An insulin-sensitive cytosolic protein kinase accounts for the regulation of ATP citrate-lyase phosphorylation

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Purified rat liver ATP citrate-lyase is phosphorylated on serine residues by an insulin-stimulated cytosolic kinase activity partially purified from rat adipocytes [Yu, Khalaf & Czech (1987) *J. Biol. Chem.* **262**, 16677–16685]. The K_m for lyase phosphorylation by this hormone-sensitive kinase activity is approx. 3 μ M. Two-dimensional tryptic-peptide mapping of the ³²P-labelled lyase reveals that the kinase-catalysed phosphorylation occurs primarily on a specific peptide. In intact ³²P-labelled adipocytes, insulin enhances the serine phosphorylation of ATP citrate-lyase by 2–3-fold. Tryptic digestion of the ³²P-labelled lyase immunopurified from insulin-treated adipocytes also yields one major phosphopeptide. ³²P-labelled lyase tryptic peptides derived from labelling experiments *in vitro* and *in vivo* exhibit identical electrophoretic and chromatographic migration profiles. Furthermore, radio-sequencing of the phosphopeptide from lyase ³²P-labelled *in vitro* indicates that serine-3 from the *N*-terminus is phosphorylated by the insulin-stimulated cytosolic kinase, in agreement with previous studies on the position of the phosphoserine residue in ATP citrate-lyase isolated from insulin-treated cells. Taken together, the similarity in site-specific phosphorylation of ATP citrate-lyase from insulin-treated adipocytes to that catalysed by the hormone-activated cytosolic kinase *in vitro* strongly suggests that this kinase mediates insulin action on lyase phosphorylation in intact cells.

INTRODUCTION

It has been well documented that, after the interaction of insulin with its cell-surface receptor, the phosphorylation state of many cellular proteins is altered. The insulin receptor itself undergoes increased phosphorylation on both tyrosine and serine residues after hormone binding [1–4]. The insulin-receptor tyrosine phosphorylation has been shown to be an autocatalytic reaction [5–7], which is essential for the full activation of the receptor intrinsic tyrosine kinase activity [8–13]. A number of specific phosphorylated tyrosine residues which appear to be linked to receptor kinase activity have been identified within the receptor β subunit [14–19]. Insulin also increases the tyrosine phosphorylation of lipocortin I [20] and several unknown cellular proteins [21–25]. However, the identity and function of the kinase or kinases mediating the insulin-stimulated serine phosphorylation of the insulin receptor remain undefined.

The large majority of the insulin-induced changes in cellular protein phosphorylation are serine/threonine-specific. For example, the serine/threonine phosphorylation of ATP citrate-lyase [26–28], acetyl-CoA carboxylase [29–31], ribosomal protein S6 [32,33], phosphatase inhibitor II [34] and a number of electrophoretically resolved but uncharacterized proteins [35–44] is increased by insulin. On the other hand, the hormone is also reported to decrease the serine/threonine phosphorylation of ATP citrate-lyase (on threonine; [45]), glycogen synthase [46], glycogen phosphorylase [47], pyruvate dehydrogenase [48], 3-hydroxy-3-methylglutaryl-CoA reductase [49] and insulin-like-growth-factor-II receptor [50]. It has been postulated that subsets of cellular protein kinases and phosphatases may be activated after insulin treatment (for reviews, see [51,52]). Evidence supporting this hypothesis has grown such that a number of specific kinase activities that are enhanced by insulin treatment of cells have been identified [32,33,53–63]. However, recent evidence

suggests at least one protein kinase is decreased in activity following insulin action [64].

