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Activation of 6-phosphofructo-2-kinase by pp60v-*src* is an indirect effect

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6-Phosphofructo-2-kinase (PFK-2) catalyses the synthesis of fructose 2,6-bisphosphate (Fru-2,6-P_2), a potent stimulator of glycolysis. In chick-embryo fibroblasts, PFK-2 activity and Fru-2,6-P_2 concentration increase upon transformation by Rous sarcoma virus. We show here that the increase in PFK-2 activity required more than 2 h after shifting fibroblasts infected with a thermosensitive mutant of Rous sarcoma virus from the restrictive to the permissive temperature. Pretreatment of the cells with actinomycin D prevented this increase in PFK-2 activity, suggesting a requirement for RNA synthesis. However, the increase in PFK-2 activity did not correspond to an increase in immunoprecipitable PFK-2. Moreover, the thermostability of PFK-2 and the affinity of this enzyme for its substrate fructose 6-phosphate were increased upon transformation by Rous sarcoma virus. Staurosporine, an inhibitor of protein kinase C, prevented the increase in PFK-2 activity brought about by the shift to the permissive temperature. This, together with a comparison of the effects of phorbol esters on PFK-2 activity, suggests that pp60v-*src* stimulates, via protein kinase C, the transcription of a gene whose products is a distinct PFK-2 isoenzyme or a protein that activates PFK-2.

INTRODUCTION

Rous sarcoma virus (RSV) contains the v-*src* oncogene whose product, pp60v-*src*, is endowed with tyrosine kinase activity and is responsible for all the biochemical and morphological changes that characterize the transformed phenotype (for reviews see Weber, 1984; Jove & Hanafusa, 1987). One of these changes is an enhanced rate of glycolysis, which is typical of chick-embryo fibroblasts (CEF) transformed by RSV (RSV-CEF) (reviewed by Hanafusa, 1977). A high aerobic glycolysis, called the Warburg effect, is a common feature of many tumours and of proliferating cells (reviewed by Weinhouse, 1976). Its mechanism remains ill-understood. The enzymes that catalyse the final four steps of glycolysis are phosphorylated on tyrosine residues in RSV-CEF (Cooper *et al.*, 1983; Presek *et al.*, 1988), but the significance of such phosphorylations for the regulation of glycolysis has not been demonstrated, since they do not affect the activity of these enzymes. On the other hand, Singh *et al.* (1974) found that the activities of the regulatory enzymes of glycolysis, namely hexokinase (EC 2.7.1.1), 6-phosphofructo-1-kinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40), were increased in RSV-CEF.

Earlier work from this laboratory (Bosca *et al.*, 1986) showed that the increased activity of 6-phosphofructo-1-kinase in RSV-CEF could be explained by a pp60v-*src*-dependent increase in the concentration of fructose 2,6-bisphosphate (Fru-2,6-P_2), which is a very potent stimulator of this enzyme (for a review, see Hue & Rider, 1987). The higher concentration of Fru-2,6-P_2 in RSV-CEF was accompanied by an increased activity of 6-phosphofructo-2-kinase (PFK-2; EC 2.7.1.105), the enzyme that catalyses Fru-2,6-P_2 synthesis from fructose 6-phosphate and ATP. We have now investigated the mechanism of this increase. The data suggest that the activation of PFK-2 by pp60v-*src* is indirect and involves protein kinase C (PKC).

MATERIALS AND METHODS

Materials

All chemicals and biochemicals were purchased from Merck, Sigma or Boehringer. 3-*O*-Methyl-D-[U-^{14}C]glucose and nitrocellulose and nylon filters were from Amersham. Protein A–Sepharose CL-4B was from Pharmacia. Cell cultures and viral strains were as described (Bosca *et al.*, 1986). The incubation medium consisted of 126 mM-NaCl, 14 mM-$NaHCO_3$, 3.8 mM-KCl, 0.9 mM-Na_2HPO_4, 0.6 mM-KH_2PO_4, 0.6 mM-$MgSO_4$, 0.3 mM-$CaCl_2$, 5.6 mM-glucose and 20 mM-Hepes, pH 7.2. Cells infected with RSV exhibited the typical morphology of transformed fibroblasts at the permissive temperature.

