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Nuclear envelope signaling-role of phospholipid metabolism

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INTRODUCTION

Since the early 1980's, there has been an explosion of research in an area often described as "signal transduction". Loosely defined, signal transduction refers to *the communication of a signal initiated by an extracellular agonist to the cell interior*. Clearly, such a process is central to the growth, development and homeostasis of multicellular organisms. Indeed, many extracellular agonists induce the stimulation of cell growth, differentiation, or the expression of specific genes required for selected responses. As a result, one of the primary intracellular targets of this communication is the cell nucleus. Signal transduction pathways must, therefore, include mechanisms for the initiation of signals at the plasma membrane, a mechanism by which these signals traverse the cytoplasm, and influence, finally, a nuclear response.

Mechanisms by which cell surface-initiated growth signals impinge upon the nucleus has been the subject of numerous reviews ((Karin and Smeal, 1992; Karin, 1996; Su and Karin, 1996). Given the enormous diversity of responses induced by growth factors, it is not surprising that multiple signal transduction cascades have been identified. Indeed, there are defects in a number of these cascades that are known to result in neoplastic transformations. Signaling molecules involved in these cascades fall into three major classes; protein kinases, lipid metabolites, and GTPases. Although this diversity adds complexity, it allows for the "checks and balances" inherent in an integrated regulation of signaling cascades important for growth.

Our laboratories have focused considerable attention on the three classes described above that are involved in mitogenic signaling cascades. Principally, we have examined α-thrombin (E.C.3.4.21.5) induced proliferation of Chinese hamster fibroblasts (IIC9 cells). α-Thrombin-induced growth in IIC9 cells is completely dependent on the catalytic activity of α-thrombin and involves the cleavage of a cell surface receptor, PAR1 (protease-activated receptor 1). Activation of PAR1 stimulates metabolism of phosphoinositides (PI) and phosphatidylcholine (PC) although with different kinetics. PI hydrolysis is rapid and transient, returning to basal rates within two miutes while PC hydrolysis shows slower kinetics, but is sustained for up to several hours. Further analyses indicate that some of the induced lipid metabolism occurs in the nuclear envelope. PLD1 (ham-PLD1b), which is located in the nucleus and Golgi apparatus is involved in PC metabolism. *In vivo* and *in vitro* analyses indicate that addition of α-thrombin to quiescent cultures results in increased PLD activity in the nuclei but not the Golgi. Interestingly, α-thrombin-induced increases in nuclear PLD activity are not found in cells transiently transfected with a dominant negative RhoA. Furthermore, treatment of nuclei isolated from α-thrombin-induced cultures with RhoGDI, which inhibits RhoA activity, reduces the level of PLD

activity. Together these data indicate that RhoA is important in the regulation of nuclear PLD activity.

Interestingly, PAR1-dependent hydrolysis of PI and PC are activated through distinct G proteins. Consistent with previous reports, in stable cells lines in which the levels of the α subunit of Gq have been reduced by over 50%, α-thrombin induces PC but not PI hydrolysis. On the other hand, ablation of the α subunit of Go results in a constitutive activation of an apparent PC-PLC, but not PC-PLD. PC-PLD and PC-PLC, therefore, are differentially regulated. Although PC-PLC but not PC-PLD is constitively activated in the Goa cells, both enzymes may be important for mitogen-regulated cell growth.

Surprisingly, ablation of Goa cells results in transformation. The constitutive activation of other molecules involved in mitogenic signaling; in particular Ras, ERK, PI 3-kinase (E.C.2.7.1.137) and CyclinD1/CDK accompany this cellular transformation . Transfection of Goa cells with the α subunit of transducin (Gαt) reverses both the constitutive activation of the above enzymes as well as the transformed phenotype indicating a critical involvement of Gβγ dimers. These data indicate that isoforms of Gβγ dimers appear to be essential and implicate a unique tumor suppressor role for specific α subunits of heterotrimeric GTPases containing these Gβγ dimers.

PAR1 COUPLES TO BOTH PI AND PC HYDROLYSIS AND MITOGENESIS IN IIC9S

Three PARs, PAR1, PAR3 and PAR4, are activated by α-thrombin (Ishihara *et al.*, 1997; Nystedt *et al.*, 1994; Vu *et al.*, 1991; Xu *et al.*, 1998). IIC9 cells posses one of these receptors, PAR1. In order to establish the mechanism(s) by which α-thrombin stimulates lipid metabolism and mitogenesis, it was essential to determine whether the endogenous PAR1 receptor couples to the hydrolysis of lipids and induced mitogenesis. Our strategy was to examine enterokinase (3.4.21.9) -induced responses in IIC9 cells stably transfected with a modified PAR1 (EKTR) in which the α-thrombin cleavage site was replaced with an enterokinase cleavage site (Hung *et al.*, 1992; Collu *et al.*, 1988). This allowed us to examine responses induced by this receptor in the absence of contributions from the endogenous α-thrombin receptor (WTR). These studies were carried out in a cell derived from IIC9 cells with a genetic background identical to wild type IIC9 cells. Therefore, the cells contained all the downstream components that are required for PAR1-induced lipid metabolism and mitogenesis.

