The EMBO Journal vol.15 no.23 pp.6525–6530, 1996

Transformation by Rho exchange factor oncogenes is mediated by activation of an integrin-dependent pathway

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Constitutive activation of growth factor receptor signaling pathways leads to uncontrolled growth, but why tumor cells become anchorage independent is less clear. The fact that integrins transmit signals required for cell growth suggests that constitutive activation of steps downstream from integrins mediates anchorage independence. Since the small GTPase Rho may mediate integrin signal transduction, the effects of serum and the Rho nucleotide exchange factor oncogenes *dbl* and *lbc* on cell growth and signaling pathways were examined. Our data show that these oncogenes induce anchorage-independent but serum-dependent growth and stimulation of signaling pathways. These results show, therefore, that anchorage-independent growth results from constitutive activation of integrin-dependent signaling events. They also support the view that Rho is a functionally important mediator of integrin signaling.

Keywords: anchorage-independent growth/integrin signaling/Rho nucleotide exchange factor oncogenes/phosphatidylinositol-4,5-bisphosphate

Introduction

Cells require both adhesion to immobilized extracellular matrix proteins and stimulation by serum or growth factors in order to proliferate (Tucker *et al.*, 1981). These requirements reflect the fact that both integrins and growth factor receptors transmit signals that ultimately converge to determine cell cycle progression (reviewed in Schwartz *et al.*, 1995). By contrast, tumor cell proliferation is generally independent of both adhesion and serum, though anchorage independence is the feature that correlates best with tumorigenicity *in vivo* (Freedman and Shin, 1974).

Oncoproteins are generally recognized as components of normal growth regulatory pathways that when overexpressed or mutated lead to irreversible activation of those pathways (Cantley *et al.*, 1991). Reexamination of this view in light of information about signaling by integrins suggests a novel model (shown in Figure 1). According to this model, activation of steps after convergence of integrin- and growth factor receptor-regulated pathways would lead to both anchorage- and serum-independent proliferation. Indeed, many or most oncogenes such as v-Ras or SV40 large T display this behavior. Activation of a step on the growth factor portion, prior to convergence, should lead to enhanced proliferation but not to anchorage-independent growth. Defects of this type would be expected to lead to formation of benign tumors. By contrast, activation of a step on an integrin pathway prior to convergence would be expected to lead to anchorage-independent but serum-dependent growth. Indeed, identification of such an oncogene would support this model and provide strong evidence that the step in question was a component of an integrin-dependent growth regulatory pathway.

Integrin-mediated adhesion activates a phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) that phosphorylates phosphatidylinositol 4-phosphate (4-PIP) to produce phosphatidylinositol-4,5-bisphosphate (4,5-PIP_2) (McNamee *et al.*, 1992). Adhesion thereby regulates the supply of 4,5-PIP_2, resulting in 3- to 5-fold changes in the total cellular levels. This effect of integrins appears to be mediated by the small GTPase Rho (Chong *et al.*, 1994). Rho activates a PIP 5-kinase in cell lysates, and can form a physical complex with a 68 kDa PIP 5-kinase (Ren *et al.*, 1996). The evidence indicating that this interaction mediates the effect of integrins on PIP_2 synthesis is somewhat indirect, however. Indeed, there is also data showing that Rho mediates the effects of growth factors and other soluble factors on the actin cytoskeleton and cell cycle. Factors in serum such as lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) can induce Rho-dependent assembly of stress fibers and focal adhesions (Ridley and Hall, 1992; Ridley *et al.*, 1992), and Rho has also been shown to play an important role in regulation of the cell cycle (Olson *et al.*, 1995).

To clarify these issues, we have investigated the effects of activation of Rho by the guanine nucleotide exchange factor oncogenes *lbc* and *dbl* and by serum. *lbc* (Toksoz and Williams, 1994) and *dbl* (Eva and Aaronson, 1985) were identified by their ability to induce focus formation

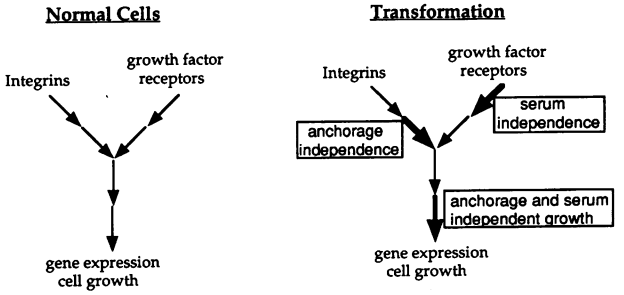

Fig. 1. Convergence of integrin and growth factor pathways. Growth regulatory signals from integrins and growth factor receptors converge at some point or points to regulate nuclear events. Constitutive activation of these events, denoted by the arrows in bold face, may lead to different phenotypes depending upon where in the pathway the event lies.

