Apoptosis induced by growth factor withdrawal in fibroblasts overproducing fructose 2,6-bisphosphate

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Abstract Fructose 2,6-bisphosphate is a potent endogenous stimulator of glycolysis. A high aerobic glycolytic rate often correlates with increased cell proliferation. To investigate this relationship, we have produced clonal cell lines of Rat-1 fibroblasts that stably express transgenes coding for 6-phosphofructo-2-kinase, which catalyzes the synthesis of fructose 2,6-bisphosphate, or for fructose 2,6-bisphosphatase, which catalyzes its degradation. While serum deprivation in culture reduced the growth rate of control cells, it caused apoptosis in cells overproducing fructose 2,6-bisphosphate. Apoptosis was inhibited by 5-amino-4-imidazolecarboxamide riboside, suggesting that 5′-AMP-activated protein kinase interferes with this phenomenon.

Key words: 5-Amino-4-imidazolecarboxamide riboside; 5′-AMP-activated protein kinase; Apoptosis; Fructose 2,6-bisphosphate; 6-Phosphofructo-2-kinase/fructose 2,6-bisphosphatase; Rat-1 fibroblast

1. Introduction

F-2,6-P2 is a ubiquitous stimulator of 6-phosphofructo-1-kinase, a key regulatory enzyme in the glycolytic pathway. Its synthesis and degradation are catalyzed respectively by PFK-2 and FBPase-2, two activities that are borne by distinct catalytic sites of a single protein [1]. Many tumor and fast growing cells exhibit a high rate of glycolysis even under aerobic conditions [2,3]. F-2,6-P2 has been reported to play a role in this phenomenon. First, the concentration of F-2,6-P2 is often higher in established lines of cancer cells than in the corresponding normal tissue [4]. Second, PFK-2 activity, F-2,6-P2 concentration and glycolysis increase in quiescent fibroblasts when they are stimulated by growth factors or phorbol esters, or are transformed by the v-src or v-fps oncogenes [4–6]. Third, PFK-2/FBPase-2 mRNA is induced in normal fibroblasts by serum or growth factors, by the v-src oncogene product and by the G1/S transition of the cell cycle [7–10].

These observations raised the question as to whether F-2,6-P2 might contribute to the mitogenic potential of the cells. If this is so, normal fibroblasts that overproduce F-2,6-P2 might keep proliferating when cultured in conditions of serum restriction. Indeed, serum or mitogen deprivation induces growth arrest in a G1-like state often termed G_o. The aim of this work was to evaluate the effect of F-2,6-P2 on the control of cell proliferation by serum in Rat-1 fibroblasts, a non-transformed diploid cell line. This was performed on clonal cell lines obtained from fibroblasts stably transfected with expression vectors for rat PFK-2/FBPase-2 in which either the FBPase-2 or the PFK-2 activity had been destroyed by site-directed mutagenesis.

2. Materials and methods

2.1. Plasmids

Plasmid pBS-KSII$^+$/PFK-2-L-H258A, kindly provided by L. Bertrand, was obtained by introducing the H258A mutation in pBS-KSII$^+$/PFK-2L [11] by site-directed mutagenesis with the primer 5′-CACTCTCACCAGCGCGGCATAGG-3′. As this plasmid lacked the original ATG start site, this site was recovered by ligating an *Eco*RI fragment from pBS-KSII$^+$/PFK-2-L to *Eco*RI-digested pBS-KSII$^+$/PFK-2-L-H258A. The S32A mutation was introduced by polymerase chain reaction [12] using the antisense 5′-CTGTGGTATG-GAGGCGCCCCTTCGC-3′ and sense 5′-TTCACTAATTCTCC-CACGATGGTG-3′ primers. An *Eco*RI fragment from a clone containing this mutation was ligated to *Eco*RI-digested pBS-KSII$^+$/PFK-2-L-H258A to generate pBS-KSII$^+$/PFK-2-L-S32A-H258A. An *Eco*RV-*Xba*I fragment of this plasmid was cloned into *Eco*RI-*Xba*I-digested pUHD-10.3 (kindly provided by H. Bujard) downstream from a minimal CMV promoter controlled by seven copies of the Tet operator, to obtain the PFK-2$^+$ plasmid. The original ATG start site was introduced into pBS-KSII$^+$/PFK-2-L-K54M [13] by ligating a *Bgl*II fragment of this plasmid to *Bgl*II-digested pBS-KSII$^+$/PFK-2-L. An *Eco*RV-*Xba*I fragment was cloned into *Eco*RI-*Xba*I-digested pUHD-10.3 to obtain the FBPase-2$^+$ plasmid. The pTet-On plasmid (pUHD17-1neo) expressing the doxycycline-inducible reverse Tet-responsive transcriptional activator under the control of the strong CMV immediate early promoter was purchased from Clontech. This plasmid also contains a cassette conferring resistance to geneticin. pBSpacΔp, a plasmid conferring resistance to puromycin, was a generous gift of J.C. Renauld.