Biochemical measurements

Fru-2,6-P_2 (Bosca *et al.*, 1986), 3-*O*-methylglucose uptake (Yamada *et al.*, 1983) and protein (Bradford, 1976) were determined as described. PFK-2 activity was assayed under $V_{max.}$ conditions after precipitation with 15% (w/v) poly(ethylene glycol) 6000 (PEG extract) (Mojena *et al.*, 1985).

PFK-2 immunotitration

Rabbit antiserum MCL-2 was raised against native PFK-2 purified (Van Schaftingen & Hers, 1986) from chicken liver. PFK-2 immunotitration was carried out in 0.2 ml containing 100 mM-KCl, 15 mM-β-mercaptoethanol, 20 mM-Tris/HCl, pH 8, 0.1% BSA, 80 μl of protein A–Sepharose suspension (1:2, v/v), a volume of PEG extract corresponding to a fixed quantity of proteins, and up to 50 μl of MCL-2 antiserum. After 3 h of agitation at room temperature, the immune complexes were precipitated (12000 *g*, 2 min) and the residual PFK-2 activity was measured in the supernatant. When the MCL-2 antiserum was replaced by non-immune serum in the incubation, no PFK-2 activity was precipitated. The MCL-2 antiserum did

Abbreviations used: AMD, actinomycin D; CEF, chick-embryo fibroblasts; Fru-2,6-P_2, fructose 2,6-bisphosphate; PEG, poly(ethylene glycol); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus; *ts*, thermosensitive.

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not modify PFK-2 activity when incubated in the absence of Protein A–Sepharose.

RESULTS

Time course of PFK-2 stimulation after pp60v-*src* expression

In CEF infected with transformation-defective, temperature-sensitive (*ts*) mutants of RSV, the tyrosine kinase activity of pp60v-*src* as well as the transformed phenotype are expressed at the permissive (36 °C) but not at the restrictive (42 °C) temperature, whereas viral replication is unaffected at either temperature (for a review, see Friis, 1978). Upon a shift from the restrictive to the permissive temperature, the pp60v-*src* tyrosine kinase activity increases immediately. Thus transformation is initiated synchronously, making it possible to study the time course of various parameters. A rapid rise in PFK-2 activity would therefore be expected under these conditions, if this rise results from a direct effect of the tyrosine kinase activity of pp60v-*src* on this enzyme.

Using one of these RSV mutants, *ts* NY68 (Kawai & Hanafusa, 1971), Bosca *et al.* (1986) demonstrated that there was a lag period before the Fru-2,6-P_2 concentration began to rise after the shift from 42 °C to 36 °C. Fru-2,6-P_2 can increase as a result of an increased concentration of fructose 6-phosphate, the substrate of PFK-2. This could occur upon stimulation of hexose uptake, as is observed following transformation of CEF by RSV (Kawai & Hanafusa, 1971). Using the same *ts* mutant, we therefore measured PFK-2 activity, Fru-2,6-P_2 concentration and 3-*O*-methylglucose uptake as a function of time after shifting the cells from the restrictive to the permissive temperature. Fig. 1 shows that PFK-2 activity increased in CEF transformed with *ts* NY68 (*ts* RSV-CEF), but only after a lag period following the temperature shift. A small initial increase in Fru-2,6-P_2 concentration preceded that in PFK-2 activity. This was probably due to the early rise in hexose uptake, which was detectable after 1 h. However, the bulk of the effect on Fru-2,6-P_2 could be ascribed to the increased PFK-2 activity, since these two parameters kept rising after hexose uptake had reached its new steady state.