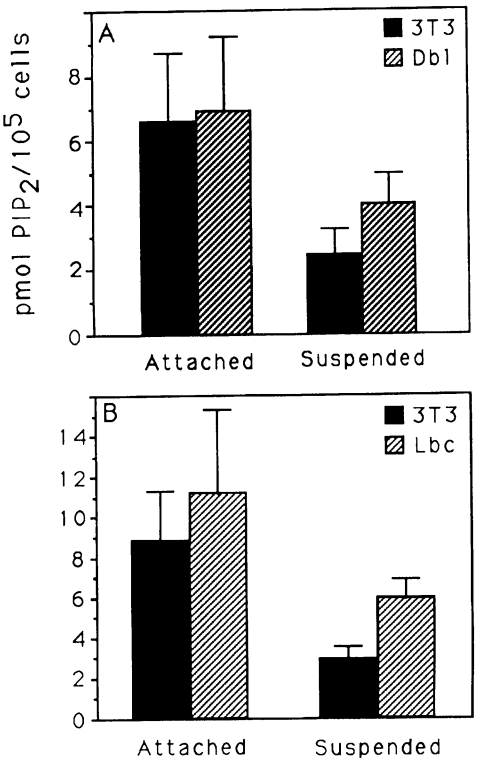

Fig. 2. Total PIP_2 mass in adherent and suspended cells. PIP_2 was assayed in cells plated on tissue culture plastic (attached) or in agarose-coated dishes (suspended) as described in Materials and methods. Values are means ± SD from 4–6 experiments. (**A**) 3T3-*dbl* cells and NIH 3T3-neo mock transfectants. (**B**) 3T3-*lbc* transfectants and NIH 3T3 parent cell line.

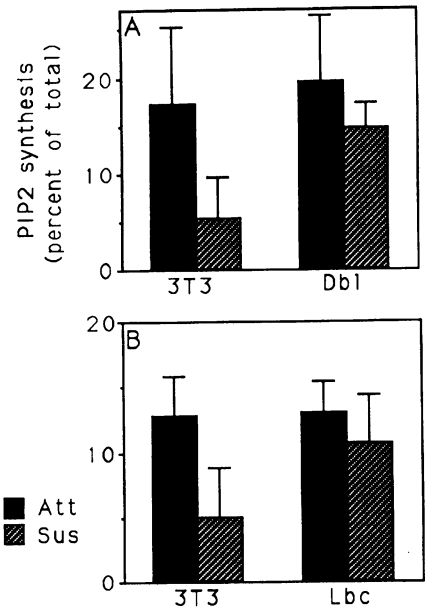

Fig. 3. PIP_2 synthesis in adherent and suspended cells. Cells that were maintained for 24 h on agarose-coated plates (Sus) or replated onto fibronectin-coated tissue culture plastic (Att) were labeled with $^{32}PO_4^-$ for 2 h and the lipid fraction separated by thin layer chromatography as described in Materials and methods. The radiolabel incorporated into PIP_2 as a percentage of total radiolabel in the lipid fraction was calculated. Values are the means ± SD from 4–6 experiments. (**A**) 3T3-*dbl* and NIH 3T3-neo transfectants. (**B**) 3T3-*lbc* transfectants and NIH 3T3 parent cell line.

and tumorigenicity *in vivo* when expressed in NIH 3T3 cells. They were shown later to induce nucleotide exchange on Rho *in vitro*, and to induce formation of stress fibers *in vivo* (Hart *et al.*, 1994; Zheng *et al.*, 1995). Furthermore, transformation by these factors is Rho dependent. Whereas Dbl activates both Rho and the related GTPase, CDC42, Lbc appears to be specific for Rho (Hart *et al.*, 1994; Zheng *et al.*, 1995).

