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Cellular Signal Transduction and the Reversal of Malignancy

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Animal cells contain only a few defined molecular systems that transduce hormonal and growth signals from the external environment to the intracellular milieu to regulate cellular growth and differentiation. Among the most ubiquitous of these "second messenger" pathways are those utilizing cyclic AMP and phosphatidylinositide turnover. The former activates protein kinase A, while the latter leads to the activation of protein kinase C and mobilization of intracellular calcium. Lesions induced by oncogenes in signal transduction systems may be responsible for the cancerous transformation of cells. In many tumor cell lines, including some transformed by the *ras* and *sis* oncogenes, activation of protein kinase A by elevation of cyclic AMP or activation of protein kinase C by addition of phorbol esters can restore many normal aspects of growth and morphology. Such "reverse transformation" is accompanied by the phosphorylation of unique cellular proteins and alterations in the phosphoinositide cycle. Molecular mechanisms by which activation of signal transduction systems can attenuate the malignant phenotype are considered in the context of cellular growth and differentiation.

Key words: protein kinase C, protein kinase A, phosphatidylinositides, sis oncogenes, reverse transformation by cAMP, ras oncogenes, phorbol esters, reverse transformation, cyclic AMP, signal transduction, cancer

The molecular mechanisms by which hormonal signals are transduced by intracellular second messenger systems to regulate cell metabolism and growth is a subject of growing interest for cancer biologists. Perturbation of second messenger systems and of the hormones and receptors that modulate them may be responsible for the oncogenic transformation of normal cells [1].

Evidence for this concept originated from studies concerning the behavior of the cyclic AMP (cAMP) signaling system as a second messenger in cancer cells. This

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model for studying the control of cell transformation is called "cAMP-mediated reverse transformation" [2,3].

The oncogenic transformation of fibroblasts by tumor viruses, oncogenes, or chemical carcinogens results in the alteration of cell growth and morphology [4]. Transformed fibroblasts grow to high saturation densities, escape from contact inhibition and substrate dependence, and have reduced serum requirements [5,6]. Morphological changes include a more rounded or spindle-like shape, altered membrane topography, loss of tight focal adhesions with the substratum, a disorganized culture morphology, and a reduction in directed motility [2]. Transformation is also frequently accompanied by a reduction in the intracellular concentration of cAMP and attenuation of the activity of the enzyme adenylate cyclase, which synthesizes cAMP.

In many such oncogenic, transformed cell lines, elevation of intracellular cAMP (for example, by addition of membrane-permeable cAMP analogs to the culture) can reverse the pleiotypic effects of transformation [7,8]. This reverse transformation by cAMP results in the rapid acquisition of normal cell growth and morphology [9]. Cells become oriented and polarized (Fig. 1). Lamellar cytoplasm and stress fibers increase, as does adhesiveness to the substratum. Density and contact inhibition of growth are restored [10]. There is an increase in cytoskeletal organization: the cytoplasmic microtubule network expands [11], microfilament bundles assemble, and there is a redistribution of cytoplasmic myosin into these bundles [12]. Thus, treatment of transformed cells with cAMP not only affects cell growth but also induces an assembly of the cytoskeletal structures that organize the cytoplasm and govern cell morphology. The ability of cAMP to restore so many normal properties to cancer cells implies that one major pathway of oncogenic transformation is the disruption of the cellular mechanisms that regulate cAMP levels.

How does cAMP exert its varied effects on the growth and morphology of the transformed cell? The only known mode of action of cAMP in higher eukaryotes is the activation of cAMP-dependent protein kinases [13-15]. As best defined in those hormone-responsive tissues where cAMP serves as a second messenger, the activation of a cAMP-dependent protein kinase (PKA) results in the phosphorylation of specific target proteins. Phosphorylation alters the activity of the proteins, and, often through a cascade, the hormonal response is evoked.

Genetic studies of tumor cells confirm that cAMP-mediated reverse transformation is also orchestrated by protein kinases. Mutants of S49 lymphoma cells defective in PKA no longer show growth inhibition by cAMP [16,17], and mutants of Chinese hamster ovary (CHO) cells defective in PKA lack a morphological and growth response to cAMP [18]. An attractive explanation of the broad spectrum of phenotypic changes induced in transformed cells by cAMP is that a cAMP-dependent protein kinase system acts pleiotypically to phosphorylate—and thereby alter the activity of—a variety of proteins important in cytoskeletal organization and cell proliferation.

Which cellular proteins are phosphorylated by the cAMP-dependent protein kinase system? Until recently, no identification of specific proteins whose phosphorylation state is altered during reverse transformation had been made. Likely candidates would be structural proteins of the cytoskeleton, the enzymes or regulatory proteins of other major second messenger systems perturbed by transforming agents, the proteins encoded by oncogenes, and the cellular proteins involved in growth regulation and cytoskeletal organization that are themselves modulated by oncogene expression.

