Mitogenic Pathways Regulated by G Protein Oncogenes

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"It is quite possible that overactivity of the cAMP system will be found responsible for hyperfunctioning benign tumors or adenomas as opposed to dedifferentiated malignant tumors where the classical oncogenes are involved."

This prediction by Dumont *et al.* (1989) has been proven correct. Recent evidence shows that G protein-coupled systems—including but not limited to adenylyl cyclase and cAMP—can induce hyperplasia and contribute to the neoplastic transformation of certain cell types. The "overactivity" of G protein-coupled systems often results from mutations that constitutively activate α subunits by reducing their GTPase activities. These mutations resemble those observed in transforming *ras* oncogenes (Barbacid, 1987). Several lines of experimental evidence indicate that GTPase-deficient G protein α subunits can behave as oncogenes. 1) GTPase inhibiting mutations in the gene encoding the α subunit (α_s) of G_s have been found in clonally derived tumors of pituitary somatotroph (Landis *et al.*, 1989; Climenti *et al.*, 1990) and thyrotroph (Lyons *et al.*, 1990; Suarez *et al.*, 1991) origin; similar G_{12} mutations (in the α_{12} subunit gene) have been identified in tumors derived from adrenal cortex and ovarian endocrine cells (Lyons *et al.*, 1990). 2) GTPase-deficient α_{12} subunits induce a transformed phenotype in Rat 1 cells (Gupta *et al.*, 1991; Pace *et al.*, 1991). 3) NIH3T3 cells may be transformed by the ectopic expression of hormone or neurotransmitter receptors that couple to the hydrolysis of phosphatidylinositol via G proteins (presumably of the G_q family), but require the continued presence of receptor activation to maintain the transformed phenotype (Julius *et al.*, 1989; Gutkind *et al.*, 1991). 4) Several fibroblast cell lines show a pertussis toxin-sensitive mitogenic response to lysophosphatidic acid or peptides such as bombesin and thrombin (Lettorio *et al.*, 1986; Murayama and Ui, 1987; van Corven *et al.*, 1989; Seuwen *et al.*, 1990), directly implicating G_i or G_o proteins in the control of cell proliferation.

How do G protein oncogenes alter cell growth regulation? The network of growth factor receptor signal pathways initiated at the plasma membrane converges in the nucleus to alter gene expression and to commit a cell to mitogenesis. As outlined below, expression of

G protein oncogene products alters cell growth by constitutively activating selected pathways within this signaling network.

ACTIVATED G_s AND THE PROLIFERATION OF PITUITARY SOMATOTROPHS AND THYROID CELLS

The ability of mutationally activated α_s to stimulate pituitary somatotroph hyperplasia is not surprising. The growth hormone-releasing factor (GRF) receptor couples to G_s and in the presence of GRF stimulates synthesis of cAMP by adenylyl cyclase. cAMP mediates the mitogenic action of GRF (Billestrup *et al.*, 1986). In response to a rise in cAMP, the cAMP-dependent protein kinase phosphorylates and activates CREB (cAMP response element-binding protein). CREB binds to the promotor of the transcription factor growth hormone factor 1 (GHF1 or pit 1) and positively regulates GHF1 transcription. GHF1 also positively regulates itself, and its expression is directly correlated with increased somatotroph proliferation (Castrillo *et al.*, 1991).

The GTPase-deficient α_s mutants found in hyperactive human pituitary somatotroph tumors (Landis *et al.*, 1989) constitutively activate cAMP synthesis and presumably maintain persistent activation of the CREB protein. This would induce GHF1 expression and activate cell proliferation. Several experiments in transgenic mice support this role for mutationally activated α_s in somatotroph tumors. First, overexpression of GRF results in somatotrophic hyperplasia (Mayo *et al.*, 1988) similar to that observed in pituitary somatotroph tumors expressing GTPase-deficient α_s . Second, expression of the cholera toxin gene driven by the growth hormone promotor leads to somatotroph hyperplasia in transgenic mice (Burton *et al.*, 1991). Like mutationally active α_s , both chronic GRF overexpression and cholera toxin catalyzed ADP-ribosylation of α_s , which inhibits its GTPase activity, persistently activate cAMP synthesis. Finally, a dominant negative nonphosphorylated CREB mutant linked to the growth hormone promotor caused pituitary hypoplasia (Struthers *et al.*, 1991), demonstrating the direct involvement of CREB and the requirement for transcriptional control by cAMP.

