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A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis

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A highly purified rat liver protein kinase phosphorylates and inactivates acetyl-CoA carboxylase, and causes rapid inactivation of microsomal HMG-CoA reductase in the presence of MgATP. Both effects are stimulated in an identical manner by AMP, and are greatly reduced by prior treatment of the kinase with purified protein phosphatase. The dephosphorylated kinase can be reactivated in the presence of MgATP, apparently due to a distinct kinase kinase, and this reactivation is stimulated by nanomolar concentrations of palmitoyl-CoA. These results show that a common, bicyclic protein kinase cascade can potently inactivate the regulatory enzymes of both fatty acid and cholesterol biosynthesis.

Protein kinase; Acetyl-CoA carboxylase; Hydroxymethylglutaryl-CoA reductase; Fatty acid synthesis; Cholesterol synthesis; Fatty acyl-CoA ester

1. INTRODUCTION

Cholesterol and fatty acids in mammals are derived either from the diet, or from de novo synthesis via branching pathways which diverge from a common pool of cytoplasmic acetyl-CoA. There are several situations in which the two biosynthetic pathways are regulated in parallel. They exhibit a synchronous diurnal rhythm in rat liver [1,2], a finding which may be explained by diurnal variation in the insulin/glucagon ratio, since insulin stimulates and glucagon inhibits both pathways in isolated rat hepatocytes [3,4]. The peak diurnal rates of both pathways are also reduced by 60–70% when rats are fed a diet high in polyunsaturated fatty acids [5].

The important sites of regulation within the pathways of fatty acid and cholesterol synthesis are believed to be those catalyzed by acetyl-CoA carboxylase and HMG-CoA reductase, respectively, and both enzymes are known to be regulated by reversible phosphorylation. Acetyl-CoA carboxylase is phosphorylated at multiple sites by a variety of protein kinases, some of which inactivate the enzyme [6–10]. These protein kinases include a cyclic AMP-independent protein kinase which we have recently partially purified from rat liver [11] and which we have tentatively termed acetyl-CoA carboxylase kinase-3 (ACK3). HMG-CoA reductase is also inactivated by multiple protein kinases [12], including a protein kinase which is stimulated by phosphorylation [13,14], and an AMP-stimulated protein kinase [15,16]. It has not been

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Abbreviations: ACK3, acetyl-CoA carboxylase kinase-3; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; HMG-CoA, hydroxymethylglutaryl-CoA; PMSF, phenylmethanesulphonyl fluoride

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clear whether these latter two HMG-CoA reductase kinases are distinct enzymes.

We now present evidence that ACK3, and the HMG-CoA reductase kinase activities that are stimulated by phosphorylation and by AMP, are all functions of the same protein. This bicyclic phosphorylation cascade allows the potential for coordinate regulation of fatty acid and cholesterol synthesis, two pathways which compete for a common precursor.

2. MATERIALS AND METHODS

2.1. *Materials*

ACK3 was purified from a 10000 × *g* supernatant fraction of rat liver in the presence of 5 mM Na pyrophosphate and 50 mM NaF as described [11], except with the addition of a DEAE-Sepharose chromatography step prior to the phosphocellulose chromatography. The kinase was purified approx. 700–1000-fold to a final specific activity of 30–50 units/mg: details of the purification will be published elsewhere. Rat liver microsomes were prepared as described in [17]. HMG-CoA reductase-overproducing hamster cells (UT-1 cells) were grown in roller bottles in 40 μM compactin [18]. They were homogenized in a Dounce homogenizer at 4°C in hypotonic buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM EGTA, 10 mM DTT, 0.25% (v/v) DMSO, 50 μg/ml leupeptin, 1 mM PMSF), and 2 M sucrose (1:7 by volume) was then added to make the medium isotonic. Membranes were collected by centrifugation (100000 × *g*, 60 min, 4°C), washed twice by resuspension and recentrifugation in 100 mM Na Hepes, pH 7.0, 250 mM NaCl, 1 mM EDTA, 5 mM EGTA, 5 mM DTT, 0.25% DMSO, 50 μg/ml leupeptin and 1 mM PMSF, and stored in aliquots at −70°C. The specific activity of HMG-CoA reductase in these membrane preparations was ~120 units/mg. Sources of other materials have been described elsewhere [9,10].

