Transformation of Rat-1 fibroblasts with the v-*src* oncogene induces inositol 1,4,5-trisphosphate 3-kinase expression

Pamela J. WOODRING* and James C. GARRISON

Department of Pharmacology and Cancer Research Center, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A.

Transformation of Rat-1 fibroblasts with the v-*src* oncogene leads to a 6- to 8-fold enhancement of the activity of the Ins(1,4,5)P_3 3-kinase in cytosolic extracts [Johnson, Wasilenko, Mattingly, Weber and Garrison (1989) Science **246**, 121–124]. This study confirms these results using another v-*src*-transformed Rat-1 cell line (B31 cells) and investigates the molecular mechanism by which pp60$^{v\text{-}src}$ activates Ins(1,4,5)P_3 3-kinase. The mRNA and protein levels for two rat isoforms of Ins(1,4,5)P_3 3-kinase were determined in the v-*src*-transformed cell line. Both the mRNA and protein levels for isoform A were elevated in v-*src*-transformed Rat-1 cells while those for isoform B were not significantly affected. Moreover, stable expression of either form of Ins(1,4,5)P_3 3-kinase in the B31 v-*src*-transformed Rat-1 cell line did not result in tyrosine phosphorylation of Ins(1,4,5)P_3 3-kinase A or B. These results suggest that at least one mechanism by which the v-*src* oncogene increases the activity of the Ins(1,4,5)P_3 3-kinase in the Rat-1 transformed fibroblast is by increasing the level of expression of Ins(1,4,5)P_3 3-kinase A.

INTRODUCTION

It is now widely accepted that activation of phospholipase C results in the cleavage of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) producing two second messenger molecules: diacylglycerol and Ins(1,4,5)P_3 [1]. The latter releases calcium from non-mitochondrial intracellular compartments by binding to and activating Ins(1,4,5)P_3-gated, calcium-permeable ion channels [2–4]. Termination of the Ins(1,4,5)P_3 signal occurs either by the action of the polyphosphate 5-phosphatase, which removes a phosphate from the inositol ring producing inositol 1,4-bisphosphate, or by the Ins(1,4,5)P_3 3-kinase, which adds a phosphate to the inositol ring producing Ins(1,3,4,5)P_4 [5]. This is an important branch point in the metabolism of inositol phosphates because this step determines whether the inositol moieties will be recycled back to *myo*-inositol or will be used as substrates for the production of more phosphorylated inositol polyphosphates such as InsP_4, InsP_5 and InsP_6. The physiological functions of many of these higher-order inositol polyphosphates remains unclear despite extensive experimentation. Some recent progress has been made in determining the role of the immediate product of the Ins(1,4,5)P_3 3-kinase, Ins(1,3,4,5)P_4. A specific Ins(1,3,4,5)P_4-binding protein was purified, cloned and demonstrated to act as a GTPase-activating protein specific for *ras* [6]. Other studies have implicated Ins(1,3,4,5)P_4 as a potential second messenger acting in conjunction with Ins(1,4,5)P_3 to maintain cellular calcium homoeostasis [7].

Cloning and characterization of Ins(1,4,5)P_3 3-kinase rat cDNAs has revealed two isoforms: the A form, originally isolated from rat brain [8,9], and the B form, originally from rat liver and thymus [10,11]. There is a tissue-specific expression of the mRNAs with the 2 kb 'A' mRNA detected in brain and testes, while the 6 kb 'B' mRNA has been found in several tissues including lung, thymus, testes, brain and heart [11]. The two proteins are 68% homologous in the C-terminal catalytic region while the N-terminal regulatory regions show little sequence similarity. In addition, the regulatory region of the B isoform is 200 amino acids longer than isoform A [10].

Several regulatory mechanisms have been implicated as means of controlling Ins(1,4,5)P_3 3-kinase activity, i.e. calcium–calmodulin stimulation, enzyme phosphorylation and proteolysis [12]. Stimulation by calcium–calmodulin has been observed in a number of different cell and tissue types including rat brain [8,13–15], rat liver [16], rat thymus [11], bovine brain [17], bovine parathyroid [18], porcine smooth and skeletal muscle [19,20], human lymphocytes [21] and human platelets [22,23]. The magnitude of the stimulation is dependent on the source of the enzyme and ranges from 1.5- to 17-fold over basal activity. Additionally, kinases such as the cAMP-dependent protein kinase and protein kinase C may regulate Ins(1,4,5)P_3 3-kinase activity. Treatment of intact cells with phorbol esters and/or cAMP analogues has been reported to stimulate Ins(1,4,5)P_3 3-kinase activity [24–26]. In contrast, *in vitro* experimentation with purified proteins exhibited a stimulation of activity by cAMP-dependent protein kinase and an inhibition of activity by protein kinase C [27,28].

In addition to the serine/threonine kinases, the tyrosine kinase pp60$^{v\text{-}src}$ has been reported to significantly induce Ins(1,4,5)P_3 3-kinase activity [29], an effect which may be the critical step leading to the overproduction of higher inositol polyphosphates in v-*src*-transformed cells [30]. Since these transformed cells offered the opportunity to explore new modes of regulation of Ins(1,4,5)P_3 3-kinase, the mechanism by which v-*src* increased the enzyme activity was characterized in this study. The results show that pp60$^{v\text{-}src}$ elevates mRNA and protein levels of Ins(1,4,5)P_3 3-kinase A to an extent which could account for the increased activity present in v-*src*-transformed Rat-1 cells. Additionally, when either of the Ins(1,4,5)P_3 3-kinase isoforms was overexpressed in v-*src*-transformed Rat-1 cells, there was no observed tyrosine phosphorylation of the protein.

