Induction of Membrane Ruffling and Fluid-Phase Pinocytosis in Quiescent Fibroblasts by *ras* Proteins

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Expression of the *ras* oncogene is thought to be one of the contributing events in the initiation of certain types of human cancer. To determine the cellular activities that are directly triggered by *ras* proteins, the early consequences of microinjection of the human H-*ras* proteins into quiescent rat embryo fibroblasts were investigated. Within 30 minutes to 1 hour after injection, cells show a marked increase in surface ruffles and fluid-phase pinocytosis. The rapid enhancement of membrane ruffling and pinocytosis is induced by both the proto-oncogenic and the oncogenic forms of the H-*ras* protein. The effects produced by the oncogenic protein persist for more than 15 hours after injection, whereas the effects of the proto-oncogenic protein are short-lived, being restricted to a 3-hour interval after injection. The stimulatory effect of the *ras* oncogene protein on ruffling and pinocytosis is dependent on the amount of injected protein and is accompanied by an apparent stimulation of phospholipase A_2 activity. These rapid changes in cell membrane activities induced by *ras* proteins may represent primary events in the mechanism of action of *ras* proteins.

ESSENTIAL TO THE UNDERSTANDING OF THE MECHANISM whereby *ras* proteins exert their effects on cell proliferation is the identification of molecular events that are directly modulated by these proteins. The mammalian *ras* family consists of three proto-oncogenes, H-*ras*, K-*ras*, and N-*ras* (*1*), each of which can acquire oncogenic properties by single missense mutations usually at either codon 12 or codon 61 (*2*). The mutated forms of the *ras* genes are prevalent in human and rodent tumor cells and have been implicated in transformation in vitro and tumorigenesis in vivo (*3*).

Mammalian *ras* genes encode homologous 21-kD proteins that are membrane-associated guanosine triphosphate–binding (GTP) proteins (*4*). The proteins have an intrinsic low guanosine triphosphatase (GTPase) activity which, in certain cases, is impaired in the mutated oncogenic protein (*5*). The GTP hydrolytic activity is a common property of all known guanine nucleotide binding (G) proteins (*6*). Members of the G-protein family regulate the activities of their cellular target by a cycle of GTP binding and GTP hydrolysis. On the basis of an analogy between *ras* proteins and G proteins, it has been proposed that the reduction in GTPase activity which accompanies mutational activation of *ras* genes impairs the regulatory function of *ras* proteins, thereby leading to the derangement of cellular signals that control cell proliferation. While the specific biochemical function of *ras* proteins has not as yet been identified, several studies have indicated that *ras* proteins may participate in the molecular events initiated by growth factors (*7*). Elucidation of the role of *ras* proteins in the acquisition of the transformed phenotype has been hampered by the pleiotropic nature of the transformation process. As has been shown (*8, 9*), microinjection of the *ras* oncogene protein into quiescent cells results in the transient stimulation of proliferation. Therefore, the transforming properties of *ras* proteins are faithfully expressed in this assay. Furthermore, the microinjection approach offers the means by which oncogenic *ras* protein can be introduced abruptly into normal cells and therefore can allow identification of the immediate effects of *ras* proteins.

H-*ras* proteins stimulate membrane ruffling and fluid-phase pinocytosis. Confluent rat embryo fibroblasts (REF-52) have a flattened polygonal shape (Fig. 1A). The cell surface structure, as revealed by scanning electron microscopy, consists predominantly of short, slender extensions and small folds. As early as 30 minutes after microinjection of the human H-*ras* oncogene protein (Fig. 1B), pronounced ruffling activity begins, as is indicated by the large lamellipodia that rise up along the periphery of the injected cells. Two hours after the cells are injected (Fig. 1C), large ruffles that form elaborate branching patterns are prominent on the dorsal surface. Ten hours after injection (Fig. 1D), the cells assume a partially rounded shape, and large regions of the membrane are now occupied by the surface ruffles. These sequential changes in surface morphology were reproducible both between different experiments and between the population of the injected cells in a given experiment. Microinjection of buffer alone or similar amounts of control protein (mouse immunoglobulin G, IgG) had no apparent effect on cell surface morphology.

The occurrence of membrane ruffling is closely associated with pinocytotic activity (*10, 11*). To test the effect of microinjection of the H-*ras* protein on fluid-phase pinocytosis, we monitored the uptake of fluorescein-conjugated dextran (FITC-dextran) by the injected cells. This substance meets the major criteria required for a marker of fluid-phase pinocytosis: it is readily soluble in aqueous medium, membrane-impermeable, stable within the intracellular milieu, and does not bind to the plasma membrane (*12*). Cells microinjected with the H-*ras* oncogene protein display a large number of intracellular vesicles containing the fluorescent marker, indicating a high rate of ongoing pinocytosis (Fig. 2B). The pinocytotic vesicles, are mostly formed in small groups and are found predominantly along the inner margin of well-developed

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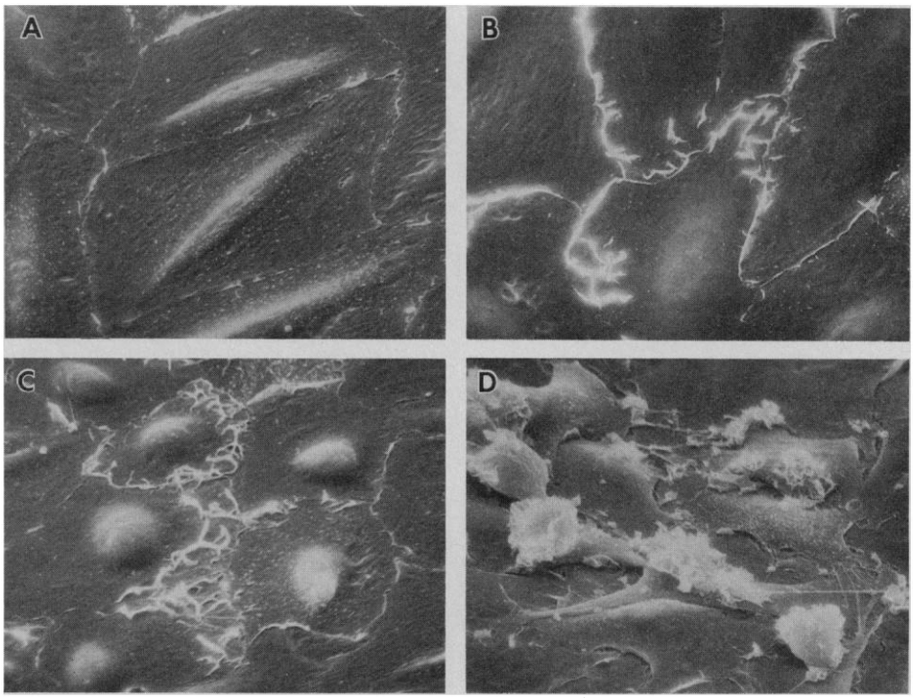