Recently, our laboratory characterized a novel cytosolic insulin-stimulated serine kinase activity in rat adipocytes [59]. The kinase activity has been partially purified by DEAE-cellulose and molecular-sieve chromatography, and has an apparent mass of about 50–60 kDa [59]. Unlike the ribosomal-protein-S6 kinase, this adipocyte cytosolic kinase is insensitive to NaF [59]. It is capable of catalysing phosphorylation of histones, Kemptide, multifunctional calmodulin-dependent protein kinase substrate peptide, ATP citrate-lyase and acetyl-CoA carboxylase, but not ribosomal protein S6. In a previous study, Denton and co-workers [28] also reported that ATP citrate-lyase and acetyl-CoA carboxylase phosphorylating activities were increased in extracts of insulin-stimulated adipocytes.

In view of previous findings that the phosphorylation of ATP citrate-lyase in intact cells [26–28] is increased by insulin, the question arises as to whether the insulin-sensitive adipocyte cytosolic kinase activity that we observed is actually responsible for the phosphorylation of the lyase enzyme *in vivo*. In this study, we show that the partially purified insulin-sensitive cytosolic kinase catalyses the serine phosphorylation of ATP citrate-lyase on a tryptic-peptide fragment which is chromatographically indistinguishable from that which occurs in intact adipocytes exposed to the hormone. Furthermore, radio-sequence analysis of this tryptic peptide derived from lyase phosphorylated *in vitro* by this kinase indicates that the position of the phosphorylated serine residue is identical with that phosphorylated *in vivo* [65].

EXPERIMENTAL

Materials

PMSF, phosphoserine, phosphothreonine and phosphotyrosine were purchased from Sigma (St. Louis, MO, U.S.A.).

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

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[γ - ^{32}P]ATP was obtained from Amersham Corp. (Chicago, IL, U.S.A.). DEAE-Sephacel, Sephacryl S-200 and Protein A-agarose were supplied by Pharmacia Biotechnology (Piscataway, NJ, U.S.A.). Precoated cellulose plastic sheets were purchased from Brinkmann Instruments (Westbury, NY, U.S.A.). Centricon-30 was obtained from Amicon (Danvers, MA, U.S.A.). Pig insulin was given by Dr. Ronald Chance (Eli Lilly, Indianapolis, IN, U.S.A.). Molecular-mass standards were from Bio-Rad. ATP citrate-lyase was purified from rat liver as described previously [36] and polyclonal antibodies against rat hepatic lyase were raised in rabbits [66].

Methods

Rat adipocyte isolation and incubation. Male rats weighing 100–125 g were used (Sprague–Dawley strain; Taconic Farms, Germantown, NY, U.S.A.). Adipocytes were prepared from the epididymal fat-pads as described by Rodbell [67] in Krebs–Ringer–Hepes buffer, pH 7.4, containing 30 mg of BSA/ml and 2 mM-sodium pyruvate, and then were equilibrated in the same buffer without collagenase at 37 °C for 10 min. After incubation, adipocytes were incubated in the absence and presence of insulin (0.1 μM) at 37 °C for various periods of time and then homogenized in accordance with the procedure described below.

Partial purification of insulin-stimulated cytosolic kinase from adipocytes. Adipocytes were homogenized in 7 vol. of 20 mM-Tris/HCl, pH 7.4, containing 225 mM-sucrose, 5 mM-EDTA, 10 mM-NaF, 200 μM -sodium vanadate, 10 mM-sodium pyrophosphate and 1 mM-PMSF. Membrane fractions were sedimented by differential centrifugation as described by McKeel & Jarett [68] with some modifications [69]. After the final centrifugation at 150 000 g for 90 min, the supernatant was mixed with 0.14 vol. of 500 mM-Hepes, pH 7.5, to increase the buffering capacity of the cytosolic extracts. The excess EDTA in the cell extracts was neutralized by 2.4-fold higher molar concentrations of MgCl_2 . Under these conditions, no change in pH was observed when the chelator was quenched.

