Biochem. J. (1991) 274, 759–767 (Printed in Great Britain)

Identification of a major maturation-activated acetyl-CoA carboxylase kinase in sea star oocytes as p44^{mpk}

Steven L. PELECH,*‡§ Jasbinder S. SANGHERA,* Harry B. PADDON,* Katherine A. QUAYLE† and Roger W. BROWNSEY†

*The Biomedical Research Centre, and the Departments of †Biochemistry and ‡Medicine, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

Maturation-activated protein-serine/threonine kinases were investigated in the high-speed supernatant fractions from sea-star oocytes harvested at the time of germinal vesicle breakdown. One of the major stimulated protein kinases able to phosphorylate acetyl-CoA carboxylase in these extracts was found to co-purify with a 44 kDa myelin basic protein kinase (p44^{mpk}) that is activated with a similar time course during oocyte maturation. Purified sea-star oocyte p44^{mpk} phosphorylated acetyl-CoA carboxylase (purified from rat liver) predominantly on serine and to a small extent on threonine. Furthermore, the phosphorylation of acetyl-CoA carboxylase occurred principally on a tryptic phosphopeptide which displayed electrophoretic and chromatographic properties very similar to those of the peptide that has previously been shown to undergo increased phosphorylation in response to insulin in rat adipocytes [Brownsey & Denton (1982) Biochem. J. **202**, 77–86]. The acetyl-CoA carboxylase was phosphorylated at a similar rate and to a similar extent by casein kinase II, which was also purified from maturing sea-star oocytes. Although casein kinase II was also activated ~ 3-fold near the time of nuclear envelope breakdown, it was responsible for only a minor component of the total enhanced acetyl-CoA carboxylase kinase activity measured in the soluble extracts from maturing oocytes. Acetyl-CoA carboxylase was a relatively poor substrate for the major S6 peptide kinase activity that was also stimulated during resumption of meiosis in the oocytes. The properties of the p44^{mpk} are reminiscent of those of a microtubule-associated protein 2 (MAP-2) kinase that is activated in response to insulin and other mitogens in mammalian cells [Ray & Sturgill (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3753–3757; Hoshi, Nishida & Sakai (1988) J. Biol. Chem. **263**, 5396–5401]. It is intriguing that several of the mammalian protein kinases that are acutely activated after mitogenic prompting of quiescent mouse fibroblasts (i.e. G_0 to G_1 transition), such as MAP-2 kinase, casein kinase II and S6 kinase II, have counterparts that are activated during M-phase in maturing sea star oocytes.

INTRODUCTION

It has become increasingly clear that cellular proliferation is extensively controlled by a network of highly conserved protein phosphorylation cascades (for reviews, see Pelech *et al.*, 1990; Maller, 1990). Alterations in the states of phosphorylation of numerous proteins reflect, at least in part, the modulation of the activities of several protein kinases that participate in the network. This is best exemplified by the mitogen-activated protein-serine/threonine kinases that act upon the 40 S ribosomal protein S6. Treatment of Swiss mouse 3T3 cells with insulin, insulin-like growth factor 1, epidermal growth factor (EGF), platelet-derived growth factor, fibroblast growth factor or phorbol 12-myristate 13-acetate (PMA) induces the activation of S6 kinases (Novak-Hofer & Thomas, 1984, 1985; Pelech *et al.*, 1986; Pelech & Krebs, 1987) and consequently leads to increased phosphorylation of S6 (Martin-Perez *et al.*, 1984; Blackshear *et al.*, 1985). Similar observations have been made in a variety of other model systems, including insulin-treated mouse BC3H-1 myocytes (Spach *et al.*, 1986), rat skeletal muscle (Hecht & Straus, 1988) and rat H4 hepatoma cells (Nemenoff *et al.*, 1986).

It is feasible that activated S6 kinases may also contribute towards the mitogen-induced phosphorylation of proteins other than S6. For example, the increased phosphorylation of ATP citrate lyase that is detected in insulin-treated adipocytes and hepatocytes (Benjamin & Singer, 1975; Alexander *et al.*, 1979) and EGF-treated hepatocytes (Holland & Hardie, 1985) might also be catalysed by an insulin-activated S6 kinase (Price *et al.*, 1989).

Although the mechanisms by which protein-serine/threonine kinases are activated by mitogens have yet to be clearly defined, it seems likely that they are stimulated by covalent modification in protein kinase cascades. At least one S6 kinase, termed S6 kinase II, appears to be activated via phosphorylation by a microtubule-associated protein 2 (MAP-2) kinase that is also stimulated in response to the aforementioned polypeptide growth factors and PMA in quiescent fibroblasts (Ray & Sturgill, 1988*a*; Sturgill *et al.*, 1988; Hoshi *et al.*, 1988, 1989). The activity of this MAP-2 kinase, in turn, appears to be stimulated when it is phosphorylated on tyrosine and threonine residues by other, as yet undefined, protein kinases (Anderson *et al.*, 1990).

In addition to S6 and ATP citrate lyase, phosphorylation of the lipogenic enzyme acetyl-CoA carboxylase is also increased in response to mitogens (for reviews, see Brownsey & Denton, 1987; Hardie, 1989). This correlates with activation of acetyl-CoA carboxylase and stimulation of fatty acid synthesis in insulin- and EGF-treated rat adipocytes (Halestrap & Denton, 1973; Lee *et al.*, 1973; Brownsey & Denton, 1982; Haystead & Hardie, 1986*a*) and in similarly stimulated hepatocytes (Holland & Hardie, 1985). The insulin-induced phosphorylation of acetyl-

Abbreviations used: EGF, epidermal growth factor; GVBD, germinal vesicle breakdown; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; p34^{cdc2}, histone H1 kinase encoded by the *S. pombe cdc2* gene and its species homologues; p44^{mpk}, maturation-activated myelin basic protein kinase; PKIP, cyclic AMP-dependent protein kinase inhibitor peptide; S6 peptide or RRLSSLRA, Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala; PMA, phorbol 12-myristate 13-acetate.

§ To whom correspondence should be addressed.

CoA carboxylase occurs at sites that are distinct from those phosphorylated by recognized protein kinases, including cyclic AMP-dependent protein kinase, AMP-activated protein kinase or protein kinase C (Brownsey *et al.*, 1981, 1984; Witters, 1981; Brownsey & Denton, 1982; Witters *et al.*, 1983; Holland & Hardie, 1985; Haystead & Hardie, 1986*b*, 1988; Davies *et al.*, 1990). At least one of these insulin-stimulated phosphorylation sites can be phosphorylated by casein kinase II *in vitro* (Tipper *et al.*, 1983; Haystead *et al.*, 1988), and it corresponds to Ser-29 in the rat mammary gland acetyl-CoA carboxylase sequence (Lopez-Casillas *et al.*, 1988). However, phosphorylation of acetyl-CoA carboxylase by casein kinase II does not lead to activation of the enzyme (Tipper *et al.*, 1983). PMA treatment of isolated adipocytes also increases the phosphorylation of acetyl-CoA carboxylase at two sites that are identical to or near the sites phosphorylated in response to insulin, although, interestingly, these sites are not phosphorylated by protein kinase C *in vitro* (Haystead & Hardie, 1988). In contrast with insulin, phorbol ester treatment of isolated adipocytes does not lead to a measurable stimulation of acetyl-CoA carboxylase activity (Haystead & Hardie, 1988). Alternative hypotheses can be advanced to explain the insulin-induced activation of acetyl-CoA carboxylase, such as the production of a low-molecular-mass activator (Haystead & Hardie, 1986*b*) and dephosphorylation of the carboxylase at the site phosphorylated by AMP-activated protein kinase (Ser-79 in rat mammary acetyl-CoA carboxylase) (Lopez-Casillas *et al.*, 1988; Haystead *et al.*, 1990). However, such dephosphorylation was not apparent within intact fat cells (Brownsey & Denton, 1982). Regardless of whether the phosphorylation of the insulin-stimulated sites in acetyl-CoA carboxylase leads directly or indirectly to modulation of the enzyme activity, identification of the kinase(s) responsible would facilitate further delineation of the protein kinase cascade that mediates insulin action.