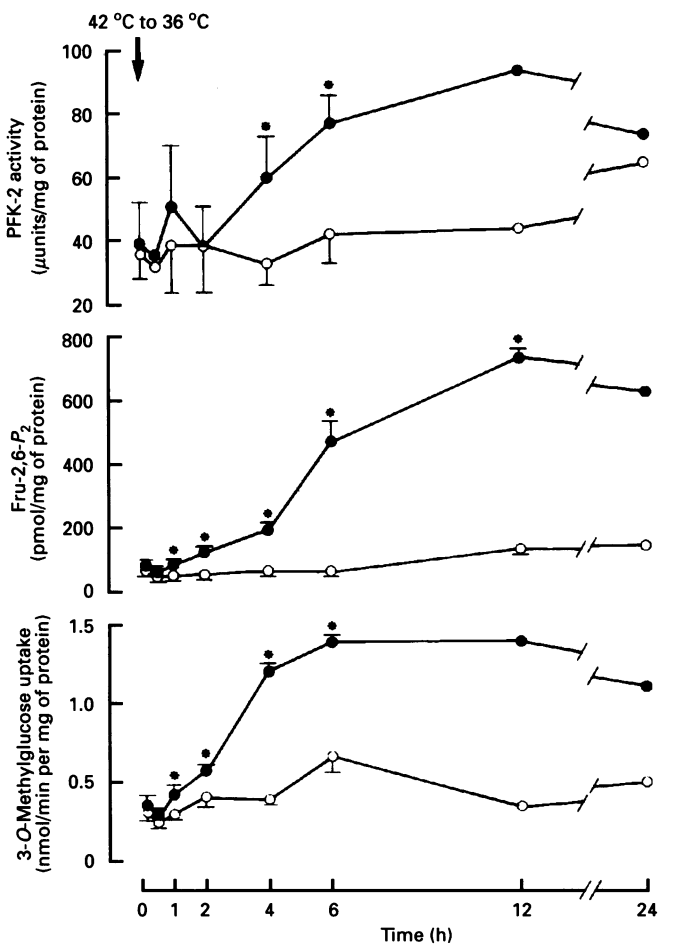

Fig. 1. Time course of the effects of pp60v-*src* expression on PFK-2 activity, Fru-2,6-P_2 concentration and 3-*O*-methylglucose uptake

Secondary cultures of CEF uninfected (○) or infected with *ts* NY68 (●) at 36 °C were incubated overnight at 42 °C and then (zero time on the Figure) returned to 36 °C while being switched from the culture medium to the incubation medium. Data are means ± S.E.M. for at least three experiments. Values without S.E.M. were obtained on single or duplicate dishes. * $P < 0.05$ when compared with corresponding control.

Changes in both PFK-2 activity and Fru-2,6-P_2 concentration were linked to transformation and not to infection, since at the restrictive temperature *ts* RSV-CEF showed a Fru-2,6-P_2 concentration and a PFK-2 activity similar to those of uninfected CEF (control CEF) (results not shown). Thus the increase in Fru-2,6-P_2 concentration caused by pp60v-*src* results from a concerted mechanism involving increases in both glucose uptake and PFK-2 activity. Since the increase in PFK-2 activity required more than 2 h after the shift to the permissive temperature, the pathway between pp60v-*src* and PFK-2 might involve one or several intermediate steps.

Effect of actinomycin D (AMD)

When *ts* RSV-CEF are shifted from restrictive to permissive temperature, pp60v-*src* tyrosine kinase activity increases even when RNA synthesis is blocked by actinomycin D (AMD) (Kawai & Hanafusa, 1971). The requirement of RNA synthesis for the stimulation of PFK-2 would confirm that pp60v-*src* acts on PFK-2 by an indirect mechanism. We therefore examined the effect of AMD on PFK-2 activity and Fru-2,6,-P_2 concentration in control and *ts* RSV-CEF shifted from restrictive to permissive temperature. The results in Table 1 show that, as expected, Fru-2,6-P_2 concentration and PFK-2 activity were higher in *ts* RSV-CEF than in control CEF at the permissive temperature, in the absence of AMD. Treatment with AMD before the temperature shift abolished this difference in PFK-2 activity. When AMD was administered to the cells after the temperature shift, PFK-2 activity was the same as in the absence of AMD, both in control CEF and in *ts* RSV-CEF (Table 1). This rules out the possibility that AMD could have lowered the activity of PFK-2 by a direct interaction with this enzyme.