2.2. *Enzyme assays*

ACK3 was assayed in incubations containing acetyl-CoA carboxylase (0.48 mg/ml) and AMP (200 μM) in Na Hepes, pH 7.0 (40 mM), glycerol (5%, v/v), NaCl (80 mM), EDTA (0.8 mM), DTT (0.8 mM), Mg acetate (5 mM) and [γ-^{32}P]ATP (0.2 mM, 1–2 × 10^5 cpm/nmol) in a final volume of 50 μl. After incubation for 10 min at 30°C, incorporation of phosphate into protein was measured by trichloroacetic acid precipitation [9]. 1 unit of ACK3 incorporates 1 nmol of phosphate into acetyl-CoA carboxylase per min at 30°C. Acetyl-CoA carboxylase was assayed as described in [19]. HMG-CoA reductase kinase activity was assayed by incubating UT-1 cell membranes (0.92 mg protein/ml) and kinase in Na Hepes, pH 7.2 (80 mM), NaCl (100 mM), EDTA (0.2 mM), EGTA (1 mM), DTT (1 mM), DMSO (0.1%), leupeptin (200 μg/ml), PMSF (0.2 mM), $MgCl_2$ (5 mM), ATP (0.2 mM). After incubation for 5 min at 0°C, 20 μl aliquots were removed for spectrophotometric assay of HMG-CoA reductase [20].

2.3. *Dephosphorylation/inactivation and reactivation of ACK3*

For the experiments shown in figs 2A and 4B, ACK3 was incubated at 30°C in 50 mM Na Hepes, pH 7.0, 10% (w/v) glycerol, 100 mM NaCl, 2.5 mM NaF, 1 mM EDTA, 1 mM DTT with or without the purified catalytic subunit of protein phosphatase-2A (20 U/ml). At various times aliquots were withdrawn and diluted in the same buffer containing 50 mM NaF. An equivalent amount of phosphatase-2A was added to controls after incubation to preclude the possibility that any fluoride-resistant phosphatase activity could interfere in the kinase assays.

For the reactivation experiments (fig.2B), ACK3 was purified as far as the DEAE-Sepharose step and was dialyzed into buffer without NaF or pyrophosphate (50 mM Na Hepes, pH 7.0, 10% (w/v) glycerol, 1 mM EDTA, 1 mM DTT, 0.02% Brij 35). The specific activity of these preparations was around 0.5 U/mg. It was then incubated at 30°C at 4.8 mg/ml in the same buffer containing 30 mM NaF with or without palmitoyl-CoA and 0.2 mM ATP/5 mM Mg acetate.

3. RESULTS

3.1. *Reversible phosphorylation and inactivation of acetyl-CoA carboxylase by ACK3*

ACK3 has been purified 700–1000-fold from the post-mitochondrial supernatant of rat liver using six purification steps. As reported [11], it phosphorylates purified rat acetyl-CoA carbox-

ylase to a stoichiometry exceeding 1 mol/mol 240 kDa subunit. This is associated with ~75% inactivation (fig.1) when acetyl-CoA carboxylase is assayed at near-saturating citrate concentration (10 mM), and >95% inactivation at a more physiological citrate concentration (0.5 mM) [11]. These effects were completely reversed by dephosphorylation with protein phosphatase-2A (fig.1).

3.2. *Regulation of acetyl-CoA carboxylase kinase-3 by phosphorylation and by AMP*

We obtained clear evidence that ACK3 is itself regulated by phosphorylation, i.e. that this is a 'bicyclic' protein phosphorylation system. Purification of ACK3 was carried out in the presence of the protein phosphatase inhibitor, sodium fluoride, and if this was omitted the activity was very labile. Fig.2A shows that enzyme prepared in the presence of fluoride was rapidly inactivated in the presence of the purified catalytic subunit of protein phosphatase-2A. Fig.2B shows that a time-dependent reactivation of the kinase occurred when a partially purified preparation, which had been inactivated by dialysis in the absence of fluoride, was incubated with MgATP.

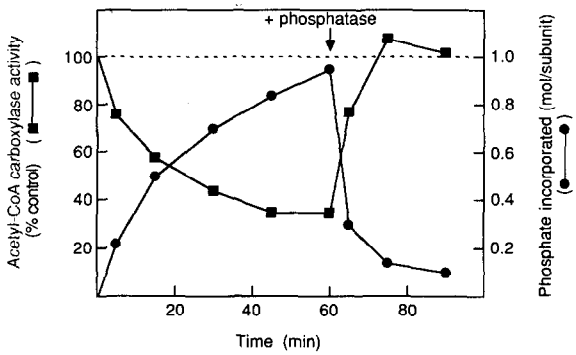

Fig.1. Effect of successive treatment with (1) ACK3 (0.03 units/ml) plus MgATP and (2) protein phosphatase-2A (20 U/ml) on the phosphorylation (●) and activity (■) of purified acetyl-CoA carboxylase (0.7 mg/ml). The protein phosphatase was added at the point shown by the arrow together with EDTA (10 mM final) to block the kinase reaction. The activity is plotted as a percentage of the activity in control incubations lacking ACK3, while the phosphorylation is corrected for slight phosphorylation (<0.1 mol/subunit) which occurred in the absence of ACK3.