We now show that *lbc* and *lbc* have little effect on cell growth or production of 4,5-PIP_2 in adherent cells, but prevent the decline in 4,5-PIP_2 and loss of responsiveness to growth factors seen in suspended cells. Thus, these oncogenes transform cells by constitutive activation of an integrin-dependent pathway.

Results

Synthesis of 4,5-PIP_2

Previous work showed that levels of 4,5-PIP_2 declined dramatically over several hours when adherent mouse fibroblasts were detached and held in suspension. To examine the role of Rho in this process, NIH 3T3 cells transfected with *lbc* or *lbc* were examined. Neither oncogene had any significant effect on PIP_2 levels in adherent cells (Figure 2). In suspension, control cells showed a decline in PIP_2 levels to approximately one-third of initial levels. A decline in PIP_2 levels was also observed when *lbc* and *lbc* cells were placed in suspension; however, this decline was less dramatic and PIP_2 levels remained consistently above those found in suspended control cells (70 and 100% for *lbc* and *lbc*, respectively). These differences are highly reproducible and statistically significant ($P<0.05$). Rates of synthesis of 4,5-PIP_2 showed a similar pattern, declining markedly in suspended 3T3 cells, but showing only a modest decline in *dbl* and *lbc* cells (Figure 3). These results are consistent with the idea that adhesion activates a Rho-dependent PIP 5-kinase, and that *dbl* and *lbc* result in adhesion-independent activation.

To test whether these differences in PIP_2 levels and rates of synthesis were functionally significant, cells were treated with thrombin to trigger hydrolysis of PIP_2, and intracellular calcium levels were monitored (Figure 4). Whereas control cells showed a sharp decline in thrombin-induced calcium mobilization after incubation in suspension, *lbc*- and *dbl*-transformed cells showed no decrease or only a slight decline, respectively. Thus, elevated levels of PIP_2 correlate with enhanced ability to undergo calcium mobilization in response to an agonist that triggers PIP_2 hydrolysis and release of IP_3.

Growth characteristics

To analyze further how activation of Rho alters signaling pathways, proliferation of *lbc*- and *dbl*-transformed cells was analyzed. When put in semi-solid medium containing 10% serum, *dbl* cells rapidly formed large colonies (Figure 5). In fact, these cells grow as well or better in suspension than *ras*-transformed cells (not shown). Cells transformed by *lbc* grew more slowly, but after 14 days also formed moderate colonies in suspension. In both cases, ~25% of the cells formed colonies, as compared with <1% for control 3T3 cells. These results were not unexpected, since these cell lines were shown previously

