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AN ACTIVATED S6 KINASE IN REGENERATING RAT LIVER

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SUMMARY: S6 kinase activity was increased in the regenerating liver 5 h after partial hepatectomy compared with sham-operated liver. The protein kinase activity was eluted from DE-52 column at approximately 250 mM NaCl and was not affected by known regulators of protein kinases. The S6 kinase was further purified by chromatography on peptide R_1A_{13}-Sepharose 4B and Sephadex G-150. The molecular weight of the enzyme was estimated to be 4.5 x 10^4 by gel filtration. The enzyme catalyzes the phosphorylation of whole histone, mainly H2B histone, at 75 mM Mg^{2+}. These properties are similar to those of a proteolytically modified Ca^{2+}/phospholipid-independent form of protein kinase C.

Phosphorylation of ribosomal protein S6 (S6) in mammalian cells and tissues is stimulated in response to growth promoting stimuli; for example, serum, hormone, growth factor or phorbol ester. In serum-stimulated Swiss 3T3 cells, the 40 S subunits containing highly phosphorylated forms of S6 have been shown to have an apparent selective advantage in entering polysomes (1,2), suggesting that the phosphorylation of S6 would participate in the initiation of protein synthesis. Although protein kinase activity which phosphorylates S6 has been detected in many cellular

Abbreviations: S6, ribosomal protein S6; Peptide R_1A_{13}, synthetic peptide Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ala; SDS, sodium dodecyl sulfate; TPA, 12-o-tetradecanoyl-phorbol 13-acetate

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systems, the regulatory mechanism of the enzyme activation upon growth promoting stimuli remains unknown. In previous reports we proposed that protease-activated form of protein kinase C was released from the rat liver plasma membrane and played an important role of phosphorylation of intracellular proteins (3,4).

In this report, we show the activation of S6 kinase in regenerating rat liver. The relationship between the S6 kinase and the protease-activated form of protein kinase C will be discussed. We have utilized a synthetic peptide Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala-Ser-Thr-Ser-Lys-Ala termed R_1A_{13} whose structure is based on the region of S6 containing the sites phosphorylated by insulin-regulated kinase and cAMP-dependent protein kinase (5).

EXPERIMENTAL PROCEDURE

40 S ribosomal subunits were isolated from rat liver ribosomes (6,7). cAMP-dependent protein kinase, its protein inhibitor and calmodulin were purified from bovine heart (8), rabbit skeletal muscle (9) and bovine brain (10), respectively. Calf thymus histones were prepared as specified earlier (11). Peptide R_1A_{13} was synthesized by the solid phase method (12,13). Peptide R_1A_{13}-Sepharose 4B was prepared by immobilization of peptide R_1A_{13} (10 mg) on activated CH-Sepharose 4B (2g).

The kinase activity with a synthetic peptide R_1A_{13} was assayed in a reaction volume of 50 µl containing 10 mM Hepes-KOH (pH 7.4), 5 mM magnesium acetate, 30 mM 2-mercaptoethanol, 5 µg of peptide R_1A_{13}, enzyme fraction and 50 µM (γ-^{32}P)ATP (200-300 cpm/pmol). After incubation at 30°C, the reaction was stopped by the addition of 20 µl of 25 % trichloroacetic acid, and subsequently 10 µl of 10 mg/ml of bovine serum albumin were added. The precipitated protein was removed by centrifugation and 40 µl aliquot of the supernatant fluid was spotted on P-81 paper. P-81 paper squares were washed four times in 75 mM phosphoric acid and counted for radioactivity in 10 ml of H_2O. Protein kinase activity with rat liver 40 S ribosomal subunit was assayed in a final volume of 40 µl containing 10 mM Hepes-KOH (pH 7.4), 5 mM magnesium acetate, 30 mM 2-mercaptoethanol, 20 µg of 40 S ribosomal subunit, enzyme fraction and 50 µM (γ-^{32}P)ATP (2,800 cpm/pmol). After incubation at 30°C, reactions were terminated by addition of 10 µl of sodium dodecyl sulfate (SDS)-electrophoresis buffer and boiling. The products of the reaction were analyzed by SDS-polyacrylamide (15 % w/v) gel electrophoresis(14). The radioactivity of the excised bands (32 K protein) was quantified by liquid scintillation spectrometry. This protein corresponded to S6 protein on the two-dimensional gel electrophoresis (1).

