for the synthetic peptide. Direct validation of this point will require regulated expression of PKI(1–31) in most or all the cells, so as to permit direct examination of the endogenous (intracellular) protein phosphorylation in the presence and absence of this peptide. The present data, however, support the specificity of the effects of PKI(1–31) on cAMP-directed transcriptional regulation and furthermore indicate the feasibility of introducing bioactive peptide fragments intracellularly via recombinant DNA techniques.

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4. The one-letter symbols used for amino acids are as follows: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine; and X, unspecified.
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14. We thank M. Comb, N. Birnberg, B. Seed, S. Aruffo, and D. Moore for helpful discussions; M. Comb, S. Aruffo, and D. Moore for gifts of plasmids pENKAT-12, πLXX, and pXGH5, respectively; J. Smith for initial synthesis of PKI(1–31); G. Curtis for tissue culture; I. Kang and M. Fitzgibbon for technical assistance; and M. Chambers for typing the manuscript. J. Corbin provided purified cGMP-dependent protein kinase. W. Wetekam (Hoechst AG) provided the β-galactosidase vector described in Fig. 1 as a gift. Supported in part by a grant from Hoechst AG.

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Rapid Stimulation of Diacylglycerol Production in *Xenopus* Oocytes by Microinjection of H-*ras* p21

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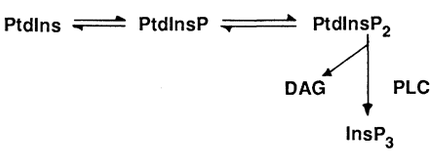
The p21 products of *ras* proto-oncogenes are thought to be important components in pathways regulating normal cell proliferation and differentiation. These proteins acquire transforming properties as a result of activating lesions that convert *ras* genes to oncogenes in a wide array of malignancies. In *Xenopus laevis* oocytes, microinjection of transforming *ras* p21 is a potent inducer of maturation, whereas microinjection of a monoclonal antibody to *ras* p21 inhibits normal maturation induced by hormones. The phosphoinositide pathway is a ubiquitous system that appears to play a key role in diverse cellular functions. By use of the *Xenopus* oocyte system, it was possible to quantitate the effects of *ras* p21 microinjection on individual components of the phosphoinositide pathway. Within 20 minutes of microinjection, levels of phosphatidylinositol 4,5-bisphosphate, inositol 1-phosphate, and inositol bisphosphate increased 1.5- to 2-fold. The most striking effects were on diacylglycerol, which increased 5-fold under the same conditions. In contrast, the normal *ras* p21 protein induced no detectable alteration in any of the metabolites analyzed. The earliest effects of the transforming p21 on phosphoinositol turnover were observable within 2 minutes, implying a very rapid effect of *ras* p21 on the enzymes involved in phospholipid metabolism.

GENES INVOLVED IN THE TRANSduction of signals required for normal cell proliferation commonly appear to be subverted in the neoplastic process (*1*). One such group is the highly conserved *ras* gene family. The p21 proteins encoded by *ras* genes bind guanine nucleotide (*2*) and have intrinsic guanosine 5′-triphosphatase (GTPase) activity (*3, 4*). They also appear to require attachment to the inner cell membrane for their function (*5*). In human tumors, *ras* oncogenes frequently have been found to be activated by point mutations at one of two major sites in their coding sequences (*6*). These activating lesions affect biochemical activities of the p21 protein, leading, for example, to impaired GTPase activity (*6*) or altered nucleotide exchange (*7*). Microinjection of bacterially expressed, mammalian transforming *ras* p21 proteins into NIH 3T3 cells leads to stimulation of DNA synthesis (*4, 8*). Thus, it has been suggested that by analogy with known G proteins, whose active state is the guanosine 5′-triphosphate (GTP)–bound but not guanosine 5′-diphosphate–bound form (*9*), *ras* p21 proteins may be regulatory proteins involved in transduction of signals that lead to DNA synthesis.

