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Underexpression of the 43 kDa inositol polyphosphate 5-phosphatase is associated with cellular transformation

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The 43 kDa inositol polyphosphate 5-phosphatase (5-phosphatase) hydrolyses the second messenger molecules inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]. We have underexpressed the 43 kDa 5-phosphatase by stably transfecting normal rat kidney cells with the cDNA encoding the enzyme, cloned in the antisense orientation into the tetracycline-inducible expression vector pUHD10-3. Antisense-transfected cells demonstrated a 45% reduction in Ins(1,4,5)P_3 5-phosphatase activity in the total cell homogenate upon withdrawal of tetracycline, and an ~80% reduction in the detergent-soluble membrane fraction of the cell, as compared with antisense-transfected cells in the presence of tetracycline. Unstimulated antisense-transfected cells showed a concomitant 2-fold increase in Ins(1,4,5)P_3 and 4-fold increase in Ins(1,3,4,5)P_4 levels. The basal intracellular calcium concentration of antisense-transfected cells (170 $\pm$ 25 nM) was increased 1.9-fold, compared with cells transfected with vector alone (90 $\pm$ 25 nM). Cells underexpressing the 43 kDa 5-phosphatase demonstrated a transformed phenotype. Antisense-transfected cells grew at a 1.7-fold faster rate, reached confluence at higher density and demonstrated increased [^{3}H]thymidine incorporation compared with cells transfected with vector alone. Furthermore, antisense-transfected cells formed colonies in soft agar and tumours in nude mice. These studies support the contention that a decrease in Ins(1,4,5)P_3 5-phosphatase activity is associated with cellular transformation.

Keywords: cell transformation/inositol 1,4,5-trisphosphate/5-phosphatase

Introduction

Agonist-induced hydrolysis of the ubiquitous membrane phospholipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] results in the generation of the second messenger molecule inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (Berridge, 1983, 1993; Berridge and Irvine, 1984; Majerus, 1992). Ins(1,4,5)P_3 binds to specific receptors, resulting in the release of calcium from intracellular stores and the initiation of many essential cellular responses including proliferation, contraction and secretion (Berridge and Irvine, 1984, 1989; Abdel-Latif, 1986). Ins(1,4,5)P_3 can be phosphorylated by the Ins(1,4,5)P_3 3-kinase to produce inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], which is implicated in gating calcium at the plasma membrane (Batty *et al.*, 1985). Both these second messenger molecules are metabolized rapidly by the inositol polyphosphate 5-phosphatase (5-phosphatase) enzymes in a signal terminating reaction (Downes *et al.*, 1982; Connolly *et al.*, 1985; Mitchell *et al.*, 1989). Ins(1,3,4)P_3, the product of 5-phosphatase hydrolysis of Ins(1,3,4,5)P_4, forms the precursor for the synthesis of the highly phosphorylated inositol phosphates, Ins(1,3,4,5,6)P_5 and InsP_6 (for review, see Menniti *et al.*, 1993).

Inositol polyphosphate 5-phosphatase activity is ubiquitous and is distributed between the membrane and cytosol of many cell types (for review, see Shears, 1989, 1992; Majerus, 1992; Verjans *et al.*, 1994a). A number of 5-phosphatase enzymes have been isolated and have been variously classified, the simplest of which designates the enzymes as either Type I or Type II (Hansen *et al.*, 1987; Mitchell *et al.*, 1989; Irvine, 1992; Jefferson and Majerus, 1995). The Type I 5-phosphatases have a molecular weight between 40 and 66 kDa, elute early from anion-exchange resins, hydrolyse both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, but do not metabolize PtdIns(4,5)P_2 (Connolly *et al.*, 1985; Hansen *et al.*, 1987; Erneux *et al.*, 1989; Takimoto *et al.*, 1989; Verjans *et al.*, 1992; Hansbro *et al.*, 1994; Hodgkin *et al.*, 1994; Matzaris *et al.*, 1994; Palmer *et al.*, 1994). In contrast, the Type II 5-phosphatases have a larger molecular weight between 75 and160 kDa, elute later from anion-exchange resins, hydrolyse Ins(1,3,4,5)P_4 poorly, but hydrolyse PtdIns(4,5)P_2 well (Hansen *et al.*, 1987; Erneux *et al.*, 1989; Mitchell *et al.*, 1989; Takimoto *et al.*, 1989; Hansbro *et al.*, 1994; Matzaris *et al.*, 1994; Jefferson and Majerus, 1995). The Type II 5-phosphatases include the protein deficient in Lowe's oculocerebrorenal syndrome, a rare X-linked syndrome associated with growth and mental retardation, cataracts and renal tubular abnormalities (Lowe *et al.*, 1952; Attree *et al.*, 1992; Zhang *et al.*, 1995). The recent cloning of members of both the Type I and Type II 5-phosphatases has demonstrated that the two classes of enzymes bear sequence homology in several highly conserved domains that may represent substrate binding sites (Laxminarayan *et al.*, 1994; Hejna *et al.*, 1995; Jefferson and Majerus, 1995; Damen *et al.*, 1996; McPherson *et al.*, 1996).

The prototype of the Type I 5-phosphatases is the 43 kDa 5-phosphatase which has been purified from both membrane and cytosolic extracts of a variety of cell types (Connolly *et al.*, 1985; Erneux *et al.*, 1989; Verjans *et al.*, 1992; Laxminarayan *et al.*, 1993; Hansbro *et al.*, 1994; Hodgkin *et al.*, 1994). Based upon kinetic analysis, the 43 kDa 5-phosphatase appears to be the most highly active

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enzyme involved in the metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 described to date (Laxminarayan *et al.*, 1993). The cloning of the human placental 43 kDa membrane-associated 5-phosphatase and the cytosolic human brain 5-phosphatase demonstrated that the enzymes are identical and contain a C-terminal CAAX motif that may mediate membrane attachment (De Smedt *et al.*, 1994; Laxminarayan *et al.*, 1994; Verjans *et al.*, 1994b).

In some human and murine leukaemia cell lines, the cleavage of inositol lipids is independent of growth factor stimulation (Geny *et al.*, 1988, 1991; Porfiri *et al.*, 1988, 1989, 1991). Circumstantial evidence suggests that the decrease in inositol polyphosphate 5-phosphatase activity in certain acute and chronic leukaemia cell extracts may play a role in the proliferating potential of the leukaemias (Nye *et al.*, 1992; Mengubas *et al.*, 1994). In this study, we have developed stable cell lines to reveal the long-term consequences of underexpression of the 43 kDa inositol polyphosphate 5-phosphatase in regulating Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and, therefore, calcium signalling. Using this approach, we have been able to study the effects of overproduction of Ins(1,4,5)P_3 in unstimulated intact cells under physiological conditions. The effects of chronically elevated Ins(1,4,5)P_3 and intracellular calcium concentration on cellular transformation have been investigated.