2.2. Cells

Rat-1 fibroblasts were maintained as monolayers in DMEM supplemented with 10% TET-FCS (Clontech), 50 IU/ml of penicillin and 50 μg/ml of streptomycin. Stable Tet-On transfectants of Rat-1 fibroblasts were obtained by lipofection using DOTAP (Boehringer, Mannheim) according to the manufacturer's instructions, with 7.5 μg of pTet-On plasmid for 1.5×10^6 cells per 10-cm culture dish in DMEM containing 0.5% Tet-FCS. The DOTAP-DNA complex was left on the cells for 18 h, at which time the cells were washed with PBS and incubated in fresh medium. Six hours later the cells were trypsinized and transferred to DMEM containing 10% Tet-FCS and G418

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Abbreviations: AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, 5′-AMP-activated protein kinase; BrdU, bromodeoxyuridine; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; F-2,6-P2, fructose 2,6-bisphosphate; FBPase-2, fructose 2,6-bisphosphatase; PBS, phosphate buffered saline; PEG, polyethylene glycol; PFK-2, 6-phosphofructo-2-kinase; PKA, protein kinase A; PP-2A, phosphoprotein phosphatase-2A; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling

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(Gibco BRL) at 450 μg/ml. The medium was replaced every 4 days until a non-clonal population resistant to G418 was obtained. When used as controls these cells were kept in a medium containing G418. To obtain the K clones and the P clones, Tet-On cells were stably transfected by lipofection using DOTAP as described above, with 7.5 μg of PFK-2$^+$ (K clones) or FBPase-2$^+$ (P clones) plasmid plus 50 ng of pBSpacΔp plasmid for 1.5×10^6 cells. After selection with puromycin (1 μg/ml), clonal populations were obtained by dilution and screened for Fru-2,6-P2 concentration in the absence or presence of doxycycline (1 μg/ml). All the data shown refer to cells treated with doxycycline.

2.3. BrdU incorporation and apoptosis

Cells were plated at 10^4 cells/well and incubated for 48 h under the conditions indicated. BrdU was added 4 h before the end of the incubation and its incorporation into DNA was determined with the BrdU labeling and detection kit of Boehringer by measuring absorbance at 450 nm. For evaluation of apoptosis, the Cell Death Detection ELISA^{PLUS} of Boehringer was used according to the manufacturer's instructions. Cells were plated at 10^4 cells/well and incubated for 48 h as indicated. Mono- and oligonucleosomes were detected in cell lysates with biotinylated anti-histone and peroxidase-coupled anti-DNA antibodies, followed by measurement of absorbance at 405 nm. When indicated, the extent of apoptosis was calculated as absorbance of test lysate divided by absorbance of lysate from control cells cultured in 10% serum containing medium. For detecting apoptosis by the TUNEL reaction, the cells were incubated on slides for 48 h under the conditions indicated, fixed for 30 min in a freshly prepared solution of paraformaldehyde in PBS (4%) and permeabilized for 2 min in an ice-cold permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate). The DNA strand breaks were identified by labeling free 3′-OH termini with fluorescein-modified dUTP in the presence of terminal deoxynucleotidyltransferase and detection with anti-fluorescein sheep antibody FAB fragments conjugated with alkaline phosphatase. After substrate reaction (fast red, Boehringer) stained cells were identified by light microscopy. For the detection of apoptosis by DNA ladders, cells were cultured for 48 h under the conditions indicated, trypsinized, centrifuged and resuspended in 200 μl of PBS (4°C) for processing according to the apoptotic DNA ladder kit of Boehringer with 2×10^6 cells per DNA extraction. After purification of DNA, RNAse was added at 80 μg/ml followed by a 30 min incubation at room temperature. The DNA samples were electrophoresed in 1.2% agarose gels and DNA fragments were visualized with ethidium bromide.