Cytosolic extracts derived from control and insulin-treated fat-cells were applied separately to 2.8 cm $\times$ 1.5 cm DEAE-Sephacel columns equilibrated in 25 mM-Hepes, pH 7.4, containing 100 mM-NaF, 200 μM -sodium vanadate, 1 mM-PMSF and 2 mM-DTT at 4 °C. The anion-exchange column was then washed with 30 ml of the same buffer. The columns were developed with a linear 0–0.5 M-NaCl gradient in the buffer at a flow rate of 1 ml/min. Fractions (1 ml) were collected, and the Kemptide-phosphorylating activity in each fraction was determined in 20 μl samples by the procedure described previously [59]. The peak fractions containing the control and insulin-stimulated protein kinase activities in the DEAE-Sephacel eluates were pooled individually. Portions (1.4 ml) of the combined samples were loaded separately on to a 50 cm $\times$ 0.9 cm Sephacryl S-200 column equilibrated in 25 mM-Hepes, pH 7.4, containing 100 mM-NaF, 200 μM -sodium vanadate, 1 mM-PMSF and 2 mM-DTT at 4 °C. The columns were eluted with the same buffer at a flow rate of 0.25 ml/min. Fractions (2 ml) were collected, and the kinase activity in a portion (20 μl) of each fraction was determined by using Kemptide as substrate and [γ - ^{32}P]ATP. The fractions containing the Kemptide-phosphorylating activity were combined. Routinely, the magnitude of insulin stimulation on the cytosolic kinase activity after these two column steps is 4–5-fold above basal. The pooled fractions were either used directly or concentrated 10-fold on Centricon-30.

Phosphorylation of ATP citrate-lyase *in vitro*. A 5 μl sample of rat liver ATP citrate-lyase (9–100 μg) was mixed with 20 μl of the

partially purified cytosolic kinase preparations from control and insulin-treated adipocytes. The phosphorylation reaction was initiated by the addition of 8 μl of 80 μM -[γ - ^{32}P]ATP (sp. radioactivity 40 $\mu\text{Ci/nmol}$), 40 mM- MgCl_2 , 12 mM- MnCl_2 and 4 mM-DTT and was continued for 30–120 min at 22 °C. The reaction was terminated by the addition of 0.1 ml of 0.17 M-Tris/HCl electrophoresis sample buffer, pH 6.8, containing 100 mM-DTT and 10 % SDS, and the sample was boiled. For tryptic-peptide mapping, the ^{32}P -labelled ATP citrate-lyase was phosphorylated with high-specific-radioactivity [γ - ^{32}P]ATP (3 mCi/nmol) and then reduced with DTT and alkylated with iodoacetamide in accordance with previously described procedures [9]. The sample was then subjected to electrophoresis on a SDS/7 % polyacrylamide gel. The gel was fixed, dried and autoradiographed at -70 °C. The ^{32}P -labelled lyase band was excised from the gel and the associated radioactivity was measured by Čerenkov counting.

^{32}P -labelling *in vivo* of adipocyte ATP citrate-lyase. Two portions of rat adipocytes (1 ml) were ^{32}P -labelled in phosphate-free Krebs–Ringer–Hepes buffer containing 30 mg of BSA/ml and 1 mCi of carrier-free [^{32}P]P_i/ml for 2 h at 37 °C. The ^{32}P -labelled adipocytes were homogenized in 6 vol. of 10 mM-Tris/HCl, pH 7.4, containing 225 mM-sucrose, 5 mM-EDTA, 50 mM-NaF, 200 μM -sodium vanadate and 1 mM-PMSF. The homogenates were fractionated into membrane and cytosol fractions by differential centrifugation [69]. A 2 ml sample of cytosol was mixed with 50 μl of rabbit anti-(ATP citrate-lyase) antiserum and incubated at 4 °C for 3 h. The lyase–antibody complexes were adsorbed to 20 μl of Protein A–agarose at 4 °C for 1 h. The resin was then pelleted and washed extensively with the homogenization buffer without sucrose. The immunoprecipitates were resolved by electrophoresis on SDS/polyacrylamide gels after reduction and alkylation as described previously [9].