Fig. 1. Cell surface ruffling induced by microinjection of the *ras* oncogene protein. REF-52 cells (*40*) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (8 percent). Cultures were maintained at 37°C in a humidified atmosphere (95 percent air and 5 percent CO_2). Cells were plated onto 35-mm culture dishes and grown to confluency. The cells were either mock-injected (A) or injected with the *ras* oncogene protein (2 mg/ml) (B, C, and D) (*41*). At 0.5 hour (B), 2 hours (C), or 10 hours (A and D) after injection; the cells were fixed and processed for scanning electron microscopy as follows. The cells were rinsed twice with phosphate-buffered saline (PBS) and were fixed in 2 percent cacodylate-buffered glutaraldehyde (*p*H 7.4) containing 4.5 percent sucrose. The samples were then postfixed in 1 percent osmium tetroxide, dehydrated through graded ethanol, and critical-point dried in a Bomar SPC 900-EX with ethanol as the transitional fluid. Specimens were sputter-coated with gold and observed with a scanning electron microscope (Amray 1000). Photographs were taken at 20 kV. Note the induction of membrane ruffles, initiating at the cell periphery (B) and progressing over the dorsal surface of the cells (C and D) following the injection of the *ras* oncogene protein. (×1400)

ruffled membranes (visible as a dark rim with many folds in Fig. 2A, arrowheads). In contrast, the level of pinocytotic uptake of FITC-dextran in cells that were microinjected with the control protein (Fig. 2, C and D) is very low and is comparable to that observed in buffer-injected cells or uninjected cells. The stimulatory effect of *ras* oncogene protein on membrane ruffling and pinocytosis was not restricted to the particular cell type used because we found that microinjection of the protein into normal rat kidney cells similarly results in the enhancement of ruffling and pinocytosis. Comparison between the rates of uptake of FITC-dextran in *ras*-injected cells and in buffer-injected cells, as determined by the rate of formation of pinocytotic vesicles, is shown in Fig. 3A. The number of vesicles containing FITC-dextran increases linearly with time of exposure of cells to the fluorescent marker. These are the kinetics predicted for FITC-dextran uptake by fluid-phase pinocytosis (*12*). Furthermore, the rate of pinocytotic activity is ten times higher in cells that were injected with *ras* oncogene protein than in cells that were injected with buffer (Fig. 3A).

In view of the difference in both the biochemical properties and the biological effects between the proto-oncogenic and oncogenic *ras* proteins, we compared their effects on fluid-phase pinocytosis after microinjection into quiescent fibroblasts. Both proteins induce rapid stimulation of fluid-phase pinocytosis (Fig. 3B). This stimulatory effect is apparent within 0.5 to 1 hour after injection. Thereafter, the pinocytotic activity of cells injected with the *ras* oncogene protein increases steadily to reach a maximum 3 hours after injection and is maintained at this level for more than 15 hours. In contrast, the stimulatory effect of the *ras* proto-oncogene protein is transient and is no longer apparent 5 hours after injection. Likewise, the *ras* proto-oncogene protein induces a rapid but transient increase in membrane ruffling. Using immunofluorescence microscopy, we found that both proteins could be detected in comparable amounts and in the same apparent intracellular distribution even 20 hours after injection (*13*). Therefore, the difference between the *ras* oncogene protein and the proto-oncogene protein in promoting and sustaining the increased membrane ruffling and pinocytosis most likely is not attributable to a difference in turnover rates of the injected proteins.

In previous cytochemical studies, various markers for fluid-phase pinocytosis were used to demonstrate that these materials are interiorized into membrane-bound, electron-transparent vesicles (*10, 14, 15*). An uninjected cell (which gave the same appearance as a cell injected with a control protein or buffer alone) contains few cytoplasmic vesicles (Fig. 4, A and C). In contrast, a cell that had been injected with the *ras* oncogene protein exhibits an increase in the number of electron-transparent intracellular vesicles (Fig. 4, B and D). To determine the pinocytic origin of these vesicles, injected cells were exposed to horseradish peroxidase, a cytochemical marker that is used for monitoring fluid-phase pinocytosis. Pinocytic vesicles containing the enzyme are clearly visualized in the *ras*-injected cell (Fig. 4D, inset). These pinocytic vesicles have electron-transparent centers and a peripheral rim of reaction product. In addition, the surface of an uninjected cell is relatively smooth (Fig. 4A), while the surface of the injected cell is irregularly contoured (Fig. 4B). This surface appearance probably reflects membrane invaginations that may give rise to the membrane-bound pinocytotic vesicles. Overall, the ultrastructural changes induced by microinjection of the *ras* oncogene proteins are consistent with the stimulation of fluid-phase pinocytosis.

Characterization of *ras* oncogene protein–induced pinocytosis and its relation to proliferation. The extent of stimulation of fluid-phase pinocytosis by the *ras* oncogene protein depends on the amount of protein introduced into the cells. A threshold concentration of protein (>0.1 mg/ml in the needle, which corresponds to approximately 10^5 molecules of *ras* protein injected per cell) is required to initiate the stimulatory effect on pinocytosis (Fig. 5C), and maximal stimulation (Fig. 5A) is produced by microinjection of the protein at a concentration of 2 mg/ml (approximately 2×10^6 molecules of *ras* protein per cell). Injection of intermediate concentrations of the protein produces an intermediate stimulation (Fig. 5B). The amounts of injected *ras* proteins that we used are within the range of those expressed in various *ras* transformed cells.

