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Activation of an insulin-stimulated S6 kinase in 3T3 L1 cell-free extracts by proteolysis

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Using chromatography on a Fast S-Sepharose column, the insulin-stimulated S6 kinase can be resolved from other S6 kinases present in 3T3 L1 cell extracts. Only one S6 kinase is greatly activated by insulin (4–5-fold) and phosphorylates S6 with a high stoichiometry (4–5 mol phosphate per mol S6). This insulin-dependent S6 kinase can be activated in cell-free extracts by incubation with high concentrations of Ca^{2+}. This activation is blocked by protease inhibitors such as leupeptin and is mimicked by trypsin. The stimulation does not require the presence of the protein kinase dependent on phospholipids and calcium (PK-C) in the cell extracts. The level of stimulation produced by proteolysis in the cell extracts is comparable to that reached in vivo by incubation with insulin.

Protein S6 kinase; Proteolysis

1. INTRODUCTION

Insulin and other growth factors promote the activation of a soluble S6 kinase present in the cytosol of 3T3 L1 cells and other cell lines [1–5]. This mitogen-activated S6 kinase shows an absolute specificity for S6 and incorporates up to 4–5 mol phosphate per mol S6, the same stoichiometry as that observed in intact cells treated with insulin [6]. S6 can also be phosphorylated 'in vitro' by several histone kinases with lower stoichiometry [7–10]. Complete purification of a mitogen-activated S6 kinase has been recently reported from different sources [11–14]. This S6 kinase is regulated by phosphorylation-dephosphorylation; treatment with phosphatase 2A inactivates the enzyme [14–16] whereas another protein kinase, MAP-2 kinase, can phosphorylate and reactivate the S6 kinase [16].

Here, I report that an S6 kinase can be activated in cell-free extracts by a calcium-dependent protease or by trypsin. This kinase has been identified as the insulin-stimulated S6 kinase according to its specificity, high stoichiometry of phosphorylation and behaviour on columns of Fast S-Sepharose.

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Abbreviations: PK-C, protein kinase dependent on phospholipids and calcium; TPA, 12-tetradecanoylphorbol 13-acetate; DME medium, Dulbecco's modified Eagle's medium

2. MATERIALS AND METHODS

Histones and casein were from Sigma. All resins were from Pharmacia. The 40 S ribosomal subunits were prepared according to [17]. Protein kinase M, the catalytically active proteolytic fragment of protein kinase C, was prepared as in [18] from purified PK-C [7]. Protein kinase assays were performed as in [2] with 0.5 mg/ml 40 S ribosomal subunits, 0.5 mg/ml casein or 0.4 mg/ml histone as substrates. Two-dimensional gel electrophoresis was carried out as in [12]. Proteins were determined with the BCA kit from Pierce using serum albumin as standard. Preparation of the 3T3 L1 cell extract was performed as described [2] using 25 mM Hepes, pH 7.5, 1 mM EGTA as homogenization buffer.

3. RESULTS AND DISCUSSION

The insulin-stimulated S6 kinase can be easily

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resolved from other S6 kinases present in 3T3 L1 cell extracts via chromatography on Fast S-Sepharose or Mono S columns. When a high-speed supernatant was loaded on a Mono S column, more than 90% of the total S6 kinase activity was retained in the column (fig.1). This kinase activity elutes in two well-defined peaks with different properties. The first, eluting at 170 mM KCl (peak I), is very strongly stimulated with insulin and constitutes 30–50% of the total S6 kinase in extracts from insulin-treated cells. The second (eluting at 260 mM KCl, peak II) is the major S6 kinase found in control cells and is very slightly increased by insulin (20–50%). Similar increases were noted in extracts of cells treated with the phorbol ester TPA (fig.1). Neither form exhibits histone kinase activity nor is able to phosphorylate proteins other than S6 in the 40 S subunits. The stoichiometry of phosphorylation of S6 by each peak is different. Peak II S6 kinase, similarly to cAMP-dependent kinase or PK-C, incorporates 2 mol phosphate per mol S6 whereas the value for peak I can be up to 5 (not shown). The stability of the two forms is also different. Incubation of an extract derived from insulin-treated cells at 30°C for 15 min produced a total loss in peak I S6 kinase and only a 10–20% decrease in peak II (not shown). The activity can be maintained in the presence of phosphatase inhibitors such as *p*-nitrophenyl phosphate, suggesting the involvement of a phosphatase in deactivation, as described for the insulin-activated S6 kinase [14–16]. Peak II, but not peak I, copurifies on the Mono S column with a casein kinase activity. The addition of excess casein to the S6 phosphorylation assay abolishes completely the S6 kinase activity present in peak II but does not affect the activity associated with peak I (not shown).