Results

Studies were conducted to investigate the effects of underexpression of the 43 kDa 5-phosphatase on Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and intracellular calcium in the unstimulated cell. We initially attempted to constitutively underexpress the 43 kDa 5-phosphatase in NIH 3T3 cells. The 2.6 kb cDNA encoding the human 43 kDa membrane-associated 5-phosphatase was cloned in the antisense orientation into the expression vector pCMV2 (Laxminarayan *et al.*, 1994). The antisense construct was stably co-transfected with a neomycin resistance cassette into NIH 3T3 cells and clones were selected for resistance to geneticin ~3–4 weeks later. Clones were assessed for underexpression of the 43 kDa 5-phosphatase by determining the Ins(1,4,5)P_3 5-phosphatase activity in the total cell homogenate and the detergent-soluble membrane fraction of the cell (results not shown). Using this constitutive expression system, only three cell lines underexpressing the 5-phosphatase were isolated and, with repeat passages (between six and eight), Ins(1,4,5)P_3 5-phosphatase activity gradually increased to the levels observed in cell lines transfected with vector alone. These studies suggested that constitutive underexpression of the 5-phosphatase could not be maintained for prolonged periods, so regulated underexpression was attempted.

Regulated expression of the antisense cDNA was established using the tet-regulatory system (Gossen and Bujard, 1992). The 2.6 kb cDNA encoding the human 43 kDa membrane-associated 5-phosphatase was cloned in the antisense orientation into the response plasmid pUHD 10-3. The antisense construct and a neomycin resistance cassette were stably co-transfected into normal rat kidney (NRK) cells, which contained the regulator plasmid pUHD 15-1. Clones were selected by their resistance to geneticin ~3–4 weeks later. Approximately 50 antisense-transfected

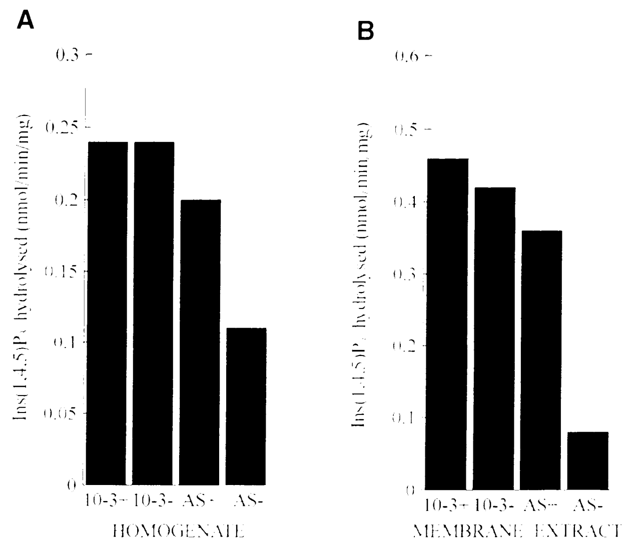

Fig. 1. Characterization of cell lines stably underexpressing the 43 kDa 5-phosphatase. The 2.6 kb cDNA encoding the 43 kDa membrane-associated 5-phosphatase was cloned in the antisense orientation into the response plasmid pUHD 10-3 of the tet-regulatory expression system. The construct, along with a neomycin resistance cassette, was transfected into NRK cells which expressed the regulator plasmid pUHD 15-1. Antisense-transfected clones were selected by neomycin resistance and isolated as described in Materials and methods. Isolated clones, transfected with the antisense construct (AS) or with vector alone (10-3) were assessed for Ins(1,4,5)P_3 5-phosphatase activity in the presence (+) or absence (–) of tetracycline (1 µg/ml) in the total cell homogenate (**A**) or in the detergent-soluble membrane fraction of the cell (**B**). Results are the mean and standard deviation of three separate experiments using three independent antisense- or vector-transfected clones.

clones were isolated, of which 10 were characterized for underexpression of the 43 kDa 5-phosphatase. As an additional control, cell lines transfected with the pUHD 10-3 vector alone were also selected.

Immunoblotting cell extracts with antibodies to the purified 43 kDa 5-phosphatase (Laxminarayan *et al.*, 1993) failed to demonstrate a 43 kDa polypeptide in vector-transfected NRK cells in either the presence or absence of tetracycline (1 µg/ml), using up to 200 µg of total cell homogenate.

To increase the sensitivity of enzyme detection, cell lines were selected on the basis of reduction in Ins(1,4,5)P_3 5-phosphatase activity in the total cell homogenate and the detergent-soluble membrane fraction of the cell in the absence of tetracycline. Withdrawal of tetracycline led to induction of antisense 43 kDa 5-phosphatase gene expression and a decrease in Ins(1,4,5)P_3 5-phosphatase enzyme activity in all antisense-transfected cell lines tested. Analysis of the enzyme activity in the total cell homogenate of antisense-transfected cells demonstrated a 45% reduction in Ins(1,4,5)P_3 5-phosphatase activity upon withdrawal of tetracycline [0.11 ± 0.035 nmol Ins(1,4,5)P_3 hydrolysed per min/mg ($n = 9$) versus 0.20 ± 0.05 nmol Ins(1,4,5)P_3 hydrolysed per min/mg ($n = 9$) for antisense-transfected cells in the presence of tetracycline] (Figure 1A). Analysis of the detergent-soluble membrane fraction of antisense-transfected cell lines demonstrated an ~80% reduction in enzyme activity upon withdrawal of tetracycline [0.08 ± 0.02 nmol Ins(1,4,5)P_3 hydrolysed per min/mg ($n = 9$) versus 0.36 ± 0.03 nmol Ins(1,4,5)P_3

hydrolysed per min/mg (n = 9) for antisense-transfected cells in the presence of tetracycline] (Figure 1B). No significant difference in enzyme activity was demonstrated in the cytosolic fraction of antisense-transfected cells in the presence or absence of tetracycline (results not shown). The reduction of Ins(1,4,5)P_3 5-phosphatase enzyme activity in the detergent-soluble membrane fraction and not the cytosol is consistent with the subcellular location of the native enzyme (Laxminarayan *et al.*, 1993, 1994). The remaining Ins(1,4,5)P_3 5-phosphatase enzyme activity in the antisense-transfected cells may be attributed to the 75 kDa 5-phosphatase, as >30% of the total cellular Ins(1,4,5)P_3 5-phosphatase activity was immunoprecipitated using antibodies to the recombinant 75 kDa 5-phosphatase (results not shown).