Microinjection of the *ras* oncogene protein in the presence of the protein synthesis inhibitor cycloheximide (15 μg/ml) does not impair the ability of the protein to stimulate pinocytosis (compare Fig. 6D to 6A). Under these conditions, the enhanced pinocytotic

activity is maintained for approximately 5 hours after injection. This indicates that the induction process that links injection of the *ras* oncogene protein to the subsequent increase in pinocytosis does not depend upon newly synthesized protein.

Previous studies (*10, 14, 16*) have shown that cell surface ruffling and fluid-phase pinocytosis are stimulated as a result of the addition of serum or purified growth factors (epidermal growth factor, EGF, or platelet-derived growth factor, PDGF) to cells maintained in serum-free medium. It was therefore possible that the stimulation of fluid-phase pinocytosis by the *ras* oncogene protein is functionally coupled to the action of serum growth factors. To test this possibility, we injected *ras* oncogene protein into cells maintained in serum-free medium for 24 hours (Fig. 6B). The *ras* oncogene protein can stimulate pinocytosis in the absence of serum to a level comparable to that observed in the presence of serum (Fig. 6A).

In recent years it has become increasingly evident that a large number of membrane-triggered cellular responses, some of which involve the stimulation of ruffling and pinocytosis, are mediated by calcium-dependent mechanisms. Well-documented examples include stimulus-secretion coupling in secretory cells (*17*) and mitogenic stimulation of lymphocytes (*18*) and fibroblasts (*19*). The calcium requirement for the fluid-phase pinocytosis activated by the *ras* oncogene protein was examined by removing calcium from the extracellular medium before microinjection. The *ras* oncogene protein failed to stimulate pinocytosis in cells that were incubated in calcium-free medium for 2 hours before injection (Fig. 6C). Similar results were obtained with EDTA-containing medium. In contrast, pinocytosis was stimulated by the *ras* oncogene protein if the cells were injected within 15 minutes of changing to calcium-free medium. This stimulatory effect was maintained, however, only for the initial 2 hours after injection. The relation of intracellular calcium to the *ras*-induced pinocytosis was studied with the use of TMB-8, a compound that inhibits mobilization of intracellular calcium (*20*). Pinocytotic activity induced by *ras* oncogene protein was suppressed in cells treated with TMB-8 only during the first 1 to 2 hours after injection (Table 1). Therefore, the calcium requirement for the stimulation of pinocytosis by *ras* oncogene protein is time-dependent; the initial stimulation appears to be mediated by calcium mobilized from intracellular stores, and the further stimulation seems to be controlled by the availability of extracellular calcium.

Microinjection of the *ras* oncogene protein stimulates DNA synthesis and cell proliferation (*8*). In an attempt to assess the causal role of fluid-phase pinocytosis in *ras*-induced mitogenesis, we examined the correlation between pinocytotic activity and the proliferative response induced by the injection of *ras* oncogene protein. Injection of *ras* oncogene protein leads to stimulation of DNA synthesis and cell proliferation only under conditions that favor the stimulatory effect of the injected protein on pinocytosis (Table 1). We have not as yet identified a case in which the *ras* protein fails to induce stimulation of pinocytosis and maintains its ability to stimulate DNA synthesis and cell proliferation. Furthermore, the proliferative response induced by *ras* oncogene protein appears to correlate with the sustained enhancement of pinocytosis,