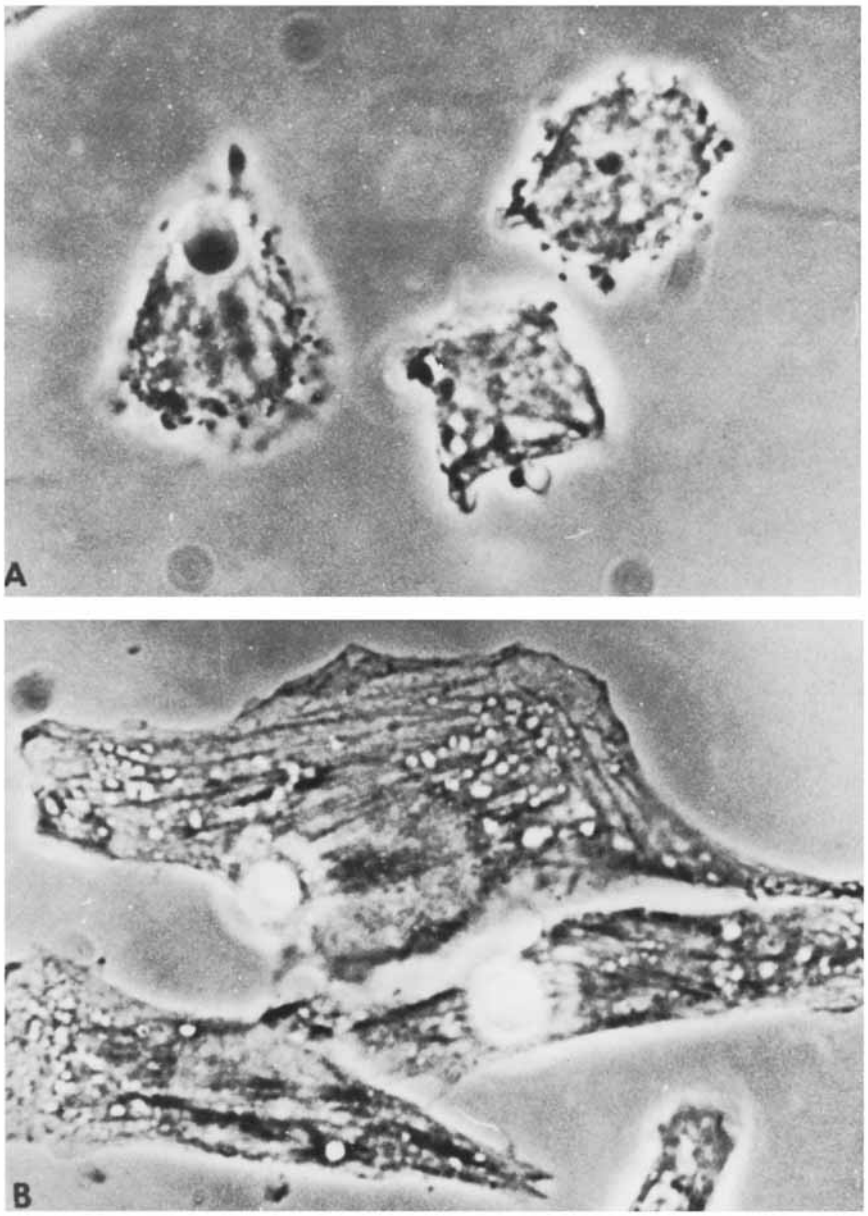

Fig. 1. Effect of db-cAMP on the morphology of CHO cells. **A:** No treatment. **B:** db-cAMP (1 mM) for 18 hr. Phase contrast micrographs.

Research in the authors' laboratory has identified a number of proteins, including a 20,000-dalton myosin light chain, unidentified 45,000- and 50,000-dalton proteins, and a 155,000-dalton protein possibly bound to microtubules, as proteins whose phosphorylation is altered during reverse transformation [3,19]. However, a specific link between the phosphorylation of these species and the phenotypic effects of cAMP on tumor cells remains to be established.

The protein products of a class of retroviral oncogenes are membrane/cytoskeleton-bound protein kinases with the unique ability to phosphorylate tyrosine residues [20,21]. Transformation often results in an elevation of total cellular phosphotyrosine and a moderate increase in the levels of phosphotyrosine in specific proteins. However, despite intensive research in several laboratories, it has so far proved difficult to establish a cause-and-effect relationship between cAMP-dependent or tyrosine phosphorylation of specific cellular proteins and modulation of the transformed phenotype [1,22]. Recently, we and others have begun to study the involvement of another major second messenger system, phosphoinositide turnover, in the events of reverse transformation. Increased turnover of a minor membrane phospholipid, the bis-phosphorylated form of phosphatidylinositol (PI), phosphatidylinositol 4,5-bisphosphate (PIP_2), is one of the earliest events following stimulation of cell proliferation by growth factors [23–25]. Phosphoinositide turnover is also increased in Rous sarcoma virus-transformed cells [26,27]. Hydrolysis of PIP_2 by a specific phosphodiesterase releases two products: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Both function as second messengers [28–30]. IP_3 appears to function as a second messenger for the mobilization of calcium [31,32], while DAG is an endogenous activator of the phospholipid- and Ca^{+2}-dependent protein kinase C (PKC) [30,33].

Protein kinase C has been implicated in cell proliferation. It also appears to be the major receptor for, and mediator of the action of, phorbol esters, the potent tumor promoters [33,34]. These compounds mimic many of the parameters of transformation in cell culture [70]. Recently, it was shown that retroviral tyrosine kinase such as the *src* and *ros* enzymes are associated in vitro with phosphoinositide kinases and can regulate phosphoinositide metabolism in vivo [27,35]. Hence, unrestricted activation of protein kinase C, either by binding of phorbol esters or by overproduction of inositol phospholipids catalyzed or stimulated by oncogene-encoded tyrosine kinases, may play a major role in regulating the transformed phenotype. This second messenger pathway is thus a likely target for modulation by cAMP and cAMP-dependent phosphorylation.

PHOSPHOINOSITIDE TURNOVER DURING REVERSE TRANSFORMATION

Our studies demonstrate that phosphoinositide metabolism is strongly affected during cAMP-mediated reverse transformation of CHO cells. They further demonstrate a dramatic influence on the morphologic phenotype of cells during reverse transformation by phorbol 12-myristate 13-acetate (PMA), the most potent known tumor promoter, and diacylglycerol (DAG), both direct activators of protein kinase C. Further, we have found major synergistic effects of PMA, DAG, and cAMP, both on phosphoinositide metabolism and on the phosphorylation of specific CHO cell proteins.

CHO cells were labeled with $^{32}PO_4$ before and after exposure to dibutyryl-cAMP (db-cAMP). Phospholipids were extracted and resolved by thin-layer chromatography [27,36]. Radioautography of the chromatograms demonstrated a substantial increase in levels of the phosphoinositides PI, PIP, and PIP_2 (Fig. 2). The correlation of these phosphoinositides and changes with time of exposure of the cells to db-cAMP is shown in Figure 3. Upon removal of cAMP, phospholipid concentrations rapidly return to those found in untreated CHO cells. The dependence of this

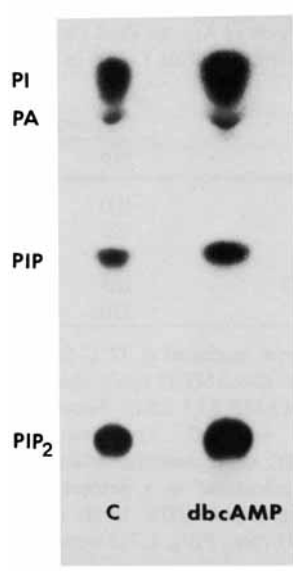

Fig. 2. Changes in phosphoinositide and phosphatidic-acid levels induced in CHO cells by db-cAMP. CHO cells were incubated at 37°C for 18 hr in the absence or presence of db-cAMP (1 mM) and testosterone propionate (15 μM). During the final 90 min the cells were radiolabeled using carrier-free $H_3\ ^{32}PO_4$ (0.1 mCi/ml). Cells were removed from the culture dish, and the lipids were extracted, resolved, and analyzed by autoradiography as described [27,36].