In the presence of tetracycline, antisense-transfected cells consistently demonstrated an ~15% reduction in 5-phosphatase enzyme activity compared with cells transfected with vector alone [0.2 ± 0.05 nmol Ins(1,4,5)P_3 hydrolysed per min/mg (n = 9) versus 0.24 ± 0.05 nmol Ins(1,4,5)P_3 hydrolysed per min/mg (n = 9), respectively, in the total cell homogenate] (Figure 1A and B). These studies suggest that antisense expression of the 43 kDa 5-phosphatase is not suppressed completely by tetracycline. Similar findings using this expression system have been described in human embryonic kidney cells (HEK 293) (Howe *et al.*, 1995). As tetracycline does not completely suppress antisense gene expression, in some experiments vector-transfected cell lines were used as controls. Three independent antisense-transfected clones, which demonstrated the lowest Ins(1,4,5)P_3 5-phosphatase enzyme activity upon withdrawal of tetracycline, were used in the subsequent studies.

Inositol phosphate formation in transfected fibroblasts

To investigate changes in inositol phosphate metabolism in cell lines underexpressing the 43 kDa 5-phosphatase, NRK cells were serum deprived in the presence of [^{3}H]inositol for 48 h and inositol phosphates extracted and analysed, as described in Materials and methods. Serum-deprived antisense-transfected cells showed a dramatically altered profile of cellular inositol phosphates as compared with vector-transfected cells (Figure 2A and B and Table I). The 43 kDa 5-phosphatase hydrolyses the 5-position phosphate from Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 with high affinity (K_m = 5 and 1.2 μM respectively), thereby forming Ins(1,4)P_2 and Ins(1,3,4)P_3 (Laxminarayan *et al.*, 1993). Therefore, in cell lines underexpressing the enzyme, the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in the unstimulated cell should increase and the products of the hydrolysis of these second messengers decrease. A 2-fold increase in the level of Ins(1,4,5)P_3 was observed in antisense-transfected cells [1764 ± 493 c.p.m./mg (n = 5)] compared with vector-transfected cells [889 ± 370 c.p.m./mg (n = 3)]. In addition, a 4-fold increase in Ins(1,3,4,5)P_4 levels was observed in antisense-transfected cells [215 ± 23 c.p.m./mg (n = 3)], compared with vector-transfected cell lines, where Ins(1,3,4,5)P_4 was virtually undetectable [57 ± 20 c.p.m./mg (n = 3)]. Ins(1,4)P_2, the product of 5-phosphatase hydrolysis of Ins(1,4,5)P_3, was marginally higher in serum-deprived vector-transfected cells compared with antisense-transfected cells; however, this was not statistically significant. Ins(1,3,4)P_3 was not detectable in either vector- or antisense-transfected unstimulated cells. The level of total inositol monophosphates in the antisense-transfected cell lines was three times that observed in vector-transfected cells.

Recent studies have demonstrated that incorporation of radiolabel into the higher phosphorylated inositol phosphates such as InsP_5 and InsP_6 requires several days to reach apparent isotopic equilibration (Menniti *et al.*, 1993). InsP_5 levels were increased 3-fold in serum-deprived antisense-transfected clones; however, no significant reproducible increase in the levels of InsP_6 were observed routinely. The increase in InsP_5 in antisense-transfected cell lines suggests that these higher phosphorylated inositol phosphates may be formed by phosphorylation of Ins(1,3,4,5)P_4, rather than Ins(1,3,4)P_3 (Irvine *et al.*, 1992). These studies clearly demonstrate that, in cell lines underexpressing the 43 kDa 5-phosphatase, there is a profound alteration in the metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, resulting in a significant increase in the levels of these second messenger molecules.

Assessment of intracellular calcium levels in the unstimulated cell

As unstimulated antisense-transfected cells demonstrated increased Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels, the impact of elevation of these second messengers on basal intracellular calcium levels was determined. Cells were serum starved for 24 h and then loaded with the fluorescent calcium indicator, Fura-2 as described in Materials and methods. The basal intracellular calcium concentration of serum-deprived vector-transfected cell lines was 90 ± 25 nM (n = 13). In contrast, basal calcium levels in antisense-transfected cells were 170 ± 25 nM (n = 14), a significant increase (1.9-fold, $P<0.05$) compared with vector-transfected cells (Figure 3A). The effects of elevated Ins(1,4,5)P_3 on depletion of the total intracellular calcium stores were determined as described (Margolis *et al.*, 1990). Calcium influx was blocked using lanthanum (200 μM), and total calcium stores were quantitated following ionomycin treatment (Figure 3B). Two populations of antisense-transfected cell lines were evident. Antisense-transfected cell lines with basal cell calcium levels <150 nM (n = 9) demonstrated a small reduction in total intracellular calcium stores compared with vector-transfected cells [660 ± 185 nM versus 825 ± 125 nM (n = 9), respectively]; however, this decrease was not statistically significant. In contrast, antisense-transfected cell lines with basal calcium levels >150 nM (n = 5) showed a marked (5-fold) reduction in intracellular stores (170 ± 70 nM, $P<0.05$).

Transforming potential of cells underexpressing the 43 kDa 5-phosphatase

It was notable during the transfection procedure that cells transfected with the antisense 43 kDa 5-phosphatase appeared to grow faster and reach confluence at a higher density than vector-transfected cells in the absence of tetracycline. To determine whether a sustained elevation in intracellular calcium levels affects cell growth, growth rates of antisense- versus vector-transfected cells were determined as described in Materials and methods. Anti-

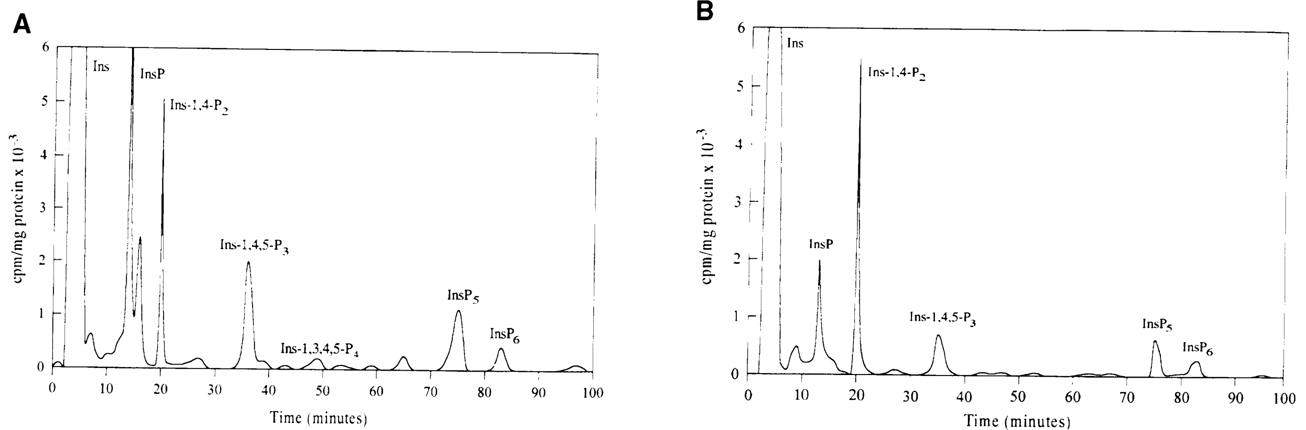

Fig. 2. HPLC elution profiles of inositol phosphates from NRK cells. Cells were labelled with [^{3}H]inositol for 48 h and the inositol phosphates were extracted and separated on HPLC as described in Materials and methods. HPLC profiles are of NRK cells stably expressing the antisense 43 kDa 5-phosphatase (**A**), or the control plasmid, pUHD 10-3 (**B**). The profiles are representative of at least three similar experiments using three different antisense- or vector-transfected clones.