One major pathway involved in DNA synthesis induced by growth factors is mediated by phosphoinositide turnover according to the following scheme:



where PtdIns is phosphatidylinositol, PtdInsP is phosphatidylinositol phosphate, $PtdInsP_2$ is phosphatidylinositol 4,5-bisphosphate, and $InsP_3$ is inositol trisphosphate. Cleavage of phosphoinositides by phospholipase C (PLC) produces 1,2-diacylglycerol (DAG) and inositol phosphates (InsPs) (*10*). Whereas DAG acts as an essential cofactor for protein kinase C (PKC), inositol (1,4,5)trisphosphate, mobilizes Ca^{2+} from nonmitochondrial intracellular stores. Recent studies have indicated that cells chronically transformed by *ras* oncogenes exhibit increased phosphoinositide turnover (*11*). Moreover, such transformants have been reported to exhibit increased levels of DAG associated with a partial activation and downregulation of PKC (*11*). These findings have suggested that *ras* p21 proteins may directly or indirectly stimulate PLC-catalyzed breakdown of $PtdInsP_2$ or PtdIns. Because such investi-

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gations have been performed with *ras* transformants developed and maintained in long-term culture, it has been difficult to establish whether *ras* p21 products have a rapid effect on phosphoinositide metabolism and, if so, at which point in the pathway.

Recently, Birchmeier *et al.* reported that microinjection of *ras* p21 proteins into *Xenopus laevis* oocytes induces meiosis (*12*). Moreover microinjection of the *ras* p21 monoclonal antibody, Y13-259 (*13*), inhibits hormone-dependent oocyte maturation (*14*). Because of the large size of the oocyte, we reasoned that this system might make it possible to quantitate different phosphoinositides in small numbers of cells. The availability of bacterially expressed, normal and transforming mammalian *ras* p21 proteins (*4, 15*) with known biologic activity in microinjected mammalian cells (*4, 8*) led us to investigate the effects of *ras* p21 proteins in the oocyte system on phosphoinositide metabolism by a direct kinetic approach.

We first established the time course of incorporation of [^{3}H]*myo*-inositol or [^{3}H]glycerol into total lipids by microinjection into *Xenopus* oocytes. The production of DAG can be quantitated with [^{3}H]glycerol, whereas inositides can be measured with [^{3}H]*myo*-inositol. Levels of phosphatidylinositols can be estimated with either isotope. Similar kinetics of incorporation were obtained with either precursor; plateau levels were achieved within 4 to 5 hours of incubation at 23°C (Fig. 1). When small numbers of oocytes (five to ten) labeled in this manner were analyzed, it was possible to readily quantitate individual components of the phosphatidylinositol pathway.

To investigate the effects of *ras* p21 microinjection on phosphoinositide turnover, oocytes were labeled with [^{3}H]*myo*-inositol before microinjection with either normal or transforming v-H-*ras* p21. Microinjection of transforming *ras* p21 had a very rapid effect on PtdIns (Fig. 2). Within 2 minutes, PtdIns levels had decreased by about 20%, and the effect persisted for several minutes. Although there was no significant alteration in PtdInsP, we observed a 50% increase in $PtdInsP_2$ levels within 2 minutes of microinjection. This effect persisted such that by 6 minutes there was a 75% increase in $PtdInsP_2$ above the control level (Fig. 2).

Previous studies indicated that the transforming *ras* p21 protein was at least 100 times as potent in inducing oocyte maturation as the normal p21 (*12*). Microinjection of normal *ras* p21 at a concentration similar to that of the transforming *ras* p21 had no detectable effect on the level of any of the three phosphatidylinositols (Fig. 2). Thus, rapid effects on phosphoinositide metabolism were specific to the transforming p21 mutant. When oocytes were microinjected with the transforming p21, we also observed an 80% increase in InsP and a 20% increase in $InsP_2$ (Fig. 3). There were slight but statistically significant increases in $InsP_3$ and inositol tetraphosphate ($InsP_4$) as well (Fig. 3). Kinetic analysis revealed that increases were first observed between 2 and 4 minutes for InsP and between 4 and 6 minutes for the others. The lesser magnitude of the responses of $InsP_3$ and $InsP_4$ may be explained by the presence of active phosphatases that rapidly degrade the $InsP_3$ released, as described in other systems (*16*).