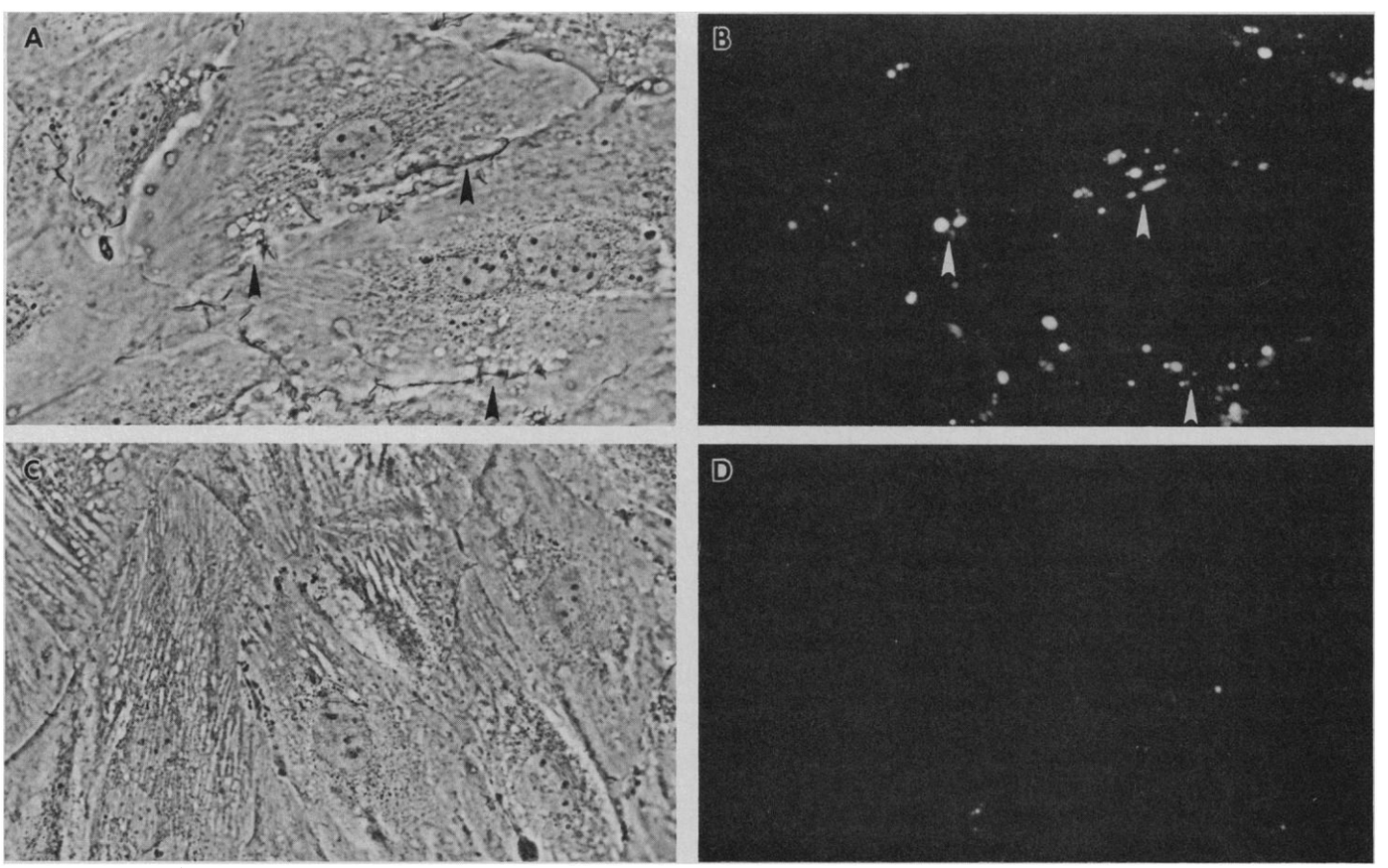

Fig. 2. Stimulation of fluid-phase pinocytosis by the *ras* oncogene protein. Confluent REF-52 cells were injected with either the *ras* oncogene protein (2 mg/ml) (A and B) or control protein (IgG, 3 mg/ml) (C and D). Four hours after injection, FITC-dextran (1 mg/ml) was added to the medium. The cells were incubated for 10 minutes, washed with PBS, and examined as living cells (a 40× water immersion lens attached to a Zeiss PMIII was used). (A and C), Phase contrast micrographs; (B) and (D), fluorescent micrographs corresponding to the same field, respectively. The arrowheads indicate the coincidence of membrane ruffling (A) and pinocytotic vesicles (B).

whereas no stimulation of DNA synthesis is observed when the *ras* oncogene protein induces transient stimulation of fluid-phase pinocytosis. These results point toward the possibility that the mechanisms by which the *ras* oncogene protein stimulates pinocytosis may contribute directly to the mitogenic activity exhibited by this protein.

Effect of microinjection of *ras* oncogene protein on phospholipid metabolism. A common characteristic of cell surface stimuli that trigger membrane-related events is their ability to alter phospholipid metabolism (*21*). We therefore decided to test whether the effects of *ras* proteins on membrane ruffling and pinocytosis are accompanied by changes in phospholipid metabolism. The phospholipid composition of the *ras*-injected cells was analyzed as described in Fig. 7. The cells injected with buffer alone showed the same phospholipid composition as mock-injected cells. Figure 7A shows the migration pattern on an oxalate-impregnated thin-layer chromatography (TLC) plate of ^{32}P-labeled phospholipids from buffer-injected cells or *ras*-injected cells at 30 minutes (track 2) and 1 hour after injection. Injection of the *ras* oncogene protein had no apparent effect on the levels of ^{32}P-labeled phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid at this level of resolution. Our attempts to quantify the levels of ^{32}P incorporated into the polyphosphoinositides PIP and PIP_2 were hampered by the very low net phosphorylation signal caused by the rapid turnover of the phosphates in these phospholipid species. Using an acidic TLC solvent system to analyze the lipid extracts (Fig. 7B), we have observed that the levels of lysophosphatidylcholine and lysophosphatidylethanolamine in *ras*-injected cells (tracks 2 and 3) are reproducibly one to one-and-a-half times higher than in buffer-injected cells (track 1). This increase is detectable as early as 30 minutes after injection and becomes more apparent by 1 hour after injection. Lysophospholipids are the products of phospholipase A_2 activity. This enzyme catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 1,2-diocyl-*sn*-phosphoglyceride, thereby producing two products: free arachidonic acid and lysophospholipids. Therefore, the increase in lysophosphatidylcholine and lysophosphatidylethanolamine detected in cells injected with the *ras* oncogene protein is an indication that the activity of phospholipase A_2 was stimulated in these cells (*22*).

Activation of phospholipase A_2 accompanies in several distinct types of responses triggered by ligands (*23*). The magnitude of the effect produced by microinjection of *ras* oncogene protein is similar to that observed upon ligand-induced activation of phospholipase A_2 (*24*). The activity of the enzyme is calcium-dependent (*25*) and leads to the release of arachidonic acid, which provides the precursor for the formation of prostaglandins and other metabolites (*26*). Free arachidonic acid, moreover, may partition into membranes and alter their structural properties by virtue of its effect on bilayer fluidity (*27*). Likewise, lysophospholipids, formed as a consequence of phospholipase A_2 activation, can exert profound effects on cell surface organization by virtue of their detergent-like properties. Since membrane ruffling and pinocytosis are inevitably associated with dynamic changes in cell surface properties, the activation of phospholipase A_2 by microinjection of the *ras* oncogene protein may be directly involved in the *ras*-induced stimulation of these cell surface activities.

The *ras*, proteins and membrane dynamics. The search for the function of *ras* proteins has recently focused on the possibility that *ras* proteins may mediate the transduction of mitogenic signals that originate at the cell surface. This hypothesis appears attractive

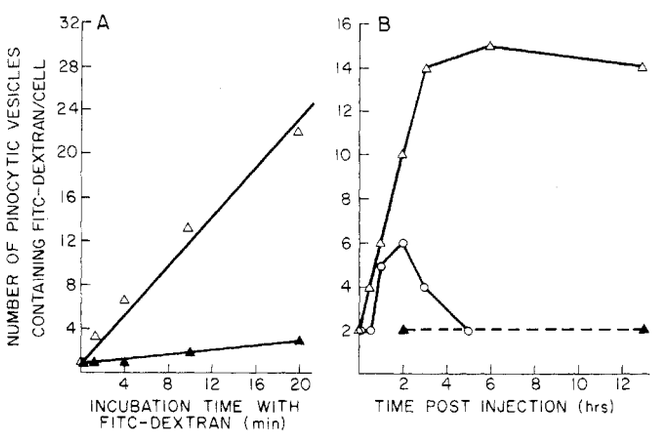

Fig. 3. Kinetics of the stimulation of pinocytosis by the *ras* oncogene protein. In (A), confluent REF-52 cells were injected with either the *ras* oncogene protein (△) or buffer alone (▲). Four hours after injection the cells were incubated with FITC-dextran (1 mg/ml) for the times indicated. The cells were washed with PBS and examined by fluorescence microscopy. For the quantification of FITC-dextran uptake, the number of fluorescent vesicles per injected cell was determined. Every fluorescent vesicle, regardless of size, was identified as a pinocytotic vesicle. Each value shown is the average of 15 counted cells. The number of pinocytotic vesicles per cell was consistent within the population of the injected cells counted (SD <3). In (B), confluent REF-52 cells were injected with either the *ras* oncogene protein (△), the *ras* proto-oncogene protein (○) or buffer alone (▲). At the indicated times after injection the cells were incubated for 10 minutes with FITC-dextran (1 mg/ml), and the number of fluorescent vesicles per injected cell was determined as described above.

