Differential Regulation of Protein Kinase C Isozymes by Bryostatin 1 and Phorbol 12-Myristate 13-Acetate in NIH 3T3 Fibroblasts*

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Bryostatin 1 and phorbol 12-myristate 13-acetate (PMA) are both potent activators of protein kinase C (PKC), although in many systems bryostatin 1 induces only a subset of the responses to PMA and blocks those which it does not induce. We report here that in NIH 3T3 fibroblasts PMA showed similar potencies for translocating PKC isozymes α, δ, and ε to the Triton X-100-soluble and -insoluble fractions and for the down-regulation of the three isozymes. Bryostatin 1 was slightly more potent than PMA for translocating PKCa and was more potent than PMA for down-regulating it. Bryostatin 1 was markedly more potent than PMA for translocating PKCβ but showed a biphasic dose-response curve for down-regulating this isozyme. 1-10 nM bryostatin 1 down-regulated PKCβ to a similar extent as PMA; lower (10-100 pM) or, unexpectedly, higher (100 nM to 1 μM) doses of bryostatin 1 caused either no or reduced down-regulation. Moreover, these high (100 nM to 1 μM) doses of bryostatin 1 inhibited the down-regulation of PKCβ by 1 μM PMA when coapplied. Bryostatin 1 caused translocation of PKCβ with slightly higher potency than PKCβ, but there was no protection of this isozyme at any of the doses examined. Bryostatin 1 induced a long-term increase in c-Jun level. The dose-response curve for bryostatin 1 was biphasic, with maximal induction at 1-10 nM bryostatin 1, coincident with the maximal down-regulation of PKCβ. We conclude that bryostatin 1 showed substantially different regulation for PKCa, PKCS, and PKCe, whereas PMA distinguished only weakly between these isozymes.

Bryostatin 1 is a very potent, non-phorbol ester activator of PKC1 currently being evaluated as a potential chemotherapeutic agent (1). Despite the widely documented dissimilarities between the biological responses induced by typical phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) and bryostatin 1, the mechanistic basis for these differences remains unclear (1).

The translocation of PKC from the cytosolic to the particulate fraction is considered a marker of PKC activation, although current understanding suggests greater complexity (2). Coupled to PKC activation is accelerated breakdown, termed down-regulation (3). In most systems this down-regulation is thought to play an inhibitory role, depleting the active enzyme (for review, see Ref. 3). However, since cleavage of the catalytic domain of PKC from the inhibitory regulatory domain represents an intermediate in the proteolytic breakdown of the enzyme, in some systems proteolysis may represent an alternative activation pathway (4).

Bryostatin 1 has two unusual characteristics as a PKC activator. First, in many systems bryostatin 1 induces only a subset of the responses to PMA and blocks those which it does not induce. In the HL-60 promyelocytic leukemia cell line, bryostatin 1 fails to induce differentiation and inhibits PMA induced differentiation (5); in Friend erythroleukemia cells, bryostatin 1 fails to inhibit differentiation and overcomes the PMA induced block of differentiation (6); in primary mouse keratinocytes, bryostatin 1 fails to induce transglutaminase activity, cornified envelope formation, and long-term down-regulation of epidermal growth factor binding, and bryostatin 1 inhibits all of these responses induced by PMA (7). A second unusual characteristic is that in many systems bryostatin 1 shows a biphasic dose-response curve. Examples include inhibition of growth in A549 human lung carcinoma cells (8), stimulation of growth of JB6 cells (9), sensitization of human cervical carcinoma cells to cis-diamminedichloroplatinum (II) (10), stimulation of erythropoiesis (11), induction of cytokine secretion in human mononuclear cells (12), and suppression of transglutaminase activity in NIH 3T3 fibroblast cells.5 Taken together, these data suggest that the regulation of PKC by bryostatin 1 is substantially different than that by PMA. Here we provide evidence for differential regulation of the PKC isozymes by bryostatin 1. We report a biphasic dose-response for down-regulation of PKCβ by bryostatin 1, and antagonism of the down-regulation of PKCβ by PMA at high bryostatin 1 concentrations. Long-term induction of c-Jun by bryostatin 1 was likewise biphasic, mirroring the down-regulation of PKCβ.