EKTR was first tested for its ability to stimulate arachidonic acid release. Enterokinase does not increase the release of arachidonic acid and its metabolites from the parental IIC9 cells expressing only WTR. In contrast to parental IIC9 cells, enterokinase induces a significant release of arachidonic acid and its metabolites in cells expressing EKTR (Cheng *et al.*, 1997). Importantly, cells expressing EKTR also respond to α-thrombin (Cheng *et al.*, 1997). α-Thrombin stimulates an essentially identical release of arachidonic acid from IIC9 cells expressing EKTR as parental IIC9 cells (Cheng *et al.*, 1997), indicating that expression of EKTR does not block the effect α-thrombin in these cells.

We then asked whether activation of PAR1 resulted in an increase in the level of diglyceride (Raben *et al.*, 1987). As was seen for arachidonic acid release response, enterokinase stimulated a slow increase in diaglycerides in EKTR cells (Cheng *et al.*, 1997). After 5 minutes, the level of diacylglycerol induced in EKTR expressing cells was approximately half of the level archived by a high concentration of α-thrombin (1.5 NIHunits/ml). After 30 minutes, the enterokinase-induced diacylglycerol level tripled (Cheng *et al.*, 1997), achieving the same level of diacylglycerol accumulation as that induced by α-thrombin (both high and low concentrations).

There is an important difference between the kinetics of diglyceride production induced by α-thrombin compared to that induced by enterokinase in EKTR cells. In contrast to α-thrombin (Wright *et al.*, 1988), only a small “shoulder” (1.4 ± 0.5 fold-increase in three experiments performed in duplicate, n=6) of diglyceride is produced in response after 15 second stimulation with enterokinase (Cheng *et al.*, 1997). Because the diglycerides produced at this time in response to α-thrombin are largely derived from PI hydrolysis (Pessin and Raben, 1989), the absence of this peak in response to enterokinase indicates either; (a) enterokinase does not stimulate PI hydrolysis, or (b) the PI induced diacylglycerol is very small and

largely masked by the diglycerides derived from PC hydrolysis. To discriminate between these possibilities, the source of the diglycerides induced by enterokinase was investigated by quantifying the water soluble head groups released in response to enterokinase. The increase in PI hydrolysis, as evidenced by the increase in IPs (IP_1, IP_2 and IP_3), observed in response to enterokinase is similar to that observed in response to a high concentration of α-thrombin (1.5 NIHunits/ml α-thrombin) in wild type cells (Cheng *et al.*, 1997). However, in contrast to wild type cells, PI hydrolysis is observed only in the presence of LiCl (data not shown). Enterokinase-induced PC hydrolysis, on the other hand, is virtually identical to α–thrombin induced PC hydrolysis (Cheng *et al.*, 1997).

Given the data indicating that induced PC metabolism is a major signal transduction component in mitogenesis (Rangan *et al.*, 1991), and the observations that PAR1 activates PC, as well as other lipid metabolism, we tested the role of PAR1 α-thrombin-induced mitogenesis. Indeed, in cells expressing EKTR enterokinase elicits a 2 fold increase in [^{3}H]thymidine incorporation with an EC_{50} of 100 U/ml (Cheng *et al.*, 1997). A comparable increase in [^{3}H]thymidine incorporation was observed in cultures treated with 0.0015 NIHunits/ml α-thrombin (data not shown). PAR1, therefore, can stimulate growth of IIC9 cells and must couple to the signaling pathway important for mitogenesis.

MITOGEN-STIMULATION RESULTS IN AN INCREASE IN NUCLEAR DIGLYCERIDES

Generation of Nuclear Diglycerides

While it was previously assumed that agonist-induced phospholipid hydrolysis, resulting in increased levels of diglycerides, occurred at the plasma membrane, there is substantial evidence demonstrating that agonists can generate diglycerides in the nucleus. Work by Smith and Wells (Smith and Wells, 1983) provided the earliest indication that nuclei posses a PI-cycle that is regulated independently from the (Dan should this be PC or PI) PC-cycle residing in the plasma membrane. This is consistent with data showing that mitogen-induced diglyceride mass at the plasma membrane does not account for the diglyceride mass observed in total cell extracts. In vasopressin-stimulated hepatocytes (Exton, 1990) and α-thrombin-stimulated fibroblasts (Wright *et al.*, 1988), the total amount of induced diglyceride represents nearly 1% of the total mass of cellular lipid. If all of this diglyceride was present in the plasma membrane, it would result in drastic and detrimental changes in the physical properties of this structure (Siegel *et al.*, 1989)). In addition, Martin *et al.* demonstrated that in GH_3 cells stimulated with thyroid releasing hormone (TRH), the small initial diglyceride production (possibly derived from PIPs) occurs at the plasma membrane while the larger later phase of diglyceride production (likely derived from PC) is produced in internal membranes (Martin *et al.*, 1990). These data demonstrate that agonist-induced diglyceride production must occur at sites other than the plasma membrane.

In view of the fact that mitogen stimulation results in a significant elevation of cellular diglyceride levels, and diglycerides play an important role in mitgenic signaling cascades, it was important to determine whether these lipids were generated in the nucleus. Indeed, α-thrombin induces a rapid albeit transient rise in nuclear diglycerides in quiescent IIC9 cells (Jarpe *et al.*, 1994). EGF also increases nuclear diglyceride levels (unpublished observation).