Table 1. Effects of *ras* proteins on fluid-phase pinocytosis, initiation of DNA synthesis, and stimulations of cell proliferation under various incubation conditions. ND, not detected.

Protein injected	Amount (mg/ml)	Stimulation of pinocytosis* (hours after injection)		Mitogenic effects	
		2	12	DNA synthesis†	Cell proliferation‡
Normal growth medium					
None (buffer)		−	−	−	−
Control (IgG)	3	−	−	−	−
ras oncogene	2	+	+	+	+
ras oncogene	0.2	+	−	ND	−
ras oncogene	0.06	−	−	ND	−
ras proto-oncogene	2	+	−	−	−
Ca^{2+}-free medium, 2 hours before injection					
ras oncogene	2	−	−	−	−
Ca^{2+}-free medium at time of injection					
ras oncogene	2	+	−	−	−
TMB-8 (50 μM at time of injection)§					
ras oncogene	2	−	+	ND	ND
Serum-free medium (24 hours before injection)					
ras oncogene	2	+	+	+	−

*Fluid-phase pinocytosis induced by microinjection of *ras* proteins was monitored by the uptake of FITC-dextran as described earlier. Pinocytotic activity that was at least threefold higher than the activity measured in buffer-injected cells or in cells injected with control protein was scored as plus. †DNA synthesis was measured by ^{3}H-thymidine incorporation and emulsion autoradiography. [^{3}H]thymidine (1 μCi/ml) was added to the medium within 1 hour after injection, and the cells were further incubated for 24 hours under the indicated conditions. After the incubation period, the cells were fixed, coated with Nuclear Track Emulsion (NTB-2, Kodak), and processed for emulsion autoradiography (exposure, 48 hours). In uninjected confluent REF-52 cells as well as in cells that were injected with buffer alone or with control protein, ^{3}H-thymidine labeling was observed in 2 percent of the cells. Initiation of DNA synthesis was scored as plus when more than 60 percent of the injected cells showed ^{3}H-thymidine labeling. ‡The mitotic index in confluent monolayers of REF-52 cells (determined by the percentage of metaphase to late telophase cells) was <1 percent. Stimulation of cell proliferation was scored as plus when the mitotic index in the injected area was >50 percent 20 hours after injection. §3,4,5-trimethoxybenzoic acid 8-(diethylamine)octyl ester.

because of (i) the membrane localization of *ras* proteins, (ii) the structural and biochemical similarities between *ras* proteins and G proteins, and (iii) the well-documented growth promoting activity of *ras* oncogene proteins. However, the molecular events subserving the biological activity of *ras* proteins are as yet unknown. Our data show that microinjection of *ras* proteins into quiescent fibroblasts results in a marked stimulation of membrane ruffling and fluid-phase pinocytosis. These effects are manifested rapidly (within 30 minutes after injection), do not require protein synthesis, and are dependent on the amount of *ras* protein injected into the cells. These findings indicate that *ras* proteins are directly responsible for the stimulation of membrane ruffling and pinocytosis. Further support comes from the observation that NRK cells transformed by v-K-*ras* oncogene show enhanced ruffling and pinocytotic activity compared to normal NRK cells (*13*).

A similar series of membrane responses follows the interaction of certain hormones, mitogens, and immunomediators with their receptors. For example, the binding of EGF (*14*), PDGF (*16*), NGF (nerve growth factor) (*28*), and insulin (*29*) to target cells is followed by the enhancement in membrane ruffling and pinocytosis. In the immune system, binding of chemotactic peptides to neutrophil membrane receptors similarly stimulates ruffling and pinocyto-