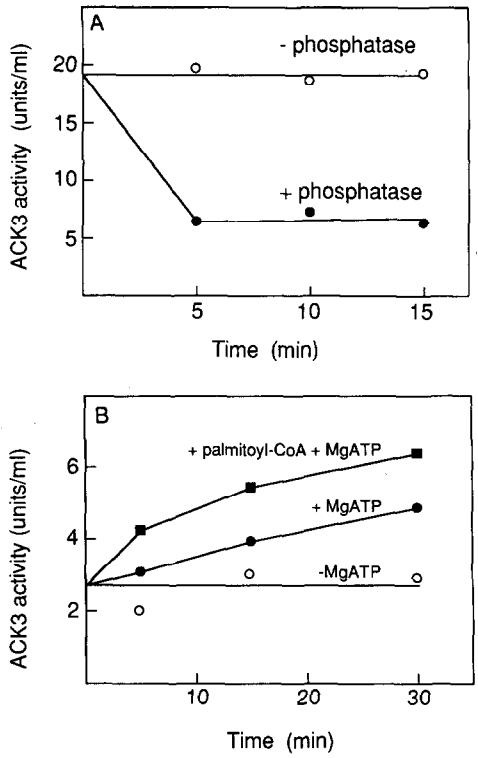

Fig.2. (A) Inactivation of ACK3 by protein phosphatase-2A; (B) MgATP-dependent reactivation of dephosphorylated ACK3 in the presence and absence of 200 nM palmitoyl-CoA.

This MgATP-dependent reactivation did not occur with more highly purified preparations, suggesting that the partially purified kinase was reactivated by a contaminating 'kinase kinase' rather than by autophosphorylation. Intriguingly, we found in three independent preparations that the reactivation of ACK3 by endogenous kinase kinase was stimulated 2–4-fold by palmitoyl-CoA in the range of 50–200 nM (e.g. fig.2B). Palmitoyl-CoA had no effect when the preincubation was carried out in the absence of MgATP (not shown), confirming that it affected the kinase kinase reaction, and not the ACK3 reaction.

In previous work in which crude preparations of acetyl-CoA carboxylase from rat liver were incubated with MgATP, Yeh et al. [21] reported that inactivation of acetyl-CoA carboxylase was stimulated by AMP. The phosphorylation of acetyl-CoA carboxylase by ACK3 was stimulated 4-fold by AMP with a half-maximal effect at ~2 μM (fig.3A).

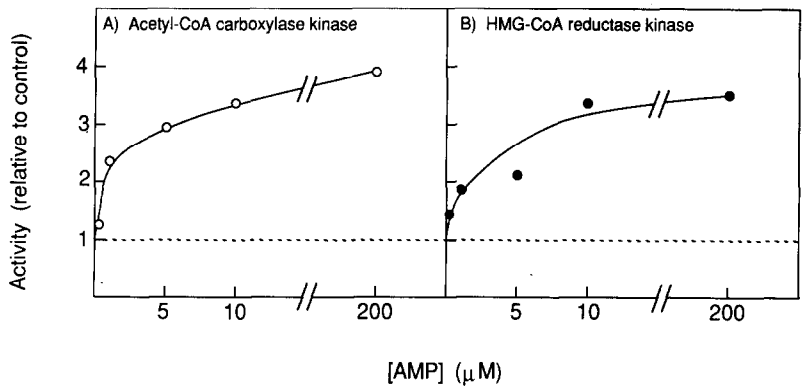

Fig.3. Activation of the acetyl-CoA carboxylase kinase (A) and HMG-CoA reductase kinase (B) activities of the enzyme preparation by AMP. Results are expressed relative to results of incubations without AMP.

3.3. *Inactivation of HMG-CoA reductase by purified acetyl-CoA carboxylase kinase-3*

Two of the properties of ACK3, i.e. stimulation by micromolar concentrations of AMP, and activation/inactivation by kinase kinase/protein phosphatase, have been reported previously for protein kinase activities which regulate HMG-CoA reductase, the regulatory enzyme of cholesterol synthesis [12–16]. To investigate the possibility that ACK3 might be related to one or both of these HMG-CoA reductase kinases, we studied the effect of ACK3 on HMG-CoA reductase activity in membranes prepared from UT-1 cells, a hamster cell line which overexpresses HMG-CoA reductase [18]. Fig.4A shows that highly purified ACK3 rapidly inactivates HMG-CoA reductase in a UT-1 cell membrane fraction. Rapid inactivation was dependent on the addition of both ACK3 and MgATP, although there was a slow inactivation in the presence of MgATP alone, due apparently to UT-1 cell HMG-CoA reductase kinase present in the membranes. Similar results were obtained using rat liver microsomes in place of UT-1 cell membranes (not shown). Fig.4B shows that the inactivation of the UT-1 cell enzyme was markedly reduced by prior treatment of ACK3 with protein phosphatase, while fig.3B shows that it was stimulated 3–4-fold by AMP with a half-maximal effect at ~3 μM.