A similar scenario appears likely in thyrotrophs, where cAMP is mitogenic also (Dumont *et al.*, 1989). The thyroid-stimulating hormone (TSH) receptor stimulates cell proliferation in thyrotrophs and FRTL5 rat thyroid cells by a cAMP-dependent mechanism (Dumont *et al.*, 1989). In the FRTL5 rat thyroid cell line (Ambesi-Impimbato *et al.*, 1980) the β -adrenergic receptor or forskolin may replace the TSH receptor in stimulating cell growth (Hen *et al.*, 1989); like TSH both agents activate cAMP synthesis and CREB transcriptional control of specific genes. At present, however, the CREB-regulated genes that control thyrotroph proliferation have not been identified.

Constitutively activated G_s mutants will not stimulate proliferation of most cell types simply because elevated cAMP is mitogenic in only a few types of cell, including somatotrophs, thyrotrophs, and parotid acinar cells (Dumont *et al.*, 1984). In the few cell types where cAMP is mitogenic, GTPase-deficient α_s mutants may indeed function as oncogenes. The identification of GTPase-inhibiting mutations in clonally expanded hyperfunctioning adenomas of the pituitary and thyroid supports the designation of the activated α_s mutants as *gsp* oncogenes (Landis *et al.*, 1989; Climenti *et al.*, 1990; Lyons *et al.*, 1990; Suarez *et al.*, 1991). In fact, the action of *gsp* oncogenes in somatotroph hyperplasia may be compared with that recently proposed for *v-ras* in fibroblast transformation. The proto-oncogene *c-jun*, a component of the AP-1 transcription factor complex, in cooperation with *v-ras* is able to transform rat embryo fibroblasts (Ruley, 1990). Transfection assays demonstrated that *v-ras* was able to cause the hyperphosphorylation and persistent activation of *c-jun*, presumably by activating serine/threonine protein kinase cascades, leading to transcriptional changes in gene expression (Binetruy *et al.*, 1991). The *gsp* oncogene product similarly sustains phosphorylation and constitutive activation of CREB, inducing the expression of the pituitary-specific GHF1 transcription factor. In combination, persistently activated CREB and the induction of GHF1 resulting from expression of the *gsp* protein would stimulate somatotroph proliferation as observed in the growth hormone hypersecreting pituitary adenomas. Presumably, *gsp* is tumorigenic in only a small subset of cells because this response requires tissue-specific transcription factors, like GHF1, that stimulate mitogenesis and are expressed in a CREB-dependent fashion. It seems likely that similar tissue-specific growth-promoting transactivation factors regulated by CREB will be identified in the cell types where cAMP is mitogenic.

TRANSFORMATION POTENTIAL OF ACTIVATED G_{12} MUTANTS

Expression of constitutively activated α_{12} polypeptides in Rat 1 cells induced a loss of contact inhibition, anchorage-independent growth, a decreased serum re-

quirement, and tumor formation in nude mice, showing that a mutant α_{12} gene can function as an oncogene (Gupta *et al.*, 1991; Pace *et al.*, 1991). In NIH3T3 and Swiss 3T3 cell lines, the mutant α_{12} , however, induced fewer changes characteristic of transformation. Thus, activated α_{12} subunits encoded by *gip2* oncogenes produce a transformed phenotype in only a subset of cell types, as was the case for the *gsp* oncogene. GTPase-inhibited α_{12} subunits are more likely to produce oncogenic effects in cell types that exhibit hormone-stimulated mitogenic responses that are blocked by pertussis toxin, which catalyzes the ADP-ribosylation of G_i and G_o α subunit polypeptides. Pertussis toxin inhibition of mitogenic responses has been described for lysophosphatidic acid (van Corven *et al.*, 1989), thrombin (Seuwen *et al.*, 1990), bombesin (Lettorio *et al.*, 1986) and serum (Murayama and Ui, 1987) in cell types where G_{12} and G_{13} are predominantly expressed with little or no G_o α subunit expression. To date, only Rat 1 cells have been extensively characterized in relation to the phenotypic consequences of expressing GTPase-inhibited α_{12} .