Abbreviations used: BCIP, 5-bromo-1-chloro-3-indolyl phosphate; FLAG®, the eight amino acid sequence DYKDDDDK; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NBT, Nitro Blue Tetrazolium; PIP_2, phosphatidylinositol 4,5-bisphosphate.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Unlabelled Ins(1,4,5)P_3 was purchased from LC Services, and [^{3}H]Ins(1,4,5)P_3 and [^{32}P]dCTP from Du Pont-New England Nuclear. [^{32}P]CTP and Sequenase were from Amersham. Dowex AG1-X2 (formate form, 100–200 mesh) and protein molecular-mass standards were from Bio-Rad. Monoflow 4 scintillant was purchased from National Diagnostics. Calpain inhibitors I and II were from Calbiochem. Pefabloc and RNA molecular-mass markers were from Boehringer Mannheim. All other protease inhibitors were purchased from Sigma. Guanidine isothiocyanate was from GIBCO/BRL. Multiple-tissue Northern blots and the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) amplification primers were from Clontech. The ribonuclease protection assay kit was purchased from Ambion. The *in vitro* transcription kit, the prime-a-gene labelling system, the altered sites *in vitro* mutagenesis kit, restriction enzymes, pGEM plasmid, anti-(mouse IgG) alkaline phosphatase-conjugated antibodies, Nitro Blue Tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP) were purchased from Promega. Formamide and anti-(M2 FLAG® epitope) antibodies were from the Eastman Kodak Company and anti-phosphotyrosine antibodies from Upstate Biotechnology Incorporated. Protein A–Sepharose was purchased from Pharmacia. The pATH plasmid was a gift from Dr. K. R. Lynch (University of Virginia). The pDoubleTrouble plasmid was a gift from Dr. J. Linden (University of Virginia).

Ins(1,4,5)P_3 3-kinase activity assay

The maintenance of Rat-1 cells was as described in [29]. Cells were grown to near confluence for all experiments unless otherwise indicated. Typically four 150-mm^2 plates of each cell line were used for each cytosol preparation. Homogenates were prepared from normal and v-*src*-transformed Rat-1 cells at 4 °C using a glass dounce homogenizer. Cells were homogenized in 1 ml of buffer containing 50 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 2.5 mM EGTA and 0.5 mM EDTA supplemented with a cocktail of protease inhibitors, (10 μg/ml calpain inhibitors I and II, 100 μg/ml pefabloc, 2.5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml bacitracin and 20 μg/ml benzamidine). For tissues, samples were pulverized in the above buffer using a Polytron (three 15 s pulses at setting 6). Unbroken cells were removed by centrifugation at 200 ***g*** at 4 °C and the resulting supernatant was centrifuged at 100000 ***g*** for 1 h at 4 °C. Typically 25–50 μg (as determined by the method of Lowry et al. [31]) of supernatant protein from either tissues or Rat-1 cells was used to initiate enzyme assays, in a final reaction volume of 100 μl. Enzyme activity was measured at 25 °C in a buffer containing 10 mM Hepes (pH 7.0), 7 mM $MgSO_4$, 110 mM KCl, 10 mM NaCl, 3 mM EGTA, 5 mM ATP, 5 mM 2,3-diphosphoglycerate (Sigma), 5 mM cysteine, 0.1 mg/ml BSA, 1–50 μM unlabelled Ins(1,4,5)P_3 and 3000 c.p.m. of [^{3}H]Ins(1,4,5)P_3. The calculated free calcium concentration was adjusted to 1 μM using the ligand-binding program, EQ-CAL (Biosoft, Ferguson, MO, U.S.A.). After a 1–10 min incubation, the enzyme reaction was quenched by the addition of ice-cold 0.4 M ammonium formate/0.1 M formic acid. Samples were briefly centrifuged and applied directly to 1 ml Dowex 1-X8 columns which were washed sequentially with 5 ml of water, 5 ml of 0.4 M ammonium formate/0.1 M formic acid to release InsP_2, 15 ml of 0.8 M ammonium formate/0.1 M formic acid to release InsP_3, and finally 15 ml of 1.5 M ammonium formate/0.1 M formic acid to elute InsP_4. Samples were diluted with scintillant and inositol phosphates were quantified by liquid scintillation counting. Formation of Ins(1,3,4,5)P_4 was confirmed by strong anion-exchange HPLC [30]. Using brain cytosolic protein, the Ins(1,4,5)P_3 3-kinase activity assay was linear with time up to 5 min (for 50 μg) and linear with protein from 10 μg to 500 μg. To measure Ins(1,4,5)P_3 3-kinase activity after SDS/PAGE, the samples were prepared and assayed as described in [22].

Cloning of rat Ins(1,4,5)P_3 3-kinases

Ins(1,4,5)P_3 3-kinase B isolated from a rat liver cDNA library was a generous gift from Dr. G. Banting (University of Bristol, U.K.) [10]. Base 1795 was changed from thymine to cytosine to correct an error in the original cDNA (gorf 34.1). Briefly, *in vitro* mutagenesis was performed using Promega's Altered Sites II *in vitro* mutagenesis system. The oligonucleotide used to convert the mistake was 5′-CCCTGGATCCAGCTGGCAGG-3′. The *Kpn*I 3.05 kb restriction fragment of gorf 34.1 was subcloned into the pALTER-1 plasmid and the procedure for the mutagenesis was followed as indicated in the instruction manual, with the modification of using the *Escherichia coli* strain BMH71-18 instead of ES1301. A 2.1 kb fragment of the coding region of gorf 34.1, from 592 bp to 2643 kb, was used for Northern blots and hybridizing bands were confirmed using an oligonucleotide designed within this region. Restriction fragments used for the ribonuclease protection assay [*Kpn*I (15 bp)–*Pst*I (235 bp) and *Sst*I (1004 bp)–*Pst*I (1456 bp)] were subcloned into the pGEM7z plasmid and linearized with an enzyme that cut at a site in the polylinker to make run-off transcripts of 255 bp and 512 bp respectively.