These results demonstrate that at least one intermediate step, inhibited by AMD, is involved in the activation of PFK-2 by pp60v-*src*. The difference in Fru-2,6-P_2 concentration between *ts* RSV-CEF and control CEF seen at the permissive temperature was only partially decreased by AMD. This is in agreement with the previous observation that AMD does not prevent the increase in glucose uptake in *ts* RSV-CEF shifted from the restrictive to the permissive temperature (Kawai & Hanafusa, 1971). As described above, an increase in glucose uptake is expected to lead, via an increase in the fructose 6-phosphate concentration, to an increased Fru-2,6-P_2 concentration even if the PFK-2 activity does not increase.

PFK-2 content and properties

To see whether the difference in PFK-2 activity between *ts* RSV-CEF and control CEF was explained by a change in enzyme content, PFK-2 activity was immunotitrated in PEG extracts from these cells. There was no difference in the amount

Table 1. Effects of AMD on Fru-2,6-P_2 content and PFK-2 activity

Secondary cultures of CEF, uninfected or infected with *ts* NY68 at 36 °C, were incubated overnight at 42 °C. At 2 h before returning to 36 °C, the cells were switched to incubation medium and AMD (1 or 2 μg/ml) was added ('AMD before t° shift'). Alternatively, the cultures were treated in the same way except that AMD (2 μg/ml) was added 5 h after returning the cells to the permissive temperature ('AMD after t° shift'). Determinations were performed 6 h after the temperature shift. Results are means ± S.E.M. for the numbers of experiments shown in parentheses. * $P < 0.01$ versus uninfected cells; † $P < 0.01$ versus infected cells not treated with AMD. N.D., not determined.

Cells	AMD	[Fru-2,6-P_2] (pmol/mg of protein)	PFK-2 activity (μunits/mg of protein)
Control	None	66 ± 4 (4)	28 ± 4 (4)
	Before t° shift	71 ± 5 (4)	20 ± 4 (7)
	After t° shift	N.D.	25 ± 1 (3)
RSV-CEM	None	277 ± 18 (4)*	52 ± 1 (2)*
	Before t° shift	149 ± 10 (4)*†	22 ± 5 (7)†
	After t° shift	N.D.	60 ± 5 (3)*

Table 2. PFK-2 activity, content and thermostability

The content is the volume of MCL-2 antiserum required to precipitate 50% of PFK-2 activity in the presence of Protein A–Sepharose, calculated from log plots of immunotitration curves. Thermostability to the time necessary to inactivate 50% of PFK-2 activity at 50 °C (see Fig. 2). * $P \leq 0.01$ versus controls. The values are means ± S.E.M. for the numbers of experiments given in parentheses.

Cells	Activity (μunits/mg of protein)	Content (μl)	Thermostability (min)
Control	30 ± 5 (15)	9.1 ± 2.6 (4)	6.3 ± 0.4 (3)
RSV	61 ± 8 (15)*	10.4 ± 2.5 (4)	10.8 ± 0.8 (3)*

of immunotitrable PFK-2 between control and *ts* RSV-CEF. Table 2 summarizes several experiments and shows that, despite the higher activity of PFK-2 in *ts* RSV-CEF, the content of PFK-2 protein was the same as in control CEF. These data suggest that the difference in PFK-2 activity results from a change in specific activity rather than a change in content.

We therefore searched for RSV-induced differences in PFK-2 properties. We first studied the thermostability of this enzyme *in vitro* by measuring the activity of PFK-2 remaining after treatment of PEG extracts at 50 °C for various periods of time. The data (Fig. 2*a*, Table 2) show that PFK-2 from *ts* RSV-CEF was more thermostable than that from control CEF. Moreover, this change was abolished by pretreatment of the cells with AMD (Fig. 2*b*).

We then determined in PEG extracts the apparent K_m for fructose 6-phosphate of PFK-2 from control and *ts* RSV-CEF. PFK-2 activity was assayed with a saturating concentration (4 mM) of the other substrate, MgATP, and with various concentrations of fructose 6-phosphate in equilibrium with glucose 6-phosphate in a ratio of 1:3 (Bartrons *et al.*, 1983). Apparent K_m values were calculated from Hanes plots. The ratio of the apparent K_m for fructose 6-phosphate for control CEF relative to *ts* RSV-CEF was 2.6 ± 0.28 (mean ± S.E.M. for four experiments, $P < 0.01$). When the cells were pretreated with AMD (2 μg/ml) 2 h prior to shifting to the permissive temperature, this ratio was equal to 0.8, indicating that the apparent affinity of PFK-2 for fructose 6-phosphate was no longer increased upon transformation by RSV. Thus both the specific activity of PFK-2 and its apparent affinity for fructose 6-phosphate are increased upon transformation by RSV, and both changes are AMD-sensitive.