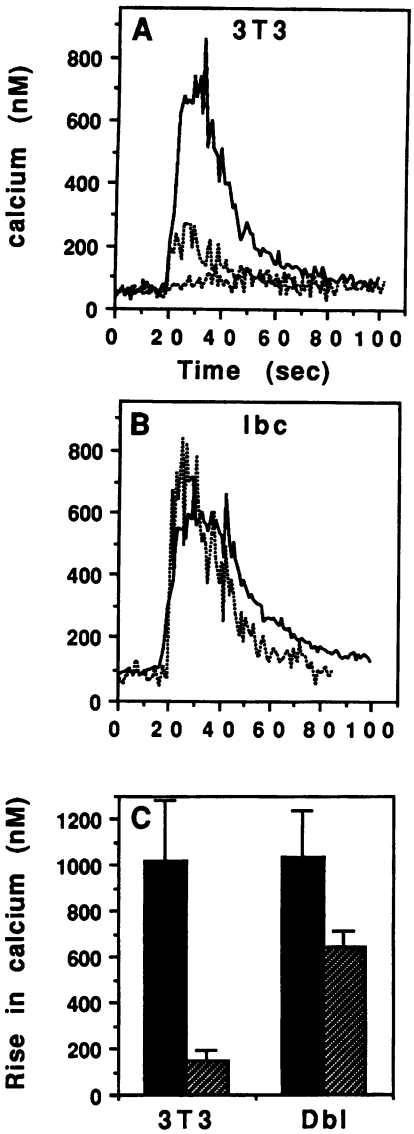

Fig. 4. Thrombin-induced calcium mobilization. Cells that were maintained for 24 h in suspension were loaded with FURA2 and replated on fibronectin-coated coverslips or kept in suspension. Intracellular calcium was monitored in single cells before and after stimulation with 0.5 U/ml thrombin. (**A**) NIH 3T3 cells. The solid line denotes a cell on fibronectin. The broken lines denote two examples of suspended cells. (**B**) 3T3-*lbc* cells. The solid line denotes a cell on fibronectin, the broken line a cell in suspension. Results are representative of 4–6 experiments. (**C**) 3T3 and 3T3-*dbl* cells. Summary of 6–12 experiments with NIH 3T3-neo and 3T3-*dbl* cells. Values are the mean increases in calcium above the baseline for cells on fibronectin (black bars) or for cells in suspension (hatched bars) ± SD.

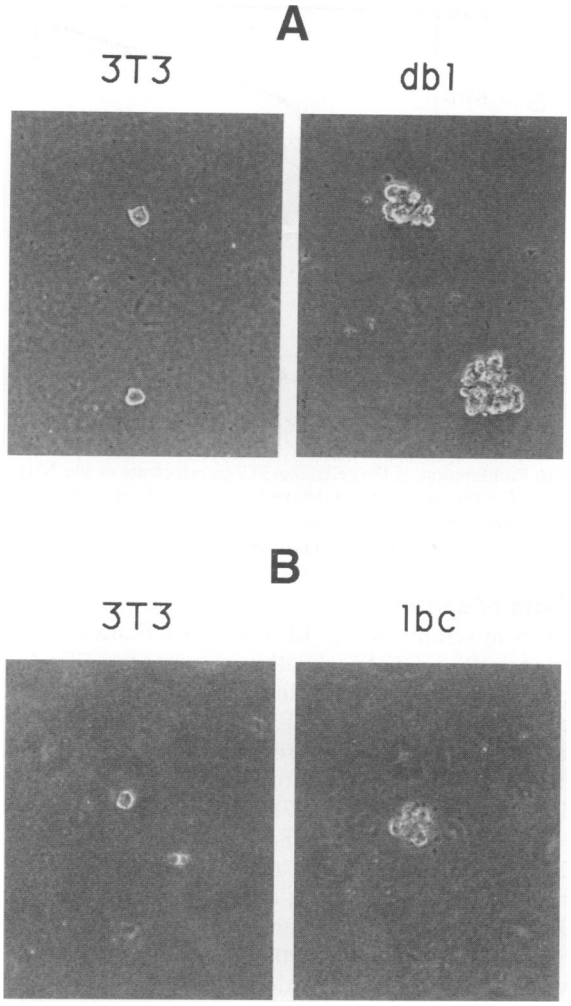

Fig. 5. Anchorage-independent growth. Cells were suspended in methyl cellulose with 10% serum for 7 days (*dbl*) or 14 days (*lbc*) and phase contrast micrographs taken of resulting colonies. Magnification = 438×.

Table I. Doubling times for proliferation of NIH 3T3-neo and 3T3-*dbl* cells

Cells	10% serum	0.5% serum	0% serum
3T3-neo	21 ± 3	49 ± 3	death
3T3-*dbl*	21 ± 1	42 ± 1	death

The doubling times were calculated from growth curves similar to those in Figure 6. Values are the means ± SD from three experiments.

to form tumors in animals. However, *dbl* and *lbc* cells showed no detectable colony formation (<0.1%) in semi-solid medium containing 0.5% serum, whereas other oncogenes including *ras* have been reported to induce colonies in low serum (Oldham *et al.*, 1996).

Growth of cells in monolayer culture was also examined. Surprisingly, adherent *lbc* cells showed a slightly slower rate of proliferation compared with parent 3T3 cells. No indication of serum-independent growth was detected (Figure 5), and both control and *lbc* cells died in serum-free medium. For *dbl* cells, death was also observed in serum-free medium though, in 0.5% serum, a modest growth advantage was detected (Table I). By contrast, *ras*-transformed cells survived under serum-free conditions and showed a substantial enhancement of proliferation in low serum (Mulcahy *et al.*, 1985; data not shown). Ras can induce production of autocrine growth factors which may mediate to some extent the serum-independent growth of certain *ras*-transformed cells (Oldham *et al.*, 1996). Conditioned medium from neither *lbc* nor *dbl* cells promoted growth of NIH 3T3 cells in low serum (data not shown), which could contribute to failure of these oncogenes to induce serum-independent growth.