Source of the Induced Nuclear Diglycerides

In order to identify the enzymes responsible for the increase in the induced nuclear diglycerides, it's critical to identify the lipids serving as the source of those diglycerides. In the NIH3T3 cell studies, a small decrease in PIP and PIP_2 levels were observed suggesting that the hydrolysis of PIs was responsible for at least part of the increase in IGF-1-induced nuclear diglycerides (Divecha *et al.*, 1991; Divecha *et al.*, 1993b). The decrease in nuclear PIs, however, did not account for all of the induced diglycerides (Cataldi *et al.*, 1990; Cocco *et al.*, 1988; Cocco *et al.*, 1989; Cocco *et al.*, 1992; Divecha *et al.*, 1993a), supporting the idea of another source. These nuclei were isolated in the presence of detergents (see above), so that examination of other potential sources, and complete quantification of all nuclear diglycerides, was not possible.

In light of these data and our results, we examined the source of nuclear diglycerides induced by α-thrombin in IIC9s. First, we took advantage of our

previous observation indicating that [^{3}H]myristate is preferentially incorporated into PC when intact IIC9s are acutely labeled (Wright *et al.*, 1992). Treatment of IIC9 cells with α-thrombin resulted in an increase in [^{3}H]myristate radiolabeled nuclear diglycerides suggesting that PC hydrolysis was a contributing source of the nuclear diglycerides. However, elevation of these radiolabeled diglycerides does not alone allow for a definitive identification of all phospholipid sources (Leach *et al.*, 1992).

Because it is not possible to selectively radiolabel nuclear phospholipids, potential sources of the induced nuclear diglycerides cannot be obtained by analysis of the release of water-soluble radiolabeled headgroups from metabolically labeled cultures. We, therefore, used a method for determining the source of the induced diglycerides developed in our laboratory (Leach *et al.*, 1992; Pessin and Raben, 1989; Pessin *et al.*, 1990). In this method, the molecular species of the induced diglycerides is compared with the molecular species of the potential phospholipid sources. This analysis has proven useful in establishing the source of diglycerides generated in response to mitogens in fibroblasts (Leach *et al.*, 1992; Pessin and Raben, 1989; Pessin *et al.*, 1990) and in response to neurotransmitters and neurotrophic factors in PC12 cells (Pessin *et al.*, 1991). In addition, similar analyses have been used to identify PC as the source of induced diglycerides and PA in a variety of systems (Augert *et al.*, 1989; Holbrook *et al.*, 1992; Lee *et al.*, 1991).

Molecular species analysis analysis of the induced nuclear diglycerides indicates that PC hydrolysis is the predominant, if not exclusive, source of the induced diglycerides at all times. We should note, however, that small increases in nuclear PI-derived diglycerides, representing ≤1% of the total nuclear lipid may escaped detection.

In view of our data , as well as data from Divecha and coworkers (D'Santos *et al.*, 1999b; D'Santos *et al.*, 1999a; Divecha *et al.*, 1997b; Divecha *et al.*, 1997a), it is tempting to speculate that mitogens activate a PC cycle in the nuclear envelope as well as a PI cycle. In support of this hypothesis, enzymes involved in these cycles have been identified in the nucleus. PI cycle enzymes; PI-PLC (E.C.3.1.4.11), diglyceride kinase, and PI 4 and 5 kinases (E.C.2.7.1.67, E.C.2.7.1.68 respectively), have been localized in the nucleus (Payrastre *et al.*, 1992). A PI-3-kinase, which phosphorylates myo-inositol at the D-3 ring position (Carpenter and Cantley, 1996b; Corvera and Czech, 1998; Toker and Cantley, 1997) has been identified in the nucleus (Neri *et al.*, 1994; Neri *et al.*, 1999). An enzyme involved in PC biosynthesis, CTP:phosphocholine cytidylyltransferase (E.C.2.7.7.15), has also been found in the nucleus (Wang *et al.*, 1993). This enzyme is particularly interesting it serves as the regulatory enzyme in PC biosynthesis and its activity is regulated by diglyceride (Kent, 1990; Kolesnick and Hemer, 1990). We should note, however, that although it is activated in response to mitogens, it appears to leave the nucleus and move to the endoplasmic reticulum (Northwood *et al.*, 1999; Jackowski, 1996). In preliminary studies, we have been identified PC hydrolyzing activity in isolated IIC9 nuclei (M.B. Jarpe and D.M. Raben, unpublished observations). These data provide strong support for the hypothesis that mitogens activate a PI and/or PC cycle in the nuclear envelope. While these data also argue that the nuclear diglycerides are derived from phospholipids in the nuclear envelope, we cannot completely rule out the possibility that at least some diglyceride may be generated in a non-nuclear membrane and then transferred to the nucleus.

It is important to emphasize, however, that PC may not be the source of nuclear diglycerides in all cases. In a recent report, Neri *et al.* demonstrated that PI may be the source of nuclear diglycerides in Swiss 3T3 cells and that these diglycerides are responsible for the activation of nuclear PKC (Neri *et al.*, 1998) (see below). As the authors point out, this may reflect agonist and cell type differences.

