Expression of the *v-src* or *v-fps* oncogene increases fructose 2,6-bisphosphate in chick-embryo fibroblasts

Novel mechanism for the stimulation of glycolysis by retroviruses

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The concentration of fructose 2,6-bisphosphate and the activity of 6-phosphofructo-2-kinase are increased after infection of chick-embryo fibroblasts with the Rous sarcoma virus, or with a temperature-sensitive mutant of this virus at the permissive, but not at the non-permissive, temperature. This is observed after transformation by retroviruses carrying either the *v-src* or *v-fps*, but not the *v-mil* and/or *v-myc*, oncogenes. Comparison of the effects of the Rous sarcoma virus with those of phorbol myristate acetate on fructose 2,6-bisphosphate suggests that both result from the stimulation of a step which is rate-limiting for 6-phosphofructo-2-kinase activation and which is also controlled by protein kinase C.

INTRODUCTION

Transformation of chick-embryo fibroblasts (CEF) by the Rous sarcoma virus (RSV) involves a series of biochemical and morphological changes that all depend on the activity of *p60v-src*, the protein encoded by the viral *src* gene (for review, see Weber, 1984). One of these biochemical changes is an increased rate of glycolysis, which is also typical of many tumours and established lines of transformed cells (Weinhouse, 1976), and is observed after exposure of quiescent cells to mitogens (Diamond *et al.*, 1978; Bruni *et al.*, 1983) and tumour promoters (Driedger & Blumberg, 1977; O'Brien *et al.*, 1979). This results in part from a stimulation of 6-phosphofructo-1-kinase (PFK-1) (Singh *et al.*, 1974). However, the mechanism by which expression of the *v-src* gene leads to PFK-1 stimulation is unknown. In view of the discovery (Van Schaftingen *et al.*, 1980) of fructose 2,6-bisphosphate (Fru-2,6- P_2), a potent stimulator of liver PFK-1, we have investigated whether the concentration of Fru-2,6- P_2 might be under the control of *p60v-src*. Indeed, we had found that Fru-2,6- P_2 is present in CEF, that it can stimulate their PFK-1, and that both Fru-2,6- P_2 concentration and the glycolytic rate are increased after treatment of CEF with mitogenic doses of insulin or with phorbol esters (Bosca *et al.*, 1985).

We have now studied the effects of CEF transformation by RSV or by retroviruses carrying oncogenes that are or not related to *src*. The parameters measured included the production of lactate, the concentration of Fru-2,6- P_2 , and the activity of 6-phosphofructo-2-kinase (PFK-2), the enzyme that catalyses the synthesis of Fru-2,6- P_2 . The changes observed were compared with those elicited after exposure of the cells to the tumour promoter phorbol 12-myristate 13-acetate (PMA).

MATERIALS AND METHODS

Materials and cell culture

The sources of chemicals and biochemical reagents are as indicated previously (Bosca *et al.*, 1985; Mojena *et al.*, 1985). [3 H]Thymidine (24 Ci/mmol) was from Amersham and [35 S]methionine (1000 Ci/mmol) was from New England Nuclear. Ham's F10 medium and methionine-free Eagle's modified minimum essential medium were from Flow Laboratories; chicken serum, newborn-calf serum and antibiotics were from Gibco; tryptose phosphate broth was from Difco.

CEF were prepared (Hunter, 1979) from virus-free group-specific-antigen-negative chick embryos (White Leghorn; Lohman, Cuxhaven, Germany) after 8–9 days of incubation. The cells were cultured in an atmosphere of air containing 5% CO_2 at 38 °C in Ham's F10 medium supplemented with penicillin/streptomycin (50 units/ml), tryptose phosphate broth (3 g/l), 7 mM- $NaHCO_3$ and 20 mM-Hepes, pH 7.2 (culture medium), to which 2.4% (v/v) chicken serum and 10% (v/v) newborn-calf serum were added. After 4–5 days of culture, the cells were collected by trypsin treatment, resuspended in culture medium supplemented with 6% newborn-calf serum (growth medium), and seeded in 60 mm-diam. plastic Petri dishes (0.7×10^6 cells in 3 ml per dish). Primary cultures were infected by addition of medium containing the indicated viruses: Schmidt-Ruppin RSV-A, Rous-associated virus type 1 (RAV-1), FSV (FAV), MC29 (RAV-1) and MH2 (RAV-1). The cells were passaged once, and after 4–5 days of secondary culture, the growth medium was removed and replaced by the same volume of incubation medium (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- $CaCl_2$, 20 mM-Hepes, pH 7.2).