4. DISCUSSION

Our results show that a highly purified preparation of a rat liver protein kinase, which

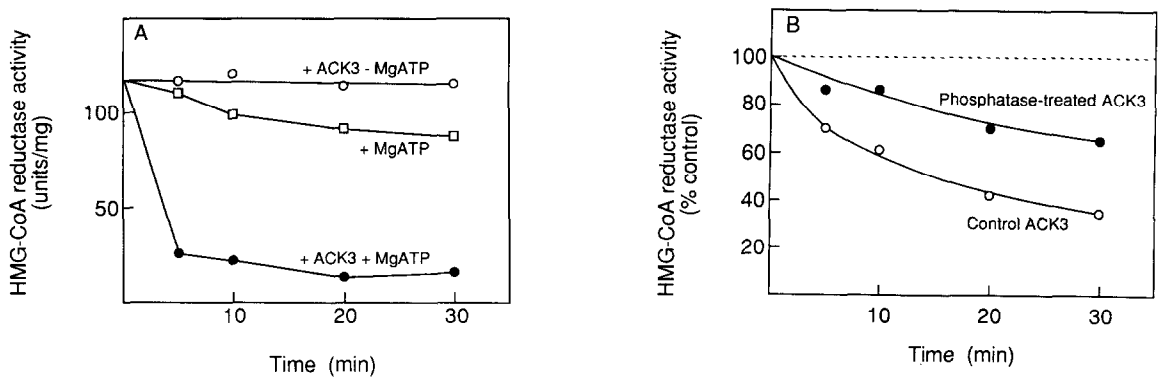

Fig.4. Time course of inactivation of HMG-CoA reductase by ACK3. (A) Using untreated ACK3 (1.7 units/ml). Controls lacked either ATP or ACK3. (B) Using ACK3 (0.9 units/ml) which had been pretreated with or without protein phosphatase-2A for 20 min, and expressing results as percentages of the HMG-CoA reductase activities in control incubations lacking ACK3.

phosphorylates and inactivates acetyl-CoA carboxylase, also rapidly and potently inactivates HMG-CoA reductase in a time-dependent manner in the presence of MgATP. That these effects are functions of the same protein is shown by the observations that both phosphorylation of acetyl-CoA carboxylase and inactivation of HMG-CoA reductase were stimulated in an identical manner by micromolar concentrations of AMP, and were reduced markedly when the protein kinase preparation had been previously treated with protein phosphatase. In addition, we have recently shown that the acetyl-CoA carboxylase kinase and HMG-CoA reductase kinase activities copurify from the crude post-mitochondrial supernatant (in preparation). Our results also suggest that the HMG-CoA reductase kinase activities which are activated by AMP and by phosphorylation are the same enzyme, a point that has not been clear from previous studies [13–16].

This bicyclic protein kinase system clearly has the potential to regulate coordinately fatty acid and cholesterol biosynthesis in rat liver. Although further experiments are necessary to prove that ACK3 phosphorylates both HMG-CoA reductase and acetyl-CoA carboxylase in vivo, several testable hypotheses can be framed regarding the function of this bicyclic phosphorylation system. The system may allow minute-by-minute modulation of the two pathways in response to hormones, and there is already evidence in rat hepatocytes that both enzymes are phosphorylated in response to glucagon [3,19]. Activation of the protein kinase by AMP could cause inhibition of these ATP- and NADPH-consuming pathways in cells depleted of ATP, e.g. by anoxia. Another function of the system may be feedback regulation, since it has been shown that phosphorylation of HMG-CoA reductase occurs in response to cholesterol feeding in vivo [22], while our results show that palmitoyl-CoA, the end-product of fatty acid synthesis, activates the phosphorylation cascade in vitro. Simultaneous inactivation of HMG-CoA reductase, via the common kinase system, would prevent acetyl-CoA (accumulated as a result of inhibition of acetyl-CoA carboxylase) from being diverted into cholesterol synthesis [23].

It is important to note that the existence of a common regulatory protein kinase does not imply that changes in the rate of fatty acid and cholesterol synthesis will always occur coordinately in vivo. Both enzymes are also regulated independently at the level of protein synthesis and/or degradation [12,24], and this is particularly important for HMG-CoA reductase, for which the half-life can be as low as 2–4 h [25]. It is also possible that phosphorylation of the two enzymes by this kinase system could be modulated independently by binding of effectors to the protein substrates. There is evidence that sterols interact with the membrane domain of HMG-CoA reductase [25] and this could conceivably promote phosphorylation of this enzyme [22] without affecting acetyl-CoA carboxylase.

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