Many of the protein kinases that are activated acutely when quiescent (G_0) fibroblasts are prompted with mitogens to re-enter the cell cycle at G_1-phase appear to have counterparts that are stimulated later at M-phase in maturing oocytes. For example, when *Xenopus laevis* oocytes blocked in prophase at the G_2/M-phase border are triggered to resume meiotic maturation with insulin or PMA, activation of S6 kinases and enhanced S6 phosphorylation ensues (Stith & Maller, 1984; Cicirelli *et al.*, 1988*b*). We have also described an activated myelin basic protein (MBP) kinase ($p44^{mpk}$) in maturing *Xenopus* (Cicirelli *et al.*, 1988*a*) and sea-star (Pelech *et al.*, 1988; Sanghera *et al.*, 1990) oocytes that may be highly related to the mammalian MAP-2 kinase. In addition, the activity of casein kinase II, which is rapidly stimulated in mitogen-treated mammalian cells (Sommercorn *et al.*, 1987; Karlund & Czech, 1988; Carroll & Marshak, 1989; Ackerman & Osheroff, 1989), is shown in this report to also be enhanced in maturing sea-star oocytes.

As acetyl-CoA carboxylase represents a powerful 'reporter' for protein kinases activated by cell stimulation, it was of interest to investigate whether it could serve as a probe for novel oocyte maturation-activated protein kinases. In a previous study with maturing *Xenopus* oocytes, a 2-fold increase in the activity of an unidentified cyclic AMP-independent acetyl-CoA carboxylase kinase was detected (Cicirelli *et al.*, 1988*a*). Herein we have characterized the M-phase-activated acetyl-CoA carboxylase kinases in maturing sea-star oocytes, and report that a major activity is distinct from casein kinase II or ribosomal S6 kinases, and instead corresponds to $p44^{mpk}$. The apparent phosphorylation by $p44^{mpk}$ of a phosphopeptide corresponding to an insulin-directed site on acetyl-CoA carboxylase further supports the concept that mitogen-stimulated protein kinases of mammalian cells may be homologous to counterparts in evolutionarily distant systems.

EXPERIMENTAL

Materials

Sea stars (*Pisaster ochraceus*) were collected locally from beaches in the Vancouver area. $p44^{mpk}$ was purified from maturing *P. ochraceus* oocytes as described (Sanghera *et al.*, 1990). Casein kinase II was also purified to homogeneity from maturing sea-star oocytes by adopting the protocol of Mulner-Lorillon *et al.* (1988). MBP was purified from bovine brain (Deibler *et al.*, 1984). Acetyl-CoA carboxylase was purified from rat liver (Brownsey *et al.*, 1988). The S6 peptide (Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala) was a gift from Dr. Edwin G. Krebs (University of Washington, Seattle, WA, U.S.A.). The cyclic AMP-dependent protein kinase inhibitor peptide (PKIP), with the sequence Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp (Scott *et al.*, 1986; Cheng *et al.*, 1986), α-casein, histone type III-S (H1), phenylmethanesulphonyl fluoride, Mops, 1-methyladenine (1-MeAde), DEAE-Sephacel and ATP were purchased from Sigma. MonoQ, phenyl-Sepharose, polylysine–Sepharose, Sephacryl S-300 and Superose 12 were obtained from Pharmacia. Hydroxyapatite and electrophoresis and gel-filtration standards were from Bio-Rad Laboratories. [γ-^{32}P]ATP was from ICN Biochemicals.

Preparation of cytosol from maturing sea-star oocytes and column fractionation

Oocytes, which were arrested at the G_2/M border during meiosis, were isolated from sea stars as described (Meijer *et al.*, 1984). The immature oocytes were induced to undergo maturation at 14 °C by the addition of 1-MeAde to the incubation medium. Germinal vesicle breakdown (GVBD) typically occurred about 80 min later. Oocytes were recovered by centrifugation at 4 °C (5 min at 1500 rev./min), and a 33% (w/v) oocyte homogenate was prepared by dispersing 1 vol. of packed cells in 2 vol. of chilled homogenizing buffer (125 mM-β-glycerol phosphate, pH 7.2, 12 mM-EDTA, 2 mM-sodium orthovanadate, 1 mM-dithiothreitol and 0.5 mM-phenylmethanesulphonyl fluoride) and then disrupting the cells with 25 strokes of a glass/glass Dounce homogenizer. The homogenates were immediately centrifuged for 15–20 min at 250000 *g* in a Beckman TL-100 ultracentrifuge, and the supernatants were quickly frozen and stored at −70 °C in portions.

Details of the various column chromatographies are provided in the Figure legends, and further details are as reported previously (Sanghera *et al.*, 1990).

Protein kinase assays

Unless stated otherwise, all protein kinase assays were performed in a final volume of 25 μl with [γ-^{32}P]ATP (50 μM; ~ 1000 c.p.m./pmol for filter paper assays and ~ 5000 c.p.m./pmol for SDS/PAGE gels), PKIP (0.5 μM), and assay dilution buffer (pH 7.2) containing Mops (20 mM), β-glycerol phosphate (30 mM), $MgCl_2$ (20 mM), EGTA (5 mM), dithiothreitol (1 mM) and sodium orthovanadate (0.5 mM). Protein substrates used were acetyl-CoA carboxylase (0.1 mg/ml), MBP (1 mg/ml), α-casein (1 mg/ml), histone H1 (Sigma Type III-S) (1 mg/ml), and the S6 peptide RRLSSLRA (0.25 mM). All reaction preincubations were carried out at 0 °C, and the kinase reactions, which commenced upon addition of [γ-^{32}P]ATP, were of 5–10 min duration at 30 °C. The reaction rates generally became non-linear when the assay duration exceeded 15 min or when the final concentration of oocyte protein was greater than 0.3 mg/ml. The filter paper assays were terminated by spotting 20 μl samples on to a 1.5 cm^2 piece of Whatman P81 phosphocellulose paper. The papers were washed 10 times by shaking for 5 min in

phosphoric acid (1 %, w/v) before being transferred into 6 ml plastic scintillation vials containing 3 ml of scintillation fluid, and radioactivity was counted in a Packard Tri-Carb 2200 scintillation counter.

Protein was estimated by the method of Bradford (1976) using BSA ($A^{1\%}_{280}$ 6.5) as a standard.

Electrophoresis

Acetyl-CoA carboxylase kinase assays were terminated by the addition of 25 μl of SDS/PAGE sample buffer (pH 6.8) containing Tris/HCl (125 mM), SDS (4 %, w/v), Bromophenol Blue (0.01 %), mercaptoethanol (10 %, v/v) and glycerol (20 %, w/v). The samples were heated in a boiling-water bath for 4 min and then subjected to SDS/PAGE on 1.5 mm thick gels, with acrylamide at 10 % (w/v) in the separating gel and 4 % (w/v) in the stacking gel, using the buffer system described by Laemmli (1970). The gels were either stained with Coomassie Blue or were silver-stained using the method of Merril *et al.* (1981). For autoradiography, dried gels were exposed to Kodak XAR-5 film at room temperature.

For phosphoamino acid analysis, the ^{32}P-labelled acetyl-CoA carboxylase was first purified by SDS/PAGE and the radiolabelled carboxylase band was excised and incubated in 300 μl of HCl (6 M) at 105 °C for 1 h. The acid-hydrolysed sample was dried under vacuum (Speed Vac), washed with water, redried and then resuspended in 5 μl of electrophoresis buffer containing pyridine/acetic acid/water (1:10:189, by vol.). Phosphoserine, phosphothreonine and phosphotyrosine standards (1 μg of each) were electrophoresed with the sample and electrophoresis was allowed to proceed for 45–60 min at 1000 V with cooling. Subsequently, the radiolabelled phosphoamino acids were detected by autoradiography and the migration of standards was confirmed by staining with ninhydrin.