Ins(1,4,5)P_3 3-kinase A was isolated from a rat testes cDNA library in the λ ZAP II vector (Stratagene) using a PCR fragment (1.42 kb) of the Ins(1,4,5)P_3 3-kinase A coding region as a probe to screen 1.5×10^6 recombinant phages. Filters were hybridized at 42 °C in a solution containing 5 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 10 × Denhardt's [1 × Denhardt's = 1 % (w/v) Ficoll, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) BSA], 50 % (v/v) formamide, 2 % (w/v) SDS, 0.1 mg/ml herring sperm DNA, and random-primed probe labelled to a specific radioactivity of 1×10^9 c.p.m./μg. Filters were washed for 1 h at room temperature in 2 × SSC/0.05 % SDS (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7), then three times for 20 min in 2 × SSC/0.05 % SDS at 50 °C, twice for 20 min in 0.2 × SSC/0.1 % SDS at 50 °C, and twice for 15 min in 0.1 × SSC/0.1 % SDS at 50 °C. Positive plaques were purified and the full-length clone was obtained and sequenced using dideoxy chain termination sequencing with Sequenase. The same probe used to isolate the clone was used to probe Northern blots. Restriction fragments used for the ribonuclease protection assay were *Bam*HI (−26 bp) to *Csp*45I (160 bp) and *Csp*45I (160 bp) to *Xho*I (443 bp).

Northern blot analysis

Northern blots, other than the multiple-tissue Northerns purchased from Clontech, were prepared in the following manner. Total RNA was extracted from whole rat brain or rat testes purchased from Pel-freez Biologicals (10 g) or tissue culture cells ($\sim 10^7$ cells) by homogenization in 10 ml of guanidine isothiocyanate solution followed by centrifugation through caesium chloride as described in [32]. Poly(A) RNA was isolated using oligo(dT) chromatography [33]. RNA was electrophoresed through 1.2 % (w/v) agarose gels containing 6.1 % (v/v) deionized formaldehyde and subsequently transferred to nylon membranes. RNA was cross-linked to the membrane by either

baking in a vacuum oven for 2 h at 80 °C, or exposing the blot to UV radiation with the Stratagene Stratalinker oven. Membranes were then incubated with random-primer-labelled DNA probes. Hybridization was performed at 42 °C in solutions containing 50 % (v/v) deionized formamide as described above. For oligonucleotide probes, the hybridization temperature was increased to 50 °C and formamide omitted. Blots were washed at 55 °C for 20 min each in 2 × SSC/0.05 % SDS, 0.2 × SSC/0.1 % SDS and 0.1 × SSC/0.1 % SDS.

Ribonuclease protection assay

Poly(A) RNA was isolated either as described above or by using Qiagen's Oligotex-dT mRNA kits. Ribonuclease protection assays were performed according to the manufacturer's instructions using the Ambion RPA II kit. RNA probes were transcribed from restriction fragments of Ins(1,4,5)P_3 3-kinase DNA subcloned in the pGEM plasmid as described in the kit. Typically, 5–10 μg of poly(A) RNA was incubated at 42 °C with 4×10^5 c.p.m. of riboprobe. Ribonuclease (A and T1)-digested samples were then electrophoresed on 7 M urea/8 % polyacrylamide sequencing gels. Gels were fixed, then dried and exposed to Kodak X-OMAT film overnight. Autoradiographs were scanned using the BioImage scanning densitometer and quantified using the Whole Band software.

SDS/PAGE and Western blotting

Whole-cell lysates were prepared from normal and v-*src*-transformed Rat-1 cells and electrophoresed on 10 %- or 12 %-polyacrylamide/SDS gels. Resolved proteins were transferred to nitrocellulose and Western blotting was performed in the following manner. Membranes were blocked for 1 h with either 5 % (w/v) BSA or 3 % (w/v) non-fat dry milk in 50 mM Tris (pH 8), 150 mM NaCl, 0.1 % (v/v) Tween-20 (TBST). Blots were then incubated at 37 °C for 2 h with the primary antibody, briefly washed in TBST, then further incubated with anti-(mouse IgG) conjugated to alkaline phosphatase, diluted 1:7500. Membranes were washed for 20 min in TBST and the alkaline phosphatase colour development substrates (NBT and BCIP) were added. The reaction was terminated by rinsing with water.

Production of polyclonal antibodies against rat Ins(1,4,5)P_3 3-kinase A

The rat Ins(1,4,5)P_3 3-kinase A cDNA was subcloned into the pATH vector in-frame with the *trpE* fusion protein at the N-terminal end. *TrpE*–Ins(1,4,5)P_3 3-kinase A fusion protein was prepared in bacteria as described in [34]. After bacterial proteins were electrophoresed on 3-mm-thick 10 % polyacrylamide/SDS gels, the fusion protein was excised from the gel and homogenized in PBS. Identification of the fusion protein was confirmed by Western blotting with a mixture of monoclonal antibodies against Ins(1,4,5)P_3 3-kinase (a generous gift from Dr. S. G. Rhee, National Institutes of Health [14]). After rocking overnight at 10 °C, the gel pieces were removed by centrifugation and the supernatant was dialysed against PBS overnight. Dialysed sample was then concentrated using an Amicon concentrator fitted with a YM30 filter and protein concentration was determined using the Pierce BCA protein assay. Rabbits were immunized with 150–300 μg of gel-purified fusion protein as described in [35]. Antiserum was collected and purified using a Protein G column then tested for recognition of the Ins(1,4,5)P_3 3-kinase A antigen present in rat brain homogenates by Western blotting and immunoprecipitation.

Overexpression and immunoprecipitation of Ins(1,4,5)P_3 3-kinase A

Ins(1,4,5)P_3 3-kinase A was subcloned into the pDoubleTrouble plasmid in-frame with the FLAG® epitope and the hexahistidine tail as described in [36]. This construct and the plasmid without the insert were each separately transfected into B31 cells [37] and stable cell lines were established using neomycin resistance as a selectable marker. Clones were purified by single-cell dilution and were tested for Ins(1,4,5)P_3 3-kinase expression using Western blotting with the anti-(FLAG® M2) antibody. Stable cell lines had 150-fold (isoform A), or 12-fold (isoform B), greater Ins(1,4,5)P_3 3-kinase activity than the vector-transfected cells. For immunoprecipitations, each 100-mm² dish was placed on ice and washed three times with PBS, then lysed with 1 ml of ice-cold RIPA buffer [50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS] supplemented with protease and phosphatase inhibitors (10 μg/ml calpain inhibitors I and II, 100 μg/ml pefabloc, 2.5 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml bacitracin, 20 μg/ml benzamidine, 1 mM sodium vanadate, 4 mM *p*-nitrophenyl phosphate). RIPA extract (0.1–1 mg of protein) was precleared with preimmune serum and then incubated for 1 h at 4 °C with 2–10 μl of Ins(1,4,5)P_3 3-kinase A immune serum. Protein A–Sepharose [10–30 μl of a 50 % (v/v) solution] was added and the incubation continued for an additional hour. The mixture was then centrifuged and the resulting pellet was washed three times with RIPA buffer and once with PBS. Typically, 20–100 μl of gel loading buffer was added to the final pellet and boiled for 5 min before loading on to protein gels.