Abbreviations used: CEF, chick-embryo fibroblasts; Fru-2,6- P_2 , fructose 2,6-bisphosphate; FSV, Fujinami sarcoma virus; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PMA, phorbol 12-myristate 13-acetate; RAV, Rous-associated virus; RSV, Rous sarcoma virus.

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Table 1. Effects of retroviruses and PMA treatment in chick-embryo fibroblasts

Secondary cultures at 38 °C were exposed for 5 h in incubation medium to PMA (100 ng/ml) or the vehicle. The values for thymidine incorporation are means from triplicate incubations. The other values are means $\pm$ s.d. for at least three experiments on duplicate dishes. PFK-2 activity was measured on the 0–15% poly(ethylene glycol) fraction. Abbreviation: ND, not determined.

Virus	Oncogene	PMA	$10^{-3} \times$ Thymidine incorporation (d.p.m./mg of protein)	Lactate release (μ mol/h per mg of protein)	Fru-2,6- P_2 (pmol/mg of protein)	PFK-2 (pmol/min per mg of protein)
None	—	—	83	0.6 ± 0.1	68 ± 3	9 ± 2
		+		1.2 ± 0.1	248 ± 15	25 ± 8
RAV-1	—	—	ND	0.7 ± 0.2	53 ± 4	10
		+		1.4 ± 0.1	292 ± 8	36
RSV	<i>src</i>	—	261	1.5 ± 0.2	201 ± 2	22 ± 1
		+		1.4 ± 0.1	218 ± 41	24 ± 1
<i>ts</i> NY68	<i>src</i>	—	234	2.9 ± 0.4	244 ± 25	28 ± 3
		+		2.5 ± 0.1	206 ± 11	33 ± 4
FSV	<i>fps</i>	—	341	1.8 ± 0.5	215 ± 10	27
		+		1.9 ± 0.2	228 ± 8	32
MC29	<i>myc</i>	—	156	1.1 ± 0.1	76 ± 8	4 ± 2
		+		1.8 ± 0.2	288 ± 12	20 ± 5
MH2	<i>mil-myc</i>	—	148	0.6 ± 0.1	96 ± 5	10 ± 1
		+		1.2 ± 0.1	263 ± 33	19 ± 3
MH2-cl.25	<i>mil-myc</i> ⁺	—	ND	0.7	77	ND
		+		1.2 ± 0.1	262 ± 14	ND
MH2-PA200	<i>mil-myc</i> [—]	—	ND	1.0 ± 0.1	100 ± 4	ND
		+		1.6 ± 0.1	272 ± 9	ND

This medium contained 5.6 mM-glucose except where otherwise stated. PMA was added to give a concentration of 100 ng/ml; the controls received the corresponding volume of the vehicle, dimethyl sulphoxide (0.01%). For the temperature-shift experiments, secondary cultures (4 days) transformed with RSV or mutant *ts*NY68 were incubated overnight at 42 °C. The next morning the dishes were returned to 38 °C and the growth medium was replaced by 3 ml of fresh incubation medium at 38 °C. Samples were taken at different times to measure Fru-2,6- P_2 as indicated below.

Biochemical measurements

Fru-2,6- P_2 and lactate were measured in deproteinized

cell extracts prepared as described elsewhere (Bosca *et al.*, 1985). PFK-2 activity was assayed under $V_{\max}$ conditions after precipitation with poly(ethylene glycol) as described by Mojena *et al.* (1985).