Analysis of the tryptic phosphopeptides of acetyl-CoA carboxylase phosphorylated *in vitro* by p44^{mpk} and casein kinase II by two-dimensional thin-layer gel electrophoresis and autoradiography was performed as described previously (Brownsey *et al.*, 1984).

RESULTS

Increased activity of an acetyl-CoA carboxylase kinase in cytosol from maturing sea-star oocytes

In a previous study with maturing *Xenopus* oocytes (Cicirelli *et al.*, 1988*a*), one of us described the marked activation of protein kinases that phosphorylate acetyl-CoA carboxylase, histone H1, MBP, casein and RRLSSLRA, a synthetic peptide modelled after the major phosphorylation site near the *C*-terminus of ribosomal protein S6 (Wettenhall & Morgan, 1984). All of these M-phase-activated protein kinases, with the exception of those acting on acetyl-CoA carboxylase and casein (which were not examined in detail in the *Xenopus* oocyte), could also be detected in maturing sea-star oocytes provided that precautions were adopted to include protein phosphatase inhibitors such as β-glycerol phosphate, vanadate and EGTA in the homogenization and assay buffers (Pelech *et al.*, 1987, 1988; Meijer *et al.*, 1987). As shown in Fig. 1, when compared with extracts from immature *P. ochraceus* oocytes (lane 3), soluble extracts from cells that had completed GVBD in response to 1-MeAde (lane 4) also exhibited (2–3-fold) enhanced phosphorylating activity towards acetyl-CoA carboxylase purified from rat liver.

When induced to mature at 14 °C with 1-MeAde, *P. ochraceus* oocytes typically undergo GVBD in ~ 75 min, and the first meiotic division (i.e. polar body emission) is observed about 2.5 h later. Peak stimulation of the acetyl-CoA carboxylase phosphorylating activity in the oocyte cytosols occurred when approx. 50 % of the cells had undergone GVBD (Fig. 2*a*). This activation was slower than that of the histone H1 and casein kinases, but somewhat faster than the rate of increase in RRLSSLRA and MBP phosphorylation, as measured in the crude extracts (Fig. 2). This implied that rat liver acetyl-CoA carboxylase might serve as a convenient probe for a unique maturation-activated protein kinase.

Column chromatography of acetyl-CoA carboxylase kinases from extracts of sea-star oocytes

To ascertain whether the activated acetyl-CoA carboxylase kinase was a single species and if it was distinct from some of the other protein kinases that are stimulated during meiosis, the oocyte cytosolic proteins were fractionated by various forms of column chromatography. At least three peaks of maturation-stimulated protein kinases, able to phosphorylate acetyl-CoA carboxylase, could be resolved by anion-exchange chromatography on MonoQ (Fig. 3*a*). The broad, minor peak of acetyl-CoA carboxylase kinase activity eluting at ~ 250 mM-NaCl did not appear to coincide with the phosphorylating activity towards MBP (Fig. 3*b*), histone H1 (Fig. 3*c*) or RRLSSLRA (Fig. 3*d*). On the other hand, a sharp and major peak of acetyl-CoA carboxylase kinase activity eluting at ~ 300 mM-NaCl also exhibited ability to phosphorylate each of these alternative substrates (Fig. 3). In previous studies (Erikson & Maller, 1986; Arion *et al.*, 1988; Pelech *et al.*, 1988), this later-eluting peak of phosphorylating activity has been shown to contain at least two

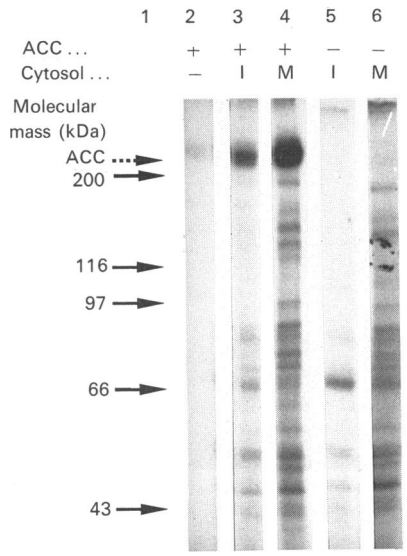

Fig. 1. Phosphorylation of rat liver acetyl-CoA carboxylase by activated protein kinase(s) in supernatant fractions from maturing sea-star oocytes

Acetyl-CoA carboxylase (ACC, 0.2 mg/ml) was incubated with [γ-^{32}P]ATP (50 μM) for 12 min at 30 °C in the absence (lane 2) or presence (lane 3) of supernatant fractions (containing 0.2 mg of protein/ml) prepared from immature oocytes (I) or from cells that had undergone GVBD ~ 80 min after the addition of 1-MeAde (2 μM) (M, lane 4). SDS/PAGE and autoradiography were performed as described in the Experimental section. Lanes 5 and 6 show phosphorylation of endogenous proteins in the supernatant fractions from immature and mature oocytes respectively (in the absence of added acetyl-CoA carboxylase). The migration of the protein standards myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa) is indicated by arrows (lane 1). Similar results were obtained in at least five independent experiments with different batches of oocytes.

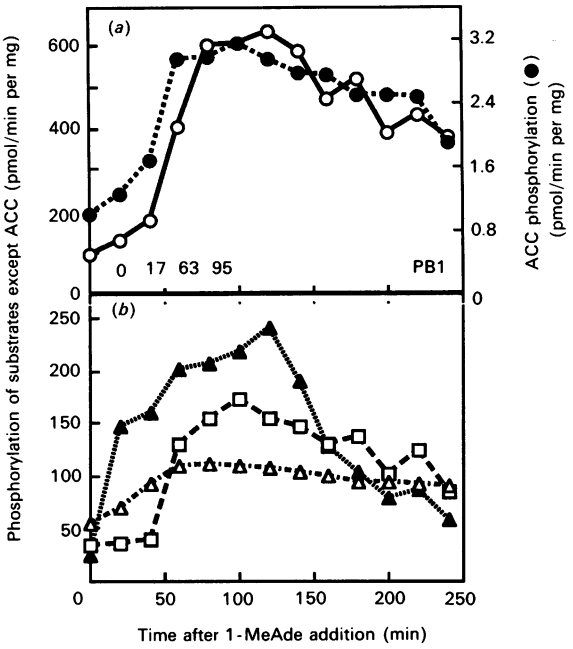

Fig. 2. Time course of the activation of protein kinases during maturation of sea-star oocytes

Oocytes from *P. ochraceus* were treated with 1-MeAde (2 μM) at 14 °C for various times up to 4 h and then the cells were harvested and supernatant fractions prepared as described in the Experimental section. Emission of the first polar body (PBI) occurred at 220–230 min after first exposure to 1-MeAde. Protein kinase activity of supernatant fractions was assessed by determination of the incorporation of ^{32}P from [γ-^{32}P]ATP (50 μM) in separate incubations employing five different protein substrates. Incubations were carried out for 10 min at 30 °C and samples were subjected to SDS/PAGE (for analysis of acetyl-CoA carboxylase) or spotted on to phosphocellulose paper (for all other proteins) before determination of ^{32}P incorporation by liquid scintillation counting. Results are shown in (*a*) for acetyl-CoA carboxylase (ACC; 0.1 mg/ml, ●) and MBP (1 mg/ml, ○), and in (*b*) for histone H1 (1 mg/ml, ▲), RRLSSLRA (0.25 mM, □) and casein (1 mg/ml, △). Similar results were obtained in at least three independent experiments. Numbers in (*a*) refer to % GVBD at the times indicated.