Two-dimensional tryptic-peptide mapping of ^{32}P -labelled ATP citrate-lyase. Gel pieces containing the reduced and alkylated ^{32}P -labelled ATP citrate-lyase were washed in 2 $\times$ 15 ml of deionized water at room temperature. The washed gel pieces were suspended in 2–3 ml of 50 mM- NH_4HCO_3 buffer containing 10 μg of Tos-Phe- CH_2Cl -treated trypsin/ml. Tryptic digestion was performed at 37 °C for 20 h. After digestion, the samples were freeze-dried, dissolved in 0.5 ml of 1 mM-HCl, freeze-dried again and finally dissolved in 50 μl of water. The solubilized digests were applied to a 20 cm $\times$ 10 cm cellulose-coated plastic sheet. Two-dimensional resolution was performed by electrophoresis in 1 % $(\text{NH}_4)_2\text{CO}_3$ (pH 8.9) for 2 h and ascending chromatography with pyridine/water/acetic acid/butan-1-ol (10:12:3:15, by vol.) as solvent. The ^{32}P -labelled lyase tryptic peptides were detected by autoradiography at -70 °C.

Radio-sequencing of ^{32}P -labelled ATP citrate-lyase tryptic peptide. The area containing the major ^{32}P -labelled lyase tryptic peptide was removed from the cellulose plate and placed into a glass test tube. The cellulose was extracted with 2 $\times$ 1 ml of 0.1 % trifluoroacetic acid each for 1 h at 22 °C. The extracts were clarified by filtration and then freeze-dried. Sequence analysis of ^{32}P -labelled peptide was performed in the presence of 4 nmol of myoglobin by using a modified Beckman 890C liquid-phase sequencer and a 0.1 M-Quadrol Program (Beckman 121078). Two precycles were performed before the first cleavage. The anilinothiazolinones were converted into phenylthiohydantoins by reaction in 25 % trifluoroacetic acid at 56 °C, and were identified and quantified by a modification of the reverse-phase h.p.l.c. procedure described by Zimmerman *et al.* [70] using

Fig. 1. Phosphorylation of ATP citrate-lyase by insulin-stimulated adipocyte cytosolic kinase

Purified rat liver ATP citrate-lyase (50 μg, *a* and *b*; 9–100 μg, *c*) was incubated with partially purified cytosolic kinase preparations from control and insulin-stimulated adipocytes for 30–120 min at 22 °C in the presence of [γ -³²P]ATP, Mn²⁺ and Mg²⁺ as described under 'Methods'. At the end of the phosphorylation reaction, 0.1 ml of electrophoresis sample buffer containing DTT and SDS was added. The sample was boiled and then resolved by electrophoresis on an SDS/7% polyacrylamide gel. The gel was fixed, dried and autoradiographed. The ³²P-labelled ATP citrate-lyase bands were then excised and the associated radioactivity was determined by Čerenkov counting. (*a*) Autoradiogram of samples after 120 min incubation. (*b*) Time course of [³²P]P_i incorporation into ATP citrate-lyase. (*c*) Kinetic analysis of ATP citrate-lyase phosphorylation.

RESULTS

Fig. 1 depicts the phosphorylation of rat liver ATP citrate-lyase by the insulin-activated cytosolic kinase from rat adipocytes. After incubation at 22 °C for 2 h in the presence of [γ -³²P]ATP, the kinase preparation from insulin-treated adipocytes catalysed a 3–4-fold higher incorporation of phosphate into the hepatic enzyme as compared with the control (Fig. 1*a*). As we previously described [59], the insulin-sensitive kinase-mediated increase in phosphorylation of ATP citrate-lyase occurs exclusively on serine residues (results not shown). The phosphorylation of the lyase by the insulin-stimulated kinase is time-dependent (Fig. 1*b*). The rate of the kinase-catalysed phosphate incorporation into the enzyme is linear up to 30 min. In terms of the absolute amount of phosphate transfer with respect to time, the activity of the hormone-stimulated cytosolic kinase towards ATP citrate lyase is rather modest. This is probably due to the fact that the cytosolic kinase preparation is only partially purified. With respect to lyase phosphorylation (Fig. 1*c*), the $V_{\max}$ values were found to be 12 (control) and 32 (insulin) fmol/min per 20 μl of cytosolic extract and the K_m value was calculated as 3 μM (control or insulin). These findings suggest that the stimulatory action of the hormone on the cytosolic kinase may be mediated through an increase in the amount of activated enzyme present in the adipocyte extracts. In terms of substrate specificity, the insulin-stimulated cytosolic kinase appears to prefer ATP citrate-lyase over Kemptide, as evidenced by the higher K_m value (100 μM) for this substrate peptide (results not shown).