Table I. Analysis of inositol phosphates in NRK cells

Compound	10-3	AS	Ratio
InsP	2530 ± 404	8645 ± 1160	3.4
Ins(1,4)P_2	4602 ± 1052	4190 ± 761	0.9
Ins(1,3,4)P_3	ND	ND	–
Ins(1,4,5)P_3	889 ± 370	1764 ± 493	2
Ins(1,3,4,5)P_4	57 ± 20	215 ± 23	3.8
$InsP_5$	510 ± 114	1534 ± 505	3
$InsP_6$	345 ± 149	465 ± 176	1.3

Cells were labelled with [^{3}H]inositol for 48 h and inositol phosphates were extracted, and separated by HPLC as described in Materials and methods. Results represent the mean and standard deviation of at least three experiments using three independent antisense- (AS) or vector-transfected clones (10-3). ND = not detected. The 'ratio' represents the fold difference between the antisense- and vector-transfected cells.

sense-transfected cells grew at a 1.7-fold faster rate than vector-transfected cells and reached confluence at a higher density (Figure 4A). The final density of antisense-transfected cells after 120 h growth was 1.6-fold higher than vector-transfected cells [$1.02 \pm 0.11\times10^6$ cells/ml (n = 9) versus $0.64 \pm 0.12\times10^6$ cells/ml (n = 9), respectively]. Growth rates of antisense-transfected cells in the presence versus the absence of tetracycline were also analysed (Figure 4B). In the presence of tetracycline, the antisense-transfected cells demonstrated similar growth characteristics to vector-transfected cell lines. The presence or absence of tetracycline did not alter the growth curves for vector-transfected cells. Further evidence supporting the increased growth rates of antisense-transfected cells was shown by incorporation of [^{3}H]thymidine, as a measure of DNA synthesis. Three independent antisense- or vector-transfected cell lines were either serum deprived or stimulated with 10% fetal calf serum (FCS) and the incorporation of [^{3}H]thymidine assessed as described in Materials and methods. Figure 4C shows the [^{3}H]thymidine incorporation of three representative serum-deprived antisense- or vector-transfected cell lines. Serum-deprived antisense-transfected cells demonstrated a 2.1-fold higher incorporation of [^{3}H]thymidine as compared with vector-transfected cells [1202 ± 314 c.p.m. (n = 4) versus 567 ± 26 c.p.m. (n = 4), respectively]. Stimulation with 10% FCS resulted in a marked increase in [^{3}H]thymidine incorporation by both antisense- and vector-transfected cells (Figure 4D). Stimulated antisense-transfected cells demonstrated a 1.4-fold increase in [^{3}H]thymidine incorporation above vector-transfected cells [6110 ± 234 c.p.m. (n = 4) versus 4418 ± 347 c.p.m. (n = 4), respectively].

Although antisense-transfected cells did not display any consistent alteration in morphological appearance, the increased cell growth rates, DNA synthesis and cell density at confluence suggested that the cells may have acquired a transformed phenotype. The ability of antisense-transfected cells to display anchorage-independent cell growth was assessed. Cells from three independent antisense- or vector-transfected clones (1×10^5/ml) were plated in soft agar, and colony formation was assessed after 30 days, as described in Materials and methods. Antisense-transfected cells developed large prominent colonies in soft agar (Figure 5A). In comparison, vector-transfected cells formed an occasional small colony in soft agar. However, the size and number of the colonies was not characteristic of cells which display anchorage-independent cell growth (Figure 5B). The mean number of colonies formed by the antisense-transfected cells was notably (6- to 10-fold) higher than that observed for vector-transfected cells (Table II).

To assess further the transforming potential of antisense-transfected cells, we investigated the ability of cells underexpressing the 43 kDa 5-phosphatase to form tumours in nude mice. Cells (5×10^6) from three independent antisense-transfected clones (six mice per antisense clone) or vector-transfected cells (10 mice) were injected subcutaneously into BALB/c nude mice and tumour formation was assessed over a 5 week period. Sixteen of the 18 mice injected with antisense-transfected cells formed large prominent tumours at the site of injection, which averaged 6 mm in diameter (Figure 6A, Table III). Of the nude mice injected with vector-transfected cells, three of the 10 mice formed tumours; however, these were extremely small (1–2 mm in diameter) and were only detectable following post-mortem analysis (Figure 6B, Table III). Histological examination of the lesions revealed an infiltrative mass of tumour with the