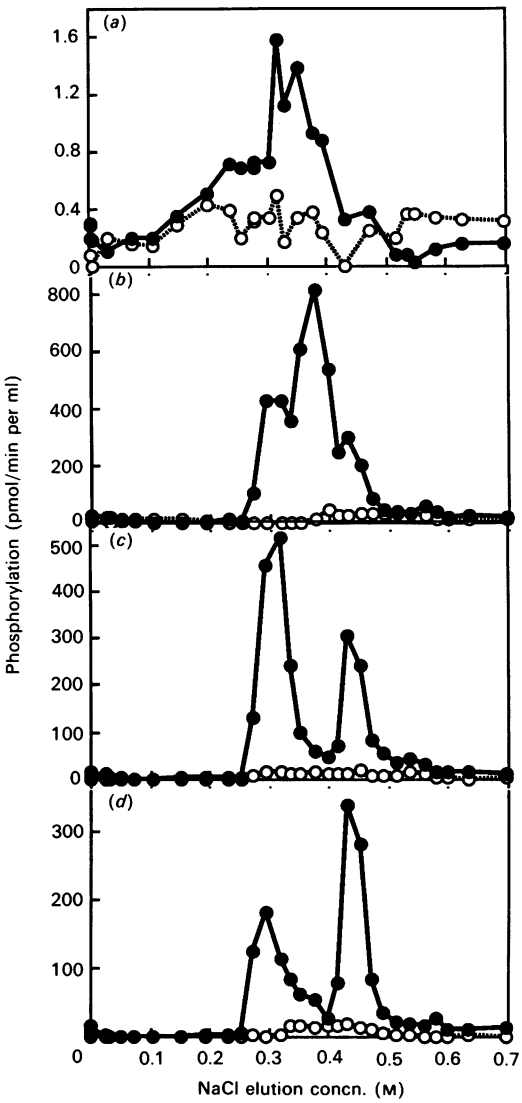

Fig. 3. MonoQ chromatography of protein kinases activated during sea-star oocyte maturation

Cytosolic protein (1 mg) from immature oocytes (○) and from oocytes exposed to 1-MeAde (2 μM) for 90 min (●) was loaded on to a MonoQ column (1 ml bed volume) in buffer A (pH 7.2) [containing Mops (5 mM), EGTA (5 mM), sodium vanadate (1 mM) and dithiothreitol (0.25 mM)] at a flow rate of 1 ml/min using a Pharmacia f.p.l.c. system. The column was developed with a 30 ml linear gradient of NaCl (0–0.8 M) in buffer A at the same flow rate. The column fractions (250 μl) were assayed for phosphorylating activity towards (*a*) acetyl-CoA carboxylase (ACC; 0.1 mg/ml), (*b*) MBP (1 mg/ml), (*c*) histone H1 (1 mg/ml), and (*d*) RRLSSLRA (0.25 mM), as described in the Experimental section. Similar results were obtained in at least three separate experiments.

distinct protein kinases, namely S6 kinase-I and the *cdc*-2-encoded/histone H1 kinase. The third major peak of acetyl-CoA carboxylase kinase activity eluted at ~ 350 mM-NaCl and coincided with the predominant peak of MBP phosphorylating activity, which contains p44^{mpk} (Pelech *et al.*, 1988; Sanghera *et al.*, 1990). The major peak of RRLSSLRA phosphorylating activity that was released from MonoQ with ~ 440 mM-NaCl probably corresponded to S6 kinase-II (Erikson & Maller, 1986; Pelech *et al.*, 1987).

All of the aforementioned protein kinases were eluted much earlier from MonoQ than was the major casein kinase that was activated ~ 3-fold at the time of GVBD in maturing oocytes (Fig. 4). This kinase, released with ~ 0.7 M-NaCl, could be identified as casein kinase II by several criteria, including its ability to phosphorylate casein, its relatively strong affinity for anion-exchange resins and its sensitivity to inhibition by heparin (2 μg/ml) (Fig. 4*b*). Furthermore, we have purified this casein kinase to near-homogeneity by the procedure of Mulner-Lorillon *et al.* (1988), and it contains the quaternary structure characteristic of this enzyme, i.e. a ~ 42 kDa α-subunit, a ~ 38 kDa α'-subunit and a non-catalytic ~ 27 kDa β-subunit (results not shown). Although casein kinase II has previously been reported to phosphorylate acetyl-CoA carboxylase *in vitro* (Tipper *et al.*, 1983; Haystead *et al.*, 1988), this substrate was comparatively poorly phosphorylated by the casein kinase II fraction from oocytes following MonoQ chromatography, perhaps because the activity level of the casein kinase was so low relative to the other maturation-activated kinases.

To further characterize the maturation-activated acetyl-CoA carboxylase kinases, cytosolic fractions from sea-star oocytes were subjected to gel-permeation chromatography. Two peaks of maturation-stimulated acetyl-CoA carboxylase kinase activity, corresponding to apparent molecular masses of 80 and 45 kDa, were resolved on Superose 12 (Fig. 5). The major histone H1 and

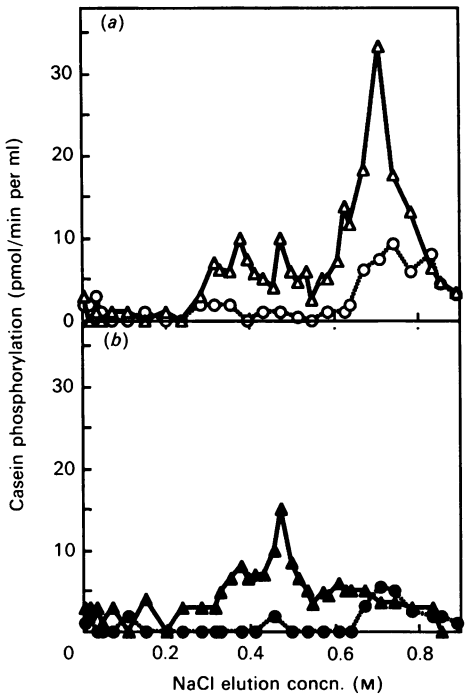

Fig. 4. MonoQ chromatography of casein kinases activated during sea-star oocyte maturation

Cytosolic protein (1 mg) from immature oocytes (○, ●) and from oocytes exposed to 1-MeAde (2 μM) for 90 min (△, ▲) was fractionated on MonoQ as described in the legend to Fig. 3. The column fractions were assayed for phosphorylating activity towards casein (0.1 mg/ml) in the absence (*a*, ○, △) and presence (*b*, ●, ▲) of heparin (2 μg/ml). Similar results were obtained in at least three separate experiments.

RRLSSLRA phosphorylating activities eluted with apparent molecular mass of 80 kDa (greater on Superose 12), but phosphorylation of MBP by p44^{mpk} coincided with the small (~ 45 kDa) acetyl-CoA carboxylase kinase on this gel-filtration column. The protein kinases able to phosphorylate acetyl-CoA carboxylase and MBP also co-eluted at ~ 45 kDa from Sephacryl S-300, although some acetyl-CoA carboxylase kinase exhibited an even higher molecular mass of 120 kDa (not shown).