Male Wistar rats (170-200 g) were partially hepatectomized under ether anaesthesia following the method as described by Higgins and Anderson (15). Rats were killed at indicated times after partial hepatectomy or sham-operation and livers were removed and homogenized with 30 strokes of a Dounce homogenizer in

2.5 volume of buffer containing 80 mM β-glycerophosphate (pH 7.4), 20 mM EGTA, 15 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 0.1 mM N-α-p-tosyl-L-lysine chloromethyl ketone, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml benzamidine, 10 μg/ml antipain, 10 μg/ml chymostatin and 10 μg/ml pepstatin. The homogenate was first centrifuged at 16,000 x g for 15 min and then 100,000 x g for 1 h. The supernatant was filtered through glass wool. For detection of S6 kinase activity, the supernatant (100 mg protein) was applied to a small DE-52 column (1.5 x 2.8 cm) that had been equilibrated in Buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 65 mM NaCl) and the column was washed with 12 volumes of Buffer A, 5 volumes of Buffer A containing 50 μM cAMP and 5 volumes of Buffer A again. Proteins were eluted with 10 volumes of linear gradient to 400 mM NaCl. Fractions of 2.5 ml each were collected and assayed for S6 kinase activity.

For purification of S6 kinase, regenerating livers (12.2 g) 5 h after partial hepatectomy from 5 rats were employed and the supernatant obtained as described above was applied to a DE-52 column (2.1 x 5.8 cm). The active fraction (47 ml) from the column was concentrated to 13.5 ml by ultrafiltration and dialyzed overnight against Buffer B (20 mM β-glycerophosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol and 20 mM NaCl). The preparation was applied to a peptide R_1A_{13}-Sepharose column (1.5 x 2.8 cm). The column, which had been equilibrated in Buffer B, was washed with Buffer B (160 ml) and then the enzyme was eluted with a linear gradient to 300 mM NaCl in Buffer B. Fractions of 1.4 ml were collected and assayed for S6 kinase activity. The active fractions were pooled and concentrated to 0.5 ml by ultrafiltration. The preparation was then subjected to gel filtration on a Sephadex G-150 column (0.7 x 97.5 cm) equilibrated in Buffer B containing 10 % ethylene glycol and 0.01 % Brij 35. Fractions of 0.78 ml were collected and assayed for S6 kinase activity. The active fractions were pooled and applied to the peptide R_1A_{13}-Sepharose 4B column again, which had been equilibrated in Buffer B. Active fractions were obtained by the same manner of elution as the first column and concentrated by ultrafiltration.

Protein was determined by the method of Bradford (16) with bovine serum albumin as a standard.

RESULTS

The supernatant (each 100 mg protein) from regenerating liver or sham-operated liver was prepared and was chromatographed on a DE-52 column. Protein kinase activity using the synthetic peptide R_1A_{13} as substrate was eluted at approximately 250 mM NaCl and was about 1.5-fold higher in extract of regenerating liver than that of sham-operated liver (Fig. 1). The phosphorylating activity of 32 K protein (corresponding to S6) in 40 S ribosomal subunit was coeluted from DE-52 column with the activity for peptide R_1A_{13}. The S6 kinase activity with peptide R_1A_{13} was not stimulated by cAMP, Ca^{2+}/phosphatidylserine/diolein or Ca^{2+}/calmodulin and not

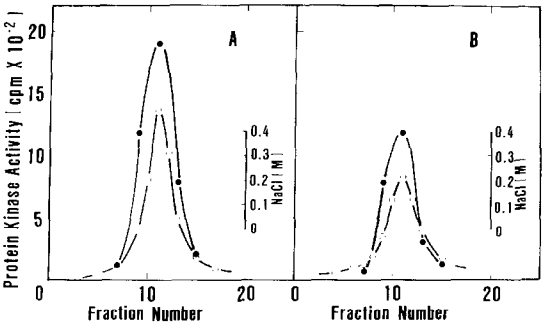


Fig. 1. Activation of S6 kinase activity in regenerating rat liver. The supernatant (each 100 mg protein) of regenerating liver (5 h after partial hepatectomy) (A) or sham-operated liver (B) was applied to a DE-52 column (1.5 x 2.8 cm). Fractions of 2.5 ml each were collected and assayed for S6 kinase activity with peptide R_1A_{13} (O) or 40 S ribosomal subunits (●) as substrate as described in EXPERIMENTAL PROCEDURE.