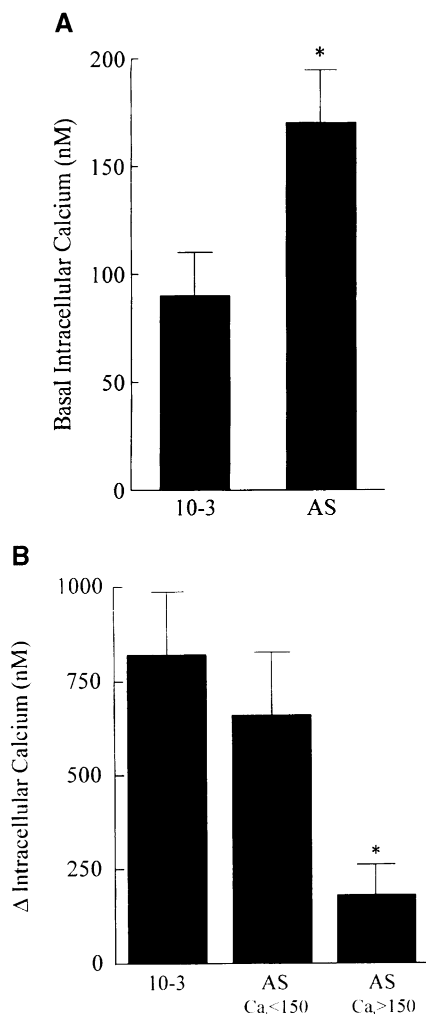

Fig. 3. Measurement of cytosolic calcium levels in NRK cells. (**A**) Basal calcium levels. Cells were loaded with 10 μM Fura-2 AM and the basal cytosolic calcium levels of antisense- (AS) or vector-transfected (10-3) clones were measured, as described in Materials and methods. Results are the mean and standard error of 14 antisense- and 13 vector-transfected cell measurements, using three independent antisense- or vector-transfected clones. * $P<0.05$. (**B**) Assessment of total intracellular calcium stores. Cells were loaded with 10 μM Fura-2 AM and basal cytosolic calcium concentrations were determined as described in Materials and methods. Lanthanum chloride (200 μM) was added to block calcium entry. Ionomycin (2 μM) was added to deplete intracellular calcium stores, and the rise in intracellular calcium was determined. Vector-transfected (10-3) cell results represent the mean and standard error of nine experiments, using three independent vector-transfected clones. The antisense-transfected (AS) cell results represent the mean and standard error of nine measurements using antisense-transfected clones with a basal calcium of <150 nM (Ca_i <150) and five antisense-transfected clones with basal calcium levels >150 nM (Ca_i >150). * $P<0.05$.

characteristics of undifferentiated sarcoma (results not shown).

These studies support the contention that a chronic elevation in Ins(1,4,5)P_3 and calcium in the unstimulated cell is associated with cellular transformation, and implicates the 43 kDa inositol polyphosphate 5-phosphatase as a potential tumour suppressor gene.

Discussion

To investigate the effect of elevated Ins(1,4,5)P_3 and intracellular calcium on cell growth, we created stable cell lines that underexpress the 43 kDa 5-phosphatase enzyme. We have demonstrated that a decrease in cellular Ins(1,4,5)P_3 5-phosphatase activity results in a significant increase in Ins(1,4,5)P_3 and calcium levels in the unstimulated cell, which is associated with cellular transformation.

We have been unable to demonstrate by Western blot analysis that the 43 kDa protein is decreased in the antisense-transfected cells due to our lack of a suitable antibody. However, the cumulative evidence suggests that the antisense cDNA specifically results in a decreased expression of the 43 kDa 5-phosphatase. First, withdrawal of tetracycline resulted in induction of the antisense 43 kDa 5-phosphatase gene leading to a decrease in Ins(1,4,5)P_3 5-phosphatase enzyme activity in the total cell homogenate and specifically the Triton-soluble membrane fraction of the cell, which is consistent with the intracellular location of the 43 kDa enzyme (Laxminarayan *et al.*, 1993). Secondly, we have demonstrated that the principal substrates of the 43 kDa 5-phosphatase, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, are increased concomitantly. Finally, three independent antisense- or vector-transfected clones were employed in each experiment, with essentially the same result. This excludes the possibility that our results were due to clonal aberrations in the signalling system.

We have shown from our studies that a 50% decrease in total cellular Ins(1,4,5)P_3 5-phosphatase activity is sufficient to increase Ins(1,4,5)P_3 (2-fold) and calcium levels (1.9-fold) in the unstimulated cell. The 43 kDa 5-phosphatase has the greatest affinity for Ins(1,4,5)P_3 (K_m = 5 μM) and highest specific activity [250 μM Ins(1,4,5)P_3 hydrolysed/min/mg] of any 5-phosphatase described to date (Laxminarayan *et al.*, 1993). The remaining enzyme activity in antisense-transfected cells may be attributed to the 75 kDa enzyme [at least 30% of Ins(1,4,5)P_3 5-phosphatase activity in this cell line], or other 5-phosphatases, which have a lower affinity and less activity in hydrolysing Ins(1,4,5)P_3.

The role of Ins(1,4,5)P_3 in mitogenesis is controversial. Blockade of PtdIns(4,5)P_2 hydrolysis using antibodies to the phosphoinositide leads to a decreased mitogenic effect (Uno *et al.*, 1988; Han *et al.*, 1992). Virally transformed cells are dependent on calcium influx for proliferation and demonstrate an altered regulation of calcium homeostasis (Hartmann *et al.*, 1986; Rasmussen and Means, 1989; Ghosh *et al.*, 1991). In addition, microinjection of phospholipase C (PLC) into quiescent NIH 3T3 cells induces cell growth and morphologic transformation of the growth-arrested fibroblasts (Smith *et al.*, 1989). However, more recent studies support the contention that growth factor-stimulated mitogenesis and Ins(1,4,5)P_3 production are distinct signalling pathways. L'Allemain *et al.* (1989) first reported that phosphoinositide hydrolysis and calcium play no major role in the short-term events mediated by growth factors. Platelet-derived growth factor (PDGF)-stimulated Ins(1,4,5)P_3 production, the rise in intracellular calcium and mitogenesis do not correlate in cells over-expressing PLC-γ (Margolis *et al.*, 1990). Furthermore, disruption of PLC-γ signalling by a specific mutation in the fibroblast growth factor (FGF) receptor abrogates Ins(1,4,5)P_3 production but has no effect on its ability to induce mitogenic responses (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). These latter studies have investigated the short-term effects of growth factor stimulation. In