To characterize further the phosphorylation of ATP citrate-lyase by the insulin-sensitive cytosolic kinase, the number of phosphorylation sites on the enzyme was assessed by two-dimensional tryptic-peptide mapping. In these experiments, the lyase was first phosphorylated with [γ -³²P]ATP in the presence of control or insulin-stimulated kinase preparations. The ³²P-labelled lyase was then purified by SDS/PAGE. After autoradiographic localization of the 110 kDa ATP citrate-lyase band, the phosphoenzyme was excised and incubated with excess Tos-Phe-CH₂Cl-treated trypsin over a prolonged period at 37 °C. The tryptic hydrolysates were then resolved by electrophoresis and t.l.c. on cellulose plates. As shown in Fig. 2, the phosphoryl-

Fig. 2. Two-dimensional tryptic-peptide mapping of ATP citrate-lyase ³²P-labelled by control and insulin-stimulated cytosolic kinase preparations

Purified rat liver ATP citrate-lyase was ³²P-labelled with high-specific-radioactivity [γ -³²P]ATP (3 mCi/nmol) by control and insulin-stimulated cytosolic kinase preparations. The phosphorylated lyase was reduced and alkylated and then isolated by electrophoresis. The gel pieces containing the phosphorylated lyase were digested with Tos-Phe-CH₂Cl-treated trypsin by the procedure described under 'Methods'. The tryptic hydrolysates were resolved by two-dimensional chromatography on cellulose-precoated plastic sheets (electrophoresis, A to B; chromatography, bottom to C). The ³²P-labelled tryptic peptides were detected by autoradiography at -70 °C.

acetonitrile. The radioactivity associated with the phenylthiohydantoin derived from the peptide that was released at each cycle was measured by Čerenkov counting.

Fig. 3. Analysis of ATP citrate-lyase phosphorylation in control and insulin-treated rat adipocytes

Rat adipocytes were labelled with [^{32}P]P_i for 2 h at 37 °C and then incubated further for 10 min in the absence or presence of 0.1 μM -insulin. The fat-cell samples were homogenized in buffer containing protease and phosphatase inhibitors, and the cytosolic fractions were prepared by ultracentrifugation. The ^{32}P -labelled ATP citrate-lyase in the cytosols was isolated by immunoprecipitation by the procedure described under 'Methods'. After reduction and alkylation, the antibody-lyase immunoprecipitates were resolved by electrophoresis on SDS/polyacrylamide gels, and the ^{32}P -labelled lyase was detected by autoradiography at -70 °C (a). For two-dimensional tryptic-peptide mapping (b), the gel pieces containing ^{32}P -labelled ATP citrate-lyase from control and insulin-treated adipocytes were processed as described under 'Methods'. The ^{32}P -labelled-lyase hydrolysates were then chromatographed on cellulose-precoated plastic sheets (electrophoresis, A to B; chromatography, B to C).

ated ATP citrate-lyase digests can be resolved into one major and two minor ^{32}P -labelled peptides. Most of the ^{32}P radioactivity appears to be associated with the most acidic tryptic peptide.