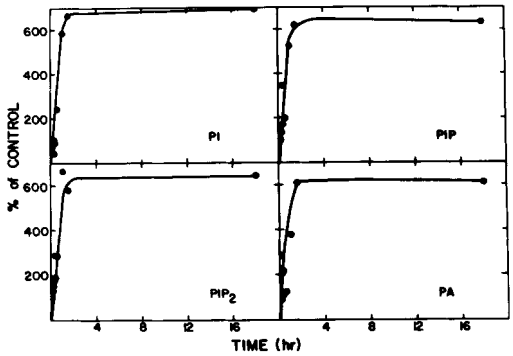

Fig. 3. Time course of db-cAMP-stimulated phosphorylation of the phosphoinositides and phosphatidic acid. CHO cells were exposed to db-cAMP (1 mM) and testosterone propionate (15 μM) at 37°C for the times indicated. Cells were radiolabeled with $H_3\ ^{32}PO_4$ (0.1 mCi/ml) for 90 min prior to lipid extraction. The phosphorylated lipids were separated by thin-layer chromatography and analyzed as described [27,36]. Results are calculated as a percentage of control (no treatment) phospholipid. Actual cpm recovered: PI, 1,580; PIP, 5,630; PIP_2, 10,560.

effect on db-cAMP concentration shows an ED_{50} of approximately 0.2 mM db-cAMP, a concentration comparable to that required for morphologic reversion. Other agents, such as cholera toxin (a cyclase activator), which elevate intracellular cAMP, induce similar increases in phosphoinositide levels (Table I).

The most straightforward explanation of these results is a cAMP-mediated inhibition of inositol phospholipid breakdown to diacylglycerol and inositol phosphates, perhaps by inactivation of the PIP_2 phosphodiesterase, although an effect on the lipid kinase is also possible.

TABLE I. Effects of Agents That Elevate or Mimic cAMP on Phosphoinositide Levels in CHO Cells*

	Percentage of control	
Addition	PIP	PIP_2
None	100	100
db-cAMP	305	330
Cholera toxin	141	176
8-bromo-cAMP	163	155
Theophylline	370	460

*CHO cells were incubated at 37°C for 18 hr with the indicated agent: db-cAMP (1 mM); cholera toxin (1 μg/ml); 8-bromo-cAMP (0.5 mM); theophylline (1 mM). Lipids were extracted, separated by thin-layer chromatography, and quantitated as described [27,36]. Values were calculated as a percent of control (no addition) phospholipid. 100% levels of phospholipids were PIP, 1,141 cpm; PIP_2, 1,743 cpm.

TABLE II. Phosphoinositide Levels in CHO Cells Treated With cAMP and Phorbol Ester*

	PMA (ng/ml) (% control)		
Phospholipid	0	1	10
PIP_2	100	315	418
PIP	100	247	352
PI	100	170	219

*CHO cells were treated with 1 mM db-cAMP for 18 hr in the presence of PMA as indicated. Phospholipids were quantitated as described [27,36]. Control values represent phospholipid levels after 18 hr exposure to db-cAMP alone. 100% levels (cpm) of phospholipids were PIP_2, 1,564; PIP, 794; PI, 331. In the absence of db-cAMP, levels of phospholipids (% control) with 10 ng PMA were PIP_2, 113; PIP, 88; PI, 92.

Activation of protein kinase C by phorbol esters can also affect phosphoinositide metabolism. There is a synergistic effect of PMA and cAMP on PIP and PIP_2 levels during reverse transformation. There are large increases in phosphoinositides when PMA is present during reverse transformation (Table II).

One implication of the experiments described above is that cAMP, by altering diacylglycerol production, may change the activity of protein kinase C. Conversely, to examine whether the activity of protein kinase C affects the induction of reverse transformation by cAMP, we determined whether exogenous diacylglycerol or PMA—both protein kinase C activators—could modulate cAMP-induced changes in protein phosphorylation, cell morphology, and growth.

SYNERGISTIC PROTEIN PHOSPHORYLATION BY cAMP, PHORBOL ESTERS, AND DIACYLGLYCEROL

We examined the effects of activation of PKC by tumor promoters of diacylglycerol on cellular protein phosphorylation during cAMP-mediated reverse transfor-

mation. An analysis by SDS-polyacrylamide gel electrophoresis is shown in Figure 4. Cyclic AMP induced the phosphorylation of proteins of 45,000 and 50,000 daltons and the dephosphorylation of a protein of 28,000 daltons. Surprisingly, the phorbol ester PMA induced similar phosphorylation changes. When cells were exposed simultaneously to cAMP and PMA there was a striking synergistic phosphorylation of both the 50,000- and 45,000-dalton proteins and a complete dephosphorylation of the 28,000-dalton protein. Exactly the same effect was observed when diacylglycerol, the natural PKC activator, replaced PMA (Fig. 5). These observations represent one of the largest reported changes in the phosphorylation of specific proteins induced in intact cells. Extraction of CHO cells with nonionic detergents in a buffer that preserves the cytoskeleton demonstrates that the 45,000- and 50,000-dalton phosphoproteins are present mainly in the cytosol/membrane fraction. To analyze further the basis for the synergism between cAMP and phorbol esters, we carried out one-dimensional peptide mapping of the ^{32}P-labeled proteins. Figure 6 shows the phosphopeptides generated from the 50,000-dalton protein. While cAMP or PMA treatment alone evokes phosphorylation of both common and unique peptides, treatment with both together results in the appearance of two new highly phosphorylated peptides not phosphorylated with either compound alone (shown by arrows in Fig. 6). This observation suggests either that cAMP and PMA synergism results from activation of a third protein kinase or, alternatively, that it results from a conforma-

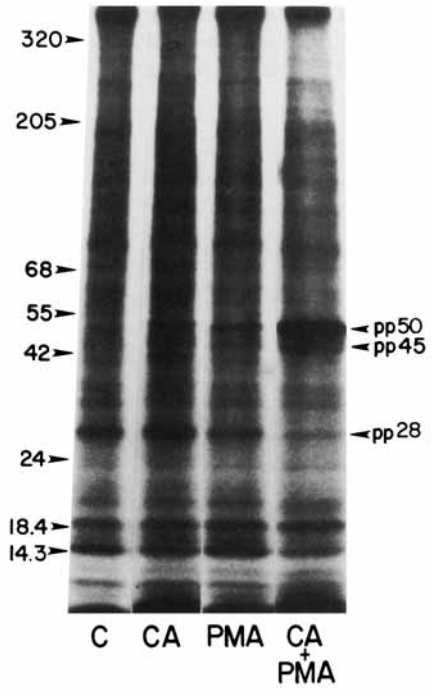

Fig. 4. Phosphoprotein distribution in CHO cells treated with cAMP and PMA. Metabolic labeling of cells with $^{32}PO_4$ and analysis of phosphoproteins on 10% SDS-PAGE was as described previously [66]. C, No treatment; CA, 0.75 mM db-cAMP, 15 μM testosterone; PMA, 200 ng PMA; CA+PMA, 0.75 mM db-cAMP, 15 μM testosterone, 200 ng PMA. Numbers and arrows on left-hand side refer to molecular weight standards (kilodaltons); those on the right indicate molecular weights of proteins whose phosphorylation is dramatically altered by CA+PMA.

