A chick embryo fibroblast protein kinase recognizing ribosomal protein S6

Activity increase after serum stimulation

Alfons Lawen and Oskar H W. Martini*

Institut fur Biochemie der Universitat Wurzburg, D87 Wurzburg, Rontgenring 11, FRG

Received 20 November 1984

We sought protein kinase(ser) activity in DEAE-Sephacel chromatography fractions of the 10000 × g supernatants of chick embryo fibroblasts using 40 S ribosomal subunits as kinase substrate, and detected a new S6-recognizing kinase activity There was one order of magnitude more enzyme activity in chromatography fractions derived from serum-stimulated than from serum-deprived cells Known protein kinase regulators and a low dose tryptic treatment did not increase the enzyme's activity

Protein kinase *Ribosomal protein S6* *Chick embryo fibroblast* *Growth control*

1 INTRODUCTION

The binding of growth factors to their receptors activates a receptor-intrinsic protein kinase(tyr) function Receptors share this particular enzymatic function with certain oncogene products (review [1]) It is likely that protein kinases(tyr) exert direct or indirect control over one or more serine-phosphorylating protein kinases Such a kinase hierarchy is suggested by the following observations. (i) Serum or growth factors elicit rapid serine-specific phosphorylations of the ribosomal protein S6 in various cells including HeLa cells, chick embryo fibroblasts (CEF) and 3T3 cells [2–8], respectively; (ii) avian sarcoma virus-transformed CEF display an unusually high level of S6 phosphorylations [9] The serine-recognizing protein kinase(s) involved in these growth-related S6 phosphorylations have yet to be identified.

We have carried out a systematic search for this enzyme(s) in DEAE-Sephacel chromatography fractions of the 10000 × *g* supernatants of a variety of cultured cells. 40 S ribosomal subunits were chosen as kinase substrate, because the S6 protein is a constituent of the 40 S subunit. By this approach we were able to detect, in S49 kin$^-$ and HeLa cells, an S6 kinase activity, which differed from other S6 kinase activities hitherto described ([10] and unpublished).

With the aim of extending our observations to non-permanent cells, we have looked for the corresponding enzyme in CEF We show here that such an enzyme exists in CEF and that preparations of the enzyme obtained from serum-stimulated CEF are one order of magnitude more active than those from serum-deprived CEF

2. MATERIALS AND METHODS

2.1 *Materials, buffers*

ATP, GTP, trypsin and soybean trypsin inhibitor were from Boehringer, Mannheim; cAMP, cGMP, spermine, spermidine, phosphatidylserine, 1,2-diolein, calmodulin, protein kinase inhibitor (P-0393) and phenylmethylsulphonyl fluoride were purchased from Sigma, Munich; DEAE-Sephacel was from Pharmacia, Freiburg; [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham-

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/85/$3 30

Buchler, Brunswick. Dulbecco's modified Eagle medium (DMEM) and foetal calf serum (FCS) were from Gibco, Karlsruhe. Ca^{2+}/phosphatidylserine/diolein-dependent protein kinase (protein kinase C), partially purified from rat brain, was a gift from U Padel, Gottingen

Lysis buffer contained 20 mM KCl, 3 mM magnesium acetate, 7 5 mM 2-mercaptoethanol, 100 μM phenylmethylsulphonyl fluoride, 13 mM Tris-HCl, 4% (w/v) sucrose, 1% (w/v) NP40, 25 mM potassium phosphate buffer, pH 7.4. Column buffer was lysis buffer without detergent and phenylmethylsulphonyl fluoride

2.2. *Methods*

CEF were prepared from 10-day-old chicken embryos and seeded in 800-ml plastic flasks They were propagated in DMEM containing 8% FCS. After 1 or 2 days of growth, secondary cultures were made. From secondary cultures protein kinase preparations were obtained as described in section 3.

Protein kinase activity was assayed in reaction mixtures (60 μl) containing 3 mM $MgCl_2$, 15 mM KCl, 10 μM EDTA, 12.5 mM Hepes-KOH buffer, pH 7.0, 1 mM dithiothreitol, 0 5 mM [γ-^{32}P]ATP (266 mCi/ml), biologically active 40 S ribosomal subunits (166 pmol/ml; prepared according to [11] from rabbit reticulocyte polysomes) and enzyme fraction (5 μl/assay), which carried over additional salts, raising the [KCl] of the assay by ~20 mM. Modified reaction mixtures differed from these standard conditions by an increased [KCl] of 100 mM and a lowered [ATP] of 50 μM. After 30 min incubation at 35°C, the reactions were stopped by the addition of an equal volume of ice-cold 96% ethanol containing 40 mM $MgCl_2$ [12], to precipitate the ribosomal subunits. The precipitated material was analyzed by SDS-polyacrylamide (15%, w/v) gel electrophoresis [13]. From the stained, dried gels autoradiogrammes were obtained with the aid of intensifying screens (Dr Goes, Suprema, Heidelberg)