EXPERIMENTAL PROCEDURES

Cells and Materials—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 4 mM glutamine and 10% fetal calf serum (complete Dulbecco’s modified Eagle’s medium). Cells were treated with bryostatin 1 (10 ps to 1 μM) or phorbol 12-myristate 13-acetate (1 nM to 1 μM) or a combination of both agents as indicated for 5 min, 2, 6, 24, and 48 h. All compounds were applied in ethanol (0.1%, final concentration). Phorbol 12-myristate 13-acetate was purchased from LC Services (Woburn, MA). Bryostatin 1 was isolated from Bugula neritina as described (13). Cells were harvested at 80–90% confluency.

Cell Lysis and Western Blot Analysis—The cells were harvested into 20 mM Tris-Cl (pH 7.4) containing 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM leupeptin and lysed by sonication. The cytosolic fraction represents the supernatant following a 1-h centrifugation at 100,000 × g at 4 °C. The Triton X-100-soluble particulate fraction was prepared by a 1-h extraction of the pellet with the same buffer containing 1% Triton X-100 and a subsequent centrifugation for 1 h at 100,000 × g. The remaining pellet is the Triton X-100 insoluble fraction. The

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

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protein samples were subjected to SDS-PAGE electrophoresis according to Laemmli (14) and transferred to nitrocellulose membranes. Western blots were stained with 0.1% Ponceau S solution in 5% acetic acid (Sigma) for determining the protein content of individual lanes. The protein staining was found to be linear up to 30 pg of proteidane. The (Sigma) for determining the protein content of individual lanes. The phosphate-buffered saline and subsequently immunostained for PKCa, -6, and -7. Monoclonal antibodies against the catalytic domain of protein kinase Ca, the regulatory domain of protein kinase Cβ, and the regulatory domain of protein kinase Cγ were purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and applied at a 2 μg/ml concentration. Affinity purified polyclonal antibody against the C terminus (PKCa amino acids 662-673) of PKCa was purchased from Research & Diagnostics Antibodies (Berkeley, CA) and applied at a 1:50,000 dilution. Affinity purified polyclonal antibody against a polypeptide corresponding to amino acids 313-326 of PKCc was purchased from Amgen antibodies (Amersham, Buckinghamshire, England) and applied at a concentration of 2 μg/ml. Polyclonal antibody was raised against the 18-amino acid C terminus of PKCy in our laboratory and applied at a dilution 1:1,000. The specificity and lack of cross-reactivity of the primary antibodies for PKC isozymes α, β, γ, δ, ε, and θ was evaluated by us for cloned PKCa, -6, -γ, -δ, -ε, -6, and -γ expressed in a baculovirus system (15). Polyclonal antibody raised against a specific sequence in the C-terminal sequence of Jun (amino acids 209-225) was purchased from Oncogene Science Inc. (Uniondale, NY) and applied at a dilution 1:2,000. The blots were incubated overnight at 4 °C with the indicated amounts of the primary antibody dissolved in 4% milk in phosphate-buffered saline. The blots were washed and the PKC isozymes were detected by horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and the ECL Western blotting detection kit purchased from Amersham (see Figs. 1 and 2). Densitometric analysis of immunoblots was performed under conditions which yielded a linear response, as analyzed using the NIH Image 1.45 program (from Dr. Wayne Rasband, National Institutes of Health). For quantitation of the PKC isozyme content of the NIH 3T3 cells, cloned PKCa, -δ, and -ε expressed in the baculovirus system (15) were used as controls. The amount of PKC for the controls was determined by measuring the maximum amount of [3H]phorbol 12,13-dibutyrate bound (16) and assuming a stoichiometry of binding of 1. The Western blots were reused one or two times after the antibodies were stripped off by incubation for 30 min at 60 °C in 3.5 M MgCl2 in phosphate-buffered saline. The amount of PKC isozyme found in each sample was normalized for the protein content of the corresponding lane.