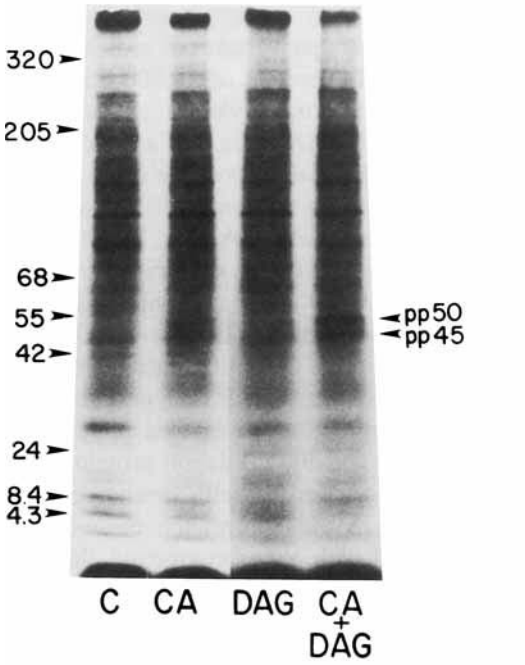

Fig. 5. Phosphoprotein distribution in CHO cells treated with cAMP and DAG. Analysis was on 10% SDS-PAGE [66]. C, No treatment; CA, 0.75 mM db-cAMP, 15 μM testosterone; DAG, 400 μg DAG; CA+DAG, 0.75 mM db-cAMP, 15 μm testosterone, 400 ng DAG.

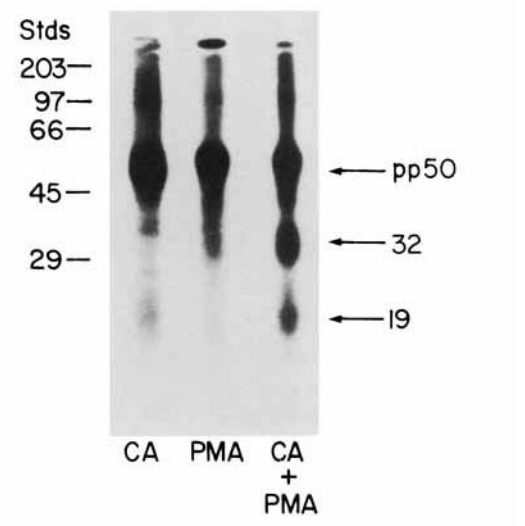

Fig. 6. Phosphopeptide map of pp50. After $^{32}PO_4$ labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Figure 4, the pp50 band was excised from the gel and subjected to one-dimensional peptide mapping with V8 protease (0.025 μg/well) on a 15% SDS-PAGE gel.

tional change in the substrate protein that makes additional phosphorylation sites accessible to PKA and/or PKC.

PHORBOL ESTERS AND DAG EXAGGERATE MORPHOLOGIC REVERSION

The influence of phorbol esters and DAG on the cAMP-induced morphological phenotype of CHO cells is shown in Figure 7. At low concentrations neither compound added alone changes cell form (Fig. 7a,c,e). The significant observation is that at these low concentrations both PMA and diacylglycerol dramatically alter the cAMP-induced morphologic phenotype. cAMP alone causes CHO cells to resume a

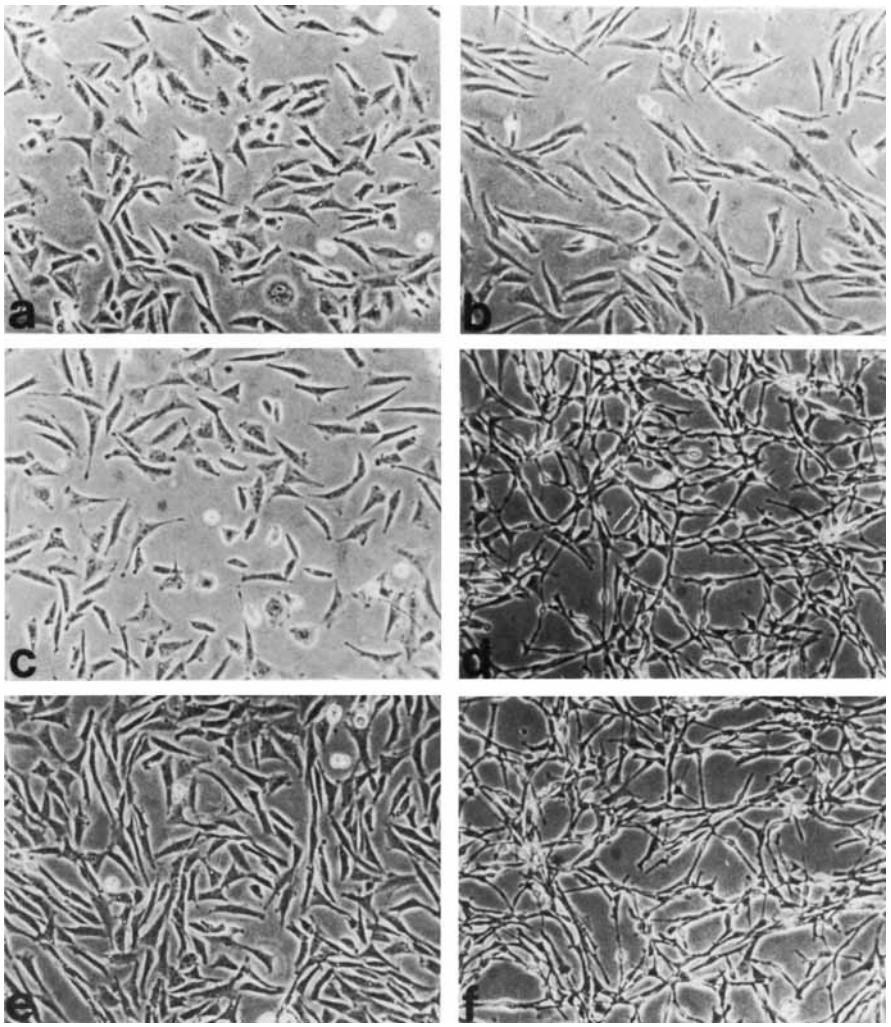

Fig. 7. Effect of phorbol esters and diacylglycerol on the morphology of CHO cells during reverse transformation. CHO cells were incubated for 18 hr at 37°C with the indicated agent. **a:** No treatment. **b:** db-cAMP (0.1 mM) + testosterone propionate (15 μM). **c:** PMA (0.5 ng/ml). **d:** PMA (0.5 ng/ml) + db-cAMP (1 mM) + testosterone propionate (15 μM). **e:** Diacylglycerol (10 μg/ml). **f:** Diacylglycerol (10 μg/ml + db-cAMP (1 mM) + testosterone propionate (15 μM).

normal fibroblastic morphology (Fig. 7b). Cells become flattened and elongated with a tranquil membrane and many actomyosin bundles (stress fibers). In the presence of either PMA (Fig. 7d) or diacylglycerol (Fig. 7f), cAMP is unable to induce cell spreading and flattening; the cell body remains rounded, and each cell extends long bilateral neurite-like processes. It is possible that continued activation of protein kinase C during reverse transformation alters the cAMP-induced assembly of actomyosin bundles. It also potentiates the cAMP-induced microtubule assembly that results in cell elongation and process extension.