Effect of staurosporine

The above results ruled out a direct effect of pp60v-*src* in activating PFK-2. The lag period and requirement for RNA synthesis were reminiscent of those seen when PFK-2 activity increases in CEF treated with phorbol esters such as phorbol 12-myristate 13-acetate (PMA). Indeed, this effect of PMA requires more than 2 h (Bosca *et al.*, 1986) and is blocked by inhibitors of RNA and protein synthesis (Rousseau *et al.*, 1988). Since this effect of PMA was shown to involve PKC activation (Bosca *et al.*, 1985), we wondered whether this might hold true for the effect of pp60v-*src*. Indeed, the stimulating effects of RSV and PMA on PFK-2 in CEF are not additive, consistent with their action through a common pathway (Bosca *et al.*, 1986). We therefore resorted to the potent PKC inhibitor, staurosporine. This compound acts by interacting with the catalytic domain of

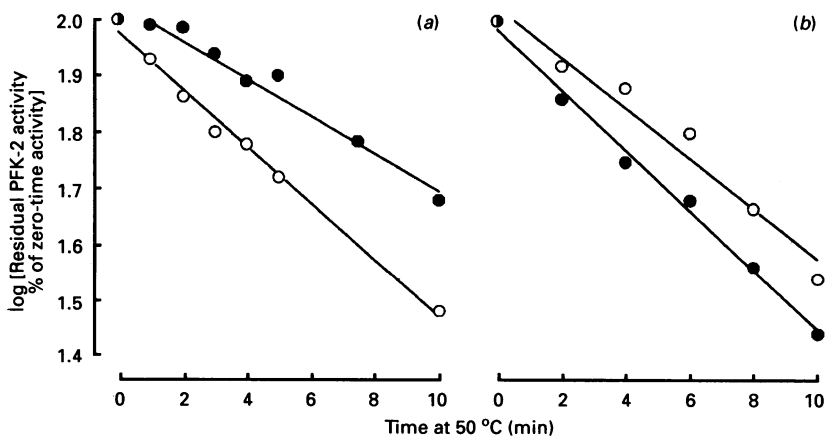

Fig. 2. Thermostability of PFK-2 from control (○) and *ts* RSV (●) CEF

(*a*) No treatment with AMD. PFK-2 activities at zero time were 6.8 and 12.5 μunits/mg of protein for control and *ts* RSV-CEF respectively. The corresponding PFK-2 half-lives at 50 °C were 5.5 and 10 min. (*b*) Cells were treated with AMD (2 μg/ml) 2 h before the shift to permissive temperature, as explained in Table 1. PFK-2 activities at zero time were 11.6 and 11.3 μunits/mg of protein respectively. The corresponding PFK-2 half-lives at 50 °C were 7.2 and 5.3 min.

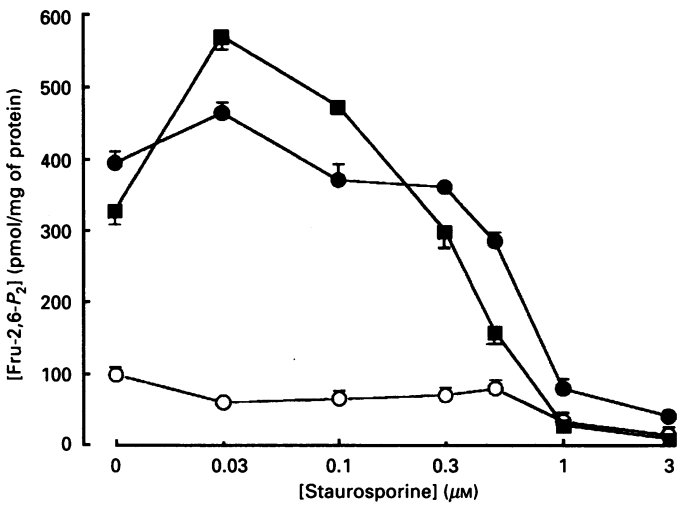

Fig. 3. Dose–response curve for the effect of staurosporine on Fru-2,6-P_2 concentration