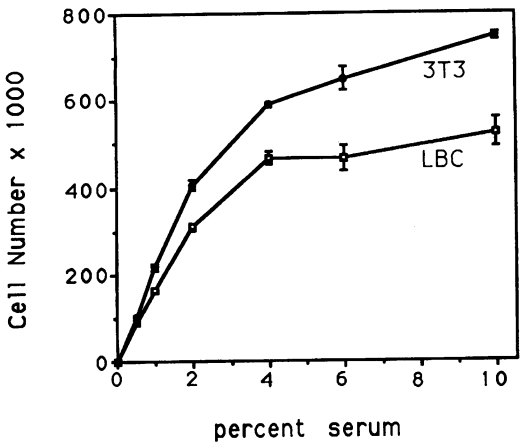

Fig. 6. Proliferation of *lbc* cells. *lbc*-3T3 transfectants or the NIH 3T3 parent cells were plated at 10 000 cells/35 mm well in medium with the indicated concentration of serum. After 7 days, cell number was determined as described in Materials and methods.

Effects of serum

Factors in serum such as LPA and PDGF are reported to activate a Rho-dependent pathway in Swiss 3T3 cells. To ascertain whether PIP 5-kinase is also activated under these conditions, Swiss 3T3 cells were maintained in varying concentrations of serum for 24 h, then stained with rhodamine–phalloidin or analyzed for total levels of PIP_2. As shown in Figure 7A, PIP_2 levels were reduced in low serum, with the half-maximal effect occurring at 0.3%. In five experiments, the level of PIP_2 in cells in serum-free medium was 59 ± 7% of control levels (i.e. cells in 1% serum; concentrations >1% had no further effect). Examination of actin stress fibers (Figure 7B) revealed that, as previously described, serum was required for their formation. The dose dependence was similar to that observed for PIP_2 levels, with the half-maximal effect occurring at ~0.3% serum, and a nearly maximal effect occurring at 1% serum. By contrast, Swiss 3T3 cells that were incubated in suspension for 24 h in 2% serum had PIP_2 levels that were 16% of those found in adherent cells (1.5 ± 0.4 pmol PIP_2/μg DNA in suspended cells versus 9.6 ± 0.3 PIP_2/μg DNA in adherent cells). Thus, serum also modulates PIP_2 levels, and the effect correlates with stress fiber formation. However, serum is a less potent regulator than adhesion.

Previous work has shown that overexpressing an activated mutant of Rho by injecting cDNAs into the nuclei of cells resulted in DNA synthesis in serum-starved, adherent cells (Olson *et al.*, 1995). To test whether activation of endogenous Rho by serum is sufficient to induce DNA synthesis, cells were incubated in varying concentrations of serum then treated with bromodeoxyuridine (BrdU) to label S phase nuclei. As shown in Figure 6B, the low concentrations of serum needed to activate Rho-dependent assembly of stress fibers stimulated DNA synthesis only slightly. The half-maximal effect for DNA synthesis was ~3%, about an order of magnitude higher than the concentration of serum required for assembly of stress fibers and enhancement of PIP_2 levels. Thus, activation of endogenous Rho is not sufficient for progression through the G_1 part of the cell cycle and entry into S phase. Rho function does appear to be necessary for entry into S phase, however, since injection of C3