3. RESULTS

Subconfluent secondary cultures of CEF were exposed to serum-free DMEM for 20 h, with one medium change after 18 h. Thereafter, one group of cultures received FCS (15%, final concentration), whereas the controls were left serum-free. 30 min later the incubations were stopped by removal of the medium and shock-cooling of the cultures with ice-cold phosphate-buffered saline. The drained cells were scraped off the wall of the flask using a rubber policeman and were then lysed with lysis buffer (6 ml/flask). From the lysates 10000 × g supernatants were prepared by 15 min centrifugation at 4°C. Of each supernatant 6 ml were chromatographed on a DEAE-Sephacel column. The column was washed with column buffer, first (15 ml) without, then (2.5 ml) with added cAMP (50 μM), to remove the catalytic subunit of the cAMP-dependent protein kinase from the column (table 1). This was to simplify the detection and identification of other S6-recognizing kinases to be eluted later. The catalytic subunit in the column effluent was identified by the fact that its activity was strongly inhibited by Walsh kinase inhibitor [14], a protein known to block this enzyme.

Following the cAMP-treatment, the column was washed once more with column buffer (15 ml) and then developed by application of a [KCl] gradient (11 ml), which raised the [K^+] from 65 mM, the basal concentration in the column buffer, to 665 mM. Twenty fractions were collected, and each one was assayed for protein kinase activity using 40 S ribosomal subunits as substrate and [γ-^{32}P]ATP as cosubstrate as described in section 2. The reaction products, analyzed by SDS-polyacrylamide gel electrophoresis, were detected by autoradiography of the gels (fig.1).

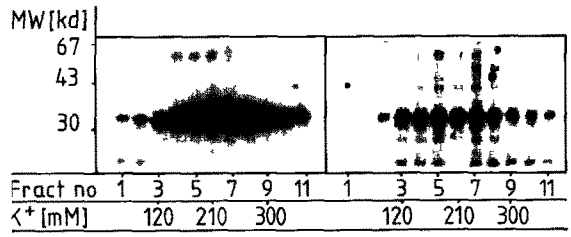

Fig 1 Protein S6-phosphorylating kinase activity in DEAE-Sephacel chromatography fractions which were prepared from the 10000 × g supernatants of either serum-stimulated (left panel) or serum-deprived (right panel) CEF 40 S ribosomal subunits served as substrate in the kinase assays [γ-^{32}P]ATP was the cosubstrate The reaction products were analyzed by SDS-polyacrylamide (15%, w/v) gel electrophoresis An autoradiogramme of the dried gel is shown.

Table 1

Quantitative comparison of protein kinase activities obtained from serum-stimulated and serum-deprived CEF

Treatment of cells	cAMP-eluted activity (cpm)	KCl-eluted activity (cpm)	(cpm × $10^{-3}/A_{280}$)
15% FCS			
1 determ	322	2128	218 3
2 determ	483	2592	265 8
3 determ	440	2310	236 9
No FCS			
1 determ	251	216	22 7
2 determ.	216	200	21 0
3 determ	309	171	18 1

10000 × *g* supernatants were chromatographed on a DEAE-Sephacel column After a wash with cAMP-containing buffer (2 75 ml), the column was developed by application of a [KCl] gradient The wash and the eluate fractions were assayed for protein kinase activity with ribosomal 40 S subunits as substrate and [γ-^{32}P]ATP as cosubstrate Fractions 4–8 (fig 1) were pooled for triplicate determinations of protein kinase activity The A_{280}/ml of the pools (2 75 ml) was 1 95 for stimulated and 1 9 for unstimulated cells The results shown were reproduced using another set of CEF cultures A 10-fold increase of kinase activity was observed after serum stimulation

Radiophosphorus was bound almost exclusively to a 33 kDa protein, which could be identified as ribosomal protein S6 by reference to earlier two-dimensional electrophoretic separations of ribosomal proteins ([15] and work cited therein), in which this same protein was designated S3.

The radioactive zones were excised from the gel, and the radioactivity was determined. The results (not shown) suggest that 60% of the kinase activity eluted within a [K^+] range of 170–240 mM (fractions 5–7) with a peak activity at 200 mM This elution behaviour agreed with that of a mammalian S6 kinase prepared and analyzed under identical conditions [10].

A comparison of the amounts of phosphate transferred to S6 by pooled fractions 4–8 prepared from either serum-stimulated or from serum-deprived CEF is shown in table 1. The enzyme preparation derived from stimulated cells was 12-times as active as that from unstimulated cells. The rates of S6 phosphorylation were near-linear during the 30 min assay period (not shown), which forms a prerequisite for the comparisons made in table 1.