RESULTS

Establishment of the system

Expression of the v-*src* protein has been previously reported to cause an increase in Ins(1,4,5)P_3 3-kinase activity. This effect remained after crude extracts were partially purified and was not affected by addition of calmodulin to the assay medium [29]. Another Rat-1 fibroblast cell line infected with the Rous sarcoma virus (B31 *src* cells [37] which will be referred to as v-*src* Rat-1 cells) shows a similar increase in activity, as shown in Table 1. This result substantiates our previous finding in v-*src*-transformed Swiss 3T3 and Rat-1 fibroblasts and is consistent with the hypothesis that the effect is specific for pp60$^{v\text{-}src}$. The increase in Ins(1,4,5)P_3 3-kinase activity in B31 *src* cells was approx. 7-

Table 1 Ins(1,4,5)P_3 3-kinase activity in rat cytosolic extracts

Cytosolic extracts were prepared from either stable cell lines or rat tissues as described in the Materials and methods section. A 25 μg sample of extract was used for each assay. Assays were performed in the presence of the 5-phosphatase inhibitor 2,3-diphosphoglycerate, for 1–3 min at an Ins(1,4,5)P_3 concentration of 1–6 μM. Data shown are the mean ± S.E.M. for at least four independent extract preparations per sample. Formation of [^{3}H]Ins(1,3,4,5)P_4 from [^{3}H]Ins(1,4,5)P_3 was confirmed by strong anion-exchange HPLC separation as previously described [30].

Extract source	Ins(1,4,5)P_3 3-kinase activity (pmol/min per mg)	Increase over Rat-1 cells (fold)
Rat-1 cells	172 ± 10	1.0
v-*src* Rat-1 cells	1229 ± 79	7.1
Rat brain	981 ± 51	5.7
Rat testes	386 ± 24	2.2

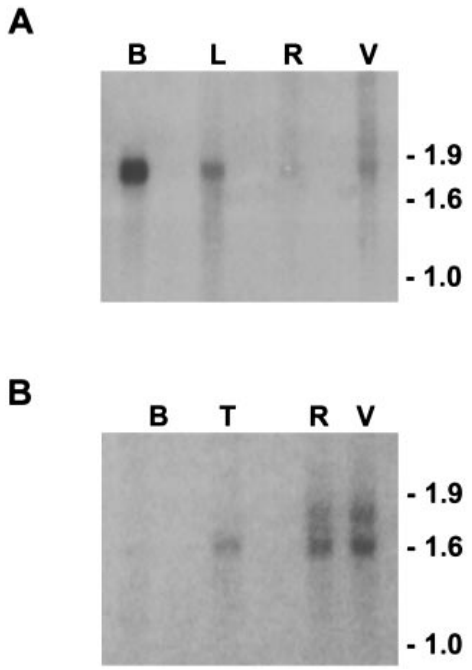

Figure 1 Comparison of Ins(1,4,5)P_3 3-kinase mRNA expression levels in Rat-1 and v-*src*-transformed Rat-1 cells

(**A**) Northern blot analysis showing Ins(1,4,5)P_3 3-kinase A mRNA expression in 7 μg of Rat-1 (R), and 7 μg of v-*src*-transformed Rat-1 (V) poly(A) RNA. Two μg of rat brain (B) and 7 μg of rat liver (L) poly(A) RNA were included to serve as positive controls. Immobilized RNA was hybridized to a [^{32}P]dCTP random-primer-labelled 1.4 kb fragment of cDNA comprised of the coding region of Ins(1,4,5)P_3 3-kinase A. The hybridizing mRNA from all four sources was 1.8 kb. Data are representative of three separate Northern blot experiments. (**B**) Northern blot analysis of Ins(1,4,5)P_3 3-kinase B mRNA expression. Two μg of poly(A) RNA each from rat brain (B), and rat testes (T), and eight μg of poly(A) RNA each from Rat-1 (R), and v-*src*-transformed Rat-1 cells (V) were blotted and probed with a 2.1 kb fragment of cDNA comprising the coding region of Ins(1,4,5)P_3 3-kinase B. Two hybridizing bands were detected, one at 1.8 kb and one at 1.6 kb. RNA molecular-mass-marker positions are indicated in kilobases on the right-hand side of the blots. Data are representative of two separate Northern blot experiments.

fold, an increase which resembles that found in tsLa29 *src*-infected cells and pp60$^{v\text{-}src}$-transfected cells [29]. In addition, 90 % of the activity was found in the cytosol and the effect was not altered by the addition of up to 10 μM calmodulin in crude cytosolic extracts. The v-*src*-transformed Rat-1 Ins(1,4,5)P_3 3-kinase activity is slightly greater than that seen in rat brain cytosolic extracts and is about 4-fold greater than that observed in rat testes cytosolic extracts. Subsequent HPLC analysis of the inositol phosphates generated in the assays confirmed that the measured inositol phosphate formed from Ins(1,4,5)P_3 was indeed Ins(1,3,4,5)P_4.

