The 25 kDa protein kinase inhibitor present in liver cell is absent in fast growing HTC cells and is induced in sodium butyrate treated cells

Marc Delpech, Florence Lévy-Favatier, Lydie Tichonicky and Jacques Kruh+

Faculté de Médecine Cochin-Port-royal, Institute De Pathologie Moléculaire, Unité Associée CNRS, 24, rue du Fg Saint-Jacques, 75014 Paris, France

Received 10 August 1985

We have recently characterized a cAMP independent protein kinase inhibitor in rat liver. This inhibitor is absent or inactive in fast growing HTC cells and is induced according to exponential kinetics by sodium butyrate, a compound which arrests cell growth at the G_1 phase of the cell cycle. It is suggested that the inhibitor could be involved in cell growth regulation.

Protein kinase *Enzyme inhibitor* *Cell growth regulation* *Sodium butyrate* *Hepatoma cell*

1. INTRODUCTION

Protein phosphorylations have been involved in the regulation of cell metabolism and of cell growth (review [1]). cAMP-independent protein kinases are able to phosphorylate many proteins, including chromosomal proteins, casein, phosvitin [2], however very little is known about the regulation of these enzymes. Several inhibitors specific for these enzymes have been described [3–6]. We have recently characterized a 25 kDa inhibitor of cAMP-independent protein kinases present in rat liver nuclei and cytoplasm which copurifies with HMG proteins [7].

To establish a possible involvement of this inhibitor in cell growth regulation we have studied its activity in fast growing hepatoma cells and in cells treated with sodium butyrate, a compound which arrests cell growth at early G_1 phase of the cell cycle [8]. We were unable to find any inhibitory activity in growing cells and we observed an induction of its activity according to exponential kinetics in sodium butyrate treated cells.

+ To whom correspondence should be addressed

Abbreviations: HMG proteins, high mobility group proteins; HTC cells, hepatoma tissue cultured cells

2. MATERIALS AND METHODS

2.1. *Cell culture*

HTC cells were grown at 37°C in suspension in Swim's 77 medium supplemented with 10% newborn calf serum as described by Hershko and Tomkins [9]. When indicated sodium butyrate was added to the medium at a final 5 mM concentration.

2.2. *Preparation of the inhibitor*

2.2.1. From liver

The inhibitor was prepared as described from rat liver [7].

2.2.2. From HTC cells

The cells were washed 3 times with phosphate buffered saline and homogenized in a Teflon potter with 0.5 ml for 10^8 cells of 0.75 M perchloric acid, 0.5 mM PMSF. The homogenate was treated as described [7].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2.3. *Preparation of chromosomal non-histone proteins*

2.3.1. From liver

Nuclei were prepared according to Chauveau et al. [10]. They were washed 3 times with 10 mM Tris (pH 7.5), 3.3 mM $CaCl_2$ and solubilized overnight by gentle stirring in 1 M NaCl, 10 mM Tris (pH 7.5). The NaCl concentration was lowered to 0.35 M by addition of 10 mM Tris (pH 7.5). The viscous solution was centrifuged at 40000 × *g* for 90 min in a Beckman rotor 30. The chromosomal non-histone proteins were present in the supernatant.

2.3.2. From HTC cells

The washed cells were homogenized in 0.25 M sucrose, 10 mM Tris (pH 7.5), 0.2% Triton X-100. The homogenate was layered on a 2.2 M sucrose, 10 mM Tris (pH 7.5) cushion and centrifuged in a Beckman rotor SW25 at 45000 × *g* for 1 h. The pellet which contains the nuclei was treated as described for liver.

2.4. *Phosphorylation of chromosomal non-histone proteins*

These proteins include cAMP-independent protein kinases and phosphoprotein substrates of these enzymes [11,12]. Chromosomal non-histone proteins, 100 μg, were incubated in a final 250 μl solution in the presence of 5 μCi [γ-^{32}P]ATP (3 Ci/mmol, Amersham, England), 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, for 30 min at 37°C. The proteins were precipitated with 1 ml of 33% trichloroacetic acid. The precipitates were collected on Millipore HAWP 0.45 μm filters, washed with 10 ml of 25% trichloroacetic acid. The filters were transferred to 5 ml of Unisolve and the radioactivity measured.

2.5. *Inhibitor assays*

The incubation medium was as described above except that 100 μg of partially purified chromosomal protein kinase preparations which include part of the enzyme substrates were used instead of total chromosomal non-histone proteins, in order to discard most of the endogenous enzyme inhibitors. Exogenous inhibitor was added at various concentrations and the incubations were carried out as described above.