Physiological role of induced nuclear diglycerides

The above data are consistant with the hypothesis that modulation of nuclear diglyceride levels are important components of mitogenic signal transduction pathways. A likely physiological target for the induced nuclear diglycerides is the activation of nuclear PKC. Indeed, the thrombin-induced rise in nuclear diglyceride in IIC9s is accompanied by an increase in the level of PKC-α found at the nucleus (Jarpe *et al.*, 1994; Leach *et al.*, 1992). Consistent with the importance of

nuclear diglycerides in mitogenesis, IGF-1 does not induce nuclear PKC in mutant 3T3 cells which contain IGF-1 receptors but fail to respond mitogenically to IGF-1 (Martelli *et al.*, 1991). Topham et al provided some of the strongest evidence that nuclear diglycerides are essential for mitogenic stimulation (Topham *et al.*, 1998). In this study, expression of DGK-ζ reduced the levels of nuclear diacylglycerol levels and attenuated the growth of Cos cells. These data suggest that DGK-ζ modulates the level of nuclear diglycerides. Taken together, these data clearly suggest a linkage between mitogen-induced nuclear lipid metabolism, PKC activation, and cellular proliferation.

While the early whole-cell studies illuminated the central role of diglycerides in the activation of PKC and identified many of the important implications of this event, it has led many investigators to assume that the activation of PKC is the *only* biological affect of these lipids. There is substantial evidence that is not the case. In addition to the fact that these lipids are a major precursor of phospholipids and triglycerides synthesized *de novo*, diglycerides also modulate other cellular processes independent of PKC activation. For example, phospholipases (Zidovetzki *et al.*, 1992), enzymes involved in lipid synthesis (Kent, 1990; Kolesnick and Hemer, 1990; Wang *et al.*, 1993), the generation of superoxide in neutrophils (Uhlinger *et al.*, 1991), membrane fusion (Siegel *et al.*, 1989), and membrane/cytoskeletal interactions (Burn *et al.*, 1985) may be modulated by diglycerides via PKC-independent mechanisms. Furthermore, there is increasing interest in proteins that bind and are modulated by diglycerides (Ron and Kazanietz, 1999). In view of our evidence that a nuclear PLD is regulated by RhoA, it is interesting to note that some of these proteins modulate the activity of small molecular weight GTPases (Ron and Kazanietz, 1999). It seems likely that many other functions affected by elevated diglyceride levels remain to be discovered.

It is also possible that the phyisological role of the induced diglycerides may depend, at least in part, by the intranuclear localization of diglycerides. In an intriguing series of studies, Divecha and coworkers found that some of the nuclear lipid metabolism occurs not in the nuclear envelope but within the nuclear matrix (Divecha *et al.*, 1993b; Divecha *et al.*, 1997b). These lipids and lipid metabolites may serve interesting roles in regulating nuclear events and definitely deserves further study.

Nuclear PLD regulated by RhoA is selectively activated in response to α-thrombin

In view of the role of nuclear diglycerides in regulating growth, it is essential that the mechanism by which the nuclear diglycerides are generated be established. One potential mechanism is via the activation of a PC-specific PLD (Exton, 1997b; Exton, 1998; Morris *et al.*, 1997). This phospholipase catalyzes the hydrolytic cleavage of PC at the phosphoglycerol bond generating free choline and phosphatidic acid (PA), that latter being converted to diacylglycerols via a PA-phosphohydrolase (E.C.3.1.3.4).

To begin delineating the enzymes involved in nuclear diglyceride production we examined the ability of α-thrombin to activate a nuclear PLD. We took advantage of the well known transphosphatidylation property of PLD. In the presence of small molecular weight primary alcohols, such as ethanol, PC-PLD utilizes the alcohol in lieu of water thereby converting PC into a phosphatidylalcohol (phosphatidylethanol, Pet, in the presence of ethanol) (Kobayashi and Kanfer, 1987). Using this assay we showed that α-thrombin stimulation of quiescent IIC9 cells results in an increase in PLD activity in IIC9 nuclei as indicated by an increase in the amount of nuclear PEt when quiescent cells incubated in the presence of ethanol are treated with α-thrombin. We also showed, that PLD activity in isolated nuclei isolated from α-thrombin-induced cultures is higher than that observed in nuclei isolated from quiescent cultures.

These data prompted us to determine which PLD isoform was present in the IIC9 nuclei and identify the components responsible for its regulation. There are at least two mammalian PLD isoforms, PLD1 and PLD2 (Hammond *et al.*, 1995; Morris *et al.*, 1996; Hammond *et al.*, 1997; Colley *et al.*, 1997; Exton, 1997a; Exton, 1997b). Western blot analysis of IIC9 nuclear extracts from IIC9 cells demonstrated that only PLD1 was present at the nucleus (unpublished observation). PLD1 is modulated by a number of small molecular weight GTPases, including RhoA. Interestingly, the α-thrombin-induced activation of nuclear PLD is accompanied by the translocation of RhoA to the

nucleus (Baldassare *et al.*, 1997), suggesting that this GTPase regulates nuclear PLD activity. This is supported by the observation that addition of RhoGDI to nuclei isolated from α-thrombin-induced cultures reduces the α-thrombin-induced elevated PLD activity to that observed in nuclei isolated from quiescent IIC9s. PLD activity is increased in these RhoGDI-treated nuclei by addition of RhoA in the presence of GTPγS. Consistent with these data, we found that expression of a dominant negative RhoA (N19RhoA) inhibits the α-thrombin-induced increase in nuclear PLD activity (unpublished observation). Addition of RhoA in the presence of GTPγS to nuclei isolated from quiescent IIC9 cells resulted in a significant increase of endogenous PLD activity similar to that found for nuclei isolated from α-thrombin-induced cells. In addition, removal of RhoA from nuclei from stimulated cells resulted in a lost of PLD activity that could be restored by the addition of recombinant RhoA (Baldassare *et al.*, 1997). These results suggests that PLD1 is found in the nuclei of unstimulated cells and that translocation of RhoA to the nucleus is involved in the its regulation.