2.4. Other methods

To determine PFK-2 activity, frozen fibroblasts were homogenized in 50 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 5 mM potassium phosphate, 15 mM β-mercaptoethanol, 20 mM HEPES, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5. After centrifugation ($20\,800\times g$ for 10 min) the supernate was made 6% (v/v) in PEG-6000, left for 30 min at 0°C and centrifuged as above. The concentration of PEG in the supernate was brought to 20% (30 min at 0°C). After centrifugation as above, the pellet was resuspended in homogenization buffer to reach a protein concentration of about 1 mg/ml. Samples (10–20 μl) were incubated at 30°C in 50 mM Tris-Cl, 100 mM KCl, 5 mM potassium phosphate, 20 mM KF, 5 mM fructose 6-phosphate, 15 mM glucose 6-phosphate, 1 mM dithiothreitol, pH 8.5. The reaction was started by adding 5 mM MgATP and stopped after 0–12 min by adding 1 vol of 100 mM NaOH followed by incubation at 80°C for 10 min. F-2,6-P2 was measured as described [14]. AMPK activity was measured by incubating 20 μl of a 6% PEG-treated cell extract with 0.2 mM SAMS peptide in the presence of 0.2 mM AMP [15]. PP-2A was purified from bovine heart as described [16] and assayed with paranitrophenyl phosphate. Lactate was measured by mixing 1 ml of culture medium with 1 ml of 10% $HClO_4$. After 10 min at 0°C and centrifugation at $2000\times g$ for 10 min, the supernate was neutralized with 3 M KOH-$KHCO_3$ and lactate concentration was determined as described [17]. Protein concentration was determined by the Bio-Rad protein assay. ATP and AMP were determined as described [18].

3. Results and discussion

3.1. Clonal selection of cells expressing PFK-2/FBPase-2 transgenes

Cells containing different amounts of F-2,6-P2 were obtained by stably transfecting them with a PFK-2/FBPase-2 expression vector together with a plasmid conferring resistance to puromycin. The PFK-2/FBPase-2 transgene chosen codes for the L isozyme, which is regulated by PKA. Phosphorylation inactivates PFK-2 and activates FBPase-2. The recipient cells were Tet-On transfectants, namely Rat-1 fibroblasts that we had stably transfected with an expression vector for a doxycycline-inducible chimeric protein in which the *Escherichia coli* reverse Tet repressor has been fused to the VP16 activation domain [19,20]. Puromycin-resistant clones called 'K' were obtained after transfecting Tet-On cells with a vector expressing a double mutant of PFK-2/FBPase-2 in which the FBPase-2 activity has been abolished by a H258A mutation [21] and the inactivation of PFK-2 by PKA has been abolished by a S32A mutation [22]. Such clones were expected to contain a high concentration of F-2,6-P2. Clones called 'P' were likewise obtained after transfecting Tet-On cells with a vector expressing a K54M mutant characterized by loss of PFK-2 activity, but persistence of FBPase-2 activity and of activability by PKA [13]. Such clones were expected to contain a low concentration of F-2,6-P2. The transfected PFK-2/FBPase-2 cDNA were driven by a promoter containing a rTet-responsive element in view of inducing the transgenes by doxycycline.

We isolated 63 K clones and 35 P clones. Each clonal cell line was tested for F-2,6-P2 concentration after culture in the presence or absence of doxycycline. For all the experiments, we used as a control a non-clonal population of the stable Tet-On transfectants. We selected eight K clones that had a higher F-2,6-P2 content, and three P clones that had a lower F-2,6-P2 content, than these control cells. Because of the leakiness [23] of the Tet-On system in our fibroblast cell lines, inducibility by doxycycline was never more than two to three-fold. Still, in the presence of doxycycline, the concentration of F-2,6-P2 in some K clones reached up to 2000–3000 pmol/mg of protein, which was 40–60 times higher than in control cells treated with doxycycline. The PFK-2 activity of the K clones ranged from 160–570 μU/mg of protein as compared to 85 for control cells and to 40–60 for the P clones. Levels of F-2,6-P2 higher than 1000 pmol/mg of protein were

Table 1
AMPK activity in fibroblasts

AICAR	10% serum		0.5% serum	
	Control	PP-2A	Control	PP-2A
−	1.21 ± 0.15	0.06 ± 0.01	0.61 ± 0.04	0.04 ± 0.01
+	1.08 ± 0.11	0.08 ± 0.01	1.25 ± 0.01	n.d.

