesis, the transformants expressing either *ras*G-G12T or *ras*G-G12T/T45Q were induced with conditioned

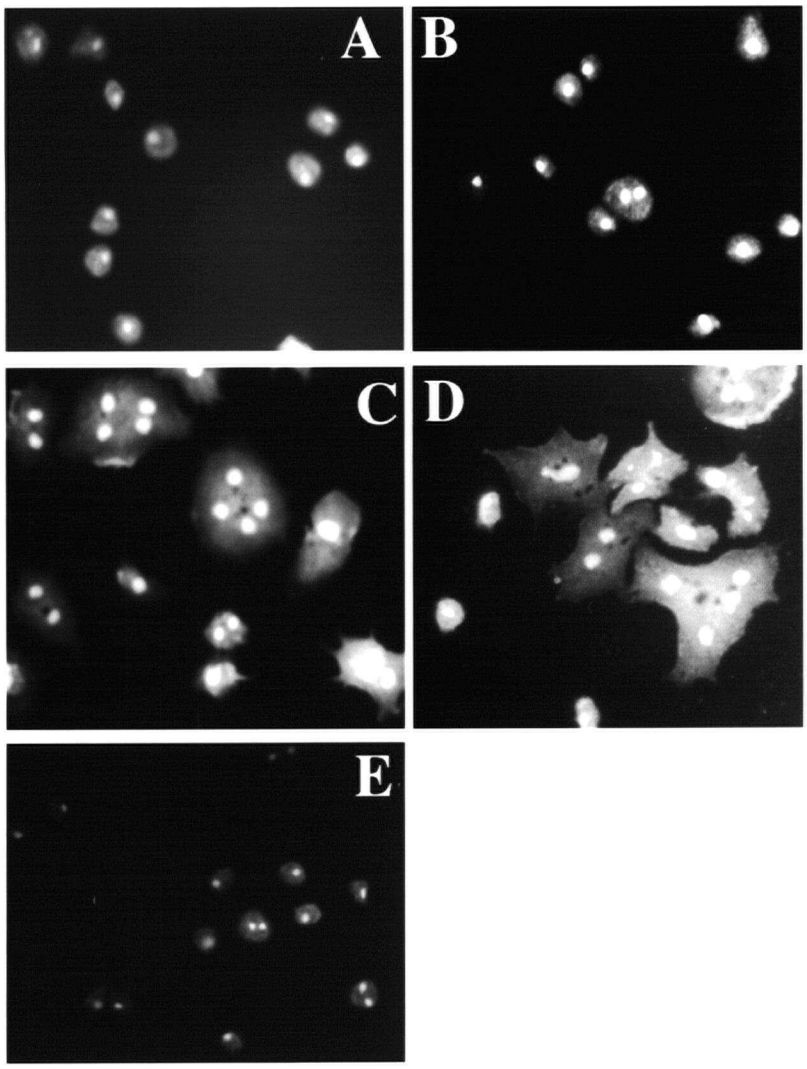

Figure 10. Nuclear staining of Ras transformed cells

pVEII-*ras*G-G12T/T35S (A), pVEII-*ras*G-G12T/Y40C (B), pVEII-*ras*G-G12T/T45Q (C), pVEII-*ras*G-G12T (D) and Ax-2 (E) cells were incubated in conditioned media for 24 hours. The cells were fixed and stained with Hoechst dye as described in the Material and Methods.

Table 1. The number of nuclei per cell

	Number of nuclei[a]		
	Suspension Growth	Surface Growth	
Strain	Induced[b]	Induced[b]	Repressed[c]
pVEII-*ras*G-G12T	1.5 (1)	1.7 ± 0.30 (4)	1.1 (1)
pVEII-*ras*G-G12T/T35S	N/D	1.2 ± 0.04 (4)	1.1 (1)
pVEII-*ras*G-G12T/Y40C	N/D	1.2 ± 0.08 (3)	1.0 (1)
pVEII-*ras*G-G12T/T45Q	2.2 (1)	2.0 ± 0.16 (3)	1.0 (1)
Ax-2	1.4 (1)	1.2 ± 0.06 (4)	1.2 (1)

[a] Mean ± standard deviation of the number of experiments shown in brackets. An average of 200 cells were analyzed per experiment.

[b] Cells were grown in conditioned HL5 media for 24 h.

[c] Cells were grown in HL5 media with 1mM folate for 24 h.

media during growth in suspension culture and then were plated on a surface for 30 minutes before nuclear staining. The average number of nuclei for Ax-2 was slightly increased to 1.4 nuclei/cell, but the number for *ras*G-G12T/T45Q transformant was still higher (Table 1).