Collectively, all these results (high stoichiometry of phosphorylation, specificity, low stability) suggest that peak I is the principal S6 kinase that is activated by insulin and responsible for complete phosphorylation of S6 following insulin addition to cells. Peak II corresponds to a casein kinase, probably casein kinase II that has been described as being increased by insulin treatment [19].

Addition of Ca^{2+} (0.4–1.0 mM) to a control cell extract promoted an increase in peak I S6 kinase (fig.2). This stimulation was prevented by the protease inhibitor leupeptin (25 μg/ml) (table 1A). Like insulin or TPA (fig.1), incubation with Ca^{2+}

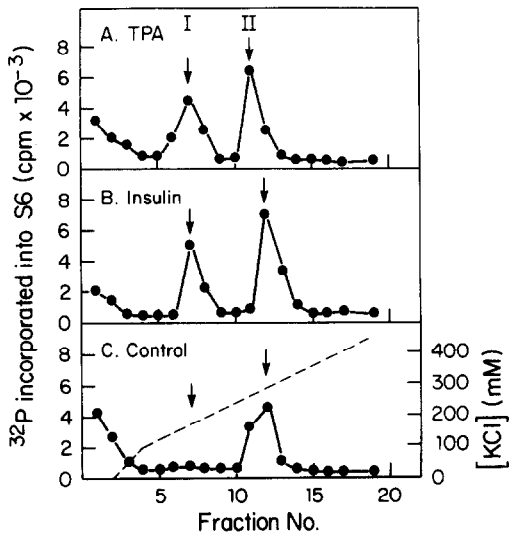

Fig.1. Separation of two forms of S6 kinase using chromatography on columns of S-Sepharose. High-speed supernatants (200 μl, 1.3 mg protein) from TPA-treated (A), insulin-treated (B) or control cells (C) were filtered (Millipore HV) and injected into an FPLC Mono S column equilibrated in 25 mM Hepes (pH 7.5), 1 mM EGTA, 1 mM DTT and eluted with a gradient of 100–500 mM KCl in this buffer. Aliquots (5 μl) of each fraction were assayed for S6 kinase activity. Cells were treated with 200 nM TPA or 400 nM insulin for 60 min before preparation of extracts.

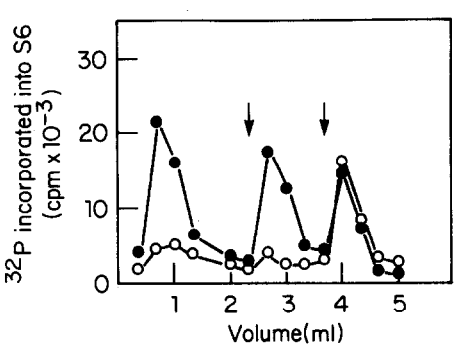

Fig.2. Increase in S6 kinase activity after incubation of cell extracts with Ca^{2+}. Extract (200 μl, 1.2 mg protein) from control cells was incubated with 1 mM $CaCl_2$ (●) or 2 mM EGTA (○) for 15 min at 30°C. After addition of EGTA to neutralize the Ca^{2+}, samples were loaded onto a column of Fast S-Sepharose (0.3 ml) equilibrated in buffer (25 mM Hepes, pH 7.5, 1 mM EGTA). The columns were washed with the same buffer plus 0.1 M KCl and the S6 kinases eluted successively with the buffer plus 0.2 and 0.3 M KCl. Arrows indicate the beginning of elution with either 200 or 300 mM KCl.