For thymidine incorporation, secondary cultures (4 days) were washed twice with culture medium, and then incubated for 30 h in culture medium containing 3 μ M-[³H]thymidine (10 μ Ci/ml). The cell layer was then washed with ice-cold incubation medium (3 $\times$ 5 ml) and lysed in 0.5 ml of 10 mM-Tris/HCl, pH 7.5, containing 0.1% SDS and 1% Triton X-100. Then 5 vol. of ice-cold 10% (v/v) trichloroacetic acid were added and, after centrifugation (Eppendorf Microfuge, 5 min), the pellets were absorbed on glass-fibre filters, washed with 5 ml of 8% trichloroacetic acid, dried, and the radioactivity was measured.

Table 2. Effect of retroviruses and PMA treatment on Fru-2,6- P_2 concentration at a non-permissive (42 °C) temperature

Experimental conditions were as in Table 1. The values are means $\pm$ s.d. for at least three experiments.

Virus	PMA	Fru-2,6- P_2 (pmol/mg of protein)
None	—	66 ± 10
	+	570 ± 64
RAV-1	—	61 ± 2
	+	336 ± 7
RSV	—	294 ± 3
	+	335 ± 26
<i>ts</i> NY68	—	88 ± 7
	+	427 ± 24

RESULTS

Expression of *p60v-src* leads to activation of the PFK-2/Fru-2,6- P_2 system

Transformation of CEF by RSV led to the expected stimulation of DNA synthesis, as measured by thymidine incorporation (Hanafusa, 1969), and of the glycolytic rate, expressed as lactate release. In addition, there was a 2–3-fold increase in PFK-2 activity (change in $V_{\max}$, with no apparent change in K_m) and a 3-fold increase in Fru-2,6- P_2 concentration (Table 1). These changes appear to depend on the expression of *p60v-src*, since they did not occur when the cells were infected with the avian leukosis transformation-defective type 1 Rous-associated virus (RAV-1), which lacks the *src* gene. To substantiate this point, we studied the effect of temperature on the

Fig. 1. Effect of PMA treatment on Fru-2,6- P_2 concentration and lactate release at different glucose concentrations

Secondary cultures at 38 °C were maintained for 5 h in incubation medium containing the indicated concentrations of glucose, with or without PMA (100 ng/ml). The rate of lactate release was calculated from samples taken after 2 h and 5 h of incubation. ●, ▲, cells treated with PMA; ○, △, controls. The values shown are means of two experiments run in duplicate.

concentration of Fru-2,6- P_2 in CEF transformed by *ts*-NY68, a temperature-sensitive transformation mutant of RSV (Kawai & Hanafusa, 1971). At the non-permissive temperature, the *v-src*-encoded protein of *ts*-NY68 loses its kinase activity and no longer maintains the transformed phenotype (Collett & Erikson, 1978). The data in Table 2 show that transformation of CEF by the wild-type RSV caused, at 42 °C, an increase in Fru-2,6- P_2 concentration similar to that observed at 38 °C (Table 1). However, although cells infected with *ts*-NY68 responded at 38 °C (permissive temperature) as did the RSV-transformed cells, they exhibited no increase in Fru-2,6- P_2 concentration at 42 °C (non-permissive temperature). Additional evidence that these effects depend on expression of the activity of *p60v-src* stems from the experiments with other oncogenic retroviruses reported below.

Mechanism of PFK-2 activation by RSV

The effects of RSV described here are reminiscent of those elicited by phorbol esters in CEF. We have shown previously (Bosca *et al.*, 1985) that the stimulation of glycolysis by PMA can be explained, at least in part, by an increase in Fru-2,6- P_2 concentration, itself resulting from an activation of PFK-2. The relationship between glycolysis and Fru-2,6- P_2 in CEF is supported by the results shown in Fig. 1. It is clear that lactate production correlated with Fru-2,6- P_2 concentration, whether glycolysis was modulated by glucose, by PMA, or by both agents.