RESULTS

Identification of PKC Isozymes in NIH 3T3 Fibroblasts—In subconfluent NIH 3T3 cultures the following PKC isozyme concentrations were measured; PKCa, 1.8 fmol/μg protein; PKCb, 1.1 fmol/μg; PKCc, 1.3 fmol/μg. We did not determine the absolute amounts of PKCd because of the lack of reliable quantitation. We did not detect any protein kinase Cβ, -γ, or -η. The distribution of the individual PKC isozymes between the fractions obtained by centrifugation was also determined based on the protein levels measured in these fractions (the soluble fraction contained about 45%, the Triton X-100-soluble particulate fraction contains about 5%, and the Triton X-100-insoluble fraction contains about 45-50% of the total protein.) The distribution of the individual isozymes between the soluble, Triton X-100-soluble particulate, and cytoskeletal fractions was 80/10/10% for PKCa, 50-55/10-15/30-35% for PKCb, and 65-75/25-35/0% for PKCc.

Translocation and Down-regulation of PKC Isozymes—Upon PMA treatment PKCα is quickly translocated from the soluble to the Triton X-100-soluble particulate and, to a lower extent, to the Triton X-100-insoluble fraction by both PMA and bryostatin 1 (Figs. 1 and 3, A and B). 1 μM PMA induced a faster translocation than an equal concentration of bryostatin 1, as shown by the amounts of isozyme left in the soluble fraction at 5 min. By 6 h translocation was maximal at this and lower concentrations. The dose-response curves for translocation were deter-
Fig. 3. Translocation and down-regulation of PKCα induced by 1 μM PMA (A) or 1 μM bryostatin 1 (B). NIH 3T3 fibroblasts were treated with 1 μM PMA or bryostatin 1 for 5 min, 2, 6, 24, and 48 h. Samples for SDS-PAGE electrophoresis were prepared and Western immunoblotting was performed as described under “Experimental Procedures.” The amount of the enzyme was quantitated in the total (□), soluble (▲), Triton X-100-soluble particulate (■), and Triton X-100-insoluble (●) fractions by densitometry and expressed as percentage of the total amount of the isozyme present in the control cells. Time is plotted on a logarithmic scale. A second experiment yielded almost identical results.

Fig. 4. PMA and bryostatin 1 induced changes in levels of PKCα in the soluble (A) and total (B) fractions of NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were treated with the indicated doses of PMA or bryostatin 1 for 6 (▲) or 24 (■) h. Samples for SDS-PAGE electrophoresis were prepared and Western immunoblotting was performed as described under “Experimental Procedures.” The amount of the enzyme was quantitated by densitometry and expressed as percentage of the amount of the isozyme present in the soluble fraction (▲) or in the total fraction (■) in the control cells. Symbols are: ■, PMA; and ▲, bryostatin 1. Each point is the average ± S.E. of three independent sets of experiments.

### Table 1

Potency of PMA and bryostatin 1 for translocating and down-regulating PKC isozymes α, δ, and ε in NIH 3T3 fibroblasts

|              | ED<sub>50</sub> for translocation (4 h after treatment) | ED<sub>50</sub> for down-regulation (24 h after treatment) |
|--------------|--------------------------------------------------------|----------------------------------------------------------|
|              | PMA          | Bryostatin 1     | PMA           | Bryostatin 1     |
| PKCα         | 21 ± 1.2 nM  | 11 ± 0.4 nM      | 48 ± 2 nM     | 2.6 ± 0.13 nM    |
| PKCδ         | 11 ± 0.3 nM  | 0.13 ± 0.003 nM  | 12 ± 0.4 nM   | 0.09 ± 0.006 nM  |
| PKCε         | 6 ± 0.8 nM   | 0.05 ± 0.004 nM  | 9 ± 1 nM      | 0.04 ± 0.006 nM  |

from the Triton X-100-soluble and -insoluble fractions showed essentially the same kinetics. Interestingly, the down-regulation of PKCα induced by 1 nM to 1 μM bryostatin 1 was faster than by equimolar PMA, in contrast to the case for translocation. The difference mainly reflected the longer persistence of PKCα in the Triton X-100-soluble particulate fraction with PMA treatment. The potencies of bryostatin 1 and PMA for down-regulation were quantitated at 24 h after treatment using the amount of PKCα remaining in the total fraction (Fig. 4B). Bryostatin 1 showed somewhat higher potency for down-regulation than for translocation. PMA displayed somewhat lower potency for down-regulation than for translocation (ED<sub>50</sub> for bryostatin 1, 2.6 nM; for PMA, 48 nM). At 100 nM PMA a significant portion of the translocated PKCα persisted in the Triton X-100-soluble particulate fraction and to a lesser extent in the Triton X-100-insoluble fraction even 48 h after treatment (data not shown).