Developmental phenotypes of the transformants expressing *ras*G-G12T/T35S, *ras*G-G12T/Y40C or *ras*G-G12T/T45Q

RasG protein levels expressed during growth in the presence of folate (Khosla *et al.*, 1996) or during growth on bacteria (Khosla, unpublished data) were found to be relatively low. However, these relatively low levels of activated RasG-G12T were sufficient to block aggregation (Khosla *et al.*, 1996). All the isolated transformants expressing *ras*G-G12T/T35S, *ras*G-G12T/Y40C or *ras*G-G12T/T45Q (twelve independent clones from each transformation) differentiated normally after growth on bacteria (data not shown). To determine if the expression of high levels of protein would produce the defect in aggregation, the *ras*G-G12T/T35S, *ras*G-G12T/Y40C and *ras*G-G12T/T45Q transformants that had been selected for the previously described morphological and cytokinesis studies were grown in HL5 medium in the absence of folate to densities of between 1×10^6 and 2×10^6 cells per ml, plated on a Millipore filter and then incubated under starvation condition to induce differentiation. These transformants also formed aggregates and differentiated normally (data not shown). In addition, normal development occurred when the transformants were incubated on non-nutrient agar.

Since cells transformed with pVEII-*ras*G-G12T exhibited reduced chemotaxis to cAMP and folate and also reduced random motility (Khosla

et al., 1996), the abilities of the various transformants to respond to a cAMP and folate concentration gradients were tested. The transformant expressing *ras*G-G12T/T35S exhibited cAMP chemotaxis that was indistinguishable from that for Ax-2. In contrast, the cells expressing *ras*G-G12T, *ras*G-G12T/Y40C and *ras*G-G12T/T45Q exhibited significantly reduced chemotaxis to cAMP compared to cells expressing *ras*G-G12T/T35S ($P<.05$) (Fig. 11)

Similarly pVEII-*ras*G-G12T/T35S transformant exhibited normal chemotaxis to folate whereas the cells expressing *ras*G-G12T and *ras*G-G12T/T45Q exhibited significantly lower chemotaxis (Fig. 11). The *ras*G-G12T/Y40C strain appeared to exhibit reduced levels of chemotaxis to folate, but the average level for three experiments was not significantly lower than that of the *ras*G-G12T/T35S strain ($P>.05$).

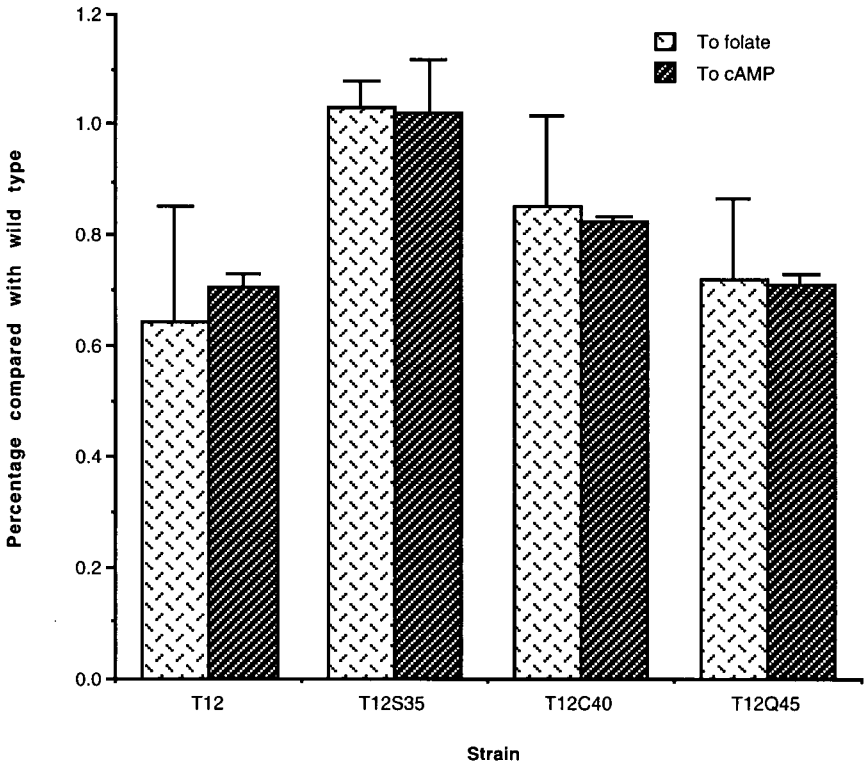

Figure 11. Chemotaxis of pVEII-*ras*G-G12T, pVEII-*ras*G-G12T/T35S, pVEII-*ras*G-G12T/Y40C and pVEII-*ras*G-G12T/T45Q transformants.