Several previous studies have demonstrated that liver ATP citrate-lyase can be phosphorylated *in vitro* by the catalytic subunit of cyclic-AMP-dependent protein kinase [65,71-75]. By h.p.l.c. it was further shown that most of the phosphate was incorporated into a serine residue on a protein fragment denoted as peptide A [65,74]. Interestingly, treatment of intact hepatocytes with glucagon [75] and insulin [65] and of adipocytes with insulin or isoprenaline [66] resulted in an increase in the phosphorylation of lyase peptide A. The similarity of phosphoamino acid content and the presence of a single major phosphorylation site in ATP citrate-lyase phosphorylated by the insulin-sensitive cytosolic kinase raised the possibility that this kinase may mediate the hormone-stimulated phosphorylation of the lyase in intact cells.

In order to address this issue, we compared the phosphorylation characteristics of ATP citrate-lyase isolated from insulin-treated intact adipocytes with those of the purified enzyme, which was ^{32}P -labelled by the insulin-sensitive cytosolic kinase *in vitro*. To obtain phosphorylated ATP citrate-lyase from intact cells, rat adipocytes were first labelled with [^{32}P]P_i, followed by a brief incubation in the presence and absence of insulin. The fat-cell preparations were then homogenized in buffer containing protease and phosphatase inhibitors, and the ^{32}P -labelled lyase was isolated by immunoprecipitation with a polyclonal anti-(ATP citrate-lyase) antiserum. As shown in Fig. 3(a), treatment of ^{32}P -labelled adipocytes with insulin resulted in a 2-3-fold increase in the ^{32}P content of ATP citrate-lyase. Phosphoamino acid analysis of the ^{32}P -labelled lyase indicated that the insulin-enhanced phosphorylation of the enzyme is serine-specific (results not shown).

To determine the number of phosphorylation sites in the ATP citrate-lyase ^{32}P -labelled *in vivo*, the lyase band was excised from SDS/polyacrylamide gels and exhaustively digested with excess Tos-Phe-CH₂Cl-treated trypsin. Upon resolution of the tryptic hydrolysates on cellulose thin-layer plates, the ^{32}P -labelled lyase from control adipocytes was found to contain one major phosphopeptide and several weakly labelled ones. It is clear that the stimulatory effects of insulin on ATP citrate-lyase phosphorylation in intact adipocytes is primarily directed at the major phosphopeptide (Fig. 3b). Owing to the very low level of ^{32}P -labelling in other phosphopeptide spots on the two-dimensional chromatogram, it is difficult to determine whether insulin has an effect on their phosphate contents. Taken together, our present results are in agreement with previous studies that insulin specifically increases the serine phosphorylation within a particular peptide sequence in ATP citrate-lyase.

Fig. 4. Co-migration of ATP citrate-lyase tryptic phosphopeptides derived from samples ^{32}P -labelled *in vitro* and *in vivo*

ATP citrate-lyase ^{32}P -labelled *in vitro* by the insulin-stimulated cytosolic kinase and that isolated from hormone-treated adipocytes were digested with Tos-Phe-CH₂Cl-treated trypsin under identical conditions as described under 'Methods'. The lyase tryptic hydrolysates were chromatographed either individually or as a mixture on cellulose-precoated plastic sheets. The ^{32}P -labelled lyase peptides were detected by autoradiography at -70 °C.

Fig. 5. Radio-sequencing of the major ATP citrate-lyase phosphopeptide derived from lyase phosphorylated by insulin-stimulated cytosolic kinase

The major insulin-sensitive lyase phosphopeptide *in vitro* as shown in Fig. 2 was recovered from the cellulose plate. The ^{32}P -labelled peptide (5400 c.p.m.) was extracted from the cellulose resin in 0.1 % trifluoroacetic acid and loaded on to a Beckman liquid-phase sequencer. Automated Edman degradation was initiated, and the quantity of ^{32}P radioactivity released at each cycle was monitored by Cerenkov counting.