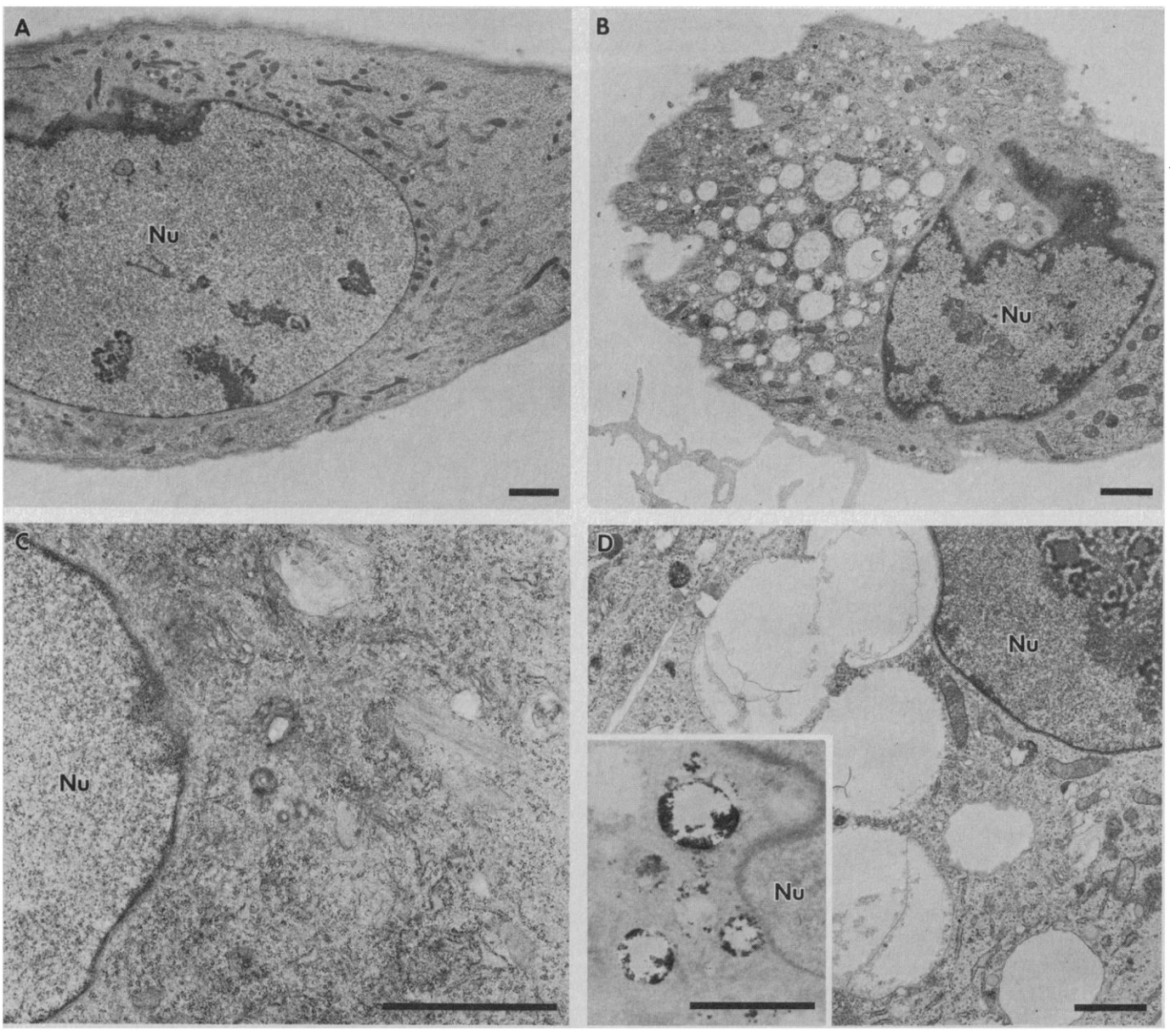

Fig. 4. Ultrastructural changes induced by injection of the *ras* oncogene protein. REF-52 cells were injected with either buffer alone (A and C) or the *ras* oncogene protein (B and D). Ten hours after injection, the cells were fixed and processed for transmission electron microscopy as described for scanning electron microscopy except that after ethanol dehydration, cells were embedded in Epon, cut, and stained with uranyl acetate and lead citrate. Electron micrographs were taken at 40 or 80 kV (Philips 201 electron microscope). Numerous cytoplasmic vesicles are seen in the cell injected with the *ras* oncogene protein (B). The electron transparent appearance of these pinocytotic vesicles is apparent at higher magnification (D). For comparison, a buffer-injected cell is shown at similar magnification (C). For the histochemical localization of horseradish peroxidase (HRP), cells were injected with the *ras* oncogene protein and 2 hours after injection exposed for 30 minutes to HRP at 1 mg/ml. Cells were then washed quickly, fixed, and processed to localize cell-bound enzyme with the diaminobenzidine hydrogen peroxide substrate mixture. The inset in (D) shows the location of HRP in a cell injected with the *ras* oncogene protein. The section was not stained with heavy metals. Nu, nucleus; bars, 2 μm.

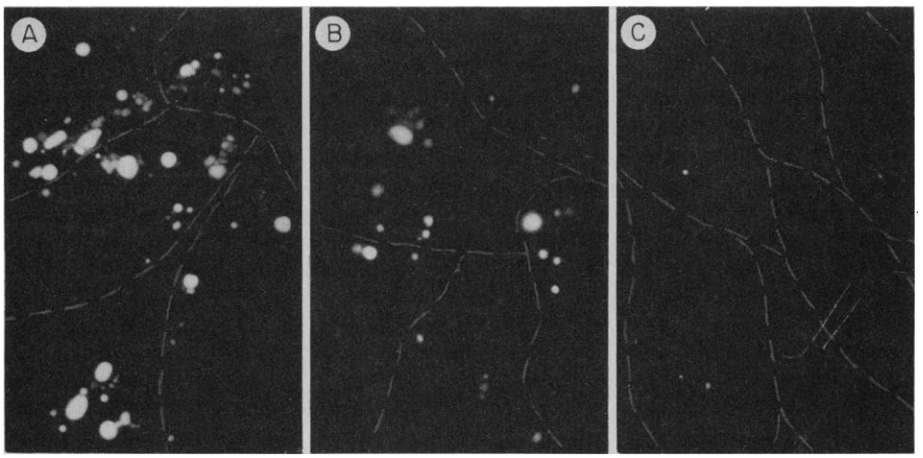

Fig. 5. Dose-dependence of the stimulation of pinocytosis by the *ras* oncogene protein. Confluent REF-52 cells were microinjected with the *ras* oncogene protein at 2 mg/ml (A), 0.2 mg/ml (B), and at 0.06 mg/ml (C). Four hours after injection, the cells were incubated with FITC-dextran (1 mg/ml) for 10 minutes and examined by fluorescence microscopy (40× lens) as described above. The boundaries of the cells are delineated on the fluorescent micrographs by the hand-drawn broken lines.

sis (*30*). While the membrane events that are triggered by *ras* protein and ligand binding are similar, several features of the *ras*-induced response deserve special consideration.

1) Membrane ruffling and pinocytosis induced by ligand binding occur within minutes after exposure of cells to ligands (*28, 30, 31*), whereas the same membrane responses when induced by *ras* proteins are first detected approximately 30 minutes after injection. The *ras* proteins that we use for microinjection have been expressed in *Escherichia coli* and, therefore, lack posttranslational modifications. Since lipidation is essential for the membrane binding and the transforming activity of *ras* proteins (*32*), injected *ras* proteins presumably have to undergo acylation in order to exert their biological effects. Therefore, the interval between microinjection of *ras* proteins and the observed membrane responses may correspond to the time required for the acylation and subsequent association with the membrane of the injected *ras* proteins.