To extend these findings, we investigated the effects of p21 microinjection on DAG levels. As shown in Fig. 4, oocytes prelabeled with [^{3}H]glycerol exhibited a rapid elevation in DAG levels, which increased fivefold within 20 minutes of microinjection with the transforming *ras* p21. In contrast, there was little or no detectable alteration in response to the normal p21 protein (Fig. 4).

Oocyte maturation is known to be stimulated in response to hormones such as progesterone and insulin as well as by Ca^{2+} ionophores (*17*). Effects of oncogene products in this system were first reported for the v-*src* protein product, which upon microinjection was found to speed the time course of progesterone-induced maturation (*18*). More recently, Birchmeier *et al.* (*12*) showed that a transforming *ras*-p21 mutant not only accelerated progesterone-induced meiosis but could induce meiosis on its own.

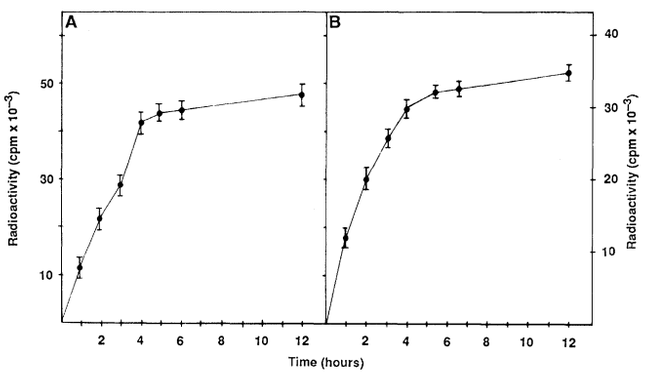

Fig. 1. Incorporation of (**A**) [^{3}H]*myo*-inositol and (**B**) [^{3}H]glycerol into total lipids after microinjection of *X. laevis* oocytes. Stage VI oocytes were manually dissected and selected from ovarian fragments by standard procedures (*21*). Oocytes were then microinjected with 40 nl of 20 m*M* tris-HCl (*p*H 7.2) that contained 10 μCi of either [^{3}H]*myo*-inositol (Amersham, 3.5 Ci/mmol) or [^{3}H]glycerol (Amersham, 450 mCi/mmol) per microliter. At indicated times of incubation at 23°C, five oocytes were homogenized, and the lipids were extracted as follows. For [^{3}H]*myo*-inositol–containing lipids, oocytes were homogenized in 440 μl of methanol:1*N* HCl (10:1; v/v), and phases were split by addition of 0.6 ml of chloroform and 160 μl of deionized water. Organic phases were collected, and aqueous phases extracted with 400 μl of chloroform. Both organic phases were collected, washed with 220 μl of methanol:1*N* HCl (10:1; v/v), and dried under nitrogen. Radioactivity was estimated by scintillation counting in 10 ml of Aquasol (New England Nuclear). For [^{3}H]glycerol-labeled lipids, oocytes were homogenized in 160 μl of deionized water before the addition of 0.6 ml of chloroform:ethanol (1:2, v/v). Phases were separated by addition of 0.2 ml of chloroform and 0.2 ml of deionized water. Samples were centrifuged, and organic phases saved. Aqueous phases were then washed with 0.5 ml of chloroform, organic phases were collected and dried, and radioactivity was determined by scintillation counting.