inhibited by GTP or the protein inhibitor of cAMP-dependent protein kinase (data not shown). Fig. 2 shows that the stimulation of the S6 kinase activity was time-dependent after partial hepatectomy. It reached a maximum at 5-7 h and the enzyme activity returned to approximately normal level after 24 h.

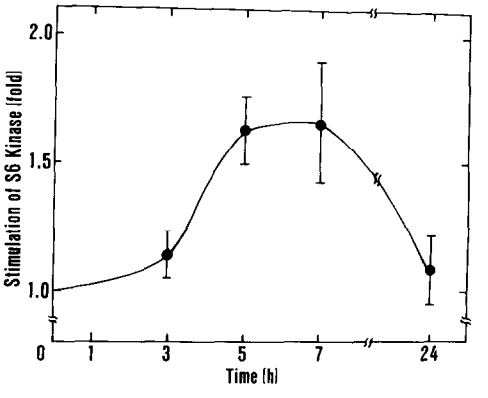
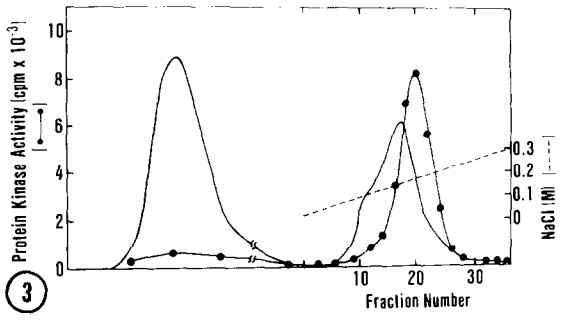


Fig. 2. The time course of the stimulation of S6 kinase activity after partial hepatectomy. The rats were killed at the indicated times after the partial hepatectomy or the sham-operation and the supernatant (each 100 mg protein) from liver homogenate was applied to DE-52 column (1.5 x 2.8 cm). Proteins were eluted as described under EXPERIMENTAL PROCEDURE and assayed for S6 kinase activity with peptide R_1A_{13}. Active fractions were collected and S6 kinase activity and protein concentration were measured. Stimulation of S6 kinase activity is defined as ratio of specific activity of the regenerating liver to that of the sham-operated liver. The points shown are the average of determinations from two separate experiments with the ranges indicated, except that the value at 5 h is the mean ± standard deviation of four determinations.



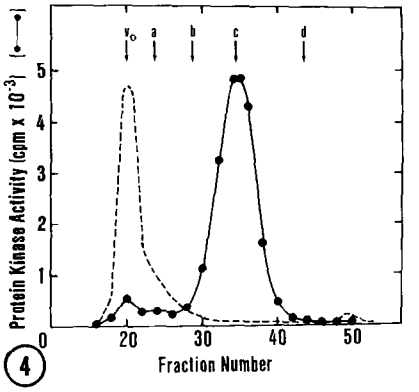


Fig. 3. Chromatography of DE-52 peak on peptide R_1A_{13}-Sepharose 4B (I). The dialyzed pool of DE-52 peak was applied to a peptide R_1A_{13}-Sepharose 4B column and the column was washed and eluted as described under EXPERIMENTAL PROCEDURE. Fractions 18-24 were pooled for further purification. A_{280} (———).

Fig. 4. Chromatography of the peptide R_1A_{13}-Sepharose 4B (I) peak on Sephadex G-150. The pooled fraction from the peptide R_1A_{13}-Sepharose 4B column was applied to a Sephadex G-150 column and the protein was eluted as described under EXPERIMENTAL PROCEDURE. Fractions 31-38 were pooled and subjected to further purification. A_{280} (-----). Molecular mass standards: a, human γ-globulin; b, bovine serum albumin; c, ovalbumin; d, horse heart cytochrome c.