A comparison of the amplitude and time course of the effects of PMA and of RSV, alone or in combination, provides strong evidence that the effect of *p60v-src* and that of PMA on the PFK-2/Fru-2,6- P_2 system involve a

common mechanism which is not a direct activation of PFK-2. As shown in Table 1, not only did PMA increase Fru-2,6- P_2 concentration and PFK-2 activity in normal or RAV-1-infected CEF to the same extent as did transformation by RSV, but also it was unable to stimulate these parameters further in CEF transformed by wild-type RSV, or by *ts*-NY68 at the permissive temperature. This is in contrast with the additivity of the effects of PMA and glucose on Fru-2,6- P_2 concentration (Fig. 1). Moreover, although the effects of RSV and PMA were again not additive at 42 °C, PMA readily increased Fru-2,6- P_2 concentration at that temperature in cells infected with *ts*-NY68 as it did in normal or RAV-1-infected CEF (Table 2).

An additional feature common to RSV and PMA treatment is the time course of their stimulatory effect on Fru-2,6- P_2 . As shown in Fig. 2, a lag period of 1.5–2 h elapsed before Fru-2,6- P_2 concentration and PFK-2 activity rose in response to PMA. Consistent with this observation, no direct effect of PMA *in vitro* on the activity of partially purified PFK-2 could be demonstrated. The time course of the effect of *p60v-src* expression could be studied by taking advantage of *ts*-NY68. When the activity of *p60v-src* from this mutant has been abolished at the non-permissive temperature, it is restored within a few minutes after shift to the permissive temperature (Kawai & Hanafusa, 1971; Sugimoto *et al.*, 1984). CEF infected with *ts*-NY68 and maintained at 42 °C were shifted to 38 °C. Fru-2,6- P_2 concentration began to rise after a lag period similar to that seen with PMA (Fig. 2). These results point to a rate-limiting step in the activation of PFK-2 by PMA and by *p60v-src*. The similarity of the lag periods as well as the lack of additivity suggest that both types of stimuli converge directly or indirectly on this rate-limiting step.

Effect of other oncogenic retroviruses

To evaluate further whether the changes in the PFK-2/Fru-2,6- P_2 system seen in RSV-transformed CEF are specific for *src*-gene expression, we resorted to five

Fig. 2. Time course of the effect of PMA and of *v-src* expression on the PFK-2/Fru-2,6- P_2 system

(a) Secondary cultures at 38 °C were switched to incubation medium and, after 2 h, were exposed to PMA (100 ng/ml) for the time periods indicated. PFK-2 activity was measured on the 6–12% poly(ethylene glycol) fraction. (b) Secondary cultures from cells infected with RSV (△) or *ts*-NY68 (▲) at 38 °C were transferred overnight at 42 °C and then (zero time on the Figure) returned to 38 °C while being switched to incubation medium. Data are from duplicate dishes.

oncogenic retroviruses other than RSV or *tsNY68*. The Fujinami sarcoma virus (FSV) contains the *v-fps* oncogene, the leukaemia virus MC29 contains the *v-myc* oncogene, and the Mill Hill no. 2 (MH2) virus contains both the *v-myc* and *v-mil* oncogenes. The oncogenes *v-src*, *v-fps*, and *v-mil* code for proteins which are structurally related. *p60v-src* and FSV *p140Agag-fps* are protein kinases specific for tyrosine residues, whereas MH2 *p100Agag-mil* appears to be associated with a serine/threonine-specific protein kinase (Moelling *et al.*, 1984). All *myc*-encoded proteins are nuclear and bind to DNA *in vitro* (for review, see Hunter, 1984). We also studied the effects of two mutants of MH2 (Bechade *et al.*, 1985) which lack either the *mil* (MH-cl.25, *myc*⁺) or the *myc* (MH2-PA200, *mil*⁺) oncogene. CEF infected by these five types of viruses were examined for their Fru-2,6- P_2 concentration and PFK-2 activity. Transformation was checked by morphological criteria and by increased thymidine uptake (Table 1). The oncogenic proteins of the respective viruses were readily detected by immunoprecipitation of lysates of [³⁵S]methionine-labelled cells followed by SDS/polyacrylamide-gel electrophoresis and autoradiography (results not shown).