G_{12} mediates inhibition of adenylyl cyclase and activation of K^+ channels; it probably regulates other effector pathways as well, although these have not yet been identified. Several observations, however, begin to define the mechanism by which G_{12} controls growth of certain cell types. First, myristylation of the α_{12} subunit is required for its efficient association with the plasma membrane (Jones *et al.*, 1990; Mumby *et al.*, 1990). α_{12} mutants that are not myristylated do not transform Rat 1a cells (Gallego and Johnson, unpublished observation), indicating that α_{12} mutants are altering signals whose origin is at the cytoplasmic surface of the plasma membrane. Second, G_{12} inhibition of adenylyl cyclase is not the mechanism for pertussis toxin-sensitive mitogenic responses. In Swiss 3T3 cells, cAMP actually promotes insulin-stimulated cell growth even though pertussis toxin inhibits mitogenic responses to bombesin, thrombin, and serum (Lettorio *et al.*, 1986; Murayama and Ui, 1987; Seuwen *et al.*, 1990; Zachary *et al.*, 1990). Overexpression of α_{12} in Rat 1a cells results in transformation (Gupta *et al.*, 1991) but not in inhibition of cAMP accumulation (Gupta and Johnson, unpublished observations). Thus, G_{12} -mediated mitogenic responses must involve signal pathways other than the inhibition of cAMP synthesis.

Transformation of Rat 1a cells by GTPase-inhibited α_{12} is significantly independent of functional *c-ras* signaling (Gupta *et al.*, 1991). The Rat 1a cell mitogens lysophosphatidic acid and thrombin, whose action is significantly inhibited by pertussis toxin, did not activate *c-ras* (Gupta and Johnson, unpublished observation). In contrast, epidermal growth factor (EGF) readily stimulated exchange of GDP for GTP bound to Ha-*ras* in Rat 1a cells. Expression of the Asn-17^{ras} dominant negative mutant (Feig and Cooper, 1988) inhibited the ability of *v-src* or *c-ras* to stimulate Rat 1a cell growth

(Gupta *et al.*, 1991). However, the dominant negative *Asn-17ras^H* mutant had little effect on the ability of α_{12} or the GTPase-inhibited α_{12} mutant to stimulate growth of Rat 1a cells. Cumulatively, the findings indicate that unlike the membrane-associated tyrosine kinase oncogene products, G_{12} -coupled growth stimulation involves pathways that do not use *c-ras* functions in initiating mitogenic signals.

Despite the *c-ras* independence of G_{12} -coupled mitogenic responses, protein kinase networks regulated by tyrosine kinase-encoding receptors, such as that for EGF or fibroblast growth factor (FGF), appear to converge with the G_{12} -linked signaling pathway in the cytoplasm. The p42 early response kinase (ERK) gene products, also referred to as mitogen-activated protein kinases, are rapidly activated by both tyrosine kinase-encoding growth factor receptors (i.e., EGF and FGF) and pertussis toxin-sensitive G protein-coupled receptors (i.e., thrombin and lysophosphatidic acid) (L'Allemain *et al.*, 1991). Activation of p42 ERK involves both tyrosine and threonine phosphorylation; both of these may be autophosphorylation events (Ferrell and Martin, 1990), even though the p42 ERK protein is a serine/threonine protein kinase for target substrates other than itself. Activation of the ERK protein seems to involve a novel protein(s) that functions as an intermediate between receptors and the ERK protein in growth factor-stimulated kinase cascades. The existence of this protein intermediate and its essential role in ERK activation are clear, but its biochemical properties have not been characterized in detail (Ahn *et al.*, 1991). Nonetheless, the ERK regulatory protein is a point for convergence in the pathways regulated by the EGF receptor and for G_{12} -coupled receptors such as that for thrombin or lysophosphatidic acid (L'Allemain *et al.*, 1991).