Golgi PLD is not induced by α-thrombin: lack of RhoA effect

PLD1 is present in both IIC9 nuclei and Golgi (unpublished observation). In contrast to the α-thrombin-induced activation of PLD activity in IIC9 nuclei, α-thrombin fails to induce PLD activity in Golgi (unpublished observation). Golgi were isolated from quiescent or cultures incubated in the presence of α-thrombin (1NIHunit/ml) and ethanol (200mM) for 30 minutes. PEt levels in the isolated Golgi, quantified as previously described (Wright *et al.*, 1992; Baldassare *et al.*, 1997) (Baldassare, Brass, Phillips, Raben, manuscript in preparation). As a positive control, PEt levels in nuclei isolated from quiescent and α-thrombin-induced cultures were also quantified and indicate that PLD was not activated in Golgi in response to α-thrombin (unpublished observation). Similar results were obtained in two other assays. Golgi were isolated from quiescent or the α-thrombin-induced cultures and PLD activity was quantified using exogenous, [^{3}H]PC in TritonX-100 mixed micelles (Baldassare *et al.*, 1997). Third, PLD activity in these Golgi was quantified using [^{3}H]PC incorporated into liposomes (Brown *et al.*, 1993). PLD activity isolated from quiescent or α-thrombin-induced cultures was again essentially identical in both assays. The PLD activity using the mixed micelle assay was; quiescent = 0.03nmol/μg/hr; α-thrombin-induced = 0.01 nmol/μg/hr. PLD activity using the liposome assay was; quiescent = 0.38nmol/μg/hr; α-thrombin-induced=0.42nmol/μg/hr). Consistent with these data, Western blot analysis (Baldassare *et al.*, 1997) shows that RhoA is not present in Golgi (Phillips, P..J., Klaus, J., Baldassare, J.J., and Raben, D.M., manuscript in preparation). These data are consistent with the agonist-induced differential localization of RhoA in Swiss 3T3 cells (Fleming *et al.*, 1996).

Ablation of Gαo results in unregulated PC metabolism and a transformed phenotype

The above data indicate that the α-thrombin receptor, PAR1, induces both a PI and PC metabolism. Furthermore, PI-PLC, PC-PLD, as well as PLA_2 and the putative PC-PLC type enzymes, are all activated by this receptor. This led us to examine whether different GTPase were selectively coupling to specific phospholipases.

Our strategy for these studies involved examining the ability of α-thrombin to stimulate PI and PC metabolism in cells in which specific Gα subunits had been selectively ablated. Gαq was known to be involved in the stimulation of PI metabolism (Exton *et al.*, 1992; Exton, 1994b; Exton, 1996). Indeed, ablation of this GTPase in IIC9 cells prevented the α-thrombin-induced activation of PI metabolism (data not shown). We ablated other α subunits using antisense RNAs to examine the effect of these ablations on PC metabolism. In studies so far, ablation of the Go α subunit (Goa cells) has yielded some surprising results.

Diglyceride level is chronically elevated in Goa cells

Given the importance of diglycerides in mitogen signaling cascades, we measured the mass of diglyceride in the Goa cells (Cheng *et al.*, 1997). Subconfluent wild type and Goa cells were incubated in serum-free medium for two days and diglyceride levels were quantified. Interestingly, the "basal" diglyceride level in the serum-starved Goa cells is twice that of quiescent wild type cells. Furthermore, while the addition of α-thrombin to the wild type cells resulted in a two fold increase

in diglyceride mass level, addition of α-thrombin to the ablated cells did not induce a significant further increase in diglyceride levels . These results, observed in two independently isolated clones, indicate that the diglyceride level in Goa cells was constitutively elevated even in the absence of any added mitogens.

The increased Diglyceride is due to a constitutively activated "Apparent" PC-PLC Activity

We have shown that PC hydrolysis is the major, if not exclusive, source of mitogen-induced diglycerides in IIC9 cells (Wright *et al.*, 1992; Pessin *et al.*, 1991; Rangan *et al.*, 1991; Pessin *et al.*, 1990; Pessin and Raben, 1989; Wright *et al.*, 1988). In view of these data, we examined the possibility that an increase in PC hydrolysis contributed to the elevated diglyceride level in the Goa cells.

In order to determine if PC hydrolysis was affected in Goa cells, the cells were radiolabeled to isotopic equilibrium with [^{3}H]choline chloride in serum-free medium for 48 hr and the intracellular [^{3}H]Choline and [^{3}H]phosphorylcholine level were quantified (Cheng *et al.*, 1997). TLC analysis of water-soluble head groups indicated that the phosphorylcholine level in the Goa cells is 5-10 fold higher than that found in wild type cells. The level of choline in the ablated and wild type cells, however, is essentially identical. These data indicate that both phosphorylcholine and diglyceride, the two products of PC-PLC, are elevated in ablated cells and strongly suggest that a PC-PLC is constitutively activated in Goa cells. These results have been observed in three independently isolated clones.