Ins(1,4,5)P_3 3-kinase mRNA distribution in rat cells and tissues

To determine whether increased levels of Ins(1,4,5)P_3 3-kinase mRNA were present in the transformed cells, mRNA was extracted from control and transformed cells and the purified poly(A) RNA was probed with DNA sequences specific for the A and B isoforms of rat Ins(1,4,5)P_3 3-kinase. The Rat-1 fibroblast contains very low levels of mRNA for the Ins(1,4,5)P_3 3-kinase A and B. Signals were barely detectable even after a month-long exposure of a Northern blot probed with Ins(1,4,5)P_3 3-kinase cDNAs labelled to high specific activities (Figure 1). The Ins(1,4,5)P_3 3-kinase A mRNA was 1.8 kb and present at high levels in rat brain. In comparison, rat liver and v-*src*-transformed cells contained much lower levels, while no signal was detected in normal Rat-1 fibroblasts (Figure 1A). The v-*src* Rat-1 cells always contained a stronger signal for the isoform A mRNA compared with normal cells ($n = 3$). In contrast, isoform B mRNA was not detectable in rat brain (Figure 1B). Rat testes contained a detectable mRNA band at 1.6 kb, and sometimes bands were observed at 4.4 kb and 6 kb (results not shown on this blot). The longer mRNAs were never present in normal or v-*src*-transformed fibroblasts; however, these cell lines did contain a 1.8 kb mRNA band in addition to the 1.6 kb band found in testes. The intensity of this 1.6 kb signal did not appear to be different between the two cell lines. Northern blots were stripped and reprobed with the G3PDH cDNA to verify that equal amounts of mRNA were loaded in each lane. No differences were observed (results not shown).

The same two Ins(1,4,5)P_3 3-kinase cDNA probes were used to examine the size and abundance of mRNA in other rat tissues including testes, kidney, skeletal muscle, liver, lung, spleen and heart. As expected from previous reports [11] the 1.8 kb Ins(1,4,5)P_3 3-kinase A mRNA was abundant in rat brain and testes on these multiple-tissue Northern blots (results not shown). Rat testes had an additional Ins(1,4,5)P_3 3-kinase A mRNA species migrating at 4.0 kb. The Ins(1,4,5)P_3 3-kinase B mRNA was 6 kb unlike the size found in normal and v-*src*-transformed rat fibroblasts. This size was apparent in all of the above rat tissues examined except brain. Some tissues contained additional shorter mRNA species: testes, 4.4 kb and 1.6 kb; kidney, 4.4 kb; skeletal muscle, 2.6 kb and 1.8 kb; and liver, 3 kb (results not shown). These may represent mRNAs which are either alternatively spliced or are similar enough in sequence to bind to the Ins(1,4,5)P_3 3-kinase B cDNA. If the latter is true, then it is probable that the 1.8 kb mRNA which bound to the Ins(1,4,5)P_3 3-kinase B probe observed in Rat-1 fibroblasts (Figure 1B) is actually the Ins(1,4,5)P_3 3-kinase A isoform mRNA because of the sequence similarity that exists between the two mRNAs.

In order to eliminate this possibility of cross-hybridization and also to increase the sensitivity of the detection method, ribonuclease protection assays were performed. Indeed, v-*src* Rat-1 cells did express higher levels of Ins(1,4,5)P_3 3-kinase A mRNA (Figure 2A) relative to normal cells, and the rat brain contained higher levels of mRNA relative to v-*src* Rat-1 cells. The Ins(1,4,5)P_3 3-kinase B mRNA levels were not significantly increased between the control and transformed cells (Figure 2B). In fact, some protection assays showed slightly higher levels of Ins(1,4,5)P_3 3-kinase B mRNA present in normal Rat-1 cells. Similar results were obtained when using a riboprobe transcribed from different regions of either the Ins(1,4,5)P_3 3-kinase A or B cDNA (see the Materials and methods section for the sequences of the riboprobes tested). Figure 2(C) shows a typical control assay performed with normal and transformed cell mRNA using the G3PDH probe to verify that equal amounts of mRNA were used between samples. In conclusion, evidence from both Northern blot analysis and ribonuclease protection assays demonstrate that expression of pp60$^{v\text{-}src}$ increased mRNA levels for isoform A and did not significantly alter levels for isoform B.

Ins(1,4,5)P_3 3-kinase A protein expression in Rat-1 fibroblasts

To examine the level of protein expression in normal and v-*src*-transformed cells, antiserum was raised against Ins(1,4,5)P_3 3-kinase A (see the Materials and methods section) and Western blots were performed. An immunoreactive Ins(1,4,5)P_3 3-kinase A protein band was visible in v-*src* Rat-1 cells, but was below the level of detection in Rat-1 cells (see arrow in Figure 3B). To