Co-purification of a major maturation-activated acetyl-CoA carboxylase kinase with p44^{mpk}

On a preparative-scale DEAE-cellulose column the acetyl-CoA carboxylase kinases and the other maturation-activated protein kinases co-eluted as a single broad peak (results not shown). However, when this peak was then further fractionated by hydroxyapatite chromatography the various protein kinases were further resolved (Fig. 6*a*). The bulk of the MBP phosphorylating activity was released at conductivities of 10–20 mS, whereas the major histone H1 phosphorylating activity was eluted at 35–40 mS. From purification studies we have found that these peaks contain p44^{mpk} and *cdc*-2-encoded/histone H1 kinase respectively (Sanghera *et al.*, 1990; S. L. Pelech, M. Daya-Makin & J. Sanghera unpublished work). S6 kinase-I and -II appear to co-elute from hydroxyapatite at conductivities between 15 and 25 mS, since further sequential fractionation of this peak on MonoQ revealed that it contains both enzymes (results not shown). The acetyl-CoA carboxylase phosphorylating activity was eluted from hydroxyapatite as an extremely broad peak (conductivities between 10 and 35 mS), which was consistent with the presence of multiple acetyl-CoA carboxylase kinases (Fig. 6*a*). Since the major histone H1 phosphorylating

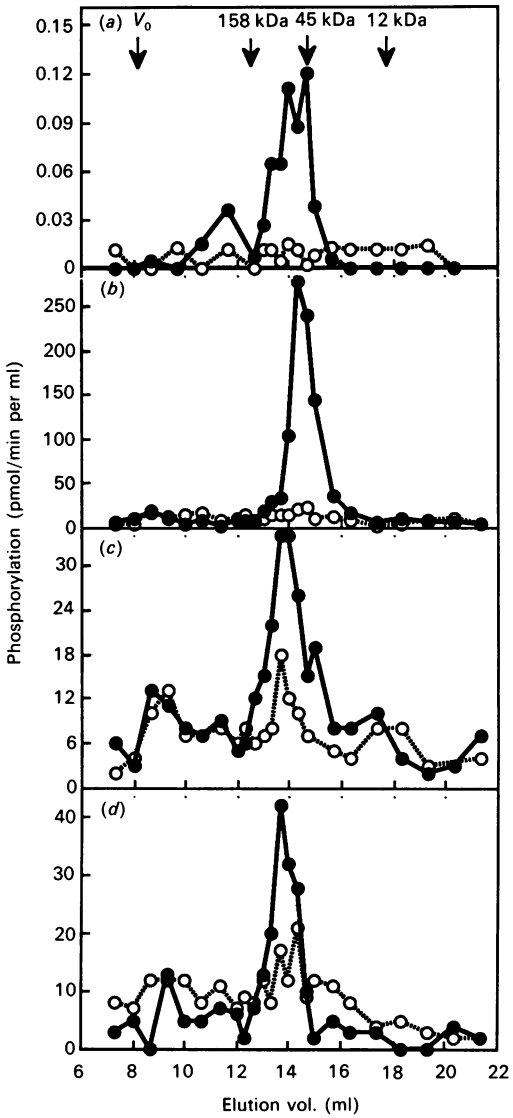

Fig. 5. Superose 12 chromatography of protein kinases activated during sea-star oocyte maturation

Cytosolic protein (0.1 mg) from immature oocytes (○) and from oocytes exposed to 1-MeAde (2 μM) for 90 min (●) was chromatographed on a Superose 12 column (22 ml bed volume) in buffer B (pH 7.2) [containing NaCl (150 mM), Mops (5 mM), EGTA (5 mM) and dithiothreitol (0.25 mM)] at a flow rate of 1 ml/min using a Pharmacia f.p.l.c. system. The column fractions (250 μl) were assayed for phosphorylating activity towards (*a*) acetyl-CoA carboxylase (0.1 mg/ml), (*b*) MBP (1 mg/ml), (*c*) histone H1 (1 mg/ml), and (*d*) RRLSSLRA (0.25 mM) as described in the Experimental section. Elution of the gel filtration marker proteins immunoglobulin G (158 kDa), ovalbumin (45 kDa) and cytochrome *c* (12 kDa), as well as Blue Dextran, which eluted in the void volume (V_0), are indicated by arrows. Similar results were obtained in at least two separate experiments.

activity required higher salt concentrations for elution from the hydroxyapatite resin than the acetyl-CoA carboxylase kinases, this implies that the *cdc*-2-encoded/histone H1 kinase did not contribute substantially to the enhanced acetyl-CoA carboxylase kinase activity detected in cytosols from maturing oocytes.

Our preliminary column fractionation studies strongly implied that p44^{mpk} might correspond to one of the major maturation-activated acetyl-CoA carboxylase kinases. Consequently the hydroxyapatite fractions containing p44^{mpk} were subjected to sequential fractionation on phosphocellulose (results not shown),

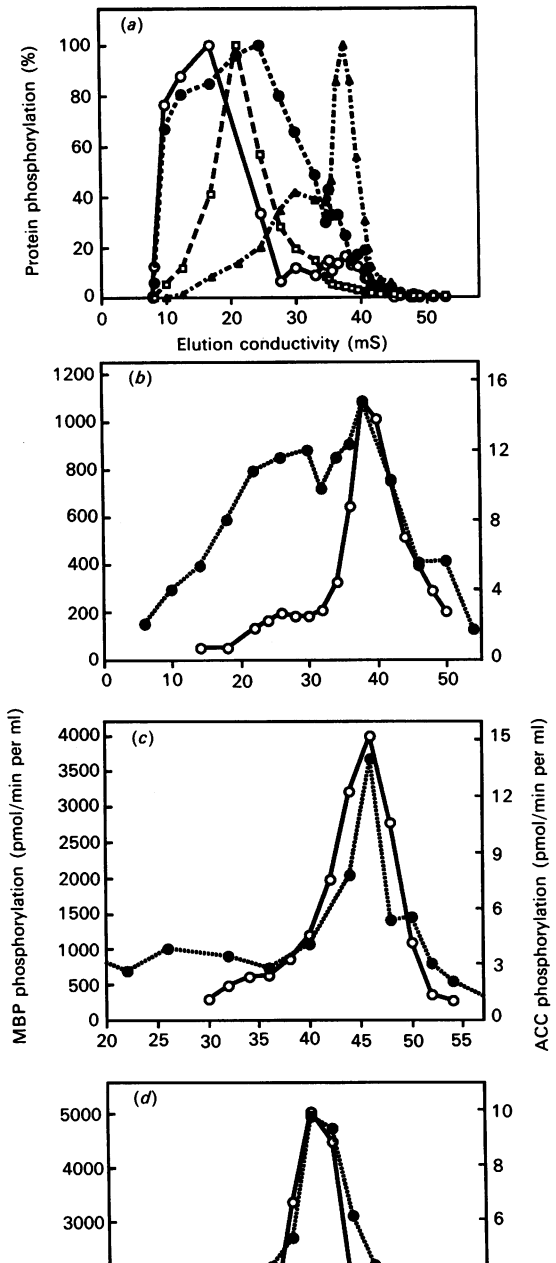

Fig. 6. Co-purification of a maturation-activated acetyl-CoA carboxylase kinase with p44^{mpk}