PHORBOL ESTERS AND cAMP COORDINATELY INHIBIT CHO CELL GROWTH

Activation of protein kinase C by phorbol esters strongly potentiates cAMP-induced growth inhibition. As shown in Figure 8, cAMP alone reduces both growth rate and saturation density of CHO cells. PMA alone has little effect on growth. However, a dramatic inhibition of growth occurs when PMA is added simultaneously with cAMP. Cells were completely growth inhibited over many days. Trypan blue exclusion and flow cytofluorometry showed that cells were still viable after PMA and cAMP treatment. Furthermore, the synergistic inhibition of growth by PMA and cAMP was completely reversible upon removal of the drugs.

The synergistic inhibition of tumor cell growth by PMA and cAMP was confirmed by testing a variety of other agents that elevate intracellular cAMP. These included 8-Br-cAMP, theophylline, cholera toxin, and isobutylmethylxanthine (IBMX). Both DAG and phorbol esters showed synergism with each compound for morphological reversion and growth inhibition. [^{3}H] Thymidine incorporation into DNA confirmed a rapid and complete inhibition of DNA replication by PMA and cAMP (95% inhibition after 25 hr) under conditions where PMA alone had no effect and cAMP was 50% inhibitory.

The preceding results suggested that PMA and cAMP inhibit CHO cell growth at a specific phase in the cell cycle. We used computerized laser flow cytospectrofluorometry to determine that cAMP imposes a G_1-specific block and that there is a very vigorous potentiation of this block by PMA. Similar results were obtained with diacylglycerol.

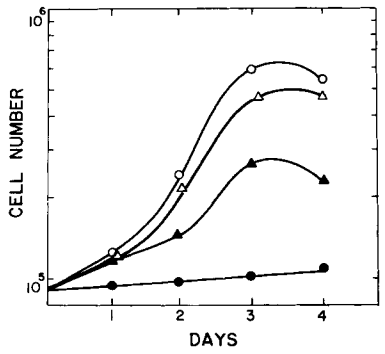

Fig. 8. Effect of PMA and cAMP on the growth of CHO cells. No addition (○—○); 0.5 mM db-cAMP (▲—▲); 0.5 mM cAMP and 20 ng/ml PMA (●—●); 20 ng PMA (△—△).

There is mounting evidence that phorbol esters can also induce differentiation to a normal phenotype in other transformed cell lines [37]. PMA has been shown to inhibit cell growth and induce differentiation of HL-60 human promyelocytic leukemia cells [38,39] and human K562 myeloid leukemia cells [40]. In fact, diacylglycerol mimics the PMA induction of HL-60 cell differentiation [41].

These results demonstrate that tumor-promoting phorbol esters, usually conceived of as mitogens and tumor promoters, can act synergistically with cAMP to reverse the transformed phenotype of cancer cells. Hence, phorbol esters must now also be conceived of as differentiating agents, which may, in some cases, actually function as antitumor drugs.

Taken together, the above observations suggest an intimate interaction in tumor cells between the signal transduction system modulated by cAMP and protein kinase A and that controlled by phosphoinositides and protein kinase C.

Cyclic AMP-mediated reverse transformation of tumor cells thus provides a unique model for dissecting these interactions and for examining the role of phosphoinositide metabolism, phorbol esters, and specific protein phosphorylation in the reversal of the transformed phenotype.

CYCLIC AMP REVERSES THE TRANSFORMATION OF NIH3T3 CELLS BY THE HUMAN H-ras ONCOGENE

Although cell lines such as CHO provide well characterized models for the study of reverse transformation, one drawback is that the transforming principle in most of these lines has not been defined. To isolate molecular pathways of reverse transformation, it would be valuable to have a cyclic AMP-responsive cell line rendered tumorigenic by a defined oncogene. We have found that the NIH3T3 cell lines transformed by the H-*ras* oncogene is an excellent model.

Human *ras* oncogenes are associated with many aspects of the transformation of normal tissue cells to cancer cells [42]. The proteins encoded by the *ras* genes are 21-kilodalton (kDa) polypeptides called p21 [43]. They are cytoplasmic proteins that are bound, in large part, to the plasma membrane [44]. Molecular cloning and biochemical characterization of the human *ras* proteins revealed that they are able to bind and hydrolyze guanosine triphosphate (GTP) in a manner analogous to protein synthesis factors [45,46], α-tubulin [47,48], and the G proteins, which transduce signals between cell-surface receptors and adenylate cyclase [49,50]. Oncogenic mutations in the human H-*ras* gene result in proteins defective in GTP hydrolysis. Such a defect may be the basis for the tumorigenic activation of the *ras* genes [51-55]. Genetic and biochemical studies in yeast have also implicated the *ras* proteins as direct or indirect modulators of cAMP synthesis [56–59]. Although the yeast studies implied that p21 can activate adenylate cyclase, our experience with the response of mammalian cancer cells to cAMP suggested that a negative regulation of cAMP levels by such oncogene proteins is also possible.