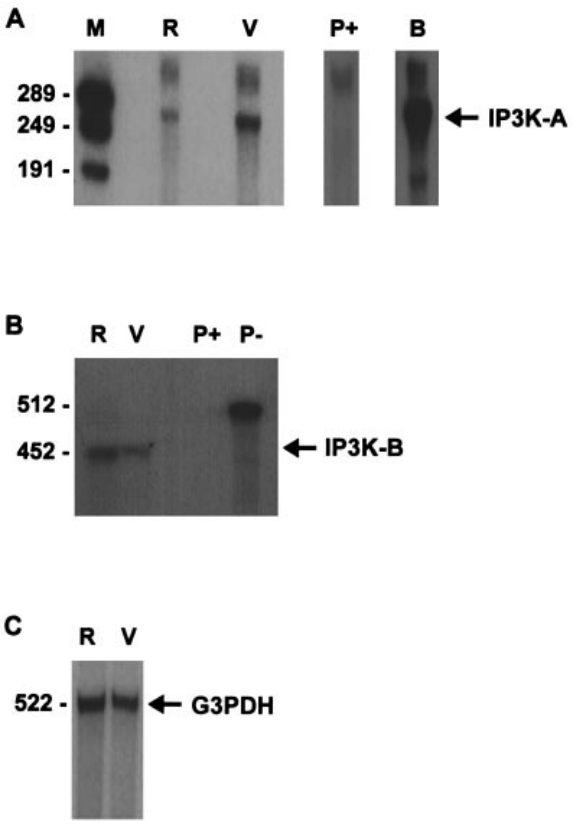

Figure 2 Ribonuclease protection assay

(**A**) Samples of 5 μg of poly(A) RNA from Rat-1 (R), v-*src*-transformed Rat-1 cells (V), and 1 μg of mRNA from rat brain (B) were each allowed to hybridize for 18 h in solution to a riboprobe specific for a 263 bp region at the 3′ end of the Ins(1,4,5)P_3 3-kinase A (IP3K) mRNA. Ribonucleases did not completely digest the probe as shown by the run-off transcript at 318 bp after the probe itself was treated with ribonucleases (P+). Migration of RNA size markers are shown in base pairs on the left-hand side. Quantification of autoradiographs showed the Ins(1,4,5)P_3 3-kinase A mRNA was increased 6.7-fold in v-*src*-transformed cells relative to control Rat-1 cells. Three separate mRNA preparations, and two different riboprobes (see the Materials and methods section) gave similar results. (**B**) Samples of 10 μg of poly(A) RNA from Rat-1 (R), and v-*src*-transformed Rat-1 cells (V) were used in a ribonuclease protection assay with the same conditions as described in (**A**). The riboprobe was a 452 bp fragment of Ins(1,4,5)P_3 3-kinase B (IP3K B). Run-off transcripts (P−) were 512 bp and were completely digested by ribonucleases in assays without mRNA (P+). For this experiment, the intensity of the signal was 1.4-fold in Rat-1 cells relative to v-*src*-transformed Rat-1 cells. Analogous results were obtained when a riboprobe designed to the 5′ end of the Ins(1,4,5)P_3 3-kinase B cDNA was used ($n = 2$). (**C**) Control assays were performed with 0.5 μg of poly(A) RNA from Rat-1 cells (R), or v-*src*-transformed Rat-1 cells (V), and a riboprobe transcribed from a fragment of the G3PDH cDNA. A 522 bp fragment was protected in both cell lines at nearly equal intensity levels. For each experiment, the quantification was corrected for any slight differences seen in the controls.

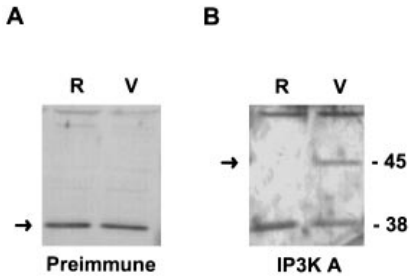

Figure 3 Comparison of Ins(1,4,5)P_3 3-kinase A (IP3K A) protein levels in normal and v-*src*-transformed Rat-1 cells

Cell lysates were prepared as described in the Materials and methods section. Eighteen μg of lysate protein from Rat-1 (R) or v-*src*-transformed Rat-1 (V) cells was used in a Western analysis with either preimmune serum (**A**) or anti-[Ins(1,4,5)P_3 3-kinase] serum (**B**) as the primary blotting antibody. The immunoreactive protein in transformed cells migrating at 45 kDa (indicated by an arrow in **B**) is approx. 8 kDa shorter than that detected in brain lysates. A signal was not detectable in normal cells even when five times more protein was loaded on to the gel. A duplicate Western blot incubated with preimmune serum shows one strongly reacting protein migrating at a molecular mass of 38 kDa which appears to provide an internal control for equivalent protein loading (arrow in **A**). These data are representative of six separate Western blots.

Table 2 Activity of the 45 kDa Ins(1,4,5)P_3 3-kinase A immunoreactive protein band after renaturation

Protein (400 μg) from five separate cytosol preparations was electrophoresed and the Ins(1,4,5)P_3 3-kinase A immunoreactive band was cut out of the gel, renatured, and assayed for activity as described [22]. Data are represented as pmol of Ins(1,4,5)P_3 converted into Ins(1,3,4,5)P_4 in each 45 kDa gel slice because protein levels could not be quantified accurately.

	Ins(1,4,5)P_3 3-kinase activity (pmol/min per slice)	
Experiment	Rat-1 cells	v-*src*-transformed cells
1	0.28	1.27
2	0.18	1.15
3	0.23	1.02
4	0.05	1.68
5	0.05	1.22
Average	0.16 ± 0.1	1.27 ± 0.3

increase sensitivity, up to 200 μg of protein was loaded on the gel and other Western blotting techniques were tested; however, preimmune bands interfered with the signal (i.e. see arrow in Figure 3A). When brain cytosol is blotted in parallel with the same antibody it is clear that the molecular mass of the protein in Rat-1 v-*src*-transformed cells is smaller (45 kDa) than that observed in rat brain cytosol (53 kDa). This could be the result of a proteolysis event since the Ins(1,4,5)P_3 3-kinase A protein is very sensitive to protease digestion, as documented in previous studies [14,15,17].

To be sure that the band which is being recognized in Rat-1 v-*src*-transformed cells was actually the Ins(1,4,5)P_3 3-kinase protein, the immunoreactive band was cut from the gel and the activity regenerated according to published methods [22]. Table 2 indicates that the band did possess Ins(1,4,5)P_3 3-kinase activity and the analogous position in the lane loaded with cytosol for normal Rat-1 cells contained levels of activity barely over background. Five separate experiments were performed using freshly isolated cytosolic extracts and the entire area from 20–65 kDa was examined for activity. The only area that contained significant activity was at 45 kDa. When duplicate lanes were blotted or the protein eluted out of the gel was re-electrophoresed and blotted on to nitrocellulose it was found that the active gel slice proteins reacted with the anti-[Ins(1,4,5)P_3 3-kinase A] antibody (results not shown). Since the 45 kDa band reacted with anti-[Ins(1,4,5)P_3 3-kinase] antibodies and contained activity after gel renaturation, we conclude that although the protein was slightly smaller than expected, this polypeptide contains at least the catalytic domain of the Ins(1,4,5)P_3 3-kinase.