Supernatant fractions (120 ml; containing ~ 30 mg of cytosolic protein/ml) from sea-star oocytes that had been stored at −70 °C were thawed and diluted to 4 litres with buffer A. DEAE-cellulose (170 ml packed volume) was added to the supernatant and the slurry was gently stirred for 1.5 h in an ice bath. The DEAE-cellulose was allowed to settle over a 30 min period, the supernatant was subsequently decanted and the settled gel was poured into a column (5 cm × 9 cm). The column was washed (4 ml/min) with 150 ml of buffer C (pH 7.2) containing β-glycerol phosphate (25 mM), Mops (10 mM), EDTA (2 mM), EGTA (5 mM), sodium orthovanadate (2 mM) and dithiothreitol (1 mM). The phosphorylating activities towards acetyl-CoA carboxylase (ACC), MBP, histone H1 and RRLSSLRA were eluted as a broad peak when the column was developed with a linear gradient of NaCl (0–0.4 M) in 800 ml of buffer C. The elution of protein kinases from hydroxyapatite is shown in (*a*). The broad kinase activity peak from DEAE-cellulose was diluted with an equal volume of buffer D (pH 7.2) [containing β-glycerol phosphate (12.5 mM), Mops (12.5 mM), EGTA (0.5 mM), $MgCl_2$ (7.5 mM), NaF (0.5 mM) and dithiothreitol (2 mM)] and loaded on to a hydroxyapatite column (2.5 cm × 5 cm). The column was washed at the same flow rate with 50 ml of buffer D, and was then eluted with a linear potassium phosphate gradient (0–0.35 M) in 300 ml of buffer D. Fractions (4 ml) were collected and assayed for phosphorylating activity towards ACC (0.1 mg/ml, ●), MBP (1 mg/ml, ○), histone H1 (1 mg/ml, ▲) and RRLSSLRA (0.25 mM, □). Values are expressed as percentages of the peak phosphorylating activity towards each protein substrate. Subsequent purification of p44^{mpk} from the hydroxyapatite column fractions was performed essentially as described (Sanghera *et al.*, 1990), except that the heparin–Sepharose column step was omitted. The MBP kinase activity eluting at conductivities between 8 and 30 mS was diluted 3-fold in buffer D and loaded on to a phosphocellulose column (2.5 cm × 5 cm). The wash-through fraction from the phosphocellulose column was directly applied to a phenyl-Sepharose column (2.5 cm × 6 cm) and the column was developed with a linear 0–3% Brij 35 gradient in 300 ml of buffer D (*b*). The MBP kinase peak around fraction 40 (~ 1.25% Brij 35) was diluted 4-fold in buffer D and loaded on to a polylysine–Sepharose column (2.5 cm × 6 cm). This column was developed with a 0–0.7 M-NaCl linear gradient in 300 ml of buffer D (*c*). The MBP kinase peak around fraction 46 (~ 0.45 M-NaCl) was diluted 4-fold in buffer D and loaded on to a MonoQ column (1 ml bed volume). This column was developed with a 0–0.8 M-NaCl linear gradient in 30 ml of buffer D (*d*). Peak MBP kinase activity in fraction 20 corresponded to ~ 0.26 M-NaCl. For (*b–d*), MBP phosphorylating activity (○) is indicated on the left vertical axis and ACC phosphorylating activity (●) is shown on the right vertical axis.

phenyl-Sepharose (Fig. 6*b*), polylysine–Sepharose (Fig. 6*c*) and MonoQ (Fig. 6*d*), and the acetyl-CoA carboxylase kinase activity was monitored throughout the purification of p44^{mpk} (Sanghera *et al.*, 1990). Essentially all of the MBP and acetyl-CoA carboxylase phosphorylating activities failed to bind to phosphocellulose (results not shown) and was retained by phenyl-Sepharose (Fig. 6*b*). However, two peaks of acetyl-CoA carboxylase kinase activity were resolved on the phenyl-Sepharose column, and only the late-eluting peak catalysed appreciable MBP phosphorylation (Fig. 6*b*). The early-eluting kinase peak was not further characterized, whereas the late-eluting acetyl-CoA carboxylase kinase peak from phenyl-Sepharose was found to purify with the MBP kinase (p44^{mpk}) on polylysine–Sepharose (Fig. 6*c*) and MonoQ (Fig. 6*d*). The final kinase preparation was purified ~ 9000-fold, with a 4% recovery of the starting cytosolic MBP phosphorylating activity and 1% recovery of the initial acetyl-CoA carboxylase phosphorylating activity. The low recovery of activity probably reflected the dephosphorylation and inactivation of p44^{mpk} by protein phosphatases during purification.

Phosphorylation of acetyl-CoA carboxylase by purified p44^{mpk} and casein kinase II

The co-purification of a major stimulated acetyl-CoA carboxylase kinase with p44^{mpk} to a single silver-stained band after the final MonoQ step (Sanghera *et al.*, 1990) strongly supports the contention that the kinases were one and the same. The association of these two activities is particularly intriguing, since it appears that a similar MBP kinase is activated in mammalian cells in response to a variety of mitogens, some of which also stimulate an acetyl-CoA carboxylase kinase which has so far not been identified (see the Introduction section). As shown in Fig. 7 (lanes 4 and 5), acetyl-CoA carboxylase was phosphorylated by p44^{mpk}, the only other ^{32}P-labelled protein being p44^{mpk} itself, which becomes autophosphorylated. The stoichiometry of phos-

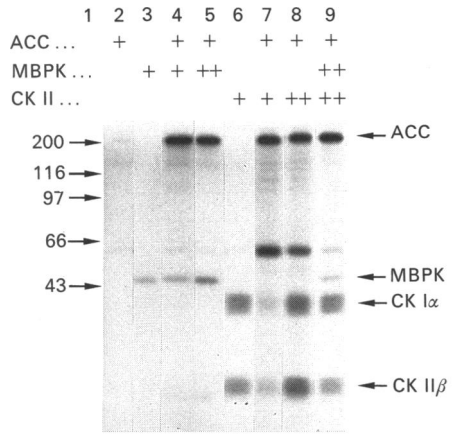

Fig. 7. Phosphorylation of rat liver acetyl-CoA carboxylase by purified p44^{mpk} and casein kinase II

Purified rat liver acetyl-CoA carboxylase (ACC, ~ 2.5 μg) was incubated for 15 min at 30 °C in a volume of 25 μl in the presence of [γ-^{32}P]ATP (50 μM) with or without p44^{mpk} or casein kinase II purified from maturing sea-star oocytes. Reactions were terminated with SDS/PAGE sample buffer and incubation of the samples at 100 °C for 4 min. After SDS/PAGE the slab gels were stained, destained, dried and subjected to autoradiography. On the autoradiogram shown, the position of marker proteins is indicated in lane 1 with appropriate subunit molecular masses (details as in the legend to Fig. 1). For all other lanes, the proteins added to the phosphorylation mixtures were as follows: 2, ACC alone; 3, p44^{mpk} (~ 1 μg/ml); 4, p44^{mpk} (~ 1 μg/ml) plus ACC; 5, p44^{mpk} (~ 2 μg/ml) plus ACC; 6, casein kinase II (~ 1 μg/ml); 7, casein kinase II (~ 1 μg/ml) plus ACC; 8, casein kinase II (~ 2 μg/ml) plus ACC; 9, p44^{mpk} (~ 2 μg/ml) plus casein kinase II (~ 2 μg/ml) plus ACC. Similar results were obtained in two separate experiments.

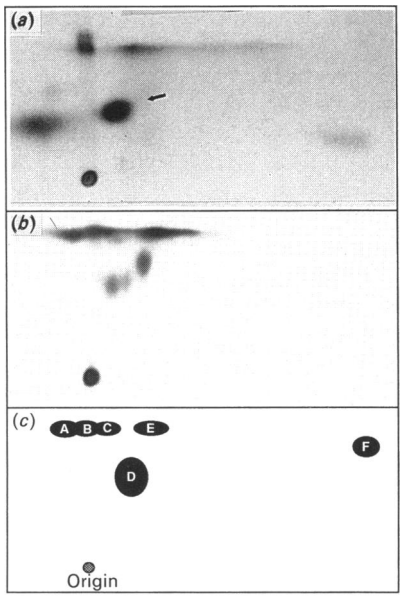

Fig. 8. Two-dimensional phosphopeptide map of rat liver acetyl-CoA carboxylase phosphorylated by purified p44^{mpk} and casein kinase II

Purified rat liver acetyl-CoA carboxylase (~ 2.5 μg) was incubated for 15 min at 30 °C in a volume of 25 μl in the presence of [γ-^{32}P]ATP (50 μM) with p44^{mpk} (*a*) or casein kinase II (*b*), both of which had been purified from maturing sea-star oocytes. After resolution of the tryptic radiolabelled carboxylase peptides by two-dimensional thin-layer gel electrophoresis, autoradiography was performed. (*c*) Migration of tryptic phosphopeptides of acetyl-CoA carboxylase that are labelled by known protein kinases. Peptides A, B, C and E are phosphorylated by casein kinase II and peptides E and F by cyclic AMP-dependent protein kinase. Peptide F is also phosphorylated by AMP-dependent protein kinase and peptide D by insulin-induced phosphorylation.

phate incorporation by p44^{mpk} into acetyl-CoA carboxylase was estimated to be ~ 0.3 mol of P/mol of 250 kDa subunit. This was very similar to the extent of phosphorylation of acetyl-CoA carboxylase by casein kinase II purified from maturing sea-star oocytes (Fig. 7, lanes 7 and 8). There was no appreciable further enhancement of the rate or extent of phosphorylation of acetyl-CoA carboxylase when it was incubated with [γ-^{32}P]ATP in the presence of both kinases simultaneously (Fig. 7, lane 9).