Using the same enzyme preparations, the effects of the following protein kinase regulators were examined· cAMP, cGMP, Ca^{2+}/calmodulin, spermine/spermidine, Ca^{2+}/phosphatidylserine/diolein. None of them stimulated the activity of the enzyme (table 2). Instead, the last combination of substances was inhibitory. Under modified assay conditions, i.e , when [Ca^{2+}] and [K^+] were increased, the inhibition was even more pronounced. It may be noted that the corresponding mammalian enzyme had a [K^+] optimum of 35 mM (unpublished). The kinase preparations were also incubated with a low dose of trypsin [16] prior to assay in the presence of a 10-fold excess of trypsin inhibitor, S6 phosphorylation could not be increased in this way

4. DISCUSSION

We have shown here that DEAE-chromatography fractions prepared from the 10000 × *g* supernatant of CEF contained an S6 kinase activity which was cyclic nucleotide- and Ca^{2+}/phos-

Table 2

Effect of protein kinase activators on S6 kinase preparations

Additions		Assay conditions	Radiophosphate bound to S6 (% of control)		
Substance	Concentration (μM)		A	B	C
None		a	100	100	100
cAMP	1	a	124	112	n d
cGMP	1	a	92	70	n.d
Ca^{2+}/CaM	50/0.6	a	116	150	n d.
Spermine/spermidine	150/0 4	a	122	136	n.d
Ca^{2+}/PS/diolein	50/11/0 7	a	82	49	107
None		b	41	12	89
Ca^{2+}/PS/diolein	50/11/0 7	b	42	19	289

Protein kinase assays were performed either under standard (a) or modified (b) conditions, and the radiophosphate incorporation into the S6 protein was measured. The kinase preparations were pooled chromatography fractions 4–8 derived from either serum-deprived (A) or serum-stimulated (B) CEF and protein kinase C (C) from rat brain The results obtained by the respective enzyme preparations in the absence of regulatory compounds served as reference value and were set 100% CaM, calmodulin, PS, phosphatidylserine, n.d , not determined

pholipid-independent The salient observation was that there was 10-times as much of this activity in S6 kinase preparations derived from serum-stimulated as in those from serum-deprived cells. One may speculate that this enzyme played a role in S6 phosphorylation in vivo, e g., in CEF treated with serum or with insulin-like growth factor [3] and in avian sarcoma virus-transformed CEF [9]; and one wonders whether it might have been the homologous murine enzyme which was highly active in S6 phosphorylation in extracts of serum- or epidermal growth factor-treated 3T3 cells [17].

A protein kinase identified as protease-activated kinase II [18] and a kinase closely resembling casein kinase I [19], both derived from 3T3-L1 cells, have been described to phosphorylate S6 and to be insulin-responsive. Different from these reports, the increase in S6 kinase activity which we have observed cannot be interpreted as an insulin effect, since the insulin concentrations of the sera which we used were $< 5 \times 10^{-11}$ M Growth factors are more likely than insulin to have caused the increase in S6 kinase activity, and we have therefore, in the sense of a working hypothesis, designated the enzyme 'mitogen-responsive S6 kinase'.

Unlike protein kinase C – and also the protein kinase described in [19] – the mitogen-responsive S6 kinase from CEF displayed decreased activity when assayed at an elevated [K^+] (table 2). The CEF enzyme also binds somewhat tighter to DEAE-Sephacel than do most other S6-phosphorylating kinases: In particular, protein kinase C [20], protease activated kinase II [21] and H4P-kinase [22] would be expected, on the basis of published data ([23,16,24] respectively), to elute before the CEF enzyme (verified for kinase C, not shown). From the 3 cited enzymes, active fragments have been generated in vitro by limited proteolysis ([25,26,24], respectively), and we are aware that it may be more difficult to distinguish the CEF enzyme from such fragments than from their progenitors. We therefore examined the effects of varying preparation conditions presumed to be important for proteolysis in vitro, e.g , absence or presence of PMSF and/or EGTA, as well as the time span allowed for the preparation. We were unable to demonstrate an effect of these variations on our results.

ACKNOWLEDGEMENTS

We are grateful to Professors H.J. Gross and B. Hamprecht for supporting this work, Professor C. Jungwirth for making available to us the necessary cell culture facilities, W. Zens for acquainting us with the CEF culture technique and U. Padel and Professor H.-D. Soling for valuable advice. A.L. is recipient of a student's fellowship from Stiftung Cusanuswerk. O.M. enjoys the hospitality of the colleagues at the Institute of Physiological Chemistry, Wurzburg.

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