We have, in fact, found that an increase in intracellular cAMP can reverse most of the morphological and growth changes associated with the malignant phenotype induced in NIH3T3 cells upon transfection with the human H-*ras* oncogene or its overexpressed cellular progenitor ([60] and manuscript submitted). NIH3T3 clones transfected either with the oncogenic form of the human H-*ras* (T-24 bladder carcinoma) gene carrying a glycine-to-valine substitution at position 12 or with the proto-

oncogene linked to a hyperactive simian virus 40 (SV40) promoter were exposed to a variety of agents that elevate intracellular cAMP. These included db-cAMP, 8-Br-cAMP, cholera toxin, IBMX, and theophylline. In each case the *ras*-transformed lines responded identically. There was a rapid alteration in cell morphology (Fig. 9). The

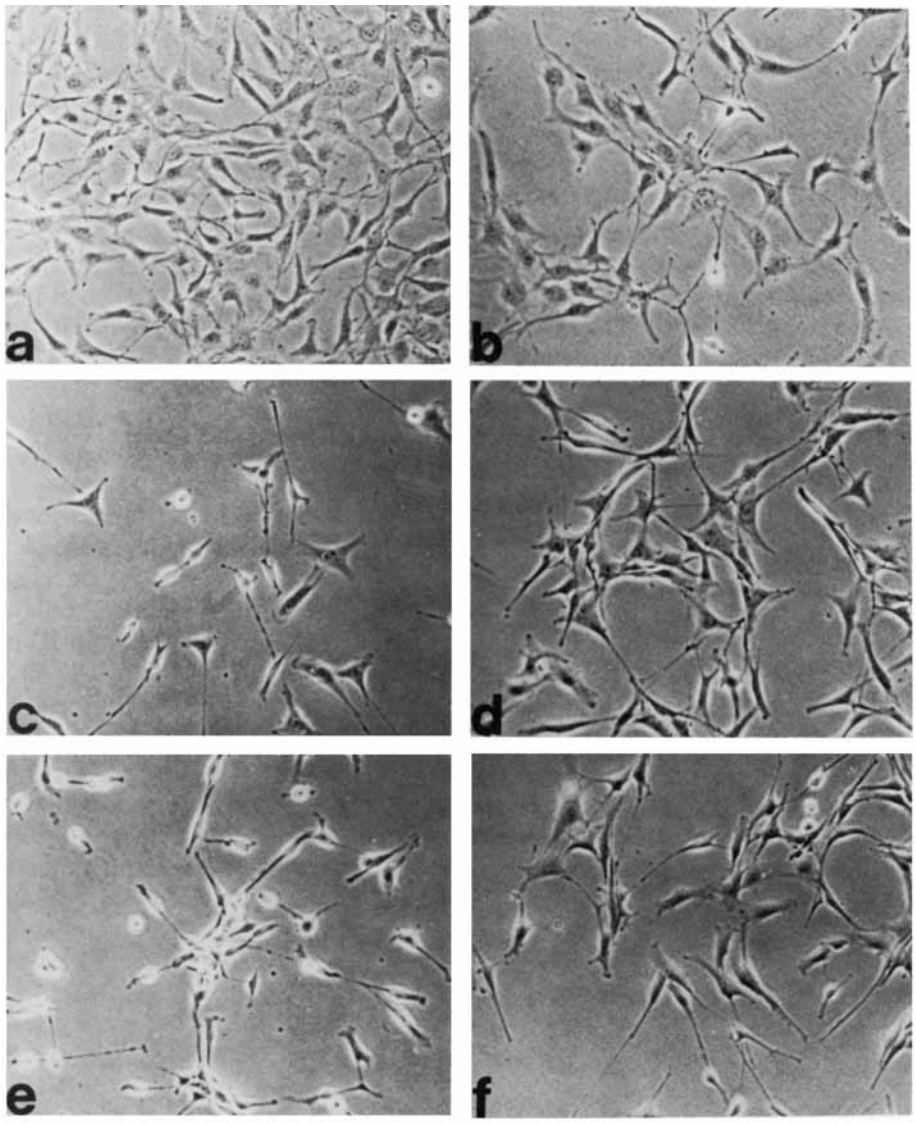

Fig. 9. Effect of elevated intracellular cyclic-AMP on the morphology of human H-*ras*-transformed NIH3T3 cells growing at low density. No addition. **a,c,e:** 0.5 mM 8-Br-cAMP and 50 mM IBMX. **b,d,f:** NIH3T3 (a,b); H-*ras* (T-24) clone 6. c,d: C-H-*ras* clone 2 (e,f). Human H-*ras*-transformed lines were established by transfection of NIH3T3 cells with plasmid cDNA encoding either the human C-H-*ras* gene or the T-24 H-*ras* mutant oncogene (carrying a glycine-to-valine mutation at position 12). The normal C-H-*ras* gene was designed to be overexpressed by insertion between an SV40 early promoter and SV40 termination and polyadenylation signals (S. Yokoyama et al, unpublished). Transformed foci were cloned using established methods.

tumor cells became less refractile, less rounded, had more tranquil membrane, were less adhesive, and spread more on the substratum. Stress fibers and actomyosin bundles were re-established. The culture morphology at confluence became typical of normal fibroblasts (Fig. 10). Contact inhibition of growth was restored (Fig. 10). As shown in Figure 11, for 3T3 cells carrying the oncogenic *ras* gene, there was a major reduction in log phase growth rate, and the saturation density was reduced to or was below that of untransformed 3T3 cells. As is typical of all tumor cell lines responsive to cAMP, the parent 3T3 cells were also partially growth inhibited by cAMP (Fig. 10). Another *ras* 3T3 line, which expresses 25–50 times the normal level of the *ras* proto-oncogene, also showed cAMP-mediated reverse transformation of morphology (Figs. 9, 10) and growth (not shown). Flow cytofluorometric analysis showed that growth arrest occurred in the G_1 phase of the cell cycle.

Thus, elevation of intracellular cAMP can reverse many of the in vitro parameters of oncogenicity associated with expression of the human H-*ras* oncogene. Although attempts to demonstrate directly an effect of *ras* proteins on mammalian adenylate cyclase activity have so far been unsuccessful [61], the concept of p21 *ras* oncogene products as signal transduction proteins that modulate second messenger pathways is, as a result of the studies described here, highly attractive.

The literature relating normal and malignant growth to cellular cAMP levels is often discordant. In certain cell types, cessation of growth is associated with increased cAMP, while in other cells cAMP may be mitogenic [62]. This is not surprising in view of the ubiquity of the cAMP pathway as a second messenger system in virtually