Phosphoamino acid analysis indicated that purified p44^{mpk} phosphorylated acetyl-CoA carboxylase predominantly on serine, and to a minor extent on threonine (by densitometric scanning, the incorporation of ^{32}P into phosphoserine represented more than 90% of the total radioactivity detected in total phosphoamino acids).

To gain insight into the site(s) on acetyl-CoA carboxylase phosphorylated by p44^{mpk}, analysis of phosphopeptides by two-dimensional mapping was carried out after complete trypsin digestion. Phosphopeptides detected by autoradiography are evident in Fig. 8. The major phosphopeptide detected after phosphorylation of purified rat liver acetyl-CoA carboxylase with p44^{mpk} is designated 'D' (Fig. 8*a*). In three separate experiments the incorporation of ^{32}P into peptide D accounted for 74 ± 2% of total phosphopeptide labelling (mean ± S.E.M.), as determined by two-dimensional densitometric scanning with a Visage analyser (Bioimage, Millipore). On the sample shown, the area of ^{32}P to the left of peptide D (migrating towards the anodic side of the origin) represents a trace of residual [γ-^{32}P]ATP. The remaining ^{32}P incorporated into phosphopeptides (approx. 25% of total) was distributed between peptides B and E. In contrast with the effects of p44^{mpk}, treatment of acetyl-CoA carboxylase with casein kinase II led to ^{32}P incorporation which could be largely accounted for by phosphopeptides A, B, C and E. Together, these phosphopeptides accounted for more than 90% of total ^{32}P incorporation. Two minor phosphopeptides below C and E were not observed consistently in all experiments and did not co-migrate with peptide D.

Phosphorylation of acetyl-CoA carboxylase by p44^{mpk} was not accompanied by any apparent change in the activity of acetyl-CoA carboxylase, despite measurement of the enzyme activity over a range of concentrations of the allosteric activator citrate (results not shown).

DISCUSSION

In the present study we have sought to identify a major maturation-activated protein kinase that is responsible for phosphorylation of acetyl-CoA carboxylase. By several criteria this acetyl-CoA carboxylase kinase appears to correspond to p44^{mpk}. The two activities display a similar time course of activation during sea-star oocyte maturation and co-purify to apparent homogeneity. It is unlikely that the carboxylase is a physiological substrate of p44^{mpk} in sea-star oocytes, since acetyl-CoA carboxylase activity is not detectable in the oocyte extracts above background levels when using the assay based upon [^{14}C]bicarbonate incorporation (even after maximal activation with albumin and citrate). Nevertheless, acetyl-CoA carboxylase is a revealing substrate, since it possesses a range of phosphorylation sites recognized specifically by different protein kinases (Brownsey & Denton, 1987; Hardie, 1989; Davies *et al.*, 1990).

p44^{mpk} seems to be highly related to the 42 kDa MAP-2 kinase, which is stimulated in response to mitogens in 3T3-L1

pre-adipocytes that do contain acetyl-CoA carboxylase (Ray & Sturgill, 1988*a*,*b*). Both protein kinases phosphorylate MBP, whereas other substrates including casein, histones and 40 S ribosomes are phosphorylated much more slowly by the two kinases. Both activities are highly selective for ATP relative to GTP, both bind tightly to phenyl-Sepharose but are not retained by phosphocellulose, and both are inhibited by NaF but are insensitive to heparin (Ray & Sturgill, 1988*a*,*b*; Sanghera *et al.*, 1990). Furthermore, the two kinases are phosphorylated on tyrosine within intact cells, and a polyclonal antibody raised against purified sea-star p44^{mpk} recognizes the 3T3-L1 MAP-2 kinase on immunoblots (A. Rossomando, M. Weber, T. Sturgill, J. Sanghera & S. Pelech, unpublished work). Until sequence information becomes available for both kinases, it remains unclear just how closely related they are. However, it is feasible that the MAP-2 kinase (or another kinase related to p44^{mpk}) may be responsible for the increased phosphorylation of acetyl-CoA carboxylase in response to insulin and PMA in mammalian cells. This may be supported by our observations on the properties of the site on acetyl-CoA carboxylase phosphorylated by p44^{mpk} (designated 'D'). The two-dimensional mapping procedure allows separation of a number of phosphopeptides, including those observed after treatment of acetyl-CoA carboxylase with casein kinases I and II, cyclic AMP-dependent protein kinase and AMP-stimulated protein kinase. These phosphopeptides are all clearly resolved from a phosphopeptide derived from acetyl-CoA carboxylase that shows markedly enhanced phosphorylation in response to insulin treatment of fat cells, previously designated 'I' peptide (Brownsey & Denton, 1982). The I-peptide and the D-peptide described here show extremely similar migration upon two-dimensional mapping, suggesting that p44^{mpk} may phosphorylate a major insulin-directed site on acetyl-CoA carboxylase. This deduction must remain tentative, however, and we have accordingly employed a different labelling to indicate insulin-directed and p44^{mpk}-directed phosphorylation. Amino acid sequencing will be required to confirm if I- and D- peptides are indeed identical. This will probably not be straightforward, since the sequencing of the I-peptide has not yet been achieved (it remains the only physiologically phosphorylated site on acetyl-CoA carboxylase that so far has not been sequenced). The availability of a protein kinase that may phosphorylate acetyl-CoA carboxylase at the insulin-stimulated site could facilitate the delineation of its surrounding amino acid sequence and therefore the location of this phosphorylation site within the primary sequence of the enzyme.

Even though casein kinase II was also stimulated during sea-star oocyte maturation, there was insufficient activity to contribute significantly towards the total measured elevated acetyl-CoA carboxylase kinase activity. Nevertheless, the activation of casein kinase II during meiosis in echinoderms is a novel finding. In *Xenopus laevis* oocytes it has been proposed that an attenuation of casein kinase II activity may be a prerequisite for acquisition of competence to mature (Osborne *et al.*, 1989). Microinjection of purified casein kinase II inhibits progesterone-triggered maturation. However, it augments maturation elicited by co-injection with maturation-promoting factor, which bypasses the earlier progesterone-induced events (Mulner-Lorillon *et al.*, 1988). In *Rana temporaria* oocytes casein kinase II activity in ribonucleoprotein particles is stimulated about 4-fold within 7 h after progesterone treatment, and this occurs independently of protein synthesis (Kandror *et al.*, 1989). Thus it would seem that casein kinase II is also activated near the time of GVBD in maturing amphibian oocytes. This activation might arise from phosphorylation of casein kinase II by another maturation-activated protein kinase as part of a phosphorylation cascade. Ackerman & Osheroff (1989) have described the reversal of EGF-stimulated casein kinase II activity in extracts from the A431 human carcinoma cell line by alkaline phosphatase, which is consistent with an activating role for phosphorylation of the kinase.

Although p44^{mpk} was a major acetyl-CoA carboxylase kinase in cytosol from maturing sea star oocytes, it can be seen from inspection of Figs. 3, 5 and 6 that additional maturation-activated protein kinases that phosphorylated this substrate were present. It would appear that a fraction of the acetyl-CoA carboxylase kinase activity was co-eluted with S6 kinase-I during MonoQ chromatography. However, after sequential fractionation of this S6 kinase (S. L. Pelech & J. S. Sanghera, unpublished work). Another, possibly related, 70 kDa S6 kinase purified from rat liver was reported to exhibit about 15-fold less activity towards acetyl-CoA carboxylase than did cyclic AMP-dependent protein kinase (Price *et al.*, 1989). Therefore the unidentified acetyl-CoA carboxylase kinases that were activated during sea-star oocyte maturation could correspond to additional novel protein kinases. Such acetyl-CoA carboxylase kinases, like p44^{mpk} and its mammalian homologues, might potentially be involved in the regulation of fatty acid synthesis in response to mitogens in mammalian cells.