Tet-On cells were cultured for 48 h in medium containing 10% or 0.5% serum without or with addition of 1 mM AICAR 1 h before harvesting. AMPK activity (nmol/min/mg of protein) was measured in cell extracts incubated for 30 min at 30°C without (control) or with 11 U/ml PP2-A. Data are means ± S.E.M. for three independent experiments. n.d., not detectable.

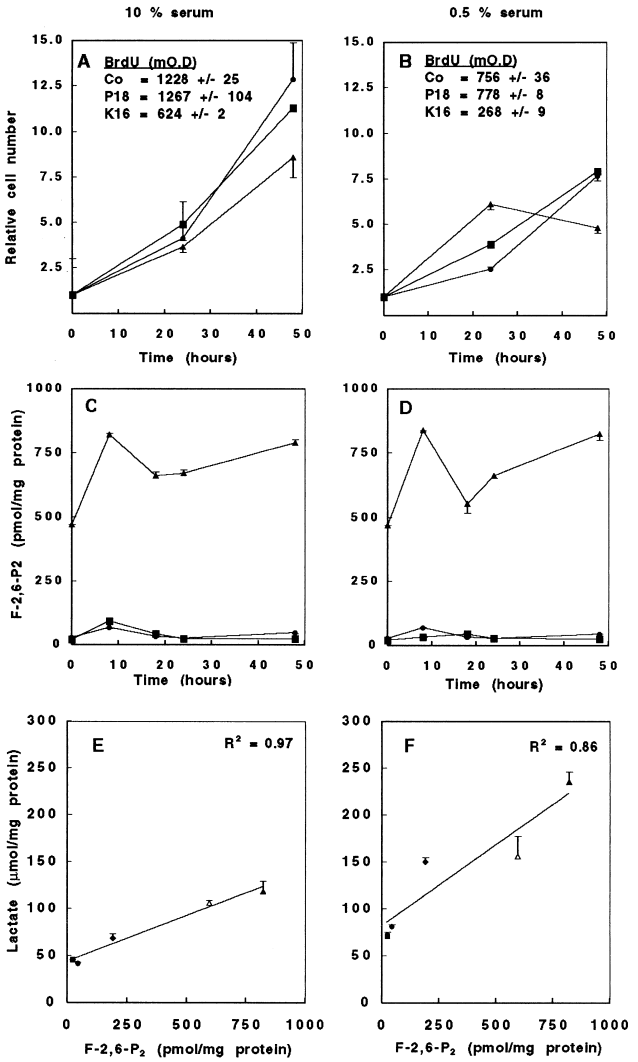

Fig. 1. Effect of serum starvation on the growth and glycolysis of fibroblasts containing different concentrations of F-2,6-P2. The culture medium containing 10% serum was replaced at time zero by fresh medium containing either 10% serum (A, C, E) or 0.5% serum (B, D, F). Cells were seeded at about 10^5 cells/6-cm dish. At the times indicated, samples were taken for measuring the number of cells (results are expressed as ratio to number of cells at time zero) and F-2,6-P2 concentration. BrdU incorporation (insets) was measured after 48 h of culture and the results are expressed as absorbance units (mO.D). The data are means ± S.E.M. for three experiments with control (●), P18 (■) or K16 (▲) cells (E, F). The data refer to lactate produced over the 48 h incubation and to F-2,6-F2 concentration determined at the end of the incubation period in the experiments described in A–D. The three values for clone K16 correspond to data obtained with cells that had been in culture for 26 (▲), 58 (Δ) or 73 (♦) days (see text).

not maintained with cell passages, in contrast to the stability of F-2,6-P2 concentration over time in the P clones. This already argued against high F-2,6-P2 providing a selective advantage for proliferation.