3. RESULTS

3.1. *Absence of the inhibitor in fast growing HTC cells*

We have prepared an inhibitor assay medium from rat liver which actively incorporated [^{32}P]-phosphate from labelled ATP into endogenous proteins and we added increasing amounts of inhibitor preparation, up to 1.0 mg, from fast growing HTC cells. No inhibition of protein kinase activity was observed. Using the same medium, 100 μg rat liver inhibitor preparation completely inhibited the enzyme activity.

3.2. *Induction of inhibitor activity by addition of sodium butyrate to HTC cells*

Sodium butyrate, at a 5 mM final concentration, was added to HTC cell culture. Inhibitor was prepared from these cells at various times after the addition of sodium butyrate and increasing amounts of the inhibitor preparation were added to the rat liver inhibitor assay medium. Fig.1

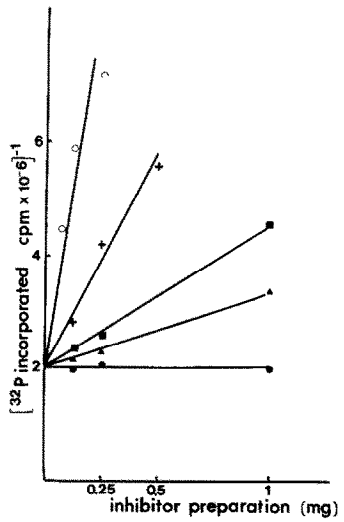

Fig.1. Effect of sodium butyrate on the inhibitor activity in HTC cells. Na butyrate, at a 5 mM final concentration, was added to HTC cell culture for various lengths of time. The inhibitor was prepared from cells treated for 6 h (▲—▲), 24 h (■—■) and 60 h (+—+) with sodium butyrate, from fast growing HTC cells (●—●) and from rat liver (○—○). Various amounts of the inhibitor preparation were added to partially purified chromosomal protein kinase preparations. The enzyme activity was measured.

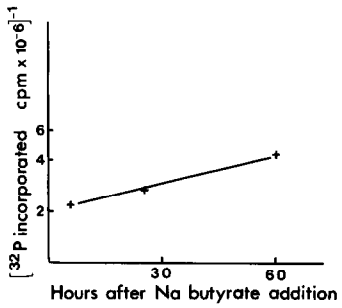

Fig.2. Kinetic of inhibitor formation after sodium butyrate addition (semi-log scale). The inhibitor was prepared from HTC cells at various times after sodium butyrate addition, 250 μg of the inhibitor preparation were added to partially purified chromosomal protein kinase preparation. The enzyme activity was measured.

shows that: (i) the inhibitor activity increased with time after the addition of sodium butyrate. However, even 60 h after this addition it was lower than that observed with liver inhibitor; (ii) for each time a linear relationship was observed between the amount of added inhibitor and the enzyme inhibition.

Fig.2 shows that the inhibitor activity is exponential as a function of time after sodium butyrate addition. Extrapolation of the curve shows that a total inhibition would require a time of presence of sodium butyrate incompatible with cell survival.

3.3. *cAMP-independent protein kinase activity in liver and in HTC cells*

We have measured the enzyme activity in the same amount of total chromosomal proteins from liver, fast growing and sodium butyrate treated HTC cells. Typical experiments show the following specific activities: liver cells, 0.9 U/mg; fast growing HTC cells, 2.8 U/mg; HTC cells treated with sodium butyrate for 24 h, 2.0 U/mg. (1 U corresponds to 1 nmol ^{32}P incorporated/h). These data show a correlation between the enzyme activity and the activity of the inhibitor which suggests a physiological role of the inhibitor.

4. DISCUSSION

In this work we show that the cAMP-independent protein kinase inhibitor we have isolated from liver cells is inactive or absent in fast growing HTC cells and that it is activated or induced by sodium butyrate, a compound which arrests cell growth at early G_1 phase of the cell cycle [8].

This observation is compatible with the role of this inhibitor in the control of cell growth in relation with protein kinase activity since nuclear protein kinases vary during the cell cycle [13] and since no other regulatory mechanism of these enzymes is known at the present time. Sodium butyrate could act either by activating the inhibitor or by stimulating its activity. It is however unlikely that the increase in inhibitor activity or synthesis results from a specific effect of sodium butyrate, a compound which is able to induce the synthesis of specific proteins (review, [14]). It is more likely that this kinase is related to cell growth, it is possible that it is cell cycle phase dependent and that it specifically occurs at the early G_1 phase.

The data presented here suggest that the 25 kDa inhibitor of cAMP-independent protein kinase is a part of the cell growth control mechanism.

ACKNOWLEDGEMENTS

This work was financially supported by the Institut National de la Santé et de la Recherche Médicale and by the Centre National de la Recherche Scientifique.

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