Peak from the DE-52 column was further purified with a peptide R_1A_{13}-Sepharose 4B column (Fig. 3) and Sephadex G-150 column (Fig. 4). The molecular weight was estimated to be 4.5×10^4. The pooled enzyme was rechromatographed on the peptide R_1A_{13}-Sepharose 4B. The final enzyme preparation was purified about 200-fold from the pool of DE-52 column, with a recovery of 15 %.

The substrate specificity of the purified S6 kinase was examined. As shown in Fig. 5, the enzyme phosphorylated S6 in 40 S ribosomal subunit (lane 1). Bovine casein was inactive as substrate (data not shown). With whole histone as substrate the enzyme was very active at 75 mM than 5 mM Mg^{2+} and H2B histone was mainly phopshorylated at 75 mM Mg^{2+}, whereas H4 histone was not phosphorylated (lanes 3-6). The protein inhibitor of cAMP-dependent protein kinase was ineffective on the phosphorylation of S6 (lanes 1 and 2). When whole histone was phosphorylated with cAMP-dependent protein kinase, the reaction was very rapid at 5 mM

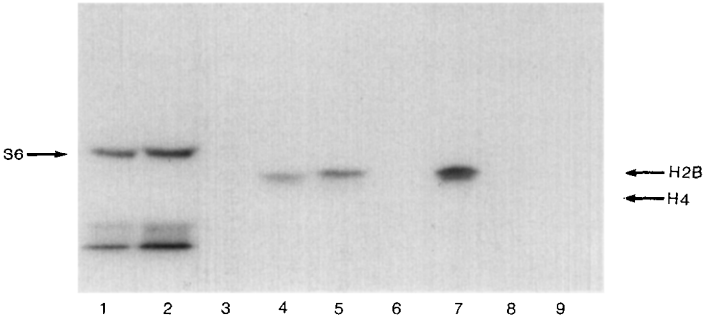


Fig. 5. Substrate specificity of partial purified S6 kinase. The partial purified S6 kinase (1.7 units) (lanes 1-6) or cAMP-dependent protein kinase (2.3 ng) (lanes 7-9), was incubated with 20 μg of 40 S ribosomal subunit (lanes 1 and 2), 40 μg of whole histone (lanes 3, 4 and 7-9), 10 μg of H2B histone (lane 5) or 10 μg of H4 histone (lane 6) as substrate in a total volume of 40 μl contaning 10 mM Hepes-KOH, pH 7.4, 30 mM 2-mercaptoethanol, 5 mM (lanes 1-3, 7 and 9) or 75 mM magnesium acetate (lanes 4-6 and 8) and 50 μM (γ-^{32}P)ATP (3,500 cpm/pmol, lanes 1 and 2; 1,060 cpm/pmol, lanes 3-9). For the assay of cAMP-dependent protein kinase, cAMP (2.5 μM) was added. In lanes 2 and 9, protein inhibitor of cAMP-dependent protein kinase (4.8 μg) was added. After incubation at 30°C for 30 min, the reactions were stopped by addition of SDS-electrophoresis buffer and boiling as described under EXPERIMENTAL PROCEDURE. The products of the reactions were analyzed by SDS-polyacrylamide gel electrophoresis using 15 % (w/v) gel (lanes 1 and 2) or 17.5 % (w/v) gel (lanes 3-9). Autoradiograms of the dried gel are shown.

than 75 mM Mg^{2+} and it was completely inhibited by the protein inhibitor of cAMP-dependent protein kinase (lanes 7-9). The apparent Km for the peptide R_1A_{13} was 18 μM and the apparent Km for ATP was 33 μM (data not shown).