Visual comparison of the phosphopeptide maps between ATP citrate-lyase ^{32}P -labelled *in vitro* and *in vivo* suggests that the major peptide fraction phosphorylated by the insulin-stimulated cytosolic kinase may be chromatographically similar to that derived from the hormone-treated adipocytes (cf. Figs. 2 and 3). To investigate this possibility, the tryptic hydrolysates derived from ATP citrate-lyase ^{32}P -labelled *in vitro* and *in vivo* were mixed and subsequently analysed by two-dimensional peptide mapping on cellulose plates. As shown in Fig. 4, the major insulin-stimulated phosphopeptide fractions derived from samples *in vitro* and *in vivo* co-migrate on the cellulose plate. The additional spots on the autoradiogram of the mixed sample are due to the presence of other minor phosphopeptides in the sample *in vitro*, and their intensities are exaggerated by over-exposing the X-ray film.

To examine the site or sites of phosphorylation of peptide A, we determined by radio-sequencing the position of the phosphoserine residue after phosphorylation of liver ATP citrate-lyase by the insulin-sensitive cytosolic kinase. Previously, Avruch and co-workers [65] demonstrated that in hepatocytes the insulin-directed phosphorylation of ATP citrate-lyase occurred on the third serine residue (from the *N*-terminus) of a tryptic peptide derived from the enzyme. In our experiments, the insulin-sensitive phosphopeptide in ATP citrate-lyase was isolated by sequential tryptic hydrolysis and two-dimensional electrophoresis and chromatography on thin-layer cellulose plates. The extracted ^{32}P -labelled lyase peptide was absorbed on Polybrene resin and sequenced by automated liquid-phase Edman degradation. The phenylthiohydantoin samples recovered at each cycle were monitored for ^{32}P radioactivity by Cerenkov counting. As shown in Fig. 5, a peak of ^{32}P radioactivity appears at cycle 3. No other radioactivity spike can be detected even when the sequencing reaction is extended to cycle 12. In comparison with the total amount of radioactivity applied, the yields at cycles 3, 4 and 5 are 2.3, 1.2 and 1.0 % respectively. These results indicate that residue 3 is the primary phosphorylation site in the lyase tryptic peptide. The low yield and the trailing of ^{32}P radioactivity after the peak fraction are common phenomena of liquid-phase radio-sequencing, owing to the incomplete extraction of the highly charged phosphate ion from the Polybrene-containing reaction

cup. Taken together, these data demonstrate that the insulin-activated kinase catalyses phosphorylation at the third serine residue from the *N*-terminus of a tryptic peptide derived from hepatic ATP citrate-lyase.

DISCUSSION

The present study provides direct evidence that the insulin-enhanced serine phosphorylation of ATP citrate-lyase in intact cells may be mediated by a novel hormone-activated cytosolic kinase that we recently discovered in rat adipocytes [59]. This conclusion is based on three key findings. First, the insulin-stimulated cytosolic kinase is capable of phosphorylating the lyase *in vitro* with high affinity (Fig. 1; $K_m = 3 \mu\text{M}$). Second, there is a marked resemblance of phosphoamino acid content and chromatographic behaviour between the primary phosphopeptides derived from ATP citrate-lyase ^{32}P -labelled *in vitro* and *in vivo* (Fig. 4). Third, using radio-sequencing techniques, we demonstrate that the insulin-sensitive cytosolic kinase catalyses the serine phosphorylation of a specific ATP citrate-lyase tryptic peptide at the same position as that reported previously for lyase isolated from hormone-treated cells (Fig. 5 and ref. [65]). This high degree of specificity exhibited by the insulin-stimulated cytosolic kinase strongly suggests that this insulin-regulated kinase is responsible for the increased phosphorylation of ATP citrate-lyase in adipocytes and hepatocytes treated with the hormone.