Table 1

Activation of insulin-dependent S6 kinase by incubation of cell extracts with Ca^{2+} or trypsin

Additions	Phosphorylation of S6 (pmol)	Activation (%)
(A)		
EGTA (2 mM)	0.9	
Ca^{2+} (50 μM)	1.5	66
(100 μM)	1.8	100
(400 μM)	2.5	178
(1 mM)	2.7	200
(1 mM) + leupeptin (25 μg/ml)	1.1	22
(B)		
EGTA (1 mM)	1.0	
+ trypsin (1 μg/mg extract protein)	1.7	70
+ trypsin (3 μg/mg extract protein)	2.4	140
(C)		
(Control cell extract)		
EGTA (2 mM)	1.5	
+ PK-M (2000 U)	1.6	7
Ca^{2+} (1 mM)	4.7	213
(PK-C depleted-cell extract)		
EGTA (2 mM)	1.7	
Ca^{2+}	4.9	188

The activation reaction was performed by incubating cell extracts with the indicated additions in a final volume of 100 μl for 10 min at 30°C. After the reaction was terminated with EGTA or trypsin inhibitor, the S6 kinase was purified by chromatography on a Fast S-Sepharose column and the material eluting with 200 mM KCl (peak I) assayed for S6 kinase activity. The depletion of PK-C in the cells of experiment C was carried out by adding 10 μM TPA to the cell medium 6 h before homogenization. After these treatments only 2.2% of the initial PK-C activity was detected in the cell extracts. One unit of PK-M activity was defined as 1 pmol ^{32}P incorporated into histone per min

stimulated peak I, but not peak II. This treatment also activated another S6 kinase which was not retained on S-Sepharose and phosphorylated histone and a 20 kDa 40 S ribosome-associated protein. In accordance with its properties, this latter kinase can tentatively be identified as PK-M (the catalytically active fragment of PK-C). Addition of trypsin to the extract (1–3 μg trypsin/mg extract protein for 15 min at 30°C) also activates the peak I S6 kinase (table 1B).

Incubation of the extract with Ca^{2+} or trypsin also promoted disappearance of PK-C activity and generation of another histone kinase activity which behaved like PK-M upon chromatography on DEAE-cellulose. PK-C is a well-known substrate for a class of calcium-activated proteases (calpains) which convert it to PK-M [20]. This reaction is abrogated by leupeptin. Although it is possible that PK-M may phosphorylate and activate the insulin-stimulated S6 kinase, several lines of evidence suggest that this is unlikely. Firstly, addition of highly purified PK-M to a control cell extract, in the absence of Ca^{2+}, did not activate the peak I S6 kinase (table 1). Secondly, Ca^{2+}-mediated S6 kinase occurs with the same efficiency in PK-C-depleted extracts derived from cells exposed to 10 μM TPA for 6 h. Such extracts contain less than 3% of the PK-C activity present in control cells and exhibit very low basal S6 kinase activity. Cells treated in this manner with TPA are, however, sensitive to insulin-dependent S6 kinase activation [21]. Incubation of these extracts with Ca^{2+} produces an increase in S6 kinase similar to that in control extracts (table 1C). No generation of PK-M was observed in these TPA-pretreated extracts. It is possible to conclude that insulin stimulates S6

kinase via a mechanism that does not involve PK-C activation and that the Ca^{2+}-dependent leupeptin-sensitive activation of S6 kinase in cell-free extracts is not mediated by either PK-C or PK-M.

All of my attempts to reproduce the activation of S6 kinase by trypsin using cytosolic fractions have been unsuccessful. These results suggest that proteolysis does not directly affect the insulin-stimulated S6 kinase but acts upon an effector of this activity. It is possible that this effector is the MAP-2 kinase that can phosphorylate the insulin-dependent S6 kinase and activate it [16]. In any case, this method of activation of this S6 kinase by proteolysis in vitro constitutes an easy and rapid way of determining the presence of the proenzyme in a cell extract.

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