2) The stimulation of membrane ruffling and pinocytosis by *ras* proteins occurs under serum-free conditions indicating that the *ras*-induced effects are not mediated by external ligands. By analogy to the proposed mechanism of action of *ras* oncogene proteins in obviating the requirements for mitogenic growth factors, it is possible that *ras* proteins bypass the requirements for external ligand for the induction of membrane ruffling and pinocytosis. While the growth-promoting activity of *ras* oncogene proteins is well documented, recent studies have demonstrated that *ras* oncogene proteins can promote the differentiation of PC-12 cells in a manner similar to the differentiation process induced by NGF (*7*). The capacity of *ras* proteins to affect diverse cellular pathways controlling cell proliferation or differentiation may be attributed to the ability of *ras* proteins to initiate cell surface events similar to those triggered by both "proliferation factors" (such as PDGF and EGF) and "differentiation factors" (such as NGF).

3) The stimulatory effects of external ligands on membrane ruffling and pinocytosis are transient and generally last only for minutes up to 1 to 2 hours after the interaction of a ligand with cell surface receptors (*29, 31*). In contrast, membrane ruffling and pinocytosis induced by the *ras* oncogene protein persist for more than 15 hours. These activities diminish only shortly before the injected cells enter mitosis, a finding in agreement with previous studies (*33*) showing that many cell surface activities including membrane ruffling and pinocytosis are reduced during mitosis. The ability of *ras* oncogene proteins to produce persistent stimulation of ruffling and pinocytosis does not simply reflect the continuous presence of the protein in the injected cells. This notion is supported

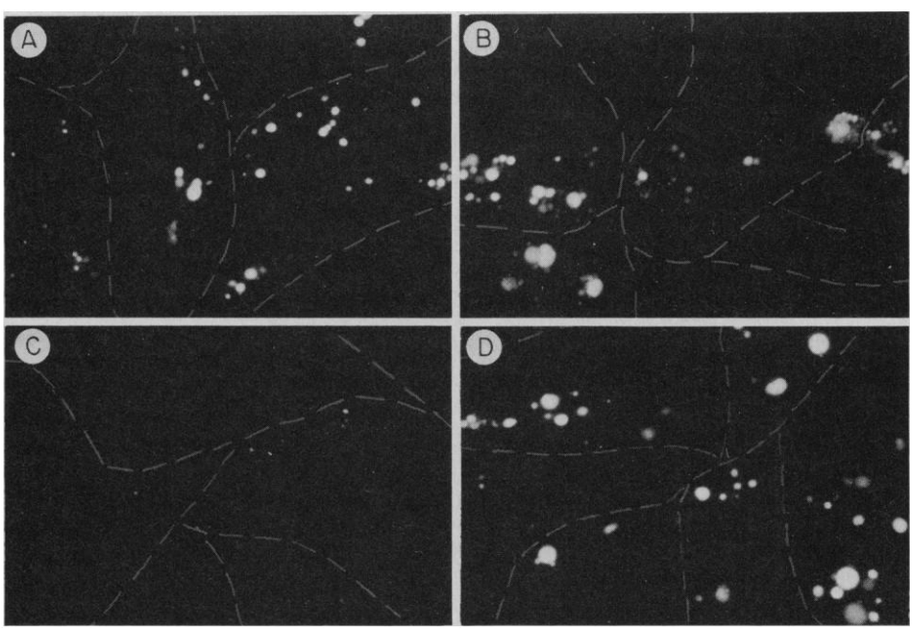

Fig. 6. Effect of serum, extracellular Ca^{2+} or cycloheximide on the stimulation of pinocytosis by the *ras* oncogene protein. Confluent REF-52 cells were maintained in normal medium (A), serum-free medium for 24 hours (B), Ca^{2+}-free medium for 2 hours (C), or normal medium plus cycloheximide at 15 μg/ml for 1 hour (D); the cells were then injected with the *ras* oncogene protein. Four hours after injection, the cells were incubated with FITC-dextran (1 mg/ml) in the indicated media for 10 minutes and examined by phase-contrast or fluorescence microscopy (40× lens) as described above. The fluorescent micrographs of the respective areas are shown, with the cell boundaries delineated by the hand-drawn broken lines.