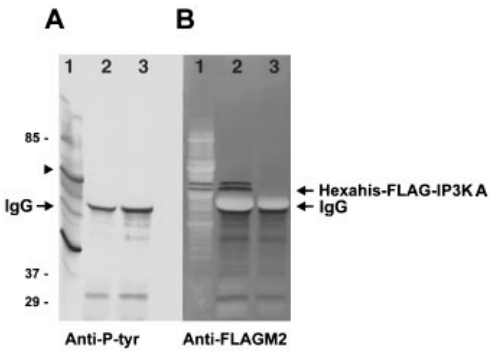

Figure 4 Absence of tyrosine-phosphorylated Ins(1,4,5)P_3 3-kinase A in v-*src*-transformed Rat-1 cells overexpressing the protein

(**A**) Anti-phosphotyrosine antibodies were used in Western blot analysis to examine the tyrosine phosphorylation state of immunoprecipitated hexahistidine–FLAG® epitope-tagged Ins(1,4,5)P_3 3-kinase A protein in v-*src*-transformed Rat-1 fibroblasts. Lane 1, 50 μg of lysate from cells overexpressing Ins(1,4,5)P_3 3-kinase A; lane 2, Ins(1,4,5)P_3 3-kinase A immunoprecipitated from 1 mg of cell lysate using 10 μl of anti-[Ins(1,4,5)P_3 3-kinase] serum and 25 μl of Protein A–Sepharose as described in the Materials and methods section; lane 3, control immunoprecipitation using 10 μl of preimmune serum. No apparent phosphotyrosine was detected on the immunoprecipitated protein in a total of four Western blots. The arrowhead indicates a 60 kDa tyrosine-phosphorylated protein, probably pp60$^{v\text{-}src}$, present in the cell lysate. The IgG heavy chain of the immunoprecipitating antibody reacts with the anti-phosphotyrosine antibody and is evident in lanes 2 and 3. (**B**) Identical samples to those shown in (**A**) were blotted and probed with the anti-(FLAG® M2) antibody. Lane 1, 50 μg of cell lysate; lane 2, Ins(1,4,5)P_3 3-kinase A immunoprecipitated from 1 mg of cell lysate protein; lane 3, control immunoprecipitation using preimmune serum. The doublet detected in lane 2 is the only specific protein immunoprecipitated when compared with the control immunoprecipitation. Analogous results are obtained when the anti-[Ins(1,4,5)P_3 3-kinase] antibody is used as the primary blotting antibody.

Overexpressed Ins(1,4,5)P_3 3-kinase A is not tyrosine-phosphorylated in Rat-1 v-*src*-transformed cells

Since many proteins become phosphorylated in conjuction with the expression of the v-*src* protein, the tyrosine phosphorylation state of Ins(1,4,5)P_3 3-kinase A was examined in v-*src* Rat-1 cells. Because of the low levels of the endogenous protein, each of the Ins(1,4,5)P_3 3-kinase isoforms was separately overexpressed in the v-*src*-transformed cells. Ins(1,4,5)P_3 3-kinase cDNA was modified by adding 5′ DNA sequences for a FLAG® antibody epitope and a hexahistidine tag via subcloning into the pDoubleTrouble mammalian expression vector (see the Materials and methods section). This construct was transfected into v-*src* B31 cells [37] and stable cell lines were purified by single-cell dilution of neomycin-resistant colonies. A stable cell line expressing isoform A at 150-fold more activity over vector-transfected cells, and another expressing isoform B at 12-fold more activity, were established. The tyrosine phosphorylation state of the overexpressed proteins was examined by immunoprecipitating the proteins with the anti-[Ins(1,4,5)P_3 3-kinase A] antibody and blotting with an anti-phosphotyrosine antibody. It was difficult to detect endogenous Ins(1,4,5)P_3 3-kinase A after immunoprecipitation because the antibody IgG heavy chain distorted the gel at the site of the Ins(1,4,5)P_3 3-kinase A protein migration. However, the overexpressed protein was slightly larger than the endogenous one because it contained both a FLAG® epitope tag and hexahistidine sequence inserted at the N-terminal end of the protein (see the Materials and methods section). The immunoprecipitated protein was detected as a doublet on Western blots using the anti-[Ins(1,4,5)P_3 3-kinase A] antibody to immunoprecipitate and the anti-(FLAG® M2) monoclonal antibody to perform the Western blot (Figure 4B). The immunoprecipitated doublet is not recognized by anti-phosphotyrosine antibodies (Figure 4A). These results were consistent with the data observed when the overexpressed protein was purified to a single band on a silver-stained gel in the presence of phosphatase inhibitors. The purified Ins(1,4,5)P_3 3-kinase A did not react with anti-phosphotyrosine antibodies on Western blots (results not shown). When analogous experiments were performed using the cell line overexpressing the B isoform of the Ins(1,4,5)P_3 3-kinase, no tyrosine phosphorylation was found. Hence, we have not found evidence of any tyrosine phosphorylation of either form of the Ins(1,4,5)P_3 3-kinase when expressed simultaneously with pp60$^{v\text{-}src}$.

DISCUSSION

Ins(1,4,5)P_3 3-kinase occupies a key position in the metabolism of inositol polyphosphates, controlling the degradation of the second messenger Ins(1,4,5)P_3 and the production of a potential regulatory molecule, Ins(1,3,4,5)P_4. Rat-1 fibroblasts transformed by the v-*src* oncogene contain elevated levels of Ins(1,4,5,6)P_4, which has been suggested to be a result of the activation of the Ins(1,4,5)P_3 3-kinase in this cell line [30]. The objective of this study was to determine the molecular mechanism by which pp60$^{v\text{-}src}$ causes the increase in Ins(1,4,5)P_3 3-kinase activity in order to understand modes of enzyme regulation which may be applicable to other systems. Regulation of the Ins(1,4,5)P_3 3-kinase has been reported to occur by both calcium–calmodulin stimulation and phosphorylation. The results from this study demonstrate that regulation can also occur by altering mRNA expression. Transformation of Rat-1 cells by v-*src* causes an increased level of Ins(1,4,5)P_3 3-kinase A protein expression that can presumably be accounted for by the observed increased mRNA levels.