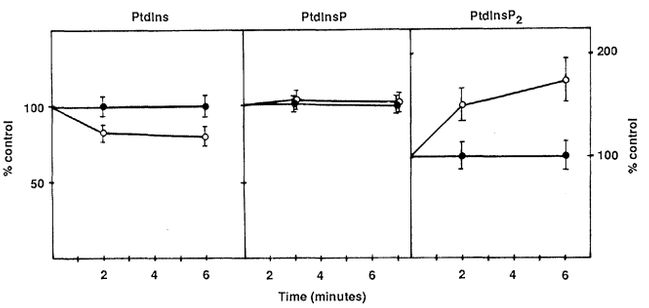

Fig. 2. Effects of microinjected *ras* p21 proteins on the levels of phosphatidylinositols in *X. laevis* oocytes. *Xenopus* oocytes were labeled with [^{3}H]*myo*-inositol for 5 hours as described in the legend to Fig. 1 and then microinjected with 40 nl of either normal (●) or transforming *ras* p21 protein (○) at 3.5 mg/ml in 20 m*M* tris-HCl and 35 mg of bovine serum albumin per milliliter of solution. At the indicated times, total lipids were extracted from ten oocytes as described in Fig. 1 and resolved by thin-layer chromatography along with corresponding standards in chloroform:methanol:water:NH_4OH (45:38:8:2). Lipids were visualized by exposing plates to iodine vapor. Radioactivity levels on individual bands were estimated by liquid scintillation counting. Results were normalized to control values (100%) that corresponded to the levels of each particular phosphatidylinositol in noninjected oocytes (PtdIns, 26,500 cpm; PtdInsP, 750 cpm; $PtdInsP_2$, 7,600 cpm). Results represent the mean values ± SD of three independent experiments performed in duplicate.

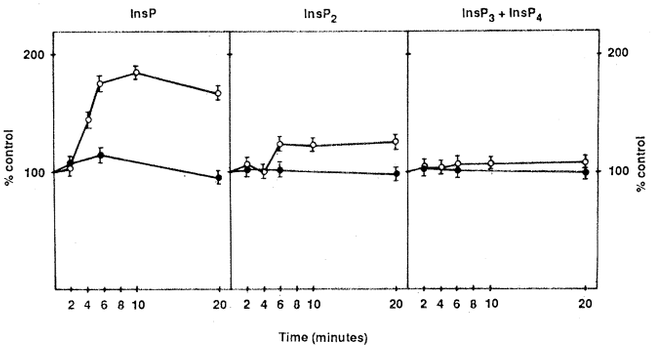

Fig. 3. Effects of microinjected *ras* p21 proteins on the release of InsPs in *X. laevis* oocytes. *Xenopus* oocytes were labeled with [^{3}H]*myo*-inositol as described in the legend to Fig. 2. After microinjection of either normal (●) or transforming (○) p21 proteins, 10 to 20 oocytes were processed as indicated, but aqueous phases were analyzed instead. Samples were passed through small (0.6-ml bed volume) Dowex 1 × 8 columns (formate form), and eluted with 8 ml of 0.2*M* ammonium formate plus 0.1*M* formic acid (InsP), 0.4*M* ammonium formate plus 0.1*M* formic acid ($InsP_2$), and 1.2*M* ammonium formate plus 0.1*M* formic acid ($InsP_3$ + $InsP_4$). Results were normalized to control values (100%) corresponding to the levels of InsPs in uninjected oocytes (InsP, 2950 cpm; $InsP_2$, 625 cpm; $InsP_3$ + $InsP_4$, 625 cpm). Results represent the mean values ± SD of three independent experiments performed in duplicate.

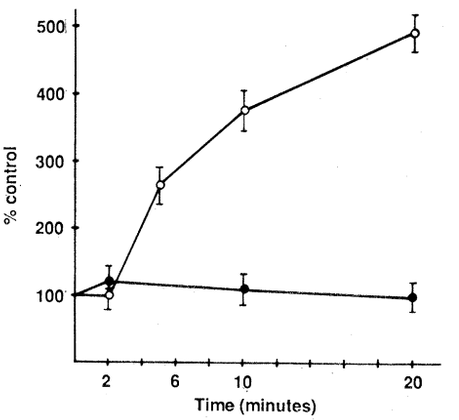

Fig. 4. Effects of microinjected *ras* p21 proteins on DAG levels in *X. laevis* oocytes. *Xenopus* oocytes were labeled with [^{3}H]glycerol as described in the legend to Fig. 1. After 5 hours of incubation at 23°C, oocytes were microinjected with 40 nl of either normal (●) or transforming *ras* p21 protein (○) as described in the legend to Fig. 2. Lipids were extracted from 10 to 20 oocytes and fractionated by thin-layer chromatography along with corresponding standards in hexane:diethyl ether:acetic acid (60:40:1; v/v/v). Lipids were visualized, and radioactivity in DAG was determined as described in the legend to Fig. 1. Results were normalized to the control level (100%) of radioactively labeled DAG in noninjected oocytes (250 cpm). Results represent mean values ±SD of three independent experiments performed in duplicate.

