pp60$^{c\text{-}src}$ is a substrate for phosphorylation when cells are stimulated to enter cycle

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The endogenous cellular oncogene products, pp60$^{c\text{-}src}$, exhibits a protein kinase activity, but is itself a phosphoprotein. Based on the assumption that pp60$^{c\text{-}src}$ might play a role in the control of cell proliferation, we have studied its behaviour as a substrate for phosphorylation known to occur when quiescent, serum-deprived cells are stimulated to enter cell cycle following addition of either serum, platelet-derived growth factor or the phorbol ester derivative, 12-*O*-tetradecanoyl-phorbol-13-acetate. For this purpose a partial purification of pp60$^{c\text{-}src}$ on DEAE ion-exchange chromatography was combined with immune precipitation. A 2–4-fold increase in serine phosphorylation of pp60$^{c\text{-}src}$ was consistently observed after stimulation of quiescent cells to growth.

Protein kinase C *pp60$^{c\text{-}src}$* *Phosphorylation* *Cell cycle* *Oncogene*

1. INTRODUCTION

pp60$^{c\text{-}src}$, the host analogue to the Rous sarcoma virus (RSV) transforming protein, pp60$^{v\text{-}src}$ [1,2], is expressed in small, but detectable amounts in cells of many tissues. The physiological role of this cellular 60-kDa phosphoprotein is unknown, but can be postulated on the basis of tissue- and developmental-stage-specific variations in levels of expression [3–10]. pp60$^{c\text{-}src}$ exhibits a protein phosphotransferase activity [11], specific for tyrosine [12] and this fact, together with its localization in association with the plasma membrane [13–15], imply a functional analogy with other such enzymatic activities which have been shown to act on/or to be modulated by growth hormone receptors [16,17]. On the other hand, previous studies have shown that the level of pp60$^{c\text{-}src}$ expression is not altered in the chick embryo cells at different stages of the cell cycle [4]. This study shows that pp60$^{c\text{-}src}$ undergoes an enhanced phosphorylation in serine shortly after quiescent cells have been stimulated to enter cell cycle by addition of either serum, platelet-derived growth factor (PDGF), or the phorbol ester derivative, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Furthermore, we report that pp60$^{c\text{-}src}$, in contrast to pp60$^{v\text{-}src}$, is not complexed by the two cellular proteins pp50 and pp90 [21,22].

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

For these experiments, normal chick embryo cells (CEC) or CEC infected with the temperature-sensitive mutant NY68 of the Schmidt-Ruppin strain of RSV (ts NY68), were employed. Cell culture was in Dulbecco's modified Eagle's minimal essential medium (MEM) with 5% calf serum, or made quiescent by culture for 24 h at 37°C in the same medium free of serum. Cultures were labeled for 6 h in phosphate-free medium using [^{32}P]orthophosphate at 1 mCi/ml. Stimulation of quiescent cells to growth was accomplished by the addition of 10% dialyzed calf serum (orthophosphate free), or PDGF (Collaborative

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Research) at 2 units/ml, supplemented with multiplication stimulating activity (MSA) (Collaborative Research) at 25 ng/ml, or of 20 ng/ml of the phorbol ester TPA (PL Biochemicals) for 7 min.

2.2. *Preparation of lysates and partial purification of pp60$^{c\text{-}src}$*

Radioactively labeled cell cultures (4×10^7), after washing once in ice-cold phosphate-buffered saline (PBS), were lyzed in 8 ml of a buffer consisting of: 4 mM KH_2PO_4, 16 mM $NaHPO_4$, 1 mM mercaptoethanol, 1 mM NaF, 1 mM EDTA and 0.5% Triton X-100, pH 7.2. The scraped cells were suspended and homogenized using 15 strokes in a tight-fitting Dounce homogenizer, followed by centrifugation at 70000 × *g* for 30 min. 9 ml of the supernatant was applied on to a DEAE–Sephacel (Pharmacia) column (1.5–8 cm) previously equilibrated with the same buffer as above, but lacking Triton X-100 and EDTA. The proteins bound were eluted with a linear gradient (total volume: 25 ml) in the same buffer, 0–0.5 M NaCl.

The total DEAE–Sephacel fractions were further purified by immune precipitation as in [23] using 5 μl pp60$^{c\text{-}src}$ reactive tumour-bearing rabbit (TBR) serum [11,18], in antibody excess for pp60$^{c\text{-}src}$. Thereafter, heat-fixed Cowan I strain *S. aureus* [19] bacteria were added, incubated at 0°C for 30 min, and the resulting solid phase washed 3 times with a buffer consisting of 10 mM phosphate, 10 mM EDTA, 40 mM NaF, 0.2% Triton X-100, 1 M NaCl, 1% Trasylol (pH 7.4). Analysis of the proteins was accomplished using autoradiography following SDS–polyacrylamide (11%) gel electrophoresis.