Secondary cultures of CEF were uninfected (○) or infected with *ts* NY68 (●) at 36 °C. After overnight culture at 42 °C in incubation medium, staurosporine was added; 10 min later, the cells were shifted to permissive temperature (36 °C), and PMA (100 ng/ml) was added to a batch of uninfected cells (■). The Fru-2,6-P_2 concentration was determined after 5 h of incubation at 36 °C. Data are means ±S.E.M. for triplicate dishes. Values without S.E.M. are from single or duplicate dishes.

Table 3. Effects of staurosporine on PFK-2 activity

Secondary cultures of CEF were uninfected or infected with *ts* NY68 at 36 °C. After overnight culture at 42 °C in incubation medium, the cells were shifted to the permissive temperature (36 °C), and PMA (100 ng/ml) was added to a batch of uninfected cells. After 5 h of incubation at 36 °C, the cells were harvested for determination of PFK-2 activity. Staurosporine (1 μM) was added (i) *in vitro* to the PEG extract prior to determination of PFK-2 activity, (ii) 30 min prior to harvesting the cells, i.e 4.5 h after the temperature shift, or (iii) 10 min before the temperature shift. Data are means ±S.E.M. for the numbers of experiments given in parentheses. * $P < 0.01$ compared with control (no treatment, no staurosporine).

Treatment	Staurosporine	PFK-2 activity (μunits/mg of protein)
None	None	29±2 (5)
	In vitro	31
	After t° shift	31
	Before t° shift	9±2 (3)*
PMA	None	81±10 (5)*
	In vitro	88
	After t° shift	70
	Before t° shift	8±2 (3)
RSV	None	72±10 (5)*
	In vitro	88
	After t° shift	113
	Before t° shift	9±3 (3)*

PKC rather than by competing with diacylglycerol or phorbol esters, calcium, ATP or phospholipids (Tamaoki *et al.*, 1986).

As expected, staurosporine prevented PMA from increasing the Fru-2,6-P_2 concentration in CEF (Fig. 3). This is consistent with the demonstration (Taylor *et al.*, 1990) that staurosporine prevents the PMA-induced increase in the Fru-2,6-P_2 concentration in human fibroblasts. Low concentrations of saurosporine potentiated the effect of PMA, suggesting that this inhibitor can behave as a partial agonist. Staurosporine also prevented the effect of pp60v-*src* on Fru-2,6-P_2, and the dose–response curve was similar to that seen for the inhibition of the PMA effect (Fig. 3). Concomitantly, staurosporine not only inhibited PFK-2 activation by PMA, it also prevented the rise in PFK-2 activity seen upon resumption of pp60v-*src* action by the temperature shift (Table 3). We verified that staurosporine did not inhibit PFK-2 activity *in vitro* at the highest concentration used. A toxic effect of staurosporine on CEF was also ruled out, since this drug did not inhibit the established effects of PMA or RSV when added to cells 30 min prior to harvesting for PFK-2 measurements (Table 3). PFK-2 activity in cells exposed to staurosporine for more than 5 h was lower than in control cells, suggesting that sustained PKC activity was required for maintaining basal PFK-2 activity. Pretreatment of *ts* RSV-CEF with a high concentration of PMA to down-regulate PKC (800 nM-PMA for 24 h at 42 °C) prevented the rise in PFK-2 activity seen when returning the cells to the permissive temperature (results now shown). These results support the idea that PKC activation mediates the activation of PFK-2 by pp60v-*src*.