Phosphotyrosine content and activity associated with the p42ERK protein is elevated in transformed Rat 1a clones expressing constitutively activated α_{12} (Gupta, Heasley, and Johnson, unpublished observation), suggesting that GTPase-inhibited α_{12} may persistently alter the regulation of this pathway. The ERK gene product whose tyrosine phosphorylation is elevated in *gip2*-transformed Rat 1a cells is similar to the enzyme recently characterized by Alvarez *et al.* (1991), which efficiently phosphorylates *c-myc* and *c-jun* and is proposed (Binetruy *et al.*, 1991) to regulate functions of transcription factors. An obvious hypothesis is that the *gip2* oncogene might modulate *c-jun* activity by persistent activation of ERK proteins. This growth-control pathway would be similar to that previously proposed for *v-ras* regulation of *c-jun* activity via increased phosphorylation of its activation domain (Binetruy *et al.*, 1991). The convergence of G_{12} and tyrosine kinase-encoding receptors in activating ERK activity by independent mechanisms could also explain how *gip2* stimulates cell growth in a fashion that is largely independent of *c-ras*. In Rat 1a cells, activation of ERKs in the network of events regulated by G_{12} may suffice to enhance the phosphory-

lation of transcription factors capable of stimulating mitogenesis in Rat 1a cells. Additional experiments will determine whether a convergence of tyrosine kinase and G_{12} systems at the level of ERK proteins and possibly other serine/threonine protein kinases regulates a common set of transcription factors controlling proliferation in different cell types.

The G_{12} mediated activation of serine/threonine protein kinases like the ERK proteins may regulate other effector proteins involved in growth control, in addition to transcription factors. A consensus ERK phosphorylation site is also found in the cellular phospholipase A_2 (cPLA₂) (Clark *et al.*, 1991). Thrombin activates cPLA₂ by a G_{12} -coupled pertussis toxin-sensitive mechanism (Gupta *et al.*, 1990) that follows a time course similar to that of stimulation of ERK activity (Gupta and Johnson, unpublished observation). Arachidonic acid and its metabolites are required for mitogenesis in several fibroblast cell lines (Handler *et al.*, 1990; Zachary *et al.*, 1991); this signal pathway may contribute to the altered growth control observed in α_{12} -transformed Rat 1a cells.

TRANSFORMATION POTENTIAL OF RECEPTOR-COUPLED G PROTEIN-REGULATED PHOSPHOLIPASE C (PLC)

NIH3T3 cells have a propensity to assume a transformed phenotype when PLC-activating receptors such as the serotonin 1c and M1- and M3-type muscarinic receptors are expressed by gene transfection (Julius *et al.*, 1989; Gutkind *et al.*, 1991). The receptor-induced transformation of NIH3T3 cells requires the continued presence of agonist (i.e., serotonin or carbachol) to sustain the transformed phenotype. It must be noted that the studies with serotonin and the muscarinic receptors expressed in NIH3T3 cells did not demonstrate altered phosphatidylinositol metabolism or PLC regulation, although these receptors are known to couple to PI hydrolysis in several cell types. It is possible that these two receptors induce the transformed phenotype via pathways independent of PLC stimulation. Inhibition of PLC γ 1 activity, however, inhibits *v-ras* transformation of NIH3T3 cells (Smith *et al.*, 1990). Although PLC γ 1 is not coupled to G proteins, these findings suggest that functional PLC activity is important in transformation of NIH3T3 cells. Whether PLC β 1-like activity, which is regulated by G proteins, is an important mediator of serotonin 1c and muscarinic receptor-induced transformation of NIH3T3 cells remains to be determined.

Expression of mutant α_o subunits also transforms NIH3T3 cells (Kroll and Iyengar, personal communication), inducing both focus formation in culture and tumors in nude mice. The GTPase-inhibited α_o mutants do not transform Rat 1a cells and mutations in α_o genes have not yet been identified in tumors. The ability of

mutationally activated α_o to transform NIH3T3 cells might be related to the ability of G_o to regulate PLC activity in certain cell types. The GTPase-inhibited α_o polypeptide would bypass the requirement for receptor activation and constitutively alter PLC regulation, but just as with the serotonin 1c and muscarinic receptor studies, this idea has not been tested. It is also noteworthy that α_o is not expressed in NIH3T3 cells; therefore its mutant form must be mimicking or altering the function of another G protein. Because mutant α_{i2} does not transform NIH3T3 cells, it is not clear which G protein this might be, but the G_q family of α subunits, which have been shown to activate PLC β 1 (Srncka *et al.*, 1991; Taylor *et al.*, 1991), are likely candidates.