In the present study, the tryptic hydrolysates of the phosphorylated ATP citrate-lyase are resolved by two-dimensional chromatography on cellulose plates. As evidenced by the absence of any significant radioactivity at the origin and the presence of a major phosphopeptide in the chromatogram, the overall lyase phosphorylation pattern documented by this method is virtually identical with that reported by other workers using h.p.l.c. techniques [65,66,74]. Thus the primary insulin-sensitive ATP citrate-lyase phosphopeptide detected in our experiments is equivalent to lyase peptide A as described previously by other laboratories [65,66,74]. A close examination of the phosphopeptide map of ATP citrate-lyase ^{32}P -labelled *in vitro* indicates that the insulin-sensitive kinase also weakly phosphorylates two other lyase peptide fractions (Fig. 2). However, the presence of these minor phosphopeptides is not consistently observed. It is quite likely that they represent larger forms of the primary lyase phosphopeptide produced by incomplete tryptic digestion. Indeed, such variability in ATP citrate-lyase phosphopeptide maps has been reported previously [66,75]. Taken together, our results indicate that the action of the insulin-sensitive cytosolic kinase on ATP citrate-lyase is directed at a specific site which is well documented to be the target site for the hormone-stimulated phosphorylation in intact cells.

In view of the use of liver ATP citrate-lyase as substrate for the adipocyte cytosolic kinase, it can be argued that our conclusion on the role of the cytosolic kinase in mediating insulin-stimulated lyase phosphorylation may not be valid if the adipose-tissue lyase interacts differently with the kinase preparation from the same tissue. Thus the insulin-stimulated adipocyte cytosolic kinase may not necessarily phosphorylate the fat-cell lyase at all, or at the same site as that in intact cells. However, this possibility appears to be remote. Benjamin and co-workers have reported that both hepatic and adipose ATP citrate-lyases contain phosphopeptides denoted as A, which are chromatographically indistinguishable on h.p.l.c. [66]. Furthermore, the same laboratory has demonstrated that purified ATP citrate-lyase preparations from liver and adipose tissue can be stoichiometrically phosphorylated to the same extent by the catalytic subunit of cyclic-AMP-dependent protein kinase and by a specific ATP

citrate-lyase kinase purified from liver [74]. Significantly, they further demonstrated that the ATP citrate-lyase kinase-directed phosphorylation of lyase from both tissues is dependent on the prior phosphorylation of peptide A by cyclic-AMP-dependent protein kinase. Taken together, these results indicate that lyase preparations from liver and adipose tissues behave identically as substrates for different kinases.

Using phosphopeptide mapping and radio-sequencing techniques, two groups have demonstrated that the insulin-stimulated phosphorylation site on ATP citrate-lyase can also serve as a substrate for the catalytic subunit of cyclic-AMP-dependent protein kinase [65,66]. The question arises as to whether the partially purified kinase preparations from control and insulin-treated adipocytes may be differentially contaminated by this catalytic subunit. This possibility seems highly unlikely. As described in the Experimental section, the cytosolic kinase preparations from control and insulin-treated adipocytes have been partially purified through DEAE-Sephacel ion-exchange and Sephacryl S-200 size-sieving columns. Our previous results have indicated that the ion-exchange-chromatography step completely separates the cytosolic kinase activity from the catalytic subunit of cyclic-AMP-dependent protein kinase [59]. Thus the simplest and most plausible hypothesis consistent with the data presented here is that the insulin-sensitive cytosolic kinase catalyses phosphorylation of ATP citrate-lyase *in vivo*. An important future question relates to the mechanism whereby the kinase is activated by insulin.

It is interesting that no apparent change in catalytic activity of ATP citrate-lyase has been detected after insulin-induced phosphorylation *in vitro*. It has been suggested that the effects of the insulin-stimulated phosphorylation of this enzyme may be quite subtle. For instance, a recent study by Stralfors [76] indicated that the insulin-enhanced ATP citrate-lyase phosphorylation correlated with the immobilization of this enzyme in digitonin-permeabilized adipocytes. Clearly, a challenging task for future studies is to determine whether lyase phosphorylation in intact cells is associated with a physiologically relevant function.

Note added in proof (received 27 April 1990)

A paper from the author's laboratory [61] recently appeared describing the purification to near-homogeneity of an insulin-stimulated serine kinase from rat liver. The enzymic properties of this liver kinase are very similar to those of the insulin-sensitive kinase from rat adipocytes described here.

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