DISCUSSION

Earlier work from this laboratory showed that PFK-2 activity increases in CEF expressing tyrosine-specific protein kinase oncogenes, but not other oncogenes (Bosa *et al.*, 1986). An obvious possibility was that this resulted from a direct phosphorylation of PFK-2 by pp60v-*src*. The data presented here rule out this hypothesis. First, PFK-2 activation by pp60v-*src* required more than 2 h. Second, AMD prevented this activation. Thus pp60v-*src* most likely induces the expression of a gene whose product directly or indirectly activates PFK-2. This activation results from a stable change of PFK-2. Indeed, the amount of immunotitratable PFK-2 did not change, while its specific activity, thermostability and K_m did. These changes, which were all AMD-sensitive, withstood partial purification of the enzyme, consistent with a covalent modification, e.g. phosphorylation. In the rat, the liver-type isoenzyme is phosphorylated by cyclic AMP-dependent protein kinase, and in cattle the two forms of the heart-type isoenzyme are phosphorylated by this kinase as well as by PKC (Rider *et al.*, 1992). Incubation of CEF extracts with purified cyclic AMP-dependent protein kinase or with PKC had no effect on PFK-2 activity (M. J. Marchand, L. Maisin, L. Hue & G. G. Rousseau, unpublished work). Thus the activation of PFK-2 by pp60v-*src* does not involve a direct effect of these kinases on PFK-2. On the other hand, we failed in our attempts to determine the state of phosphorylation of PFK-2 from CEF incubated with [^{32}P]P_i by using the anti-PFK-2 antibody. Indeed, although this polyclonal antibody efficiently precipitates PFK-2 activity, it was not suitable for purifying the enzyme with sufficient specific activity.

Our results do not rule out the possibility that pp60v-*src* induces the expression of a PFK-2 mRNA encoding an isoenzyme (induced PFK-2) whose properties differ from those of the PFK-2 expressed in non-infected CEF (constitutive PFK-2). The apparent lack of change in PFK-2 content in transformed CEF despite the increase in PFK-2 activity would then be explained by a decreased content of the constitutive isoenzyme in transformed CEF, or by the fact that the induced PFK-2 is more immunotitrable than the constitutive PFK-2. At least two genes and three PFK-2 mRNAs and isoenzymes have been characterized in the rat (Crepin *et al.*, 1989; Darville *et al.*, 1989, 1991). Since we failed to detect, by Northern blotting with all available rat

cDNA probes, PFK-2 mRNA in control or transformed CEF, the clarification of this problem awaits the cloning of CEF PFK-2 mRNA.

In any case, the present data point to another intermediate step in RSV-induced PFK-2 activation, namely PKC activation. It is well established that PMA activates PKC, and our earlier work has shown that the increase in the Fru-2,6-P_2 concentration seen in CEF treated with PMA indeed involves PKC activation (Bosca *et al.*, 1985). Thus, albeit indirect, the evidence for an involvement of PKC in RSV-induced PFK-2 activation is as follows. (i) The effect of pp60v-*src* is not additive to that of PMA, consistent with the existence of a common pathway (Bosca *et al.*, 1986). It has (ii) the same lag period and (iii) the same requirement for RNA synthesis. It is prevented by (iv) a PKC inhibitor and (v) PKC down-regulation. (vi) Similarly to PFK-2 from RSV-treated CEF, PFK-2 from PMA-treated CEF displays an increased thermostability, without an apparent change in total content (Y. Fischer, M. A. Gueuning, X. Testar & G. G. Rousseau, unpublished work). (vii) RSV-transformed cells exhibit increased phosphatidylinositol turnover (Diringer & Friis, 1977; Johnson *et al.*, 1989). (viii) The concentration of diacylglycerol, the physiological activator of PKC, increases within 30 min following pp60v-*src* expression (Martins *et al.*, 1989). Finally, Spangler *et al.* (1989) have demonstrated that pp60v-*src* uses PKC to induce the expression of a transformation-related gene. This, together with our present and earlier work, suggests that in fibroblasts the PKC transduction pathway integrates a variety of signals, e.g. tyrosine kinase oncogenes, growth factors and tumour promoters, to control PFK-2 activity and therefore glycolysis. This effect of PKC is indirect and involves the expression of a gene whose product is a distinct PFK-2 isoenzyme or a protein that stably activates PFK-2 directly or indirectly.

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