Chemotaxis to folate and cAMP was measured as described by (Browning *et al.*, 1995). For each of the transformants, the height of the bar represents the mean distance migrated in response to folate (cross bars) and to cAMP (hatched bars), relative to Ax-2. The error bars indicate the standard error of the mean from three independent experiments.

General Discussion

The overexpression of an activated *ras*G gene in vegetative cells of *Dictyostelium* has multiple effects. *ras*G-G12T transformants fail to aggregate upon starvation due to an impairment in cyclic AMP signal relay (Khosla *et al.*, 1996), suggesting RasG-GTP is a negative regulator of the signal transduction pathway involved in cAMP relay. In addition part of the activated RasG transformant population exhibit morphological abnormalities that are accompanied by alterations in cytoskeletal function (Rebstein, 1996). These morphological abnormalities range from cells that are flattened and spread with numerous filopodia, lateral and dorsal ruffles to cells that are less flattened but display large circular dorsal ruffles (Cardelli and Bush, unpublished data). The cells that have the flattened morphology have increased F-actin located around the cell periphery (Rebstein, 1996). The expression of RasG-G12T also causes a slight increase in the average number of nuclei per cell (Rebstein, 1996).

A *ras*G null mutant in which expression of RasG was completely abolished was capable of undergoing normal development but exhibited a wide range of defects in the control of the actin cytoskeleton, including defective cell movement, a loss of cell polarity, absence of normal lamellipodia, formation of unusual small, punctuate polymerized actin structure and a large number of abnormally long filopodia. However, the most dramatic defect is in cytokinesis. *ras*G null cells become multinucleate when grown in suspension (Tuxworth *et al.*, 1997). Since both the activated *ras*G transformants and *ras*G null transformants have altered morphology and exhibit defective cytokinesis, RasG clearly has a role in the regulation of cytoskeletal function in *Dictyostelium*. However, the *ras*G null transformants differentiate normally whereas the

activated *ras*G transformants fail to aggregate, suggesting the defect in aggregation and the defects in cytoskeletal function and cytokinesis involve different downstream pathways of RasG.

In mammalian cells, the most thoroughly studied Ras-dependent pathway involves the activation of the MAPK cascade. Upon receptor-activation, GTP-bound Ras binds cytoplasmic Raf-1 and translocates it to the plasma membrane where Raf-1 kinase becomes activated. Activated Raf results in activation of a Raf→MEK→ERK, kinase cascade. This activation is clearly required for transformation of rodent fibroblasts because expression of dominant-negative versions of MEK (Cowley *et al.*, 1994), or the use of a synthetic inhibitor of MEK, blocks transformation by Ras (Dudley *et al.*, 1995).

Ras proteins have been associated with control of the cytoskeleton in mammalian cells. Activated Ras proteins have been implicated in triggering actin filament accumulation at the plasma membrane: the formation of membrane ruffles via a Rac-dependent process, and the formation of actin stress fiber and focal adhesion development via a Rho-dependent process (Ridley and Hall, 1992; Ridley *et al.*, 1992). Recent results also support the existence of a Ras effector-mediated Rac/Rho signaling pathway which is distinct from Raf/MAP kinase pathway which is required for full Ras transformation (Rodriguez-Viciana *et al.*, 1997).

Ras proteins with relatively subtle mutations in the effector region (32-40) exhibit a partial loss of function in that interaction with some effectors is maintained but interaction with others is lost. These mutants have been used to correlate effector interaction with biological function, providing further evidence of branch points in Ras signaling (White *et al.*, 1995; Joneson *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997). For example, V12S35 Ras binds to and activates Raf-1 and the MAP kinase

cascade but does not activate PI 3 kinase and does not cause membrane ruffling. In contrast, V12C40 Ras binds and activates PI 3-kinase and induces membrane ruffling but does not activate Raf and does not activate MAP kinase. When introduced into NIH 3T3 cells, neither of the mutant genes alone were able to induce transformation, but expression of both V12C40 and V12S35 Ras in the same cell resulted in transformation. (Rodriguez-Viciana *et al.*, 1997). This indicates that membrane ruffling and activation of MAP kinase represent distinct Ras effector pathways, through PI 3-kinase and Raf respectively, that both are required for cell transformation.

In fission yeast *S. pombe*, the Ras protein has been shown to regulate two effectors: Byr2 which functions in an analogous manner to Raf as a MAP kinase kinase kinase, in the pheromone MAPK pathway (Wang *et al.*, 1991), and Scd1, a guanine presumptive nucleotide exchange factor for the Rho family protein Cdc42 and therefore involved in the regulation of the actin cytoskeleton (Chang *et al.*, 1994). Thus the Scd1-Ras interaction may be analogous to the interaction between Ras and Rho signaling pathways in mammalian cell systems.