High doses of PMA and bryostatin 1 translocated PKCδ from the soluble to the Triton X-100-soluble particulate fraction (Figs. 1 and 5, A and B) (No translocation was observed to the Triton X-100-insoluble fraction.) 1 μM PMA translocated PKCδ more slowly than PKCα, whereas bryostatin 1 showed the opposite effect; the translocation of PKCδ induced by 1 μM bryostatin 1 was somewhat faster than that of PKCα (Figs. 1
reached completion at fractional was subsequently down-regulated. Down-regulation of bryostatin 1 was 0.13 nM, for PMA was 11 nM. The portion of the amount of isozyme remaining in the soluble fraction (Fig. 5B). Bryostatin 1 showed about 2 orders of magnitude higher potency for translocating PKCα than did PMA (ED_{50} for bryostatin 1 was 0.13 nM, for PMA was 11 nM). The portion of PKCζ translocated by PMA from the soluble to the particulate fraction was subsequently down-regulated. Down-regulation reached completion at 24 h after treatment with an ED_{50} of 13 nM for PMA (Fig. 6B). Surprisingly, complete time and dose-response curves for bryostatin 1 (over a concentration range of 10 pm to 1 μM) demonstrated that a very high dose of bryostatin (1 μM) failed to down-regulate PKCζ from the Triton X-100-soluble particulate fraction (Fig. 1), as was also the case for the lowest dose applied (10 pm) (Fig. 6B). The amount of PKCζ in the Triton X-100-soluble particulate fraction reached its peak at 5 min after treatment (Figs. 1 and 2). Subsequently the amount of the isozyme steadily decreased, leading to complete down-regulation at the doses of 1 and 10 nM (Fig. 2) and significant down-regulation at doses of 0.1 and 100 nM. PKCζ returned to the initial level after 24 h when 1 μM or 10 pm bryostatin 1 was applied. The biphasic down-regulation of PKCζ at 24 h in the Triton X-100-soluble particulate fraction is shown in Fig. 6B. If total PKCζ was quantitated, bryostatin 1 showed similar biphasic down-regulation with about 40% (approximately equal to the portion of PKCζ originally associated with the Triton X-100-soluble particulate fraction) of PKCζ still persisting after 24 h treatment with 1 μM bryostatin 1 (Fig. 6C). PKCe was quickly translocated from the soluble to the Triton X-100-soluble and -insoluble fractions by both PMA and bryostatin 1. Translocation was complete by 2 h after treatment (Figs. 1 and 7). Bryostatin 1 showed slightly higher potency for translocating PKCe than for PKCζ, having an ED_{50} of 0.06 nM, as determined from the amount of PKCe left behind in the soluble fraction at 6 h after treatment (Fig. 7C). As was the case for PKCζ, PMA was about 100-fold less potent than bryostatin 1 for translocating PKCe (ED_{50} for PMA was about 6 nm). The overall down-regulation of PKCe quantitated by the amount of isozyme remaining in the total fraction showed slower kinetics than that of PKCα or PKCζ (Fig. 7, A and B). This reflected the longer persistence of PKCe than of the other two isozymes in the Triton X-100-soluble and -insoluble fractions after translocation. Unfortunately, the breakdown of PKCe during the preparation of the Triton X-100-soluble and -insoluble fractions precluded precise quantitation; this problem has also been reported by others (17). PKCε showed neither translocation nor down-regulation in response to any of the treatments applied (Fig. 1). Coapplication of Bryostatin 1 with 1 μM PMA—The cotreatment with 1 nM to 1 μM bryostatin 1 and 1 μM PMA translocated and down-regulated PKCα in a fashion similar to treatment with 1 μM PMA alone (data not shown). For the translocation of PKCζ from the soluble to the particulate fraction the additive effect of the two compounds was observed, i.e. translocation was faster upon cotreatment than upon treatment with either compound alone (Fig. 2). In the Triton X-100-soluble particulate fraction increasing doses of bryostatin 1 inhibited the down-regulation of PKCζ. When 1 μM bryostatin 1 was coapplied with 1 μM PMA, after an initial increase the amount of PKCζ returned to the original level by 24 h (Fig. 2). This effect was dose-dependent, with an ED_{50} of 165 nM (Fig. 8A). Similarly, in the total fraction bryostatin 1 inhibited the down-regulation of about 25–35% of the total amount of PKCζ (which corresponds to the original amount of the membrane bound enzyme per total cell mass). The ED_{50} for inhibition was 170 nM (Fig. 8B). Like PKCe, PKCa was translocated and down-regulated by the cotreatment with 1 nM to 1 μM bryostatin 1 and 1 μM PMA in a fashion similar to treatment with 1 μM PMA alone (data not shown).