This result is not surprising since there are a number of examples of proteins that show an alteration in gene expression as a result of *src* transformation (see [38] for a review). Genes such as the epidermal growth factor receptor gene [39,40], cytoskeletal protein genes [41–43], platelet protein genes [44,45] and the glucose transporter gene [46–48] are among those reported to have altered expression following *src* transformation. In principle, the possible mechanisms for the increased mRNA levels are either an increased transcription of the gene or an increased stability of the mRNA (decreased turnover). Both mechanisms have been utilized by pp60$^{v\text{-}src}$ for increasing expression of proteins in transformed cells [39–48] and either mechanism could be employed by pp60$^{v\text{-}src}$ to increase Ins(1,4,5)P_3 3-kinase A mRNA. These mechanisms were not investigated in this study due to the difficulties encountered in quantifying very low levels of Ins(1,4,5)P_3 3-kinase poly(A) RNA in ribonuclease protection assays.

Evidence presented here shows that one mode of Ins(1,4,5)P_3 3-kinase regulation used by pp60$^{v\text{-}src}$ is to increase its mRNA and protein levels. This type of Ins(1,4,5)P_3 3-kinase regulation has not been reported previously but has been alluded to in a recent publication [49]. A 3 h stimulation of rat thyroid FRTL-5 cells with thyroid-stimulating hormone leads to a significant inhibition of Ins(1,4,5)P_3 3-kinase activity. Takazawa et al. [49] speculate that this might be the result of decreased Ins(1,4,5)P_3 3-kinase expression and/or activation of cAMP-dependent protein kinase. Similarly, regulation of Ins(1,4,5)P_3 3-kinase activity in v-*src*-transformed Rat-1 cells may involve changes in phosphorylation state as well as changes in expression level. Further complexity is added when we consider many different protein kinases may control Ins(1,4,5)P_3 3-kinase activity, and each isoenzyme may be expressed and regulated differentially.

Ins(1,4,5)P_3 3-kinase A does not appear to be a substrate for the v-*src* tyrosine kinase based on our results from stable transfection experiments (Figure 4B). However, the serine/threonine phosphorylation state was not examined in this study. Several kinases including those in the MAP kinase cascade [50] are known to be activated in association with *src* transformation. In addition, protein kinase C and cAMP-dependent protein kinase have been reported to phosphorylate Ins(1,4,5)P_3 3-kinase A to stoichiometric levels *in vitro* [27,28], resulting in alterations of enzyme activity. Changes in the activity of these kinases may occur in the B31 cells and be responsible for the increased Ins(1,4,5)P_3 3-kinase activity. Consistent with this suggestion, several investigators have reported that kinase activators or phosphatase inhibitors affect Ins(1,4,5)P_3 3-kinase activity [24–26,49,51,52]. This aspect of regulation is currently being examined in our laboratory.

The Ins(1,4,5)P_3 3-kinase A protein in v-*src*-transformed Rat-1 cells migrates at 45 kDa on SDS/PAGE as opposed to the 53 kDa protein Ins(1,4,5)P_3 3-kinase A isolated from rat brain and expressed in COS-7 cells [9]. Three possible explanations could account for this size discrepancy. First, since the Ins(1,4,5)P_3 3-kinase is susceptible to proteolysis, especially to digestion by calpain [14], the 45 kDa species could be an active proteolytic cleavage product of the full-size protein. However, when cells are lysed immediately in boiling SDS/PAGE sample buffer, or are homogenized in the presence of several protease inhibitors, a larger-sized immunoreactive protein is not evident. This does not rule out the possibility that the proteolysis event may be the result of normal processing that occurs in Rat-1 fibroblasts, and not a result of the lysate preparation. Secondly, there could be alternatively spliced forms of the protein expressed in Rat-1 fibroblasts. Preliminary evidence from our laboratory indicates that rat testes may contain an alternatively spliced Ins(1,4,5)P_3 3-kinase A which eliminates 160 amino acids at the N-terminus of the protein. The protein product of this mRNA is expected to be active since the first 185 amino acids of Ins(1,4,5)P_3 3-kinase A are not required for enzymic activity [53]. Thirdly, expression of pp60$^{v\text{-}src}$ may cause the removal of the regulatory N-terminal regulatory region of Ins(1,4,5)P_3 3-kinase A resulting in a smaller, unregulated form of the enzyme. Any of these explanations (proteolysis, an alternatively spliced isoform or removal of the regulatory region) could explain the smaller-sized protein in v-*src*-transformed Rat-1 cells.

Data presented here show that the levels of Ins(1,4,5)P_3 3-kinase mRNA and protein in rat brain are high relative to those in v-*src*-transformed cells, yet the activity levels in the respective cytosolic extract preparations are similar. The reason for this is unknown but may be the result of the following possibilities: (a) the existence of an inhibitory factor present in brain extract preparations (or an activating factor in fibroblast preparations), and/or (b) the fibroblast Ins(1,4,5)P_3 3-kinase has a higher specific activity. The first possibility seems unlikely because the specific activity of the enzyme in brain cytosol does not increase when the assay is performed at high dilutions of the extracts. Thus, it appears that the Ins(1,4,5)P_3 3-kinase as assayed in extracts from v-*src*-transformed cells has a higher specific activity for unknown reasons.

The physiological significance of the increased Ins(1,4,5)P_3 3-kinase activity in v-*src*-transformed Rat-1 cells is unknown but it is tempting to postulate that it may be related in some way to cell growth. There appear to be a number of instances in which cell growth and mitogenic signalling are correlated with phosphatidylinositol metabolism [54–58]. In this regard, it is interesting to note that an inhibition of Ins(1,4,5)P_3 3-kinase activity has been correlated with decreased cell density and DNA synthesis in adriamycin-treated Jurkat T-lymphocytes [59]. Furthermore, Ins(1,3,4,5)P_4, the product of the Ins(1,4,5)P_3 3-kinase, has been linked to the *ras* mitogenic signal-transduction pathway by virtue of its capability to bind to and stimulate a *ras* GTPase-activating protein [6]. These results provide a connection between Ins(1,4,5)P_3 3-kinase activity and pathways involved in cell growth and mitogenesis, suggesting that alterations of the Ins(1,4,5)P_3 3-kinase could result in unregulated signalling such as that observed in the transformed cell [60].

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