This response occurred within 6 to 12 hours after microinjection with low levels of the mutant protein. The amounts of p21 proteins microinjected in our studies were tenfold in excess of that required for a maximal response to the transforming p21 molecule. Moreover, at the p21 concentrations utilized for oocyte microinjection, our transforming p21 protein induced DNA synthesis in more than 50% of NIH 3T3 cells, whereas little, if any, detectable increase in DNA synthesis was observed with the normal p21 protein (*4, 8*). Thus, the inability of the normal p21 protein to induce any detectable rapid alteration in phosphoinositide metabolism correlates with its much less potent activity in inducing maturation in oocytes or DNA synthesis in mammalian cells.

We were able to reproducibly detect perturbations in phosphoinositide metabolism using as few as five to ten oocytes microinjected with 150 ng of our transforming *ras* p21 protein. There are several reports indicating that phosphoinositide pools may exist in separate subcellular compartments such that complete equilibration of all intracellular pools may not be fully achieved within a 5-hour labeling period. Nonetheless, our findings of rapid perturbations in the phosphoinositide pool, which is labeled to steady state within 5 hours argues that at least this subcomponent of the total phosphoinositide pool responds dramatically to transforming p21 microinjection.

Ras proteins as genetically engineered into *Escherichia coli* and purified lack posttranslational palmitoylation required for translocation to the inner surface of the plasma membrane. This localization is known to be required for their function (*5*). There is evidence that p21 is so modified after microinjection of oocytes, although kinetic analysis has argued that the appearance of detectable levels of the modified protein required several minutes (*12*). Thus, our ability to detect alterations in some metabolites at the earliest time point analyzed, 2 minutes, could reflect very early effects of the transforming *ras* p21 on enzymes involved in phosphoinositide metabolism.

Evidence that chronic *ras*-transformed cells exhibit increased DAG and InsPs levels (*11*) have led to speculation that *ras* p21 may directly or indirectly influence phosphoinositide turnover at the level of PLC. Supporting these findings is evidence that a G protein may regulate PLC activity since InsPs release is stimulated in various cell types by GTP-γ-S (*19*). The marked increases in DAG observed in our present studies imply that an increased level of PLC breakdown of phospholipids is an early result of transforming *ras* p21 microinjection. However, our findings that DAG increases to a much higher level than the InsPs suggests the possibility that other sources of DAG may be activated in response to *ras* p21 as well. In this regard, recent evidence has been presented for the existence of agonist-activated phosphodiesteratic hydrolysis of phosphatidylcholine through a GTP-dependent step that could reflect involvement of a G protein (*20*). More detailed analysis will be required before unambiguous conclusions can be drawn concerning the step or steps in phosphoinositide metabolism most rapidly affected by *ras* p21 proteins. However, our studies establish the oocyte system as a useful means for investigating the effects of microinjected oncogene products on these important signaling pathways.

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Mutations in Diphtheria Toxin Separate Binding from Entry and Amplify Immunotoxin Selectivity

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Monoclonal antibodies linked to toxic proteins (immunotoxins) can selectively kill some tumor cells in vitro and in vivo. However, reagents that combine the full potency of the native toxins with the high degree of cell type selectivity of monoclonal antibodies have not previously been designed. Two heretofore inseparable activities on one polypeptide chain of diphtheria toxin and ricin account for the failure to construct optimal reagents. The B chains (i) facilitate entry of the A chain to the cytosol, which allows immunotoxins to efficiently kill target cells, and (ii) bind to receptors present on most cells, which imparts to immunotoxins a large degree of non–target cell toxicity. This report identifies point mutations in the B polypeptide chain of diphtheria toxin that block binding but allow cytosol entry. Three mutants of diphtheria toxin have 1/1,000 to 1/10,000 the toxicity and 1/100 to 1/8,000 the binding activity of diphtheria toxin. Linking of either of two of the inactivated mutant toxins (CRM103, Phe^{508}; CRM107, Phe^{390}, Phe^{525}) to a monoclonal antibody specific for human T cells reconstitutes full target-cell toxicity—indistinguishable from that of the native toxin linked to the same antibody—without restoring non–target cell toxicity. This separation of the entry function from the binding function generates a uniquely potent and cell type–specific immunotoxin that retains full diphtheria toxin toxicity, yet is four to five orders of magnitude less toxic than the native toxin is to nontarget cells.