CONCLUDING REMARKS

Figure 1 shows the pathways triggered by G proteins that we propose as regulators of proliferation in different cell types. The common denominator for each is the activation of a selected set of cytoplasmic serine/threonine protein kinases capable of altering activity and expression of specific transcription factors, and thereby regulating expression of other genes. In essence, this is

similar to the control of cell growth by tyrosine kinase-encoded growth factor receptors and the *c-ras* proto-oncogene. With respect to growth regulation, the major difference between the G protein and tyrosine kinase/*c-ras* systems is that each of the G proteins is restricted to a smaller number of signaling pathways than are *c-ras* and receptor tyrosine kinases. For example, existing evidence suggests that G_s and G_q predominantly couple to adenylyl cyclase and PLC β , respectively. Although other effectors probably exist for these G proteins, the immediate second messenger responses are obviously fewer than are observed for a tyrosine kinase such as the platelet-derived growth factor (PDGF) receptor. Activation of the PDGF receptor minimally leads to the recruitment of PLC γ 1, GAP (GTPase-activating protein), PI-3 kinase, and *src*, resulting in rapid activation of *c-ras* and several second messenger pathways (Anderson *et al.*, 1990; Kazlauskas *et al.*, 1991). Aside from inhibition of adenylyl cyclase, the effectors for G_{i2} are unknown for most cell types, but they are probably fewer in number than observed for tyrosine kinases. For example, G_{i2} activation does not stimulate a transient activation of *c-ras*. The partial overlap of signal pathways regulated by G proteins with the tyrosine kinases will therefore allow a proliferative response to occur

Figure 1. Proposed G protein-regulated pathways involved in the control of cell growth and transformation. G_s is the stimulatory G protein for adenylyl cyclase. G_{i2} is the inhibitory G protein for adenylyl cyclase but also regulates selected K^+ channels and unidentified effector(s) capable of mediating the activation of p42 ERK (MAP kinase). G_q is the G protein coupled to PLC β 1 that catalyzes the hydrolysis of PtdInsP2. G_o may also regulate PLC activity in some cell types. R, receptor for respective agonists; $\uparrow$, denotes increased expression; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; calmodulin PK, calmodulin-dependent protein kinases.

only in cells where a subset of changes in gene expression, relative to the larger set observed with the tyrosine kinase-encoding receptors, suffices to promote DNA synthesis. Established fibroblast cell lines like Rat 1a and NIH3T3 are differentially primed to convert to a transformed-like phenotype when the regulation of a limited set of cytoplasmic response pathways is activated, making them useful models to study G protein control of cell growth. However, because of their restricted regulation of signal pathways to the nucleus, it is unlikely that GTPase-deficient G protein α subunits (α_{i2} , α_o , α_q , etc.) will be found to cooperate with transcription factors to transform primary fibroblasts.

Although the screening of different tumors for G protein mutants is presently limited in scope (Landis *et al.*, 1989; Lyons *et al.*, 1990), the fact that activating G protein α subunit mutations have only been found in hyperfunctioning differentiated cell types underlines the difference in oncogenic potential between them and the "classical" membrane-associated oncogenes. It seems indeed, that G proteins are primarily involved in controlling functions of differentiated cells (Dumont *et al.*, 1989), which predictably includes regulation of tissue-specific transcription factors such as GHF1 in the pituitary. This is consistent with the nonmetastatic character of the hyperplastic tumors found to contain mutations that activate G proteins. Growth-promoting tissue-specific transcription factors, under the control of G protein-regulated signal pathways, probably contribute to tumors that originate in thyroid, adrenal cortex, and ovarian endocrine cells. The transcription factors promoting growth in these tissues must be identified to define the oncogenic process involving mutationally active G proteins.

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