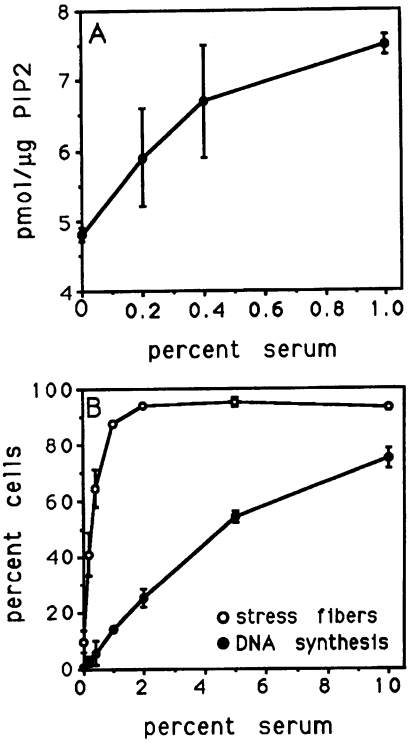

Fig. 7. Effect of serum on Swiss 3T3 cells. (**A**) Cells maintained for 24 h in 0–1% serum were harvested and total PIP_2 levels determined. (**B**) Cells maintained for 24 h in medium with varying concentrations of serum were fixed, labeled with rhodamine–phalloidin, and the fraction of cells showing stress fibers scored (○). Values are means ± range from two experiments. Alternatively, cells were labeled with BrdU for 12 h, stained and the fraction of cells with labeled nuclei scored (●). Values are means ± range from two experiments.

transferase blocks DNA synthesis in control NIH 3T3, *lbc* and *dbl* cells by ~95% (not shown).

Discussion

Our results show that expression of Lbc or Dbl does not alter PIP_2 levels or synthesis significantly in adherent cells where PIP_2 is already high, but elevates PIP_2 levels significantly in suspended cells where PIP_2 levels are otherwise low. These changes correlate with the ability of thrombin to elicit calcium mobilization. The results are therefore consistent with our model that Rho mediates the integrin-dependent increase in PIP 5-kinase activity, and provide the first evidence that activation of Rho can increase PIP_2 levels *in vivo*.

Examination of cell growth showed that activation of Rho by Lbc is not sufficient for induction of DNA synthesis, as *lbc*-transformed cells show the same serum dependence as control 3T3 cells. Adherent *lbc* cells in fact grow at a slightly slower rate than the parent NIH 3T3 cells. This result is supported by the additional observation that low concentrations of serum that are sufficient to induce Rho-dependent formation of actin stress fibers are only weakly mitogenic. Previous results, however, have shown that overexpressing activated Rho by injecting cells with cDNAs did induce DNA synthesis in serum-starved cells (Olsen *et al.*, 1995). This result is most likely a consequence of the very high expression levels that can be obtained using microinjection. Under

such conditions, cellular responses to highly over-expressed, activated proteins may not reflect normal physiological functions.

Our data do indicate that activation of endogenous Rho by Lbc or Dbl is sufficient to induce colony formation in methyl cellulose. This result is consistent with the idea that constitutive activation of an important integrin-dependent pathway should enable cells to grow without adhesion, in much the same way that constitutive activation of growth factor-dependent pathways enables cells to grow without serum. Thus, the fact that *lbc* induces anchorage- but not serum-independent growth places Rho on an integrin pathway, prior to its convergence with serum-dependent pathways.

Rho function is evidently modulated by serum factors, at least in Swiss 3T3 cells. It should be noted, however, that measurements of PIP_2 levels suggest that adhesion is a more potent activator of Rho than serum. Unlike Swiss 3T3 cells, serum-free medium induces only a partial disassembly of stress fibers in ~50% of the population of NIH 3T3 cells. Moreover, while serum-free medium induces a decrease in PIP_2 levels in Swiss 3T3 cells, only a slight and inconsistent decrease was detected in the NIH 3T3 line (data not shown). Hotchin and Hall (1995) proposed that NIH 3T3 and other cell lines show constitutive activation of Rho because of secretion of autocrine growth factors; however, NIH 3T3 cells in serum-free medium do show nearly complete cessation of DNA synthesis. This observation argues against the autocrine secretion of mitogens but, whatever the explanation, the result provides further evidence that activation of Rho is not sufficient for cell growth.

The simplest model that is consistent with all of the data discussed above is that Rho is activated by integrins, and is an important mediator of anchorage dependence. This pathway can be modulated by serum factors, at least in some cells, but it is not sufficient for induction of DNA synthesis. Whether PIP_2 or the actin cytoskeleton are crucial mediators of these events or only convenient markers is presently unknown. Rho appears to have other potential effectors (Amano *et al.*, 1996; Reid *et al.*, 1996; Watanabe *et al.*, 1996), and the roles of these proteins in cell cycle progression have not been determined. Our data do clearly show that anchorage independence and serum independence can be separated, a concept that is likely to be crucial for understanding tumorigenesis.