The aim of the work presented here was to determine if *Dictyostelium* RasG acts through more than one downstream effector. If the effects of Ras were transmitted through a single effector, then appropriately chosen secondary mutations of *ras*G-G12T should inhibit all effects. Overexpression of RasG-G12T with amino acid substitutions at either positions 35, 40, or 45 did not induce the defect in aggregation, indicating that both the effector domain (amino acids 35 and 40) and the effector distal flanking domain (amino acid 45) were required for activated RasG to prevent aggregation. In contrast, overexpression of RasG-G12T with a substitution at position 45 induced the cytoskeletal

changes whereas overexpression of RasG-G12T with substitutions at positions 35 and 40 did not. Cells expressing *ras*G-G12T/T45Q exhibited similar morphology and cytokinesis defects to those seen in cells expressing *ras*G-G12T. This result indicates that the effector domain is important for inducing the cytoskeletal defect whereas the effector distal flanking domain is not.

The fact that the position 45 change affects the disruption of aggregation by RasG-G12T but not the disruption of the cytoskeleton, suggests that the change at position 45 does not induce a general deleterious conformational change in the Ras protein. Consistent with this conclusion is the finding that replacing V45 with E in mammalian Ras has little effect on the intrinsic GTPase activity of Ras and does not effect its interaction with GAP, but prevents an activated *ras* gene from producing foci in NIH 3T3 fibroblast cell or neurite outgrowth of PC12 cells (Marshall *et al.*, 1991; Fujita-Yoshigaki *et al.*, 1991).

Residue 45 is different in most of the *Dictyostelium* Ras subfamily proteins. The change from T to Q substitutes the amino acid present at position 45 in RasB into RasG. RasG and RasB share identical effector domain Cells expressing *ras*B-G12T aggregate normally and have abnormal morphology (Delehanty, Spiegelman and Weeks, unpublished observation), suggesting that RasG-G12T/T45Q might be reacting with the same effector as RasB-G12T. However, the cells expressing RasB-G12T and RasG-G12T/T45Q have different morphological defects and it is therefore unlikely that the RasG-G12T/T45Q protein interacts with RasB effector.

In mammalian Ras, residue 35 is required for membrane ruffling and residue 40 is required for activation of MAP kinase activity (Joneson *et al.*, 1996). Both of these residues are required for the abnormal

development and cell morphology of *Dictyostelium*, that is induced by activated RasG. However, there was a slight difference between the effects of RasG-G12T/Y40C and RasG-G12T/T35S in *Dictyostelium*. The pVEII-*ras*G-G12T/Y40C transformant exhibited reduced chemotaxis to cAMP whereas there was no reduction in cAMP chemotaxis for the pVEII-*ras*G-G12T/T35S transformant. Further analysis will be needed to address the question as to whether residues 35 and 40 interact with identical or distinct signaling pathways.

Overexpression of an activated RasG-G12T protein during growth inhibited the ability of cells to aggregate upon starvation while overexpression of wild type RasG had no effect on aggregation (Khosla *et al.*, 1996). These data suggested that GTP bound RasG negatively regulates aggregation, and that the cell has sufficient regulatory capacity to compensate for the overexpression of wild type RasG, but not activated RasG. Consistent with this idea, the *ras*G null mutant has no aggregation defect. The available data suggests that *ras*G-G12T interferes with the cAMP relay (Khosla *et al.*, 1996). ERK2, which is the homologue of mammalian MAP kinase, is required for aggregation. ERK2 activation is inhibited in cells overexpressing activated RasD (Aubrey *et al.*, 1997) or RasG (Kosaka *et al*, 1998). Recent results suggest ERK2 functions as a negative regulator of the cAMP-specific phosphodiesterase RegA, and therefore down regulates intracellular cAMP levels and blocks the cAMP relay (Lu *et al*, unpublished observations). These data suggest that there might be a negatively regulated Ras signal transduction pathway to control initiation of aggregation in *Dictystelium* . It would be informative to determine if a *reg*A null mutant could rescue the aggregation defect phenotype of cells expressing *ras*G-G12T. Further more, protein-protein interaction studies could be performed to try to

show direct interactions between RasG and ERK2 or some other downstream effector that might couple RasG to ERK2. It will also be important to determine if ERK2 directly interacts with RegA.