The increased PC metabolism is not due to PLD/PAPH/CK activity

The existence of a mammalian PC-PLC remains controversial (see below). It is therefore important to examine alternative possible pathways for the elevation of diglycerides and phosphorylcholine in Goa cells. One such possibility is that PC is hydrolyzed via a PLD and the resulting PA is dephosphorylated to diglyceride, via PAPH, while the free choline is phosphorylated via choline kinase (CK, E.C.2.7.1.32). As a result of the combined action of all three enzymes, PLD, PAPH and CK, an apparent PC-PLC activity would be detected similar to that observed in *v-ras* transformed cells (Carnero *et al.*, 1994b; Carnero *et al.*, 1994a; Preiss *et al.*, 1986).

In view of the above, PLD activity was quantified in Goa cells and wild type IIC9 cells by taking advantage of the unique transphosphatidylation activity of PLD and the ability to preferentially label PC by acute labeling with [^{3}H]myristate (Wright *et al.*, 1992; Cheng *et al.*, 1997). PLD activity is indistinguishable in the ablated and wild type cells in the absence of mitogen. Furthermore, the addition of thrombin to both cell types results in comparable increases in PLD activity. These data indicate that both basal and thrombin activated PLD activity are unaltered in Goa cells.

To further examine the possible involvement of PLD/PAPH/CK activities, CK activity was quantified in wild type and Goa serum-deprived cells. These cells were incubated with [^{3}H]choline for 15 and 30 minutes and the level of radiolabeled phosphorylcholine was quantified. The conversion of choline to phosphorylcholine was essentially identical in both cell types demonstrating that the CK activity was not elevated in the Goa cells (Cheng *et al.*, 1997).

Taken together, the above data eliminate the involvement of PLD/PAPH/CK as a mechanism for the chronic elevation of diglyceride and phosphorylcholine levels in Goa cells. In addition, they indicate that $G_o\alpha$ ablation induced transformation is different from *v-ras* induced transformation, since the later involves PLD/PAPH/CK activities (Carnero *et al.*, 1994b; Carnero *et al.*, 1994a; Preiss *et al.*, 1986).

It is important to realize that while a number of prokaryotic PC-PLC enzymes have been cloned (Titball, 1993), there are no data that definitively identify a mammalian PC-PLC. There is, however, considerable evidence implicating a role for this enzyme in signal transduction cascades as indicated by the induced generation of diglycerides and phosphorylcholine (Cai *et al.*, 1993; Cheng *et al.*, 1997; Exton *et al.*, 1991; Exton *et al.*, 1992; Irving and Exton, 1987; Larrodera *et al.*, 1990; Laviada *et al.*, 1995; Li *et al.*, 1995). Additional evidence has been provided by studies employing D609, a fungal metabolite which has been shown to inhibit bacterial PC-PLC (Buscher *et al.*, 1995; Halstead *et al.*, 1995; Schutze *et al.*, 1992). It should be noted, however, that this inhibitor also blocks sphingomyelin synthase (E.C.3.1.4.12) (Luberto and

Hannun, 1998) which may account for some of the reported PC-PLC activity. Further studies, including the isolation and cloning of a mammalian PC-PLC, are certainly required.

Goa cells acquire a transformed phenotype

Cells transformed as a consequence of a defect in a signal transduction component normally associated with the regulation of mitogenesis often show changes in the concentrations of second messengers under their control (Macara, 1989; Weinstein, 1990; Waterfield, 1989). Many of the signaling cascades known to be involved in mediating mitogenic signals involve the stimulation of lipid metabolism and G proteins are known to play a role in some of these cascades (Exton, 1994a; Seuwen and Pouyssegur, 1992; Noh *et al.*, 1995). In addition, the elevation of diglyceride levels plays a central early role in transducing the mitogenic signal in these cascades.

In view of this and the above observations of elevated diglyceride levels in the Goa cells, it is perhaps not too surprising that these cells obtained a transformed phenotype. In contrast to wild type IIC9 cells which are flat and extended, Goa cells appear round, retracted, and form multiple foci in confluent monolayer cultures (Cheng *et al.*, 1997). This morphology, observed in three independently isolated clones, suggest that the Goa cells have lost contact inhibition and acquired a transformed phenotype.

An important characteristic of transformed fibroblasts is their ability to grow in an anchorage independent manner. In view of this and the above data, we assessed the ability of the Goa cells to grow in soft agar (Cheng *et al.*, 1997). Goa cells formed 20-30 fold more colonies in soft agar than wild type cells and this has been observed in a second, independently isolated, clone (data not shown). Furthermore, each of the colonies formed by the ablated cells were much larger and more dense than the colonies formed by the wild type cells. Cells transfected with control vectors (vectors without inserts) formed colonies similar to those seen with wild type cells (data not shown).

To further investigate the possibility that the ablated cells were transformed, we assessed the "basal" level of thymidine incorporation (Cheng *et al.*, 1997). Wild type IIC9 cells are after serum-deprivation for 48 hr and the level of [^{3}H]thymidine incorporation is low. In contrast, serum-deprived Goa cells display a 10 fold higher level of [^{3}H]thymidine incorporation than the quiescent wild type cells. FCS (10%) or thrombin (2 NIHunits/ml) stimulates only a modest increase in [^{3}H]thymidine incorporation in the ablated cells while these treatments stimulate a 10 fold increase in [^{3}H]thymidine incorporation in the quiescent wild type cells. Consistent with these data, Goa cells survive in serum free media for an extended period of time while the wild type cells do not. These data indicate that the Goa cells are not growth arrested in serum-free medium and are consistent with the transformed phenotype of these cells.