DIPHTHERIA TOXIN (DT) AND RICIN are potent toxins composed of two disulfide-linked polypeptide chains (*1*). The B chains bind the toxin to the cell surface and facilitate transport of the A chain to the cytosol. The A chains catalytically inhibit protein synthesis, and a single molecule of either DT A chain (*2*) or ricin A chain (*3*) in the cytosol is sufficient to kill a cell. The combination of these three activities—binding, translocation, and catalysis—produces the extreme potency of these proteins.

Monoclonal antibodies specific for tumor-cell surface antigens have been linked to toxins or toxin subunits to generate a new class of therapeutic drugs called immunotoxins (*4*). Toxin A chains linked to monoclonal antibodies show a high degree of cell type selectivity in vitro but little toxicity to solid tumors in vivo (*5*). This low toxicity may be due, in part, to slow transport of the A chain to the cytosol (*6, 7*).

Target cell toxicity of immunotoxins can be increased by including the toxin B chain in the antibody-toxin complex (*8*) or by adding it separately (*7, 9*). To achieve maximal in vitro target cell selectivity with immunotoxins containing intact ricin, lactose must be added to the medium to block non–target cell binding and toxicity of the immunotoxin via the ricin B chain. This approach is feasible in those clinical settings, such as bone marrow transplantation (*10*), where the target cell population can be incubated in vitro in the presence of lactose. Without blockage of the B-chain binding domain, however, whole-toxin conjugates have a high degree of non–target cell toxicity, thereby reducing their usefulness in vivo to that of A-chain immunotoxins (*11*).

Construction of reagents that combine the potency of intact toxin conjugates with the cell type selectivity of toxin A-chain conjugates may be possible if the binding site on the toxin B chain could be irreversibly blocked. Covalent and noncovalent chemical modifications that block the binding activity of ricin intracellularly also block its entry function, which suggests that the binding and translocation functions may be inseparable (*12*); however, inactivation of ricin binding by steric hindrance or by oxidation blocks non–target cell toxicity more than target cell toxicity (*13*).

Previously, domain deletion was used in an unsuccessful attempt to separate the translocation and the binding functions of the B chain of DT (*14*). Immunotoxins made with DT A chain, intact DT, and a

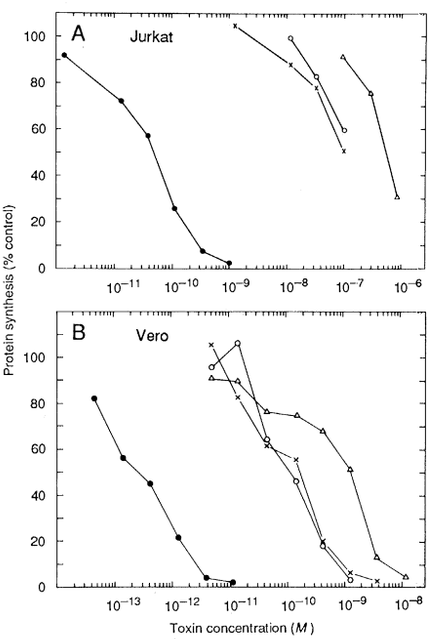

Fig. 1. Toxicity of CRM102, CRM103, CRM107, and native DT to Jurkat cells (**A**) and Vero cells (**B**). Protein synthesis was assayed by incubating 5×10^4 Jurkat cells in 100 μl of leucine-free RPMI 1640 medium containing 2% fetal bovine serum (FBS) in 96-well microtiter plates. DT (●), CRM102 (x), CRM103 (○), or CRM107 (△) was added in 11 μl of buffer and incubated with cells for 16 hours at 37°C. Cells were then pulsed with 20 μl of phosphate-buffered saline containing 0.1 μCi of [^{14}C]leucine, incubated for 1 hour at 37°C, harvested onto glass fiber filters by means of a PHD cell harvester (Cambridge Technology), washed with water, dried, and counted. The results are expressed as a percentage of the [^{14}C]leucine incorporation in mock-treated control cultures.

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