As shown in Table 1, PFK-2 activity, Fru-2,6- P_2 concentration and lactate release all increased upon transformation by FSV, as was the case with RSV. Consistent with a mechanism common to both viruses, none of these changes was amplified further by PMA treatment (Table 1). By contrast, after transformation by MC29 or MH2, lactate release increased only slightly or not at all, and there was no evidence of an activation of the PFK-2/Fru-2,6- P_2 system. An inhibition by *v-myc* of a putative effect of *v-mil* in MH2-transformed cells can be ruled out in view of the negative results also seen after infection with the two mutants of MH2. Furthermore, PMA clearly increased lactate release, Fru-2,6- P_2 concentration and PFK-2 activity in CEF infected with MC29 or MH2 as it did in normal cells. Thus retroviral activation of PFK-2 and its metabolic consequences seem to correlate with the expression of the oncogenic proteins that are tyrosine-specific kinases.

DISCUSSION

Transformation of CEF by RSV correlated with an activation of PFK-2 resulting in a 3-fold increase in the concentration of Fru-2,6- P_2 . This increase spans the range of Fru-2,6- P_2 concentrations that are effective in stimulating PFK-1 of CEF at physiological concentrations of other effectors and substrates (Bosca *et al.*, 1985). Thus our finding provides a novel clue for the increased glycolysis seen in response to the expression of the viral *src* gene. An involvement of the PFK-2/Fru-2,6- P_2 system in the control of glycolysis by mitogens and phorbol esters has been demonstrated previously (Bosca *et al.*, 1985). A lack of effect on the PFK-2/Fru-2,6- P_2 system correlated with a lack of increase in lactate production, as seen after transformation by MC29, or by MH2 or its mutants. These results draw attention to the fact that the phenotype of transformed cells does not always include an increased glycolysis or hexose uptake, in keeping with previous observations (Pouyssegur *et al.*, 1980; Royer-Pokora *et al.*, 1978).

The changes in the PFK-2/Fru-2,6- P_2 system occurring in CEF transformed by RSV may not be the only determinants of the 'glycolytic phenotype', since glucose

transport is also stimulated (Hatanaka & Hanafusa, 1970; Weber *et al.*, 1984). The relative contribution of these two phenomena to the overall control of glycolysis in CEF is unknown. Enolase, phosphoglycerate mutase and lactate dehydrogenase have been shown to be phosphorylated on tyrosine residues in cells transformed by viruses whose transforming proteins are tyrosine-specific kinases (Cooper *et al.*, 1983). The significance of these phosphorylations for the regulation of glycolysis is unknown, because they involve only 10% of the enzyme molecules, because no functional alteration in enzyme activity has been reported, and because the reactions catalysed by these enzymes are near equilibrium.

As to the mechanism of PFK-2 activation by *p60v-src*, our previous (Bosca *et al.*, 1985) and present data raise the question as to whether it involves protein kinase C, an enzyme which catalyses the phosphorylation of threonine and serine residues on proteins and has been implicated in the action of many mitogens (Nishizuka, 1984). Binding of these mitogens to their receptor is thought to lead to the release of endogenous inositol 1,4,5-trisphosphate and diacylglycerol (Berridge *et al.*, 1985), the latter being a stimulator of protein kinase C. PMA stimulates protein kinase C activity by mimicking diacylglycerol (Castagna *et al.*, 1982). In CEF, phorbol ester analogues and glycerol derivatives influence Fru-2,6- P_2 concentration in keeping with their known effect on protein kinase C (Bosca *et al.*, 1985).

Since the effects of PMA and *p60v-src* expression occurred after the same lag period and were not additive, it is likely that both stimuli share a common mechanism. This mechanism could be the direct or indirect activation of protein kinase C. The latter possibility is consistent with the finding that *p60v-src* influences polyphosphoinositide metabolism (Sugimoto *et al.*, 1984).

The finding that PFK-2 can be activated as a result of *p60v-src* expression should yield novel clues for the identification of the physiologically relevant substrates of *p60v-src*, which remain elusive. Also, determination of Fru-2,6- P_2 could prove useful as an early biochemical index of effective cell transformation by oncogenic proteins that are tyrosine-specific kinases.

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