Ablation of $G_o\alpha$ results in the mitogen-independent activation of Ras

IIC9 cells overexpressing constitutively activated Ras form multiple foci when grown in soft agar and do not growth arrest when serum-depleted (data not shown). To examine whether the Ras pathway was involved in the transformation of Goa cells we first examined Ras activation (Weber *et al.*, 1997). In growth-arrested IIC9 cells levels of activated Ras as determined by the ratio GTP/(GTP + GDP) associated with Ras is quite low and increases 6 fold within 5 minutes after the addition of PDGF or several other growth factors (data not shown). In contrast, Goa cells exhibit high levels of activated Ras in the absence of mitogen similar to levels found in IIC9 cells treated with PDGF. Addition of PDGF does not increase the level of activated Ras above the levels seen in serum-depleted Goa cells. These results are consistant with the observed inability of these cells to growth arrest with the removal of mitogen and suggest that in certain cell types loss of $G_o\alpha$ could result in neoplastic transformation.

Goa cells exhibit constitutively active ERK

Data from several laboratories has suggested the importance of ERK activation in Ras-dependent growth. We previously have demonstrated that suppression of PDGF-induced ERK activation blocked G1 progression in IIC9 cells. To determine whether ablation of $G_o\alpha$ resulted in constitutive activation of the ERK pathway, we next examined the endogenous activity of ERK in Goa cells (Weber *et al.*, 1997). Addition of PDGF and several other growth factors to growth-arrested IIC9 cells increases ERK activity approximately

7-8 fold within 15 minutes (data not shown). As previously found in CCL39 cells (Vouret-Craviari *et al.*, 1993) and IIC9 cells[2] addition of thrombin or PDGF to growth-arrested cells induces a biphasic increase in ERK activity. A rapid 8-12-fold increase of ERK activity within 5-10 minutes is followed by a sustained 4-6-fold increase in ERK activity. Asynchronous Goa cells grown in serum-free media express levels of ERK activity similar to the levels of ERK activity of IIC9 cells stimulated with PDGF (7-9-fold).

Elevated expression of cyclin D1 in absence of mitogen

An ERK-responsive region has recently been identified in the cyclin D1 promoter (Albanese *et al.*, 1995) and we have previously shown that addition of PDGF to IIC9 cells induces cyclin D1 mRNA and protein expression. In addition, we and others have shown that inhibition of mitogen-induced ERK activity blocks expression of cyclin D1 and progression of IIC9 cells through G1 (Grana and Reddy, 1995). Many tumor cell lines express elevated levels of oncogenic Ras and cyclin D1 (Hinds *et al.*, 1994; Li and Lieberman, 1989; Lovec *et al.*, 1994). To identify a possible downstream target of the Goa constitutively active Ras/ERK pathway, we measured levels of cyclin D1. Ablation of $G_o\alpha$ conferres an increase in cyclin D1 protein expression by approximately 3-4-fold. The levels of cyclin D1 in Goa cells remained constitutively elevated in the absence of mitogen.

We next investigated the effect of aphidicolin arrest and release on cyclin D1 expression (Weber *et al.*, 1997). Treatment of Goa cells with aphidicolin for 12 hours resulted in sustained levels of cyclin D1 protein in the absence of mitogen. Matsushime *et al.* (Matsushime *et al.*, 1991) previously showed that Bac1.2F5A macrophages released from aphidicolin arrest required the presence of growth factor (CSF-1) to sustain the expression of cyclin D1. Release of IIC9 cells from aphidicolin arrest in the absence of PDGF resulted in the rapid (within 5 hrs) decrease in the levels of cyclin D1 protein. However, cyclin D1 protein levels in Goa cells did not decrease significantly when released from aphidicolin in the absence of PDGF suggesting a significant difference in the requirement of sustained presence of growth factor for cyclin D1 expression. It is clear that the constitutive activation of the Ras/ERK pathway provides the sustained mitogenic signals required for the continued expression of cyclin D1. Cyclin D1 protein expression remained high through the next round of replication (approximately 24 hours after aphidicolin release). In the absence of mitogen, cyclin D1 mRNA levels in Goa cells were similar to the levels found in IIC9 cell treated with PDGF. Aphidicolin-treated and released Goa cells exhibited a 1.6-fold decrease in cyclin D1 mRNA expression although these levels were still 3-fold higher that serum-deprived IIC9 cells providing further evidence for the positive role of the Ras/ERK pathway in sustaining cyclin D1 expression in Goa cells in the absence of mitogen.

Cyclin D1/CDK complexes are constitutively active

Phosphorylation of Rb by active cyclin D1/CDK complexes has been demonstrated to be required for progression through G1 in several cell types (Grana and Reddy, 1995; Kato *et al.*, 1993; Resnitzky *et al.*, 1994; Sherr and Roberts, 1995). Although several transformed tumor cells express abnormally high levels of cyclin D1, the role of cyclin D1 in tumor formation is unclear. However, cyclin D1/CDK activity is thought to play an important role in mitogen-induced progression of cells through G1. In the absence of mitogen, IIC9 cells contain low levels of cyclin D1/CDK activity (Weber *et al.*, 1997). PDGF treatment induced a 6-fold increase in cyclin D1/CDK activity within four hours (data not shown) and sustained this level of activity through 24 hours (Weber *et al.*, 1997). In contrast to IIC9 cells, Goa cells display significant cyclin D1/CDK activity in the absence of mitogen (Weber *et al.*, 1997). In addition, treatment of Goa cells with PDGF did not increase further cyclin D1/CDK activity (data not shown). Treatment of Goa cells with aphidicolin as well as release from aphidicolin arrest in serum-free media did not result in a decrease in cyclin D1/CDK activity further correlating the mitogen independence of Goa cells with the activation of the cyclin D1/CDK complexes in the absence of mitogen. Preliminary results from our lab also suggest that high levels of cyclin D1/CDK activity are required to retain the transformed phenotype of Goa cells. These data further suggest that in certain cell types Ras transformation involves the up-regulation of cyclin D1/CDK activity.