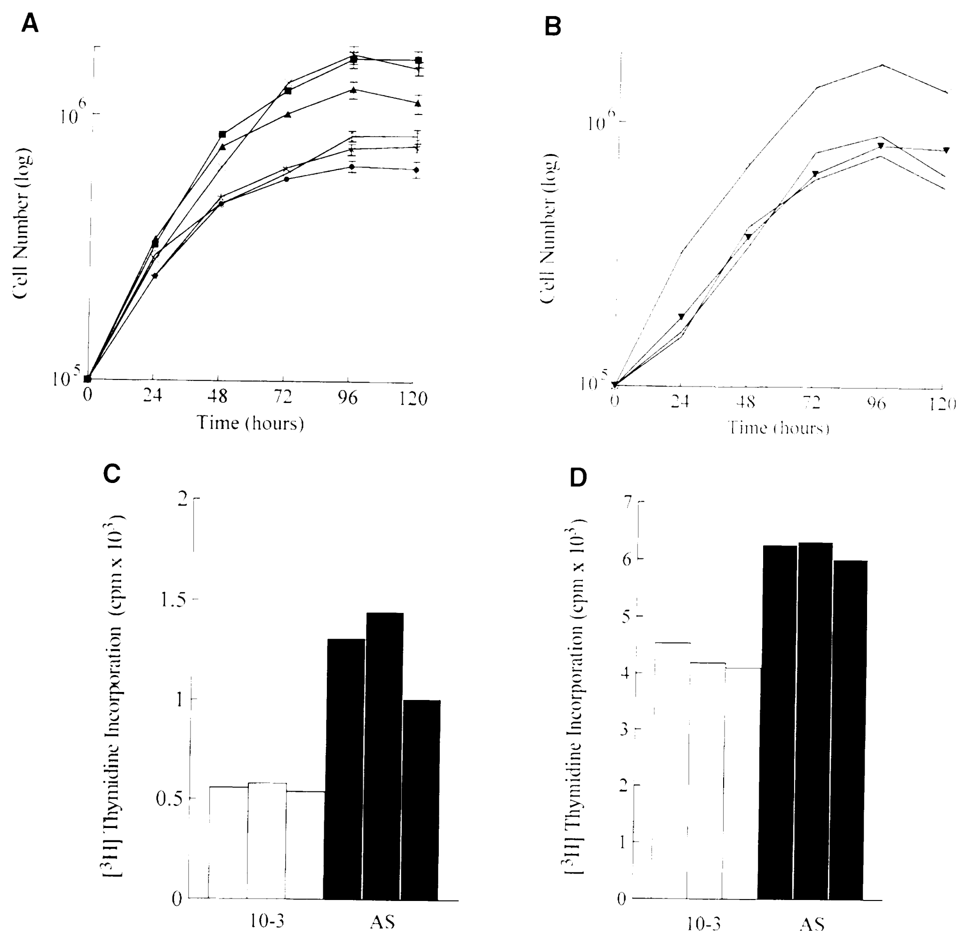

Fig. 4. Growth properties of cells underexpressing the 43 kDa membrane-associated 5-phosphatase. (**A**) Cells were plated at 1×10^5/ml and grown in DMEM with 10% FCS over a period of 120 h, as described in Materials and methods. Cell numbers were determined by counting in triplicate every 24 h using trypan blue exclusion. Cell counts are expressed as the mean and standard deviation of three separate experiments using three independent antisense- (■, ×, ▲) or vector-transfected (○, *, ●) clones. (**B**) Cells were plated as described above in the presence or absence of tetracycline (1 µg/ml). Cell numbers were determined by counting in triplicate every 24 h using trypan blue exclusion. Cell counts are from antisense cells in the presence (□) or absence (×) of tetracycline and vector-transfected cells in the presence (▼) or absence (○) of tetracycline. The results are representative of three similar experiments using three independent antisense- or vector-transfected clones. (**C**) Cells were cultured in DMEM with 0.5% FCS prior to labelling with 0.5 µCi/ml [^{3}H]thymidine for 3 h, as described in Materials and methods. Incorporation of [^{3}H]thymidine into DNA was determined by TCA precipitation and quantified by scintillation counting. The results are representative of at least three similar experiments, using three independent antisense- (AS) or vector-transfected (10-3) clones. (**D**) Cells were stimulated with 10% FCS for 18 h prior to labelling with 0.5 µCi/ml [^{3}H]thymidine as described above.

contrast, we have shown that chronic elevation of Ins(1,4,5)P_3 in the unstimulated cell is associated with an increase in the basal calcium level associated with mitogenesis, independent of growth factor stimulation. Overexpression of mutant Ins(1,4,5)P_3 receptors results in markedly suppressed cell growth in long-term growth cultures stimulated by serum, but does not effect epidermal growth factor-induced DNA synthesis (Fischer *et al.*, 1994). Human fibroblasts have been shown to proliferate independently of growth factor stimulation when the extracellular calcium concentration is increased from 0.1 to 1 mM (Huang *et al.*, 1995). Finally, it has been demonstrated recently that stable cell lines underexpressing the Type I Ins(1,4,5)P_3 receptor are unable to increase intracellular calcium which is required for antigen-specific T cell proliferation (Jayaraman *et al.*, 1995).

Recent studies have demonstrated that the intracellular calcium concentration is essential for several critical cell cycle events (reviewed in Means, 1994; Berridge, 1995). These include calcium-dependent activation of early genes responsible for induction of resting cells to re-enter the cell cycle. Calcium also stimulates completion of the cell cycle. Mitogen-activated calcium influx in early G_1 has been shown to be necessary for cell cycle progression in BALB/c 3T3 fibroblasts (Barbiero *et al.*, 1995). Finally, Ins(1,4,5)P_3 has been implicated as a mediator of the cell cycle calcium transients that trigger mitosis (Clapa *et al.*, 1994).

We have shown that the 43 kDa inositol polyphosphate 5-phosphatase plays a critical role in regulating the metabolic pathways that control intracellular calcium levels and thereby cell growth. Absence or loss of this enzyme activity from the cell is associated with cellular transformation, implicating the enzyme as a potential tumour suppressor gene. Decreased inositol polyphosphate 5-phosphatase activity has been associated with several human leukaemias and is implicated as a possible mechanism by which increased growth of leukaemia cells is achieved (Nye *et al.*, 1992; Mengubas *et al.*, 1994). We recently have mapped the human 43 kDa 5-phosphatase

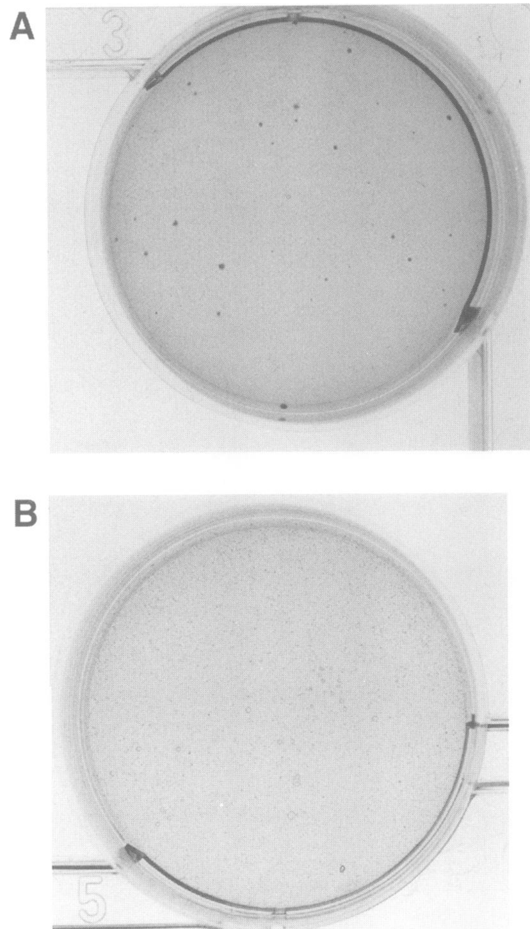

Fig. 5. Anchorage-independent growth in soft agar. Cells (1×10^5/ml) transfected with the antisense 43 kDa 5-phosphatase (**A**) or the pUHD 10-3 vector (**B**) were plated in semi-solid agar and assessed for their ability to grow anchorage independent over a 30 day period at 37°C, as described in Materials and methods.