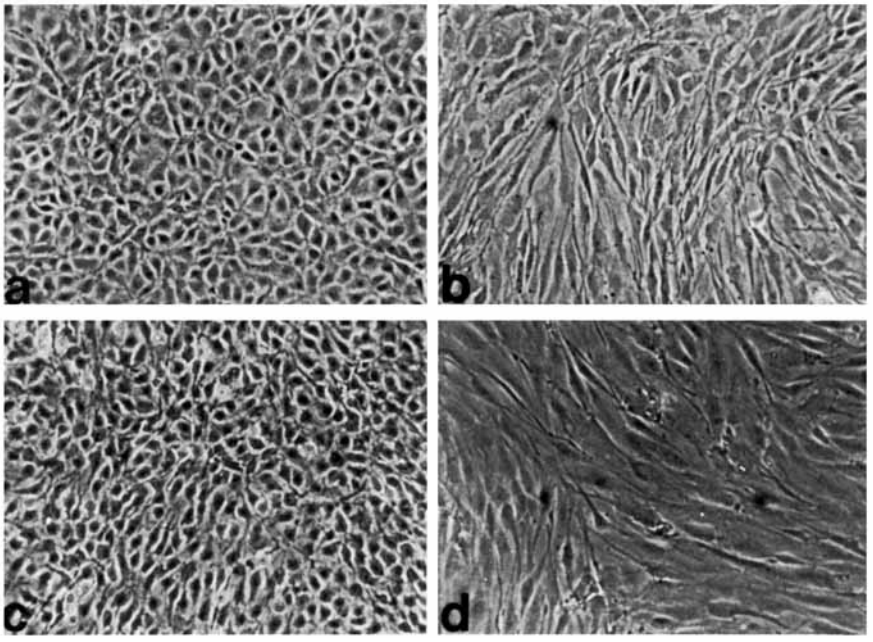

Fig. 10. Effect of elevated intracellular cyclic-AMP on the morphology of human H-*ras*-transformed NIH3T3 cells at confluence. **a:** NIH3T3 H-*ras* (T-24) clone 6 at confluence. **b:** NIH3T3 H-*ras* (T-24) clone 6 at confluence in the presence of 0.5 mM 8-Br-cAMP and 50 μM IBMX. **c:** NIH3T3 C-H-*ras* clone 2 at confluence. **d:** NIH C-H-*ras* clone 2 at confluence in the presence of 0.5 mM 8-Br-cAMP and 50 μM IBMX.

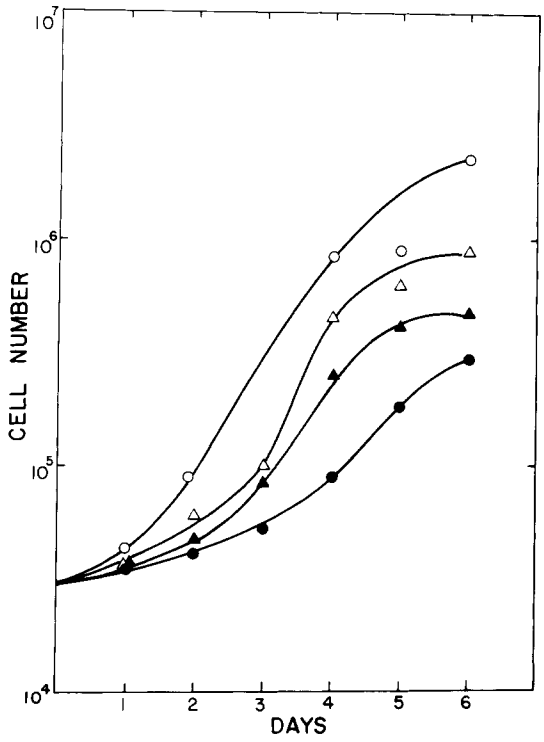

Fig. 11. Effect of cyclic AMP on the growth kinetics of H-*ras* (T-24) oncogene-transformed NIH3T3 cells. Human H-*ras* (T-24) clone 6 cells: No addition (○—○); plus 0.5 mM 8-Br-cAMP (●—●). NIH3T3 cells: No addition (△—△); plus 0.5 mM with 8-Br-cAMP (▲—▲).

all tissues. One would expect that, depending upon the nature of the hormone receptor and final effector targets, elevation of cAMP could serve as either a positive or a negative regulator of growth and malignant transformation.

In NIH3T3 cells, cAMP levels increase as cells become confluent and division ceases [63]. Our preliminary analysis of the human H-*ras* NIH3T3 transformants indicates a reduction in cAMP levels compared to untransformed NIH3T3 cells, particularly at confluence (unpublished data). Such regulation of cAMP levels by H-*ras* expression is supported by early studies that demonstrate a fall in intracellular cAMP in cells transformed by the Harvey sarcoma virus, whose oncogene is v-H-*ras* [64]. Hence an attractive hypothesis is that *ras* oncogene expression acts upon the cAMP pathway most strongly at confluence (or at least upon cell-cell contact), when cAMP levels would normally rise. The resulting decrease in intracellular cAMP would lead to escape from contact inhibition of growth and the loss of normal cell and culture morphology, which is typical of untransformed cells. Artificial elevation of cAMP, as described here, would reverse the effects of *ras* expression at confluence and restore normal saturation density and fibroblastic morphology to the transformed cells.

The only known mode of action of cAMP in eukaryotes is the activation of cAMP-dependent protein kinases, which results in the phosphorylation of specific target proteins [65]. We have previously shown that cAMP-mediated reverse transformation of CHO tumor cells is accompanied by the phosphorylation of unique

cytoskeletal and cytoplasmic proteins [66,67]. It is likely that a similar mechanism governs cAMP-mediated reverse transformation of *ras*-transformed NIH3T3 cells. Our initial results indicate that specific protein phosphorylation in response to cAMP does, in fact, occur (Lockwood et al, submitted).

The H-*ras* transformed cells show increased phosphorylation of proteins of molecular weight (MW) 80,000 and 50,000 and decreased phosphorylation of a protein of MW 97,000. Exposure of the H-*ras* cells to cAMP results in enhanced phosphorylation of proteins with MWs of 45,000, 50,000, and 80,000 daltons. Characterization of these proteins offers the promise of identifying the molecular sites of H-*ras* modulation of growth and morphology.

We conclude that, in some mammalian cells, the human H-*ras* p21 oncogene protein must function to reduce cellular cAMP levels. Such regulation could be via direct interaction with components of the adenylate cyclase or cAMP phosphodiesterase system or, indirectly, by modification of another signal transduction pathway such as the phosphatidylinositol cycle [68]. We have, in fact, recently demonstrated an association of phosphatidylinositide turnover with cAMP-mediated reverse transformation [69]. We aso infer that the human c-H-*ras* gene is part of a regulatory system that modulates cAMP levels in normal cells during growth and development.