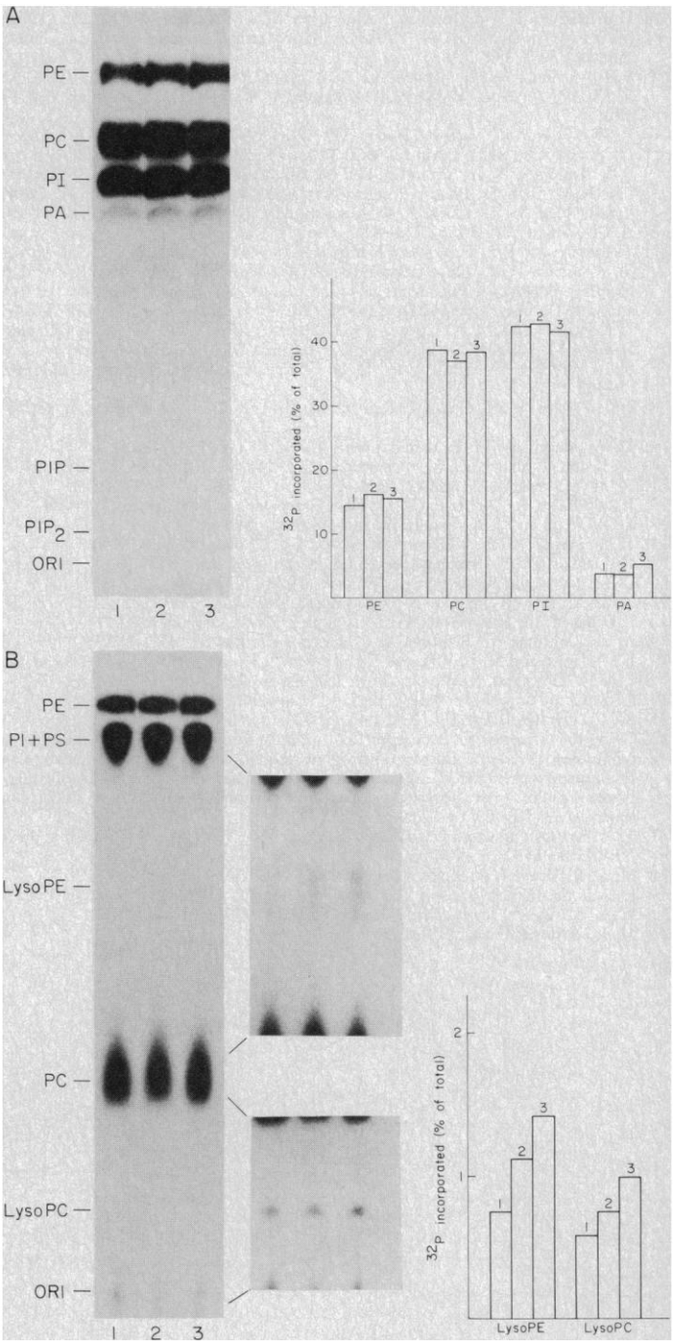

Fig. 7. Effect of microinjection of the *ras* oncogene protein on the composition of ^{32}P-labeled phospholipids. (A) Confluent REF-52 cells grown on glass chips (<1 mm^2) (100 cells per chip) were labeled with [^{32}P]P_i (carrier free, Amersham; 10 mCi/ml) in phosphate-free medium for 2 hours. The chips were washed free of excess label and transferred to normal medium. All the cells on a given chip were injected with either buffer alone (track 1) or with *ras* oncogene protein (tracks 2 and 3). At 30 minutes (track 2) and 60 minutes (tracks 1 and 3) after injection, phospholipids were extracted. Chromatographic separation of the major phospholipid subclasses was carried out on TLC plates impregnated with 1 percent potassium oxalate and activated for 15 minutes at 115°C, with the solvent system of chloroform, methanol, ammonia, and water (45:30:3:5, by volume). (B) For the separation of lysophosphatidylcholine and lysophosphatidylethanolamine TLC plates were developed in a solvent system of chloroform, methanol, acetic acid, and water (75:45:12:3). Lysophosphatidylserine and lysophosphatidylinositol run together with phosphatidylcholine in this system. The ^{32}P-labeled phospholipids were visualized by autoradiography and identified by co-chromatography with standards detected with iodine vapor. The TLC plates were exposed to film for 12 hours (A and B) or for 2 days (B, insets). Quantification of ^{32}P incorporated into phospholipids was determined by scraping the labeled phospholipids off the plates and liquid scintillation counting. Total ^{32}P incorporation into lipids was approximately 50,000 cpm under all conditions. Radioactivity applied at the origin of the TLC plates (15,000 cpm per track) was used as a measure of total ^{32}P-labeled phospholipids. Results are the average of two determinations from a single representative experiment. Similar relative values of the levels of ^{32}P-phospholipids were obtained in two independent experiments. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; LysoPE, lysophosphatidylethanolamine; LysoPC, lysophosphatidylcholine.

by the observation that the same effects when produced by the proto-oncogenic *ras* protein are transient. Therefore, it appears that, while the proto-oncogenic and oncogenic *ras* protein share a common cellular target, the regulatory function of *ras* oncogene protein is deranged thereby leading to the constitutive stimulation of membrane ruffling and pinocytosis. If these membrane activities are linked to the transforming activity of *ras* proteins, this could account for the observation that either mutational activation or overexpression of the normal *ras* genes (*34*) can induce the transformed phenotype.

We have found that the formation of lysophosphatidylcholine and lysophosphatidylethanolamine is stimulated in response to microinjection of *ras* proteins. Since these phospholipids are the products of phospholipase A_2 activity (*25*), our observations indicate that microinjection of *ras* oncogene protein results in the stimulation of phospholipase A_2 activity. From our in vivo experiments it is impossible to determine if *ras* proteins have a direct role in the stimulation of phospholipase A_2 activity. This possibility is intriguing in view of the recent evidence implicating guanine nucleotide–binding protein in the regulation of Ca^{2+}-dependent phospholipase A_2 activity (*35, 36*). In this context, both the membrane and the proliferative responses induced by *ras* proteins appear to be mediated by Ca^{2+}-dependent mechanism. Moreover, the stimulation of phospholipase A_2 activity has been implicated in the initiation of cell proliferation by serum and a number of other growth factors (*37*). Thus, the apparent effects of *ras* protein on the activity of phospholipase A_2 may reflect a critical aspect of the mitogenic activity of *ras* proteins.

Despite the recent emphasis on surface events that may control cell proliferation, little is known about the extent to which membrane-related events contribute directly to the phenotypic differences between normal and transformed cells. The ability of *ras* oncogene protein to stimulate cell proliferation, concomitant with a persistent enhancement in ruffling and pinocytosis, suggests that the proliferative effects of *ras* proteins may be closely associated with these cell surface activities. In that pinocytosis is a fundamental process in maintenance of cell homeostasis, particularly in relation to plasma membrane recycling (*38*) and internalization of fluid-phase components (*39*), alterations in the rate of pinocytosis may exert profound effects on intracellular processes controlling cell proliferation. Therefore, the stimulation of ruffling and pinocytosis may be a primary event in the chain of cellular responses triggered by *ras* proteins, eventually leading to cell proliferation. Furthermore, identification of the molecular mechanisms that mediate the effect of *ras* proteins on membrane ruffling and pinocytosis should provide insight into the biochemical function of these proteins.

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42. Chips were transferred into individual tubes containing 0.5 ml of DMEM and 10^4 unlabeled cells as carrier. Phospholipids were extracted by the addition of 1.83 ml of an ice-cold mixture of methanol, chloroform, and HCl (100:50:1, by volume). Samples were then vortexed, and phases were separated by the addition of 0.6 ml each of chloroform and 2*M* KCl. The organic and aqueous phases were separated by brief centrifugation. The lower (organic) phase was removed, and the aqueous phase was reextracted with 0.4 ml of chloroform. Pooled organic phases were then backwashed twice with 0.35 ml of a mixture of methanol and 1*M* HCl (1:1, by volume) to reduce background radioactivity from water-soluble metabolites. The organic phase was dried under nitrogen, dissolved in chloroform and methanol (2:1) and applied to silica gel LK6D TLC plates.

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