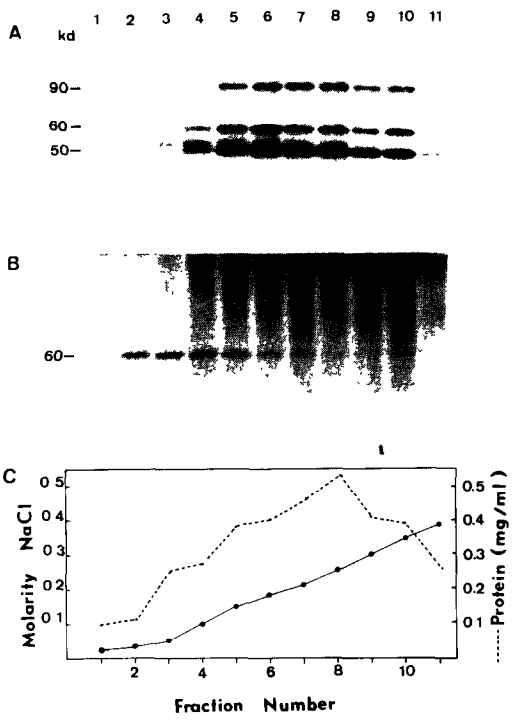

Fig.1. Partial purification of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$. Normal CEC and CEC infected with *ts*-Ny68 virus at 37°C were labeled for 6 h with [^{32}P]orthophosphate, lyzed, and the cytosol component chromatographed on DEAE–Sephacel (Pharmacia). The pp60^{src} in the fractions was further analyzed using immunoprecipitation with pp60$^{c\text{-}src}$ reactive TBR-serum. The samples were electrophoresed on SDS–polyacrylamide (PAGE) (11%) gels, followed by autoradiography. (A) Autoradiogram of the DEAE-fractions obtained from *ts*-NY68-infected cells (pp60$^{v\text{-}src}$). (B) Comparable result with normal CEC (pp60$^{c\text{-}src}$). (C) DEAE–Sephacel profile. NaCl concentrations (conductivity) and protein concentrations (method of [34]) of the individual fractions from (A) above.

3. RESULTS AND DISCUSSION

Our approach was to follow the phosphorylation of pp60$^{c\text{-}src}$ itself as a substrate in order to detect possible changes occurring after stimulation of cells to proliferate. Detection of pp60$^{c\text{-}src}$ using just immune precipitation gave a band barely distinguishable against a high background (not shown). Hence, after a trial experiment (fig.1A,C) in which Rous sarcoma virus pp60$^{v\text{-}src}$ was used to establish the purification methods, DEAE–Sephacel chromatography was adopted as a one-step procedure in order to achieve a pp60$^{c\text{-}src}$ purification of approximately 20-fold prior to immune precipitation with TBR serum. The combination of these two steps yielded unambiguous, reproducible data on the phosphorylation of pp60$^{c\text{-}src}$ (fig.1B,C).

Interestingly, as shown in fig.1B, the pp60$^{c\text{-}src}$ does not form a complex with the 50- and 90-kDa phosphoproteins as has been shown for part of viral pp60^{src} [21,22], and instead is eluted from DEAE like the uncomplexed pp60$^{v\text{-}src}$ between 50 and 150 mM sodium chloride. Since the amino acid sequences of pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ are almost

identical except for the carboxy terminus [24], and since previous studies using antibodies to individual pp60^{src} determinants prepared with synthetic peptides suggested that the binding site for pp50 and pp90 should be at the carboxy terminus [23,25,26], it is reasonable that pp60$^{c\text{-}src}$ should fail to show the complexing typical of pp60$^{v\text{-}src}$. The binding of pp60$^{v\text{-}src}$ to DEAE was strongly influenced by whether or not the 50- and 90-kDa phosphoproteins were complexed to the pp60$^{v\text{-}src}$. The modest fraction of pp60$^{v\text{-}src}$ (10%) eluting early in the salt gradient (fig.1A, lane 3) behaved as a monomer upon glycerol gradient centrifugation, peaking just before the hemoglobin standard, while the mass of material in the immunoprecipitates (fig.1A, lanes 5–10) obviously associated with the 50- and 90-kDa phosphoproteins, moved just ahead of an IgG standard upon centrifugation (not shown).

To analyze the phosphorylation of pp60$^{c\text{-}src}$ after stimulation to enter cell cycle, [^{32}P]orthophosphate-prelabeled normal chick embryo cells arrested in the G_o phase by complete serum deprivation for 24 h were compared with sister cultures stimulated for different times by the addition of 10% phosphate-free calf serum (fig.2b,c). An increased phosphorylation in pp60$^{c\text{-}src}$ was apparent by 5 min after serum addition (fig.2b), which by 120 min had returned to control levels (fig.2c). Additional experiments showed that phosphorylation was maximal between 5 and 15 min stimulation (not shown).