Table II. Assessment of colony formation in soft agar

Cell clone	No. of colonies (mean ±SD)
10-3	5 ± 2
10-3	4 ± 2
10-3	5 ± 1
AS	27 ± 1
AS	42 ± 2
AS	34 ± 6

Colonies containing >50 cells were scored by staining with 0.1% gentian violet for 1 h. Results are expressed as the mean and standard deviation (SD) of three separate experiments, using three independent antisense- (AS) or vector-transfected (10-3) cell lines.

gene to chromosome 10q23.6 (Mitchell *et al.*, 1996). Acute and chronic leukaemias are not commonly associated with gene deletions or rearrangements of this chromosome, indicating that other mechanisms may be responsible for the observed decrease in 5-phosphatase enzyme activity in these human leukaemias. The only human disease described to date that results from an abnormality in an inositol polyphosphate 5-phosphatase is Lowe's oculocerebrorenal syndrome, which is associated with proximal tubular dysfunction, congenital cataracts and mental and growth retardation (Lowe *et al.*, 1952; Attree *et al.*,

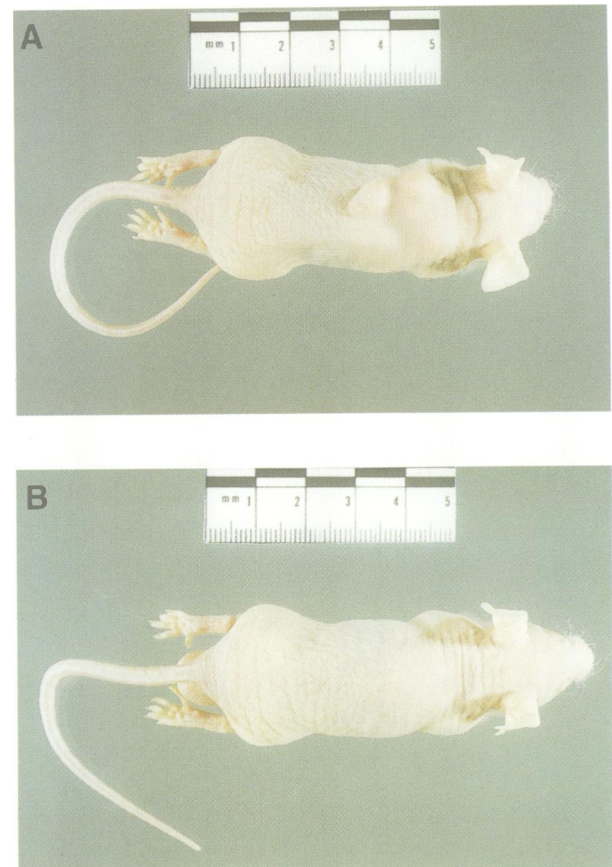

Fig. 6. Tumour formation in nude mice. Cells (5×10^6) from antisense- (**A**) or vector-transfected (**B**) clones were resuspended in 200 µl of serum-free DMEM and injected subcutaneously into the back of 6-week-old nude mice (BALB/c nu nu). Tumour formation was monitored over a 5 week period.

Table III. Assessment of tumour formation in nude mice

NRK cells	No. of mice injected	No. of mice with tumours	Tumour diameter (mm)
Vector-transfected (10-3)	10	3	1.5 ± 0.5
Antisense-transfected (AS)	18	16	6 ± 2

Tumour formation in mice injected with antisense- (AS) or vector-transfected (10-3) cells was assessed at 5 weeks post-injection. Tumours were excised and measured.

1992). Malignant transformation is not a feature of this disease. However, the Lowe's protein appears to be a Type II 5-phosphatase in that it predominantly hydrolyses PtdIns(4,5)P_2 rather than Ins(1,4,5)P_3 (Zhang *et al.*, 1995). Loss of function of the Type II 5-phosphatases should result in an increase in PtdIns(4,5)P_2 levels in the unstimulated cell, but whether Ins(1,4,5)P_3 levels are affected remains to be determined. In contrast, the Type I 5-phosphatases, of which the 43 kDa 5-phosphatase is a member, preferably hydrolyse Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and do not hydrolyse PtdIns(4,5)P_2. These observations imply that the regulation of Ins(1,4,5)P_3 and intracellular calcium, rather than PtdIns(4,5)P_2, is critical for cellular transformation.

Materials and methods

Materials

DNA-modifying enzymes were purchased from New England Biolabs, Inc. Dulbecco's modified Eagle's medium (DMEM) and tetracycline-HCl were purchased from ICN Biomedicals, Inc. Geneticin (G418 sulfate) was obtained from Gibco. NRK cells (clone 49F) were obtained from American Type Culture Collection. Ortho [^{32}P]phosphoric acid, [^{3}H]*myo*-inositol (20.5 Ci/mmol) and [methyl-^{3}H]thymidine (6.7 Ci/mmol) were obtained from Dupont-New England Nuclear. Partisal-10 SAX HPLC columns were from Waters Corp. Fura-2 AM was from Molecular probes. All other chemicals were obtained from Sigma Chemical Company, USA.

Transfection of NRK fibroblasts with the 43 kDa inositol polyphosphate 5-phosphatase.

The cDNA encoding the 43 kDa membrane-associated 5-phosphatase (Laxminarayan *et al.*, 1994) was stably underexpressed in NRK cells utilizing a tetracycline-inducible expression system (tet-regulatory system) (Gossen and Bujard, 1992; Resnitzky *et al.*, 1994). Briefly, the tet-regulatory system consists of three major components: the regulator plasmid (pUHD 15-1), which encodes a cytomegalovirus promoter/enhancer, the coding sequence for tetracycline and a SV40 polyadenylation site; the response plasmid (pUHD 10-3), which contains a tetracycline-dependent promoter and cloning sites for the insertion of foreign DNA; and the effector substance, tetracycline. The presence of tetracycline-HCl (1 μg/ml) in the cell culture medium renders the system inactive, hence the gene of interest is not expressed. Induction of the cloned gene is obtained by withdrawing tetracycline from the cell culture medium for 24 h.

The 2.6 kb 43 kDa 5-phosphatase cDNA was ligated in the antisense orientation into the *Eco*RI site of the response plasmid, pUHD 10-3. NRK fibroblasts (previously stably transfected with the functional regulator plasmid pUHD 15-1 and kindly donated by Graham Baldwin and Qun-Xing Zhang, Ludwig Institute for Cancer Research, Melbourne) were co-transfected with the pUHD 10-3–antisense 43 kDa 5-phosphatase (AS) construct and a neomycin resistance cassette, by the calcium phosphate precipitation method (Sambrook *et al.*, 1989). Control cells were co-transfected simultaneously with the pUHD 10-3 plasmid alone (10-3) and the neomycin resistance cassette. Cells were cultured in selection medium containing 1 μg/ml tetracycline-HCl and 0.4 μg/ml geneticin and antibiotic-resistant clones were selected 3–4 weeks later and assessed for Ins(1,4,5)P_3 5-phosphatase activity.