COMMON PROTEIN PHOSPHORYLATION INDUCED BY GROWTH FACTORS, cAMP, AND PHORBOL ESTERS

Recent studies in the authors' laboratories indicate that NIH3T3 cells transformed by the cloned *sis* oncogene also undergo cAMP-mediated reverse transformation of growth and morphology (manuscript submitted). The *sis* oncogene encodes a chain of platelet-derived growth factor (PDGF) [71]. PDGF 13 known to stimulate PIP_2 breakdown, inositol triphosphate formation, and calcium mobilization in 3T3 cells [68,71]. This effect of cAMP indicates a link between growth factor action and cAMP-mediated protein phosphorylation in transformed cells. Indeed, the patterns of cellular protein phosphorylation induced by PDGF, phorbol esters, and cAMP suggest an intriguing connection. As described earlier in this review, cAMP and PMA show synergistic phosphorylation of proteins in CHO cells with molecular weights of 45,000 and 50,000 daltons and dephosphorylation of a 28,000-dalton protein. In *ras* transformed 3T3 cells, cAMP induces phosphorylation of 50,000- and 80,000-dalton proteins; this phosphorylation is stimulated by PMA. In quiescent 3T3 fibroblasts both PMA and PDGF rapidly stimulate phosphorylation of an 80,000-dalton protein [72]. Both growth factors and PMA stimulate phosphorylation of tyrosine residues of a 42,000-dalton protein in chick embryo fibroblasts [73]. In addition to these common phosphorylations, both cAMP and PMA induce unique phosphoprotein changes [3,66,74]. Hence all of these systems may function, in part, via the phosphorylation of a common set of endogenous cellular substrates. In many cases, phorbol esters decrease the affinity of cell surface receptors for growth factors or hormones including epidermal growth factor [75,76], insulin [77], and transferrin [78]. This is accompanied by, and may be a consequence of, protein kinase C-mediated receptor phosphorylation [79–82]. Although cAMP-mediated receptor phosphorylation has not yet been demonstrated, it would seem a likely event in view of the above studies.

These observations suggest biochemical pathways by which signal transduction systems can interact and by which the pleiotropic biological effects of cAMP, phorbol esters, and oncogenes can be reconciled.

REVERSAL OF MALIGNANCY BY ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS—POSSIBLE MECHANISMS

The diverse biological effects of the protein kinase A and protein kinase C signal transduction pathways on cellular differentiation and oncogenic transformation may have underlying biochemical mechanisms in common. It is unlikely in cancer cells that all the cellular mechanisms controlling growth and differentiation have gone awry. Rather, it is more probable that oncogene expression has selectively modified specific second messenger pathways and that malignant cells retain the biochemical and genetic capacity for differentiation.

If this is so, then the potential exists for overcoming the specific malignant lesion, for example by restoration of normal cellular cAMP levels or direct activation of protein kinase C with phorbol esters. The presence of only a few recognized signal transduction systems in all cell types suggests that in each cell type, at specific stages of development, the cAMP/protein kinase A and phosphoinositide/protein kinase C systems can function as components of cellular pathways programmed for specific aspects of growth or differentiation. Depending on the cell type and the stage of maturation at which an oncogene suborns normal function, the biological result will differ. For example, interference with cAMP production might lead to uncontrolled growth in certain cell types such as fibroblasts, yet have little effect on or even induce differentiation in others such as certain endothelial lines.

Restoration of cAMP could then potentially have diverse effects depending on the nature of the transformed cell. Similarly, in the hematopoietic cell lineage, phospatidylinositide turnover and protein kinase C activation might be controlled by normal agents such as colony-stimulating factors. Oncogene-mediated interference with the function of these factors, by alteration in their receptors in the coupling of receptors to the phospholipid cycle, could result in leukemogenesis and defective hematopoietic differentiation. In a manner directly analogous to cAMP action, phorbol esters, by activation of protein kinase C, could circumvent the polyphosphoinositide lesion and induce differentiation of malignant cells.

The studies reviewed here make it obvious that there are intimate molecular interactions between signal transduction pathways and oncogenes in cancer cells. Possible sites at which these systems interact are indicated in Figure 12. Cyclic AMP, by activation of protein kinase A, might induce phosphorylation alterations in components of the phosphoinositide pathway such as the lipid kinases or the PIP_2 phosphodiesterase (phospholipase C). The result would be altered activity of protein kinase C. PKA might also directly phosphorylate, and thereby regulate, growth factor receptors such as that for the *sis* oncogene product (PDGF).

A protein kinase cascade involving PKA might potentially regulate expression of the *ras* oncogene at the level of the genome as well as by cytoplasmic protein phosphorylation. Protein kinase C, activated by phosphoinositide turnover or phorbol esters, may phosphorylate protein components of the cytoskeleton and the cAMP pathway to influence growth and differentiation, depending on the nature of the transformed cell. At least in the cancer cell lines reviewed here, these two major signal transduction systems are able to cooperate to inhibit cancer cell growth. Current evidence indicates that both PKA and PKC can phosphorylate a unique and common set of cellular proteins. Identification of these substrates and their function may lead to an understanding of the biochemical mechanisms by which cAMP and protein

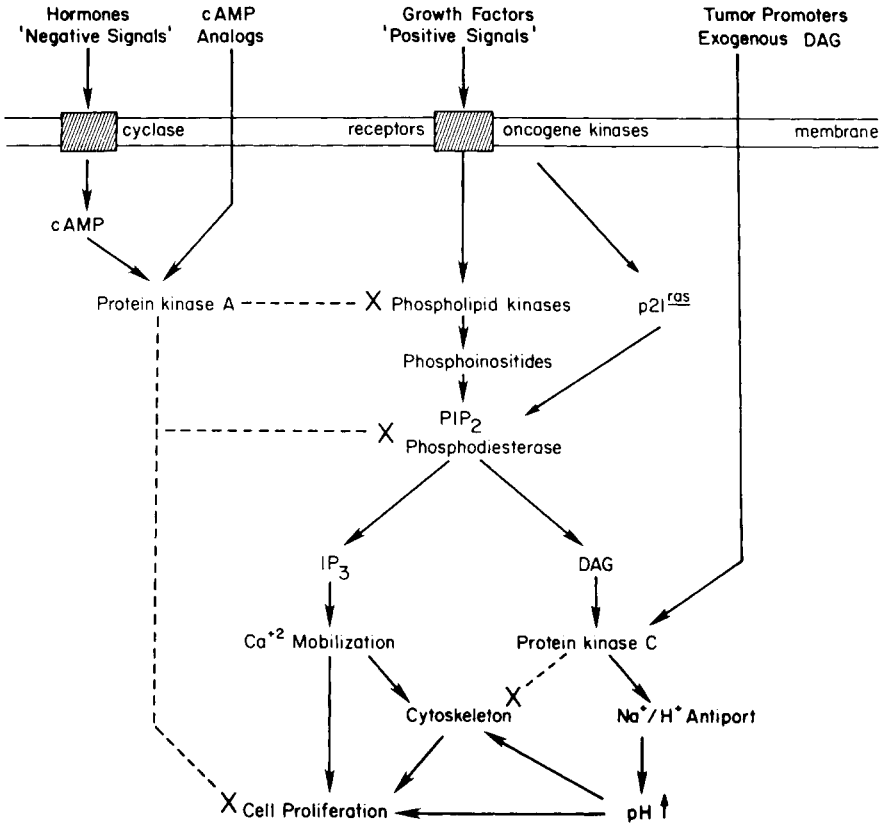

Fig. 12. Model of interactions between cellular signal transduction systems for the regulation of growth and morphology.

kinase C can attenuate oncogene function and reverse malignancy in a variety of cancer cells.

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