Regulation of c-Jun by PMA or Bryostatin 1 Treatment—The
levels of c-Jun were determined for the total cell fractions and were normalized to total cell protein. PMA induced a transient increase in the level of c-Jun with a peak at 2 h, and a return to near original levels by 48 h (Fig. 9A). Bryostatin 1 increased the amount of c-Jun to a similar extent by 2 h after treatment. However, when 1–100 nM bryostatin 1 was applied, the elevated c-Jun level decreased significantly more slowly and remained several fold higher than the control level up to 48 h. In contrast, with 1 μM bryostatin the amount of c-Jun returned to the initial level by 24 h (Fig. 9A). Complete dose-response curves showed
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FIG. 8. PMA/bryostatin 1 cotreatment induced changes in levels of PKCβ in the Triton X-100-soluble particulate fraction (A) and total fraction (B) of NIH 3T3 fibroblasts. Cells were treated with 1 μM PMA and different doses (1 nM to 1 μM) of bryostatin 1 for 24 h. Samples for SDS-PAGE electrophoresis were prepared and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the enzyme was quantitated by densitometry and expressed as percentage of the total amount of the isozyme present in the control cells. Two more sets of independent experiments yielded similar results.

FIG. 9. A, PMA and bryostatin 1 induced increase in c-Jun level in NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were treated with 1 μM PMA ( ■), 10 nM ( ●) or 1 μM (▲) bryostatin 1 for 5 min, 2, 6, 24, and 48 h. Samples for SDS-PAGE electrophoresis were prepared and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the protein was quantitated in the total fraction by densitometry and expressed as percentage of the total amount of the protein present in the control cells. B, dose-dependent changes in c-Jun level induced by bryostatin 1. NIH 3T3 fibroblasts were treated with the indicated doses of bryostatin 1 for 24 h. Samples for SDS-PAGE electrophoresis were prepared and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the enzyme was quantitated by densitometry and expressed as percentage of the amount of the protein present in control cells. Each point is the average ± S.E. of three independent sets of experiments.

that at longer time points (24–48 h) the increase of c-Jun level was clearly biphasic, mirroring the biphasic down-regulation of PKCβ (Fig. 9B).

**DISCUSSION**

Our results show that in NIH 3T3 fibroblasts bryostatin 1 was more potent than PMA for translocating and down-regulating PKCa, -δ, and -ε (Table I). This observation extends previous reports that, in the case of several human breast cancer cell lines, bryostatin 1 was more potent than PMA for inducing PKCa down-regulation (18). Likewise, down-regulation of PKCβ in mouse JB6 cells had been reported to occur more rapidly in response to bryostatin 1 than to equimolar PMA (9). The greater potency of bryostatin 1 for inducing translocation and down-regulation presumably reflects its greater potency for binding to protein kinase C (1). Obviously, down-regulation provides an attractive mechanism for the dominant inhibitory action of the bryostatins. Different kinetics of down-regulation of PKCa, PKCδ, and PKCε by PMA have been shown in Swiss 3T3 fibroblasts (19). Our data significantly extend the range of diversity of down-regulation shown by PKC isozymes since, in addition to the different kinetics for different isozymes, here we report significantly different dose-response curves for different ligands and an unusual biphasic pattern of down-regulation.