3.2. *Effect of serum starvation on cell proliferation in PFK-2/FBPase-2 transfectants*

We first compared the growth curves of a representative K clone (K16) and P clone (P18), and of control cells. In the presence of 10% serum the growth rates were similar for the P clone and control cells and were slightly reduced for the K clone (Fig. 1A). When serum concentration was reduced to 0.5%, the growth rate of control cells was reduced, as expected. This was also the case for the P clone, while the growth of the K clone did stop after 24 h. In fact, the number of K cells decreased after 48 h of serum starvation which was suggestive of cell death (Fig. 1B). These results ruled out the hypothesis that F-2,6-P2 stimulates proliferation or can prevent the decrease in DNA synthesis induced by serum starvation. To confirm this, BrdU incorporation was measured under the same conditions. The extent of DNA synthesis in 10% serum for the K clone was half that of the P clone or of control cells (inset of Fig. 1A). Moreover, serum starvation decreased DNA synthesis almost twice as much in the K clone as in the P clone or control cells (inset of Fig. 1B). In view of these results, we verified that serum starvation per se did not influence transgene expression. It did not (Fig. 1C,D), as F-2,6-P2 concentration remained high in the K clone and low in the P clone under these conditions. The decrease in cell number observed for K clones upon serum starvation raised the possibility that this was due to apoptosis. Indeed, while normal fibroblasts reduce their growth in this situation, transformed fibroblasts have been reported to undergo, instead, programmed cell death [24,25].

3.3. *Effect of serum starvation on apoptosis in PFK-2/FBPase-2 transfectants*

As a measure of apoptosis, we detected free nucleosomes in cell lysates from clone K16, clone P18 and control cells. Even under standard culture conditions, i.e. with 10% serum, apoptosis was 4–5 times higher for clone K16 than for clone P18 or for control cells (Fig. 2A). This difference was dramatically amplified (up to 23-fold) in 0.5% serum. This phenomenon held true for the eight K clones tested (Fig. 2A), showing that it was not the mere consequence of a chance genomic event caused by the transgene in clone K16. Moreover, the extent of apoptosis in serum starved cells was correlated to F-2,6-P2 concentration (Fig. 2B). This was confirmed by measuring apoptosis by two other methods, namely electrophoretic separation of DNA fragments and TUNEL (not shown). Consistent with these findings, microscopic examination showed that 48 h after serum starvation the cells from clones containing the highest F-2,6-P2 concentration were round and refractile and lost adhesiveness and they exhibited cytoplasmic shrinkage and chromatin condensation. The same morphological changes were seen after three days of confluence in 10% serum for the K clones, but not for the P clones or for control cells. Another characteristic of the clonal cell lines containing very high F-2,6-P2 concentrations is that they did not survive

Table 2
Anti-apoptotic effect of AICAR

AICAR	Apoptosis (mO.D) $n=4$	BrdU incorporation (mO.D) $n=4$	F-2,6-P2 (pmol/mg protein) $n=3$
−	987 ± 6.2	684 ± 15	273 ± 32
+	297 ± 67	633 ± 27	241 ± 21

Cell from clone K16 were incubated for 48 h in 0.5% serum in the absence or presence of 1 mM AICAR and the parameters were measured as described in Section 2. Data are means ± S.E.M. for the number (n) of experiments in each of which measurements were taken in quadruplicate.

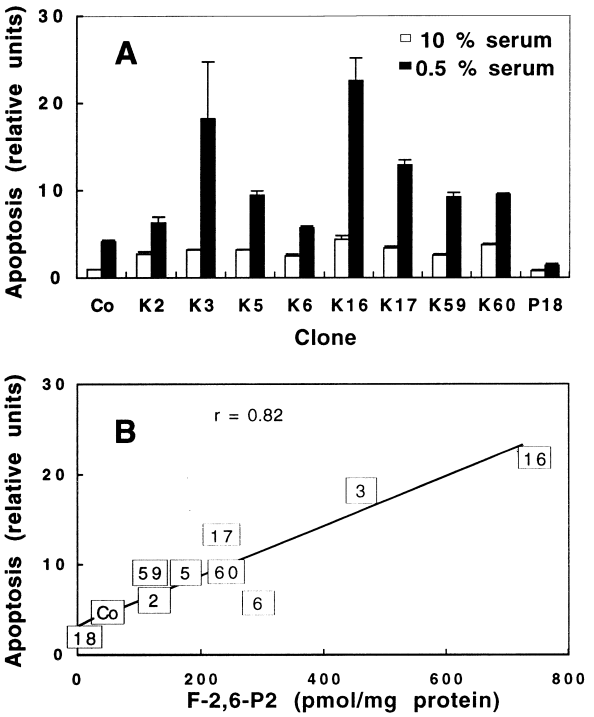

Fig. 2. Effect of serum starvation on apoptosis in fibroblasts containing different concentrations of F-2,6-P2. A: For each clone, cells were cultured in 96-well plates at 10^4 cells/well in fresh medium containing 10% or 0.5% serum. After 48 h, apoptosis was measured by the detection of nucleosomes in the cytosol. A relative value of 1 was assigned to apoptosis measured in the same experiment on control cells (Co) incubated with 10% serum. Data are means ± S.E.M. for four independent experiments in quadriplicate. B: Correlation between apoptosis in serum-starved (0.5%) cells (data from A) and F-2,6-P2 concentration (means ± S.E.M. for three experiments). The numbers inside the squares correspond to the clones in A.