DISCUSSION

Liver regeneration after partial hepatectomy has been employed as an in vivo system to investigate the regulatory mechanisms that stimulate resting cells to proliferate. Although it was reported that the phosphorylation of S6 was increased during liver regeneration (17), the enzyme responsible for the S6 phosphorylation has not previously been identified. In this report we describe the activation of S6 kinase in regenerating liver. From the results, the S6 kinase is distinct from cAMP-dependent protein kinase, protein kinase C, calmodulin-dependent protein kinases, casein

kinases or H4 histone-specific protein kinase. The S6 kinase activity is eluted from DE-52 column at approximately 250 mM NaCl and the molecular weight of the enzyme is 4.5×10^4 on gel filtration. H2B histone is a good substrate of the enzyme in the presence of high concentration of Mg^{2+}. These properties are similar to those of a proteolytically modified Ca^{2+}/phospholipid-independent form of protein kinase C, designated protein kinase M (18). Treatment of quiescent cells with the tumor promoter 12-o-tetradecanoyl-phorbol 13-acetate (TPA) activates the phosphorylation of S6 and the phosphopeptide maps of S6 are identical with that obtained following insulin treatment (19). As protein kinase C is considered to be the receptor of TPA (20), it is supposed that protein kinase C has relations to the phosphorylation of S6. Previous reports from this laboratory showed that in rat liver plasma membrane trypsin-like protease could attack protein kinase C and generate a Ca^{2+}/phospholipid independent form (3,4). Parker et al. have shown that this independent form could phosphorylate S6 *in vitro* (21). Furthermore it has been proposed that treatment of cells with TPA results in protein kinase M activity in soluble fraction (22,23). From the evidence it might be possible that protein kinase M phosphorylates S6 *in vivo*. On the other hand, Traugh and co-workers have found that protease activated kinase II (PAK II) is activated in 3T3-L1 cells in response to insulin (24) and that PAK II and protein kinase C are closely related, but unique enzymes (25). The S6 kinase of the regenerating liver is similar to protein kinase M rather than PAK II on the basis of substrate specificity; that is, both regenerating liver S6 kinase and protein kinase M phosphorylate H2B histone, while PAK II is specific for H1 (25). Further studies are required to prove the relationship between the S6 kinase in regenerating liver and protein kinase M or PAK II.

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REFERENCES

1. Thomas, G., Martin-Perez, J., Siegmann, M. and Otto, A.M. (1982) Cell 30, 235-242.
2. Duncan, R. and McConkey, E.H. (1982) Eur. J. Biochem. 123, 535-538.
3. Hashimoto, E., Mizuta, K. and Yamamura, H. (1985) Biochem. Biophys. Res. Commun. 131, 246-254.
4. Mizuta, K., Hashimoto, E. and Yamamura, H. (1985) Biochem. Biophys. Res. Commun. 131, 1262-1268.
5. Wettenhall, R.E.H., Cohen, P., Caudwell, B. and Holland, R. (1982) FEBS Lett. 148, 207-213.
6. Martin, T.E., Rolleston, F.S., Low, R.B. and Wool, I.G. (1969) J. Mol. Biol. 43, 135-149.
7. Wettenhall, R.E.H. and Wool, I.G. (1972) J. Biol. Chem. 247, 7201-7206.
8. Rubin, C.S., Erlichman, J. and Rosen, O.M. (1974) Methods Enzymol. 38, 308-315.
9. Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1977-1985.
10. Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313-1320.
11. Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K. and Iwai, K. (1976) J. Biol. Chem. 251, 6287-6293.
12. Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149-2154.
13. Kondo, H., Uno, S., Komizo, Y. and Sunamoto, J. (1984) Int. J. Peptide Protein Res. 23, 559-564.
14. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
15. Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186-202.
16. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
17. Gressner, A.M. and Wool, I.G. (1974) J. Biol, Chem. 249, 6917-6925.
18. Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603-7609.
19. Trevillyan, J.M., Perisic, O., Traugh, J.A. and Byus, C.V. (1985) J. Biol. Chem. 260, 3041-3044.
20. Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40.
21. Parker, P.J., Katan, M., Waterfield, M.D. and Leader, D.P. (1985) Eur. J. Biochem. 148, 579-586.
22. Tapley, P.M. and Murray, A.W. (1984) Biochem. Biophys. Res. Commun. 122, 158-164.
23. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986) J. Biol. Chem. 261, 8309-8313.
24. Perisic, O. and Traugh, J.A. (1983) J. Biol. Chem. 258, 9589-9592.
25. Gonzatti-Haces, M.I. and Traugh, J.A. (1986) J. Biol. Chem. 261, 15266-15272.