In contrast to the effect of bryostatin 1 treatment on PKCa and -ε, the biphasic down-regulation of PKCδ at low bryostatin 1 concentrations and the failure of PKCδ to down-regulate at higher concentrations is an unexpected finding. As with other bryostatin 1 effects, the action of the higher doses of bryostatin 1 (>10 nM) was dominant over that of PMA. The difference in behavior of bryostatin 1 on PKCa, -δ, and -ε does not reflect a difference in binding selectivity. Analysis of binding in vitro to the recombinant PKC isozymes expressed in the baculovirus system revealed similar affinities for PKCα, -δ, and -ε.

For biological responses there are multiple examples of biphasic dose-response curves for bryostatin 1, with suppression of PMA responsiveness at higher concentrations. These responses include inhibition of growth in A549 human lung carcinoma cells (8), stimulation of growth in JB6 cells (9), sensitization of human cervical carcinoma cells to cis-diamminedichloroplatinum (II) (10), stimulation of erythropoiesis (11), induction of cytokine secretion in human mononuclear cells (12), and suppression of transglutaminase activity in NIH 3T3 fibroblasts. An attractive explanation for such biphasic responses is that ligand at moderate concentrations activates PKC and induces the biological response, whereas ligand at higher concentrations causes down-regulation of PKC and thereby blocks response. Our results with bryostatin 1 suggest just the opposite situation, that down-regulation of PKCδ in-

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duces the response and that restoration of PKC\(\alpha\) blocks the response. Potentially, the ratio of PKC\(\alpha\) to other PKC isoforms, e.g. PKC\(\beta\), rather than just the absolute amount of PKC\(\alpha\) may be the critical determinant.

In any case, further experimentation will be required to clarify the mechanistic basis and functional significance of the suppression of PKC\(\alpha\) down-regulation. Preliminary results of ours show that the PKC\(\alpha\) resistant to down-regulation is enzymatically active.\(^4\)

At least three previously described mechanisms could contribute to the unusual effect of bryostatin 1 on PKC\(\alpha\). First, PKC\(\alpha\) possesses two phorbol ester binding domains (20), which appear to bind with high and low affinity, respectively (21). Occupancy of the second low affinity site and the nature of the ligand at that site might control susceptibility to down-regulation. Second, the high affinity and slow rate of release of bryostatin 1 may, at higher concentrations, drive PKC to a cellular subcompartment where it is sequestered from its usual substrates and degradative enzymes (22). Third, indirect evidence suggests a low affinity (relative to PKC) target for bryostatin 1 which leads to enhanced phosphorylation of two 70-kDa proteins (23). PKC\(\alpha\) might be a target of this pathway.

c-Jun is a major component of the AP-1 transcription factor which is positively regulated in response to cell stimulation with phorbol esters (24). In resting cells it is phosphorylated on serine and threonine at five sites negatively regulating its DNA binding activity. Activation of PKC by PMA leads to dephosphorylation of some of these sites restoring the AP-1 binding activity (25). c-jun expression is transiently induced in NIH 3T3 fibroblasts by PMA (26), with a peak of the mRNA level at 60 min after treatment. Bryostatin 1 was also shown to induce c-jun gene expression in HL-60 cells (27). Our results show that the dose-dependent regulation of long-term Jun induction by bryostatin 1 fits the biphasic down-regulation of PKC\(\alpha\). It suggests that one of the key factors involved in the regulation of AP-1 might be PKC\(\alpha\). This suggestion is further supported by the fact that PKC\(\alpha\) is translocated partly to the nuclear membrane in NIH 3T3 fibroblasts\(^5\) and that in vitro PKC\(\alpha\) phos- 

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\(^4\) Z. Szallasi, M. F. Denning, C. B. Smith, A. A. Dlugosz, S. H. Yuspa, G. R. Pettit, and P. M. Blumberg, manuscript in preparation.

\(^5\) Z. Szallasi and P. M. Blumberg, unpublished results.

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