Cell culture

Cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 0.1% streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Following stable transfection, NRK cells were cultured in the presence of 1 μg/ml tetracycline–HCl.

Inositol polyphosphate 5-phosphatase enzyme assay

Hydrolysis of Ins(1,[^{32}P]4,[^{32}P]5)P_3 was measured by extraction of released $^{32}PO_4$ as previously described (Connolly *et al.*, 1985). The concentration of Ins(1,[^{32}P]4,[^{32}P]5)P_3 used in assays was 30 μM, and it was isolated from erythrocyte ghosts as previously described (Downes *et al.*, 1982).

Isolation of cell cytosol and detergent-soluble membrane fractions

Cell culture media were aspirated and the adherent cells were washed three times in imidazole buffer (10 mM imidazole pH 7.2, containing 1 mM $MgCl_2$, 1 mM EDTA, 0.3 M sucrose, 5 mM 2-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride). The cells were scraped from the plate, resuspended in 500 μl of imidazole buffer and sonicated on ice for 15 s to obtain the total cell homogenate. The sample was then subjected to ultracentrifugation at 100 000 *g* for 1 h and the supernatant collected as the cell cytosol fraction. The pellet was resuspended in 200 μl of imidazole buffer containing 1% Triton X-100 and detergent-soluble membrane proteins were extracted at 4°C with mixing for 1 h. The solution was ultracentrifuged at 100 000 *g* for 1 h and the supernatant collected as the Triton-soluble membrane fraction. The cell samples were then assayed for Ins(1,4,5)P_3 5-phosphatase activity.

Isolation of ^{3}H-labelled inositol phosphates in [^{3}H]inositol-labelled fibroblasts

Cells were seeded onto 35 mm dishes at a density of 5×10^4 cells/ml and cultured for 24 h in the absence of tetracycline in DMEM containing 10% FCS. Cells were then labelled with 2 μCi/ml [^{3}H]inositol in inositol-free DMEM containing 0.5% FCS for 48 h. Extraction of inositol phosphates was based on the method described by Ross *et al.* (1991). Cells were lysed by the addition of 250 μl of water-saturated phenol. The phenol extract was placed into a 1.5 ml tube and the plates were washed with another 250 μl of phenol and placed into a separate tube. The dishes were washed with two 500 μl volumes of 10 mM Tris–HCl pH 7.5, 1 mM EDTA and this was added to the phenol samples. Chloroform (250 μl) was added to each tube and the extracts were mixed vigorously for 1 min. Organic and aqueous phases were separated by centrifugation (35 000 g for 5 min) and the top phase was washed a further twice with 500 μl of chloroform to remove residual phenol. The inositol phosphates were separated on a Partisal-10 SAX HPLC column at a flow rate of 1 ml/min (0–0.18 M ammonium phosphate over 5 min; 0.18 M for 45 min, 0.18–1 M over 40 min). Radioactive peaks were identified by comparison with ^{3}H-labelled inositol phosphate standards. Protein measurements were made on unlabelled cells cultured under identical conditions.

Intracellular calcium measurements

Cell monolayers (on coverslips) were serum deprived for 24 h in the absence of tetracycline in DMEM containing 0.5% FCS prior to loading with 10 μM Fura-2 AM in 10% Pluronic detergent for 40 min at 37°C. The cells on coverslips were washed three times in physiological salt solution (20 mM HEPES pH 7.4, 4 mM KCl, 140 mM NaCl, 1 mM $MgSO_4$, 10 mM glucose, 1.8 mM $CaCl_2$ at 37°C) and kept in the dark at room temperature until use, which was within 1 h of loading. The coverslip was inserted into a standard cuvette containing physiological salt solution and a stirring device. The cuvette was placed into a thermostatically controlled chamber at 37°C in a SPEX dual-wavelength 1681 fluorolog spectrometer. Excitation wavelengths were 340 and 380 nm, and emitted light collected at 505 nm. Readings were recorded using dM3000 software (Spex Industries, Inc., Edison, NJ). Agonists were added by direct injection of 10 μl volumes of stock solutions into the cuvette. Calcium values were corrected for cell autofluorescence at each wavelength by the addition of 2 μM ionomycin and 2 mM $MnCl_2$. Total intracellular calcium stores were determined based on the method described by Margolis *et al.* (1990). Lanthanum chloride was added to a final concentration of 200 μM to block calcium entry. Total intracellular calcium stores were quantitated following the addition of 2 μM ionomycin. The intracellular calcium concentration was determined as previously described (Neylon *et al.*, 1992).

Cell growth studies

Cells were seeded onto 35 mm dishes at a starting density of 1×10^5 cells/ml in the absence of tetracycline in DMEM with 10% FCS. Cells were counted daily in triplicate by trypan blue exclusion over a 5 day period or until confluent.

Measurement of [^{3}H]thymidine incorporation

Cells were seeded onto duplicate wells of a 24-well tray at a density of 1×10^3 cells/ml in DMEM with 10% FCS in the absence of tetracycline. Cells were grown for 24 h prior to serum starving for 48 h in DMEM containing 0.5% FCS. One of the duplicate dishes was stimulated with 10% FCS for 18 h prior to [^{3}H]thymidine incorporation. The cells were then pulsed with 0.5 μCi/ml [^{3}H]thymidine for 3 h and harvested by washing in ice-cold phosphate-buffered saline (10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4, 140 mM NaCl, 3 mM KCl) prior to fixing for 10 min with acetic acid:methanol (3:1). The soluble radioactivity was extracted with 10% trichloroacetic acid (TCA) for 30 min at 4°C and precipitates lysed in 0.2 M NaOH at 37°C for 2 h. The incorporated radioactivity was quantitated by scintillation counting. Cell counts were performed on unlabelled cells cultured under identical conditions.

Colony formation in soft agar

Cells (1×10^5/ml) were plated in 6-well trays with DMEM containing 10% FCS and 0.3% agar. The plates previously had been coated with a bottom layer of DMEM containing 10% FCS and 0.5% agar. Colonies were scored after 30 days at 37°C by staining in 0.1% gentian violet for 1 h at room temperature. Only colonies with >50 cells were counted. Assays were performed in triplicate.

Tumour formation in nude mice

Cells (5×10^6) from three independent antisense-transfected or three vector-transfected cell lines were resuspended in 200 μl of serum-free DMEM medium and injected subcutaneously into the back of 6-week-old athymic nude mice (BALB/c nu nu strain). Tumour formation was

monitored over a 5 week period, after which time the mice were killed, the tumours excised, measured and prepared for histological examination (as described by Prophet *et al.*, 1994).

Miscellaneous methods

Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

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