Whereas the aggregation defect appears to be specific for *ras*G-G12T transformants, both *ras*G-G12T, gain of function, transformants and *ras*G null, loss of function, transformants exhibit abnormal morphologies. Although the morphological defects are not the same in the two cells, these results are consistent with the fact that the inhibition of aggregation involves a different effector than the defect in cytoskeleton. However, is not known how the *Dictyostelium* cytoskeleton is regulated by RasG.

The *Dictyostelium* strain expressing RasG-G12T/Y40C didn't have an abnormal cell morphology, whereas a similarly mutated H-Ras protein produced an abnormal cell morphology when expressed in mammalian cells. These results suggest that there might be novel Ras effectors that regulate cytoskeleton in *Dictyostelium*. Attempts should be made to identify direct RasG downstream effector(s), using yeast two-hybrid, GST-fusion proteins or Immunoprecipitation to help address the possible pathway(s) that regulate cytoskeleton in *Dictyostelium*.

Ras proteins have been associated with both control of the cytoskeleton and cell proliferation in mammalian cells but not with cytokinesis. However since cytokinesis defects are more difficult to determine in mammalian cells than in *Dictyostelium* , it therefore is conceivable that Ras does play a role in mammalian cell cytokinesis. The most distinctive phenotype of the *ras*G null cells is the defect in cytokinesis. Although the suspension culture growth rate of the *ras*G null transformants was reduced, this was attributed to the defect in cytokinesis. However, more recent results have suggested that the

growth and cytokinesis defects may be distinct. When either *ras*G or *ras*D were transformed into the *ras*G null cells, a wild type phenotype was produced. However, when the *ras*B gene was transformed into *ras*G null cells, it rescued the cytokinesis defect but not the growth defect, suggesting that the defect in growth in the *ras*G null cells is independent from the defect in cytokinesis. (Khosla, unpublished data).

None of the phenotypes of the three double *ras*G mutants strains obtained during this work demonstrated a separation of the cytokinesis and cytoskeletal defects and there is, therefore, no evidence for an involvement of two different signaling pathways. Thus the defect in cytokinesis may be a manifestation of general disruption of the actin cytoskeleton. However the possibility that RasG controls a signaling pathway that specifically regulates cytokinesis can not be ruled out. An *Dictyostelium* homologue of mammalian Rac, RacE, has been characterized (Larochelle *et al.*, 1996). *rac*E null cells exhibit defective cytokinesis, but the actin cytoskeleton rearrangements involved in phagocytosis, receptor capping, cortical contraction, and chemotaxis appear normal. This suggests that RacE might act on only one of the downstream RasG effector pathways that is involved specifically in cytokinesis. However, when undergoing cytokinesis, the *rac*E null mutant fails to complete contraction whereas the *ras*G null mutant fails to complete cytokinesis occurs later during the process(reviewed in Chisholm *et al.*, 1997). These observations suggest RacE is not directly regulated by RasG. Genes that encode homologues of the mammalian p110 PI 3-kinase have also been cloned in *Dictyostelium* (Zhou *et al.*, 1995). PI 3-kinase null cells do not grow in shake culture (Zhou *et al.*, 1995), but exhibit nearly normal growth on a surface. These results suggest that the PI 3-kinase null cells have defects in cytokinesis that

are similar to those exhibited by the *ras*G null cells and this raises the possibility that PI 3-kinase is downstream of RasG. However, there is as yet no direct evidence for this hypothesis. The PI 3-kinase null mutant has an additional developmental defect that is not exhibited by the *ras*G null cells: aggregates produce multiple tips and abnormal fruiting bodies. However during development PI 3-kinase might act downstream of one of the other Ras proteins.

Although both the loss of RasG function and the gain of function produced cytokinesis defects in transformants, the changes were quite distinct. The abnormalities in the activated *ras*G transformants were more obvious when cells were grown on a plastic surface while the abnormalities in *ras*G null cells were most clearly found when cells were grown in suspension. The very large and multinucleate *ras*G-null cells that are observed in suspension culture divided by traction mediated cytokinesis soon after being plated on a surface (Tuxworth *et al.*, 1997). In contrast, cells expressing *ras*G-G12T exhibit a slight increase in nuclear number (1.7 nuclei/cell) (Rebstein, 1996) grown on a surface. Clearly regulation of the correct input signals through RasG is essential for cytoskeleton. Since gain of function *ras*G-G12T and loss of function *ras*G null transformants have quite distinct morphological defects, either too much or too little stimulation through the RasG mediated signal transduction pathway causes changes to cell shape.

In combination, the results suggest that RasG-G12T is capable of interacting with two distinct downstream effector molecules, one of which interacts with the effector distal domain and one of which does not.

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