Since pp60$^{v\text{-}src}$ can act to phosphorylate itself [27], a determination of whether pp60$^{c\text{-}src}$ had undergone phosphorylation in tyrosine (autophosphorylation) or in serine (exogenous kinase) was essential. A direct phosphoamino acid determination was deemed inappropriate, since during acid hydrolysis phosphotyrosine is more labile than phosphoserine. Hence, we took a different approach. pp60$^{v\text{-}src}$ is known to form 4 proteolytic digestion products after V8-protease treatment [28,29]. Of these, V-2 is the phosphotyrosine-containing fragment comprising the carboxy-terminal half, while the other fragments contain only phosphoserine (V-3 and V-4 are secondary cleavages of V-1) [29]. To establish that the phosphoamino acids phosphorylated in the fragments obtained after partial proteolysis by V8 protease of pp60$^{c\text{-}src}$ were analogous to those known

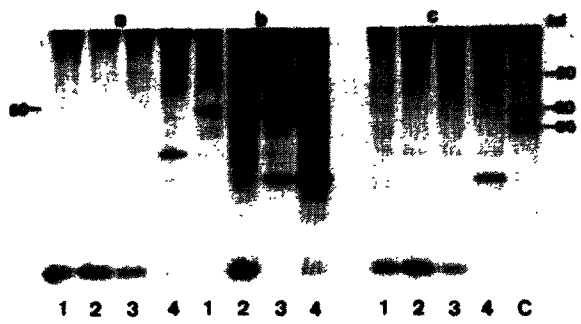

Fig.2. Analysis of phosphorylation of pp60$^{c\text{-}src}$ in quiescent normal CEC following serum addition. CEC cultures were incubated for 24 h at 37°C in Dulbecco's modified MEM without calf serum, followed by labeling for 6 h with [^{32}P]orthophosphate (1 mCi/ml). Phosphate-free calf serum was then added to 10% to some of the sister cultures. (a) Normal CEC synchronized by serum deprivation. (b) Sister cells of (a) after 5 min stimulation with serum. (c) Sister cells of (a) after 120 min stimulation with serum. Cells were treated as described in the legend to fig.1 and after centrifugation the supernatants were chromatographed through DEAE–Sephacel. The elution was stepwise, with 50 mM NaCl (1), 100 mM NaCl (2), 200 mM NaCl (3), and 500 mM NaCl (4). An immunoprecipitate of pp60$^{v\text{-}src}$ from RSV-infected cells is shown for comparison (C).

for pp60$^{v\text{-}src}$, phosphoamino acid determinations of the ^{32}P-labeled pp60$^{c\text{-}src}$ V8 fragments were carried out. It is apparent that the pp60$^{c\text{-}src}$ fragments have the same phosphoamino composition as the corresponding pp60$^{v\text{-}src}$ fragments (fig.3).

Fig.4 shows the V8 analysis of pp60$^{c\text{-}src}$ purified from unstimulated CEC (a), and from CEC stimulated by serum for 5 (b), 15 (c), and 45 (d) min. It is already apparent from the autoradiogram that the elevated phosphorylation must be due mainly to increased phosphoserine, since the changes are in the sum of V-1, V-3, and V-4, while V-2 shows the least effect upon stimulation. For purpose of quantitation, the individual fragments were excised from the gels and the radioactivity was determined as shown in table 1.

The experiments were repeated in the same way using PDGF and TPA stimulation in order to be better able to interpret the results in the light of possible molecular mechanisms. Table 2 shows that effects similar to those produced with serum were observed, except that the average fold-

stimulation was somewhat less for PDGF and TPA than for serum.

Short-lived changes in pp60$^{c\text{-}src}$ phosphorylation might have consequences for the tyrosine-specific protein kinase activity; thus, an in vitro protein kinase assay using immunoprecipitated IgG was performed. However, no significant changes in protein kinase activity could be detected (not shown).

These studies show that pp60$^{c\text{-}src}$ behaves as a substrate for phosphorylation in serine which occurs very soon after the initial stimulation to growth by either serum, PDGF or TPA. The elevated phosphorylation is short-lived and already declines by 45 min after stimulus. No corollary change in protein kinase activity could be detected

