Abstract

The effects of expression of the Ha-*ras* oncogene on the growth of murine 3T3-NRC fibroblasts were examined. Doubling times and rates of protein synthesis and degradation were measured in six cell lines. Three cell lines were Ha-*ras* transformed (as determined by Southern and Western Blotting), two were parent lines (3T3-Swiss Webster and 3T3-NRC), and the other was found to carry the Ha-*ras* oncogene but was not expressing it.

The transformed cell lines, NRC-NR#6.6-A, NRC-NR#6.6-B, and NRC-NR#6.6-C, were all found to have lower serum requirements than the nontransformed cells. Also, the transformed cells exhibited higher rates of protein synthesis than the non-transformed cells (in 1% and 10% serum), while the rates of protein degradation were virtually identical for all cell lines. In addition, the effect of platelet-derived growth factor (PDGF) and insulin on protein synthesis and degradation were determined. It was found that PDGF at a concentration of 1ng/ml induced a slight increase in overall protein accumulation (synthesis - degradation), but the increase was the same for all cell lines. The effects of insulin varied, both at 10^{-9} M and 10^{-7} M, with the cell line NRC-NR#6.6-C showing the only substantial increase in protein accumulation.

Introduction

Oncogenes are genes whose protein products act to disrupt the normal controls over cell growth. There are generally two types of oncogene, cellular and viral. Both types are thought to have arisen from normal growth regulatory genes, called proto-oncogenes, present in vertebrate genomes. Cellular oncogenes are believed to have originated by mutation of existing proto-oncogenes, while viral oncogenes were probably picked up during recombination events when a virus replicated in a host cell.(1)

Considerable evidence has been accumulated which links the mechanisms by which oncogenes transform cells to growth factors and growth factor receptors. Growth factors are hormone like peptides present in serum that recognize and bind to specific protein receptors on cell surfaces and initiate or maintain the normal biochemical events required for cellular proliferation.(2) It is conceivable that a mutation in one of the genes (proto-oncogene?) involved in regulating cell growth and differentiation could lead to the expression of an altered gene product and subsequent loss of control over this process. One of the first pieces of evidence relating the action of oncogene products to growth factors was the observation that normal chicken and mouse fibroblasts in culture require serum whereas their virus-transformed counterparts are capable of growing under serum-free conditions.(3,4)

Stronger evidence in support of this view comes from the finding that the protein product of the *erb-B* oncogene (the transforming gene of avian erythroblastosis virus), 9 p 65, has extensive amino acid homology with the cell surface receptor for epidermal growth factor (EGF).(5) The normal

receptor for EGF consists of an extracellular domain of 621 amino acids, a transmembrane domain of 23 amino acids, and a cytoplasmic domain of 542 amino acids which exhibits tyrosine kinase activity when the extracellular domain is bound to EGF.[6] The product of erb-B is a truncated form of the receptor which lacks a functional extracellular binding domain but has retained the transmembrane and cytoplasmic portions. Lack of an EGF binding domain may result in a constitutive activation of the cytoplasmic (kinase) domain.[5] EGF has been shown to cause the eventual phosphorylation of a 40 S ribosomal protein called S6,[7] an event which is known to parallel the activation of protein synthesis in many biological systems.[8] Therefore, the presence of the erb-B protein could cause a loss of growth control by artificially stimulating protein synthesis.

A further link between growth factors and oncogenes was established by the finding that the protein product of the sis oncogene closely resembles a mitogen called platelet-derived growth factor (PDGF).[9,10] PDGF is considered the major growth factor of human and animal serum and is thought to play an important role in the process of wound healing.[11] PDGF, which consists of two disulfide-bonded peptides, has the ability to stimulate several metabolic processes, including protein, lipid, and prostoglandin synthesis and tyrosine kinase activity.[12] Studies on the mode of action of PDGF have suggested that it is involved in the progression of cells through the cell cycle. The transition between the G_0/G_1 phase and the S phase of the cell cycle can be divided into two stages. The first stage, called competency, is controlled by PDGF and allows cells to enter the G_0/G_1 phase of the cycle. The other stage, called progression, is controlled by factors other than PDGF

that allow the progression of the PDGF-induced competent cells into the S phase.[13] Expression of the sis oncogene, therefore, seems to induce transformation by facilitating the passage of cells through the cell cycle.

Another growth factor present in serum is insulin. Although it has not been linked to any oncogene in particular, insulin has been studied extensively in an effort to better understand its role in controlling normal cellular processes. The receptor for insulin, like those for EGF and PDGF, exhibit tyrosine kinase activity. Furthermore, insulin has been found to regulate the phosphorylation of the S6 protein of the 40S ribosomal subunit,[14] as does EGF. Therefore, even though no oncogene products have been associated with insulin or its receptor, it is nevertheless a likely target for transformation-inducing mutations.