when plated at very low density in medium containing 10% serum. We ruled out a toxic effect of high concentrations of F-2,6-P2, e.g. an inhibition of glycolytic enzymes, as the direct correlation observed between lactate production and F-2,6-P2 concentration in 10% serum was maintained in 0.5% serum (Fig. 1E,F). In fact, the glycolytic rate was higher under the latter condition, consistent with the absence in the transgenes of the serum response element contained in the promoter of the PFK-2/FBPase-2 gene [8]. All these experiments showed that overproduction of F-2,6-P2, far from maintaining cell proliferation upon growth factor withdrawal, does increase susceptibility to apoptosis.

3.4. Involvement of AMPK

Work by others [26] has provided evidence that AMPK can inhibit the apoptotic cascade triggered by glucocorticoid treatment of thymic lymphocytes. Moreover, in mouse fibroblasts AMPK phosphorylates Raf-1 [27], a protein kinase which is thought to inhibit apoptosis by interacting with Bcl-2 and BAG-1 [28]. AMPK serves as a 'metabolic sensor' or 'fuel gauge' that is activated by an increased AMP:ATP ratio [29,30]. This enzyme switches on ATP-producing pathways, switches off ATP-consuming pathways and protects the cell against nutritional stress. On the other hand, stimulation of AMPK activity interferes with the induction of gene transcription by glucose metabolites [31,32].

These observations led us to test whether decreased AMPK activity could be involved in the apoptotic effect of F-2,6-P2. We first verified that Rat-1 fibroblasts contain AMPK activity. This was the case (Table 1). As expected, AMPK activity was decreased after incubation of the cell extracts with PP-2A, a phosphoprotein phosphatase that inactivates AMPK [29,30]. Consistent with our hypothesis, AMPK activity in control cells decreased by about 50% upon serum starvation (Table 1), a situation shown above (Fig. 2) to be accompanied by increased susceptibility to apoptosis. This decrease in AMPK activity was not due to a decreased AMP:ATP ratio, as this ratio was 0.04 in cells cultured in 10% serum and 0.06 in 0.5% serum. Moreover, AMPK activity was assayed with a saturating concentration (0.2 mM) of AMP and after removal of allosteric inhibitors and activators. Thus, serum starvation could either decrease the amount of AMPK protein or the activity of AMPK kinase, which activates AMPK by phosphorylation [29,30].

To validate our hypothesis, we tested whether an agent that increases AMPK activity can protect the cells against the apoptotic phenomenon described here. AMPK kinase is, like AMPK, stimulated by AMP. We therefore incubated the cells with AICAR (Z-riboside). AICAR is converted into ZMP, an AMP analog known to activate AMPK [30]. Treatment of serum starved cells with AICAR restored AMPK activity to the level seen with 10% serum (Table 1). AMPK activity in extracts from AICAR-treated cells was decreased by PP-2A, as expected (Table 1). Preliminary data showed that in keeping with our hypothesis, AMPK activity was smaller in serum starved K clones than in serum starved control cells. We therefore determined whether AICAR treatment could protect clone K16 against apoptosis induced by serum starvation. This was the case (Table 2). If AICAR was added to the cultured K clone when serum concentration was reduced, apoptosis measured 48 h later was 3.3-fold lower than in the absence of AICAR. This was confirmed by TUNEL analysis (not shown). There was no effect of AICAR treatment on F-2,6-P2 concentration or on DNA synthesis (Table 2).

In conclusion, the data presented here discount the hypothesis that F-2,6-P2 protects the cells against growth factor withdrawal. Instead, they show that a high F-2,6-P2 concentration correlates with increased susceptibility to apoptosis and suggest that AMPK can interfere with this phenomenon. These results should shed light on the ill-explained finding that hyperglycaemia can induce apoptosis in several systems [33,34].

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