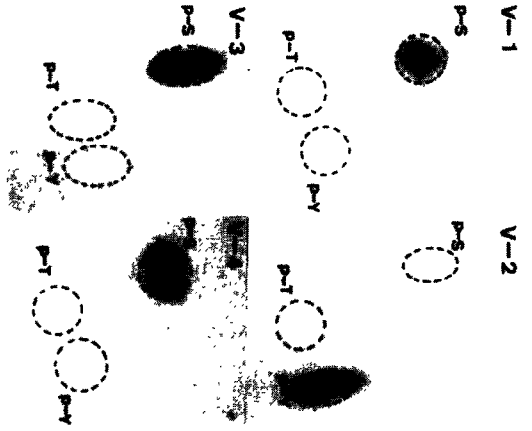

Fig.3. Phosphoamino acid analysis on purified pp60$^{c\text{-}src}$ after stimulation by serum addition. pp60$^{c\text{-}src}$ was radiolabeled with [^{32}P]orthophosphate and purified as described in fig.2. Partial proteolysis using V8 proteases was performed on the pp60$^{c\text{-}src}$, and individual PAGE fragments were cut out, eluted, and the radioactive phosphoamino acids resolved in two dimensions on cellulose thin-layer plates, according to [12]. p–S: phosphoserine; p–T: phosphothreonine; p–Y: phosphotyrosine. The positions of the stained marker phosphoamino acids are indicated by broken lines. Each PAGE fragment was associated with a particular phosphoamino acid, precisely as would be expected from data for pp60$^{v\text{-}src}$.

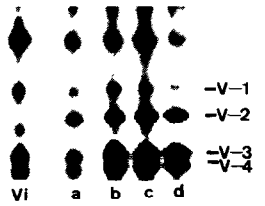

Fig.4. pp60$^{c\text{-}src}$ was prepared as described in the legend to fig.2 from [^{32}P]orthophosphate-labeled quiescent CEC cultures which were stimulated with 10% dialyzed calf serum for 0, 5, 15 and 45 min. The pp60$^{c\text{-}src}$ band from the polyacrylamide gel was subjected to partial proteolysis using V8 protease, and the resulting autoradiogram is presented. Vi, partial proteolysis of ^{32}P-labeled pp60$^{v\text{-}src}$, as marker; (a) unstimulated, (b) 5 min, (c) 15 min, (d) 45 min stimulation with 10% calf serum.

Table 1

Quantitative comparison of the phosphorylation of the V8 digestion products from fig.4

V8 fragment	CEC unstimulated	Relative radioactivity (cpm stimulated)/(cpm unstimulated) CEC after serum stimulation for:		
		5 min	15 min	45 min
V-2 (phosphotyrosine)	1	1.4	1.6	1.2
V-1 (phosphoserine)	1	2.7	2.5	0.8
V-3 (phosphoserine)	1	3.8	3.9	2.5
V-4 (phosphoserine)	1	3.2	2.9	2.1
Sum of V-1, V-3, V-4 (phosphoserine)	1	3.3	3.2	1.9

The fragments resulting from partial proteolysis [20] shown in fig.4 were cut out from the gel, and Cerenkov radioactivity determined. Data are expressed as relative radioactivity. This experiment has been repeated 5 times, always with a similar result

Table 2

Effect of PDGF and TPA on phosphorylation of $pp60^{c\text{-}src}$ [a]

Sample	Relative radioactivity (cpm stimulated)/(cpm unstimulated)		
	Un-stimulated	PDGF	TPA
$pp60^{c\text{-}src}$ band	1.0	1.9	2.4
V_8 fragments			
V_2	1.0	1.0	1.9
$V_1 + V_3 + V_4$	1.0	2.0	2.6

[a] CEC made quiescent following serum deprivation for 24 h were prelabeled with [^{32}P]orthophosphate as described in the text, and were stimulated with either PDGF (2 units/ml) together with multiplication stimulating activity (25 ng/ml) for 7 min or with TPA at 20 ng/ml for 8 min. The purification of the radioactively labeled $pp60^{c\text{-}src}$ and the representation of data are as in table 1. The data shown represent the average of 2 independent experiments

in vitro using IgG as a substrate. Therefore, the sharp change in phosphorylation of the amino terminal half of $pp60^{c\text{-}src}$ may be related to a function of this part of $pp60^{c\text{-}src}$ which is independent of the tyrosine-specific protein kinase. There are several indications that this region of $pp60^{v\text{-}src}$ is essential for transformation, not necessarily in relation to effects on protein kinase [15,30]. A possible candidate for the kinase responsible for the phosphorylation of $pp60^{c\text{-}src}$ would be the protein kinase type C, which has recently been implicated in phosphorylation of a variety of substrates upon serum, PDGF, and TPA treatment [31–33].

This study does not shed light on the actual role of $pp60^{c\text{-}src}$ in cell growth, but two useful points have emerged: that the complex of the 50- and 90-kDa phosphoproteins which binds $pp60^{v\text{-}src}$ in RSV-infected cells is not seen with $pp60^{c\text{-}src}$, and that a pivotal event in cell physiology, the transition from G_0 to G_1 of the cell cycle, has consequences for the phosphorylation state of the endogenous $pp60^{c\text{-}src}$. The latter is a hint that $pp60^{c\text{-}src}$ might well play a role in the control of cell proliferation.

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