The protein products of the ras oncogenes also seem to alter the cellular response to some component of serum. The ras oncogenes are the ones most commonly found in human tumors and were first identified as the transforming genes of the Harvey and Kirsten strains of murine sarcoma virus.[16,17] The protein coded for by the ras oncogenes, a 21,000 dalton G protein (p21), is found loosely bound to the inner cell membrane and has guanine nucleotide binding activity.[18,19] DNA sequence analyses have shown that a single nucleotide change in the codon corresponding to position 12 in the protein is sufficient to confer transforming properties on the ras protein.[20,21] In particular, any amino acid substitution at position 12, other than proline or glycine (which is encoded by the proto-oncogene), will activate the ras protein.[22]

The fact that genes related to ras have been identified in such evolutionarily diverse species as humans, rodents, fruit flies[24], and bacteria[23] suggests that the ras proteins may play a fundamental cellular physiology. Indeed, the fact that

ras proteins bind GTP and GDP suggests that they may be related to G regulatory proteins such as the elongation factors of protein synthesis[25], the stimulatory and inhibitory components of adenylate cyclase[26], or the retinal activator of cGMP phosphodiesterase[27]. The possibility of the ras proteins being associated with elongation factors of protein synthesis seems to have been ruled out by the observation that the ras proteins have an affinity for GTP that is slightly greater than that for GDP,[28] while the elongation factor EF-Tu has an affinity for GDP that is 100 times higher than that for GTP.[25,26]

A common property of all known G proteins is a GTP hydrolytic activity that is central to their regulatory function.[25,26,27] It has been found that the ras p21 mutants with valine or arginine residues at position 12 have a GTP hydrolytic activity that is 10-fold less than that exhibited by the normal ras protein.[28-31]

In an effort to understand the biological consequences of the decrease in hydrolytic activity caused by oncogenic mutations of the ras protein, Gibbs, et al. (1985) compared p21 to the G protein of the adenylate cyclase system. In this system, an activated receptor causes the G protein to bind GTP. This complex in turn stimulates adenylate cyclase activity until the reaction is terminated by GTP hydrolysis to GDP. By introducing hydrolysis-resistant GTP analogs or by inhibiting the GTP hydrolytic activity with cholera toxin, adenylate cyclase can be permanently activated. Gibbs, et al. have hypothesized that the mutant forms of p21, by virtue of their reduced GTP hydrolytic activity, would remain in the "active" form and thus maintain any biochemical system with which they are associated in a less regulated state.[32] This hypothesis is also consistent with the observation that

overexpression of normal p21 causes transformation[33], since an increase in the total p21 population would result in a greater number of p21-GTP complexes.

On the other hand, a substantial amount of evidence has been published which suggests the *ras* p21 protein may work through the phosphatidylinositol turnover cycle rather than through the adenylate cyclase system. Phosphatidylinositol (PI) and its phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2), are hydrolyzed by the enzyme phospholipase C. This enzyme has been shown to be stimulated by hormones, neurotransmitters, serum, and purified growth factors.[34-36] The breakdown products of PIP_2, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), appear to act as regulators of various intracellular processes. For example, DAG activates a calcium and phospholipid-dependent protein kinase (kinase C)[37], while IP_3 stimulates the release of calcium from intracellular stores.[38] Both the DAG-activated kinase C and IP_3-induced release of calcium result in the activation of a Na^+-H^+ exchanger in the cell membrane, which causes an increase in cytoplasmic pH and Na^+ concentration.[39,40] Fleischman, et. al. (1986) found that alterations in the steady-state levels of the phosphatidylinositol turnover cycle components is characteristic of *ras* transformation, suggesting that the *ras* protein may be involved in the regulation of PIP_2 breakdown. Specifically, *ras*-transformed cells had lower levels of PIP_2 and higher levels of DAG and IP_3 than their non-transformed counterparts.[41] They propose that the *ras* protein may directly activate phospholipase C, resulting in a decrease in PIP_2 and an increase in IP_3 and DAG.

Further evidence concerning the biochemical action of the *ras* protein comes from Ben-Sagi and Feramisco (1986)[45] of

Cold Spring Harbor laboratory. These investigators examined the effects of the ras-protein on cellular events by microinjecting oncogenic and proto-oncogenic proteins into rat embryo fibroblasts. They found that injection of Ha-ras proteins stimulated membrane ruffling and pinocytosis. The increased rate of pinocytotic activity in cells injected with the Ha-ras protein was maintained for at least 15 hours, while the effect produced by injection of the proto-oncogenic protein was transient and lasted no more than 5 hours. Similarly, the proto-oncogenic protein caused a rapid but transient increase in membrane ruffling, while the oncogenic ras protein induced membrane ruffling which lasted more than 15 hours.

Since previous results (42-44) indicated that cell surface ruffling and pinocytosis are stimulated as a result of the addition of serum or purified growth factors (EGF or PDGF) to cells maintained in serum-free medium, they next tested the possibility that the stimulation of pinocytosis and membrane ruffling by the ras oncogenic protein might be coupled to the action of serum growth factors. They found, however, that the increase in ruffling and pinocytosis induced by injection of the ras protein was the same in the presence and absence of serum. Furthermore, the stimulatory effects of external ligands on membrane ruffling and pinocytosis are transient and usually last no more than 1 to 2 hours, while the same effects induced by injection of the ras protein persist for more than 15 hours. This indicates that ras-induced effects are not mediated by external ligands. In fact, they found in further experiments that the stimulation of pinocytosis by the ras protein is dependent on calcium in a time-dependent fashion. The initial stimulation (first 1-2 hours) appears to be mediated by calcium mobilized from intracellular stores, while further stimulation

seems to be controlled by the availability of extracellular calcium. Combining these observations with those of Fleischman, et al. (1986) that *ras*-transformed cells have higher levels of IP_3 (which stimulates release of calcium from intracellular stores), we have considerable evidence in support of the view that the *ras* oncogene protein acts through the phosphoinositol turnover cycle.

I have measured the effects of two different serum concentrations (1% and 10%), platelet-derived growth factor (PDGF), and insulin on the rates of protein synthesis and degradation in Ha-*ras* transformed mouse 3T3-NR6 fibroblasts. Also, doubling times were measured in 1% and 10% serum. The following results indicate that expression of the Ha-*ras* protein in these cells enhances their response to some component of serum. This enhanced response, however, does not appear to be mediated by PDGF or insulin.

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