Materials and methods

Reagents

Cell culture medium was purchased from GIBCO-BRL (Gaithersburg, MD). Bovine serum was from Gemini Bioproducts (Calabasas, CA). Bovine adrenal cortex was from Pel-Freez (Rogers, AR). Radioactive $[^3H]IP_3$, cold IP_3 and $^{32}PO_4$ were from Amersham (Arlington Heights, IL). Thin layer plates were from Whatman (Clifton, NJ). FURA2-AM and rhodamine–phalloidin were purchased from Molecular Probes (Eugene, OR). BrdU and anti-BrdU were from Becton-Dickinson (San Jose, CA). Other reagents were purchased from Sigma Chemicals (St Louis, MO).

Cells

NIH 3T3 and Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum. For anchorage-independent growth, cells were suspended at 2500 cells/ml in DMEM with 2% methylcellulose (4000 centipoises) and 10% bovine calf serum. Three ml of suspension were added to 35 mm wells coated with 0.3 ml 1% agarose that had been equilibrated with DMEM/10% serum. Cells were examined by phase contrast microscopy to detect colony formation. For proliferation assays, cells were plated in 35 mm wells at 10 000 cells/well, in medium with varying concentrations of serum, and re-fed every 3 days. Cell number was estimated using an acid phosphatase assay (Schwartz and Denninghoff, 1994), and absolute cell number was calculated by comparison with a calibration curve.

PIP_2 mass

Total PIP_2 in cells was assayed as we have done previously (McNamee *et al.*, 1992), except that instead of using the Amersham PIP_2 assay kit, we prepared membranes from bovine adrenal cortex and assayed $[^3H]IP_3$ binding as described (Godfrey, 1992). DNA in the cellular fraction was quantitated using the diphenylamine reaction (Burton, 1956).

PIP_2 synthesis

Cells were detached from the substratum and held in suspension for 24 h on agarose-coated plates in DMEM with 0.5% serum and 0.4% methylcellulose (methylcellulose was added to reduce clumping). Cells were then either kept in suspension or plated on tissue culture plastic dishes coated with 20 μg/ml fibronectin, and incubated for 2 h in phosphate-free medium with 50 μCi/ml $^{32}PO_4$. Lipids were extracted and chromatographed as described (McNamee *et al.*, 1992). Thin layer plates were analyzed by autoradiography and spots were scraped and counted for ^{32}P by liquid scintillation counting. The radioactivity incorporated into PIP_2 was normalized for total radiolabel incorporated into the lipid fraction, and is expressed as a percentage of total $^{32}PO_4$.

Calcium mobilization

Cells were kept in suspension for 24 h in 0.5% serum/0.4% methylcellulose. They were loaded with FURA2-AM as described (Schwartz and Denninghoff, 1994) and either replated on fibronectin-coated coverslips or collected by centrifugation and resuspended on bovine serum albumin (BSA)-coated coverslips (to which they do not adhere during the course of the experiment). Cells were rinsed into HEPES-buffered saline (20 mM HEPES pH 7.25, 140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM glucose). Thrombin at 0.5 U/ml was added and calcium levels monitored in single cells as described (Schwartz and Denninghoff, 1994).

Cell labeling and immunofluorescence

Cells on coverslips were fixed in 1% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100 in PBS and stained with rhodamine–phalloidin in PBS at 1:100 for 30 min. Fluorescence was visualized using an inverted Nikon Diaphot microscope with a 60× oil-immersion lens. For each sample, at least 200 cells were scored for the presence of actin stress fibers. For assaying DNA synthesis, cells were labeled with BrdU reagent at 1:1000 for 13 h, then fixed with 1% formaldehyde and permeabilized with 1% Triton X-100. Cells were incubated in 1 M HCl for 30 min, rinsed with PBS and stained for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU diluted 1:5 into PBS. For each sample, at least 200 cells were scored for nuclear FITC staining.

Acknowledgements

We thank Drs Gary Bokoch, Sim Winitz, Mark Renshaw and Xiang-Dong Ren for helpful discussions. This work was supported by NIH grant #RO1 GM27214 to M.A.S. This is publication #10107-VB from The Scripps Research Institute.

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Received on May 28, 1996; revised on August 1, 1996