A PI-3 kinase is elevated in Goa cells

Another activity known to be involved in growth and transformation of a variety of cell types is a p85-associated PI-3 Kinase activity (Carpenter and Cantley, 1996a). In view of this, we examined this PI-3 kinase activity in our Goa cells. PI-3 kinase activity is constitutively elevated in the Goa cells (unpublished observation). This is consistent with the notion that this activity is involved in the generating the transformed phenotype of these cells. In these studies, PI-3 kinase activity was quantified in anti-p85 immunoprecipitates from cellular lysates.

Inhibition of PI-3 kinase inhibits DNA in Goa cells

In previous studies, we demonstrate that associated with the transformed phenotype of Goa cells is the constitutive elevation of DNA synthesis and growth in the absence of any mitogen (Cheng *et al.*, 1997; Weber *et al.*, 1997). Our preliminary studies above indicate that a p85-associated PI-3 kinase, shown to be involved in the regulation of cell growth in many systems (Carpenter and Cantley, 1996a), is also constitutively elevated (unpublished observation). We therefore determined whether inhibition of this activity would correlate with an inhibition of DNA synthesis in Goa cells. DNA synthesis in Goa cells is suppressed in the presence 10μM LY294002, a specific PI-3 kinase inhibitor (Fruman *et al.*, 1998) (unpublished observation). These data support the notion that the increase in the p85-associated PI-3 kinase activity is important for the generation of the transformed phenotype.

α transducin reverses the increase in PC-PLC, PI 3-kinase and ERK acitivity in Goa cells

Gβγ dimers have been shown to modulate various effectors (Clapham and Neer, 1993; Neer, 1994). Given this, the data above inspired an intriguing hypothesis. It is possible that the observed elevations of the above activities, and the transformed phenotype may result from free Gβγ dimers elevated as a consequence of ablating the α subunit of Go. To test this notion, Goa cells were transfected with α subunit of transducin (Gαt) which has been used to sequester these dimers (Crespo *et al.*, 1994).

As we recently reported, Gαt reduces the apparent PC-PLC activity in Goa cells to that observed in wild type IIC9s (Cheng *et al.*, 1997). Similarly, Gαt expression reduces PI-3 kinase activity, and ERK activities to that observed in quiescent wild type IIC9s (6). Similarly, Gαt expression reduced the elevated Ras activity in Goa cells to that observed in wild type IIC9s (data not shown). These data demonstrate that an increase in Gβγ dimers is involved in the increase in these activities observed in Goa. We should note that expression of a dominant negative Ras did not inhibit elevated PI-3 kinase activity in the Goa cells (data not shown). These data suggest that the Gβγ-sensitive, p85-associated, isoform of PI-3 kinase (p110β) is elevated in Goa cells.

Given the role of the above activities in mitoigenesis, and the unrestrained growth of Goa cells, we tested the effect of Gαt expression on Goa cells growth. Serum deprived Goa incorporate a high level of thymidine, consistent with their transformed phenotype (Cheng *et al.*, 1997). Expression of Gαt in Goa cells reduces the thymidine incorporation to that observed in the parental IIC9s (unpublished observation).

Taken together, the data provide strong support for the notion that Gβγ dimers modulate a number of signaling molecules and that aberrant elevation of these dimers results in a transformed phenotype. Furthermore, the ability of Gβγ dimers to modulate these activities is dependent on a specific isoform(s) of the dimers present. Consistent with this, ablation of specific Gβ or Gγ, subunits selectively affect specific effector activities (Kleuss *et al.*, 1992; Kleuss *et al.*, 1993). Ablation of $G\beta_1$ or $G\beta_3$, but not $G\beta_2$ or $G\beta_4$, prevents the inhibition of the voltage-dependent Ca^{+2} channel activity induced by carbachol or somatostatin in GH_3 cells (Kleuss *et al.*, 1992). It is interesting to note that in GH_3 cells all four Gβ isotypes are associated with Go. Co-transfection experiments in COS-7 cells clearly demonstrate that expression of only specific Gβ or Gγ subunits significantly activate PI-PLCβ_2 (Wu *et al.*, 1993). Whereas expression of $G\beta_1\gamma_1$, $G\beta_1\gamma_5$ or $G\beta_2\gamma_5$ stimulate PI-PLCβ_2 activity, expression of $G\beta_2\gamma_1$ had no effect. Recent data on the regulation of G protein receptor kinases (GRK) lend further strong evidence for specific Gβγ dimers inducing a specific cellular response (Daaka *et al.*, 1997). Of the Gβ subunits examined, $G\beta_1$ and $G\beta_2$ but not $G\beta_3$ activate GRK2 while GRK3 was activated by all three Gβ isoforms. In addition, using a yeast two hybrid system, only certain pairs of Gβ and Gγ subunits were found to form complexes (Yan *et al.*, 1996).

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