S.L.P. was the recipient of a Medical Research Council (M.R.C.) of Canada Scholarship. This research was supported by operating grants from the M.R.C. of Canada to S.L.P. and R.W.B. We thank Dr. Edwin G.Krebs for the use of his laboratory facilities during the early stages of this project, and for supplying the RRLSSLRA peptide.

REFERENCES

Ackerman, P. & Osheroff, N. (1989) J. Biol. Chem. **264**, 11958–11965
Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T. & Avruch, J. (1979) J. Biol. Chem. **254**, 8052–8056
Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) Nature (London) **343**, 651–653
Arion, D., Meijer, L., Brizuela, L. & Beach, D. (1988) Cell **55**, 371–378
Benjamin, W. B. & Singer, I. (1975) Biochemistry **14**, 3301–3309
Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. & Quamo, S. N. (1985) J. Biol. Chem. **260**, 13304–13315
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Brownsey, R. W. & Denton, R. M. (1982) Biochem. J. **202**, 77–86
Brownsey, R. W. & Denton, R. M. (1987) in The Enzymes, 3rd edn. (Boyer, P. D. & Krebs, E. G., eds.), vol. 18, part B, pp. 123–146, Academic Press, New York
Brownsey, R. W., Belsham, G. J. & Denton, R. M. (1981) FEBS Lett. **124**, 145–150
Brownsey, R. W., Edgell, N. J., Hopkirk, T. J. & Denton, R. M. (1984) Biochem. J. **218**, 733–743
Brownsey, R. W., Dong, G. W., Lam, V. & McGreer, W. (1988) Biochem. Cell Biol. **66**, 296–308
Carroll, D. & Marshak, D. R. (1989) J. Biol. Chem. **264**, 7345–7348
Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. A., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986) J. Biol. Chem. **261**, 989–992
Cicirelli, M. F., Pelech, S. L. & Krebs, E. G. (1988*a*) J. Biol. Chem. **263**, 2009–2019
Cicirelli, M. F., Pelech, S. L. & Krebs. E. G. (1988*b*) FEBS Lett. **241**, 195–201
Davies, S. P., Sim, A. T. R. & Hardie, D. G. (1990) Eur. J. Biochem. **187**, 183–190
Deibler, G. E., Boyd, L. F. & Keis, M. W. (1984) Prog. Clin. Biol. Res. **146**, 249–256
Erikson, E. & Maller, J. L. (1986) J. Biol. Chem. **261**, 350–355
Halestrap, A. P. & Denton, R. M. (1973) Biochem. J. **132**, 509–517
Hardie, D. G. (1989) Prog. Lipid Res. **28**, 117–146
Haystead, T. A. J. & Hardie, D. G. (1986*a*) Biochem. J. **234**, 279–284
Haystead, T. A. J. & Hardie, D. G. (1986*b*) Biochem. J. **240**, 99–106
Haystead, T. A. J. & Hardie, D. G. (1988) Eur. J. Biochem. **175**, 339–345
Haystead, T. A. J., Campbell, D. G. & Hardie, D. G. (1988) Eur. J. Biochem. **175**, 347–354
Haystead, T. A. J., Moore, F., Cohen, P. & Hardie, D. G. (1990) Eur. J. Biochem. **187**, 199–205

Hecht, L. B. & Straus, D. S. (1988) Biochem. Biophys. Res. Commun. **152**, 1200–1206
Holland, R. & Hardie, D. G. (1985) FEBS Lett. **181**, 308–312
Hoshi, M., Nishida, E. & Sakai, H. (1988) J. Biol. Chem. **263**, 5396–5401
Hoshi, M., Nishida, E. & Sakai, H. (1989) Eur. J. Biochem. **184**, 477–486
Kandror, K. V., Benumov, A. O. & Speanov, A. S. (1989) Eur. J. Biochem. **180**, 441–448
Karlund, J. K. & Czech, M. P. (1988) J. Biol. Chem. **263**, 15872–15875
Laemmli, U. K. (1970) Nature (London) **227**, 680–684
Lee, K.-H., Thrall, T. & Kim, K.-H. (1973) Biochem. Biophys. Res. Commun. **54**, 1133–1140
Lopez-Casillas, F., Bai, D.-H., Luo, X., Kong, I.-S., Hermodson, M. A. & Kim, K.-H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 5784–5788
Maller, J. L. (1990) Biochemistry **29**, 3157–3166
Martin-Perez, J., Siegmann, M. & Thomas, G. (1984) Cell **36**, 287–294
Meijer, L., Pondaven, P., Guerrier, P. & Moreau, M. (1984) Cah. Biol. Mar. **25**, 457–480
Meijer, L., Pelech, S. L. & Krebs, E. G. (1987) Biochemistry **26**, 7968–7974
Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) Science **211**, 1437
Mulner-Lorillon, O., Marot, J., Cayla, X., Pouhle, R. & Belle, R. (1988) Eur. J. Biochem. **171**, 107–117
Nemenoff, R. A., Gunsalus, J. R. & Avruch, J. (1986) Arch. Biochem. Biophys. **245**, 196–203
Novak-Hofer, I. & Thomas, G. (1984) J. Biol. Chem. **259**, 5995–6000
Novak-Hofer, I. & Thomas, G. (1985) J. Biol. Chem. **260**, 10314–10319
Osborne, H. B., Mulner-Lorillon, O., Marot, J. & Belle, R. (1989) Biochem. Biophys. Res. Commun. **158**, 520–526
Pelech, S. L. & Krebs, E. G. (1987) J. Biol. Chem. **262**, 11598–11606
Pelech, S. L., Olwin, B. B. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5968–5972
Pelech, S. L., Meijer, L. & Krebs, E. G. (1987) Biochemistry **26**, 7960–7968
Pelech, S. L., Tombes, R. M., Meijer, L. & Krebs, E. G. (1988) Dev. Biol. **130**, 28–36
Pelech, S. L., Sanghera, J. & Daya-Makin, M. (1990) Biochem. Cell Biol. **68**, 1297–1330
Price, D. J., Nemenoff, R. A. & Avruch, J. (1989) J. Biol. Chem. **264**, 13825–13833
Ray, L. B. & Sturgill, T. W. (1988*a*) Proc. Natl. Acad. Sci. U.S.A. **85**, 3753–3757
Ray, L. B. & Sturgill, T. W. (1988*b*) J. Biol. Chem. **263**, 12721–12727
Sanghera, J. S., Paddon, H. B., Bader, S. A. & Pelech, S. L. (1990) J. Biol. **68**, 1297–1330
Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1613–1616
Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. B. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8834–8838
Spach, D. H., Nemenoff, R. A. & Blackshear, P. J. (1986) J. Biol. Chem. **261**, 12750–12753
Stith, B. J. & Maller, J. L. (1984) Dev. Biol. **102**, 79–89
Sturgill, T. W., Ray, L. B., Erikson, E. & Maller, J. L. (1988) Nature (London) **334**, 715–718
Tipper, J. P., Bacon, G. W. & Witters, L. A. (1983) Arch. Biochem. Biophys. **227**, 386–396
Wettenhall, R. E. H. & Morgan, F. J. (1984) J. Biol. Chem. **259**, 2084–2091
Witters, L. A. (1981) Biochem. Biophys. Res. Commun. **100**, 872–878
Witters, L. A., Tipper, J. P. & Bacon, G. W. (1983) J. Biol. Chem. **258**, 5643–5648

Received 17 May 1990/5 November 1990; accepted 12 November 1990