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Sequence of Sites on ATP-Citrate Lyase and Phosphatase Inhibitor 2 Phosphorylated by Multifunctional Protein Kinase (a Glycogen Synthase Kinase 3 Like Kinase)†

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ABSTRACT: Multifunctional protein kinase (MFPK) phosphorylates ATP-citrate lyase on peptide B on two sites, B_T and B_S, on threonine and serine, respectively, inhibitor 2 on a threonyl residue, and glycogen synthase at sites 2 and 3. The phosphorylation sites B_T and B_S of ATP-citrate lyase are dependent on prior phosphorylation at site A whereas site A phosphorylation is decreased by prior phosphorylation at sites B_T and B_S. To study the MFPK recognition sites and the site–site interactions, the amino acid sequences of ATP-citrate lyase peptide B and inhibitor 2 were determined and compared to each other and to glycogen synthase sites 3–5. The sequence of the tryptic peptide containing the two phosphorylation sites of peptide B is -Phe-Leu-Leu-Asn-Ala-Ser-Gly-Ser-Thr-Ser-Thr(P)-Pro-Ala-Pro-Ser(P)-Arg-, and the sequence of the MFPK phosphorylation site of inhibitor 2 is -Ile-Asp-Glu-Pro-Ser-Thr(P)-Pro-Tyr-. This inhibitor 2 site is identical with the site phosphorylated by glycogen synthase kinase $3/F_A$. These results suggest that at least some of the sites phosphorylated by MFPK (B_T of ATP-citrate lyase, Thr 72 of inhibitor 2, and sites 3b and 4 of glycogen synthase) contain a Ser/Thr flanked by a carboxyl-terminal proline. However, as MFPK did not phosphorylate a series of peptides containing the -X-Thr/Ser-Pro-X- sequence, this minimum consensus sequence is not sufficient for phosphorylation by MFPK. MFPK was able to phosphorylate glycogen synthase synthetic peptide only after it had first been phosphorylated by casein kinase II at site 5, supporting the hypothesis that the phosphorylation of natural substrates by MFPK depends on prior phosphorylation at other sites. ATP-citrate lyase sites B_T and B_S resemble glycogen synthase sites 3b and 3c, suggesting that MFPK recognizes a Ser/Thr in the following pattern: -Ser/Thr()-X-X-X-Ser/Thr(P)-. Additional evidence for this four amino acid spacing was obtained when the sequence about all three phosphorylation sites of ATP-citrate lyase was found to be -Thr(P)-Pro-Ala-Pro-Ser(P)-Arg-Thr-Ala-Ser(P)-, a configuration that resembles glycogen synthase sites 3b, 3c, and 4.

How insulin produces an increase in the phosphorylation of some proteins (Benjamin & Singer, 1974, 1975; Alexander et al., 1979; Ramakrishna & Benjamin, 1979, 1983a; Smith et al., 1980; Belsham et al., 1982; Petruzelli et al., 1984; Kahn et al., 1985; Denton, 1986) and a decrease in others (Engstrom, 1980; Huges et al., 1980; Lawrence et al., 1983, 1986; Denton, 1986) is not understood. Recent studies on ATP-citrate lyase phosphorylations have shed considerable knowledge on this problem. Insulin and isoproterenol both increase the phosphorylation of ATP-citrate lyase on peptide A whereas insulin decreases the phosphorylation of peptide B of ATP-citrate lyase, but isoproterenol increases its phosphorylation (Pucci et al., 1983; Ramakrishna et al., 1984). While looking for a protein kinase that phosphorylates peptide B of ATP-citrate lyase, a multifunctional protein kinase (MFPK)[1] that is similar to rabbit muscle glycogen synthase kinase/F_A was isolated from rat liver (Ramakrishna & Benjamin, 1981, 1985; Sheorain et al., 1985b). MFPK phosphorylates peptide B of ATP-citrate lyase and sites 2 and 3 of glycogen synthase (Ramakrishna & Benjamin, 1985; Sheorain et al., 1985b). MFPK was found to be an insulin-regulated kinase as insulin at physiological concentrations rapidly decreased its activity

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[1] Abbreviations: TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; MFPK, multifunctional protein kinase; TFA, trifluoroacetic acid; PKI, cAMP-dependent protein kinase inhibitor.

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when ATP-citrate lyase, acetyl-CoA carboxylase, and glycogen synthase were used as substrates (Ramakrishna & Benjamin, 1988). Interestingly, recent observations suggest that in addition to cAMP-dependent protein kinase another cytosolic kinase that phosphorylates peptide A of ATP-citrate lyase also has been found to be under hormonal control (Yu et al., 1990).

Peptide A phosphorylation by cAMP-dependent protein kinase is essential for peptide B phosphorylation (Ramakrishna et al., 1983). This observation is similar to that described for glycogen synthase in which the phosphorylation of site 5 by casein kinase II enhances the ability of sites 3a, 3b, 3c, and 4 to be phosphorylated in a sequential fashion by glycogen synthase kinase $3/F_A$ (Picton et al., 1982; DePaoli-Roach et al., 1983; Fiol et al., 1987). It was also demonstrated that an increase in peptide B phosphorylation by MFPK leads to a decrease in peptide A phosphorylation by cAMP-dependent protein kinase (Ramakrishna et al., 1989).

Insulin controls the activity of MFPK which has specificity for peptide B of ATP-citrate lyase (Ramakrishna & Benjamin, 1988). As peptide B is phosphorylated both in vivo and in vitro (Pucci et al., 1983; Ramakrishna et al., 1984) and peptide A and B phosphorylations affect each other (Ramakrishna et al., 1983, 1989), it was important to determine the primary amino acid sequence of peptide B of ATP-citrate lyase.

Materials and Methods

Materials. MFPK and ATP-citrate lyase were purified from rat liver as described previously (Ramakrishna & Benjamin, 1983b, 1985). The catalytic subunit of cAMP-dependent protein kinase and glycogen synthase were purified from rabbit skeletal muscle (Bechtel et al., 1977; Ramakrishna et al., 1981; Sheorain et al., 1984). Glycogen synthase synthetic peptide (Ac-PRPASVPPSPSLSRHSSPHQSEDEEEP-amide) was a gift from Dr. P. J. Roach (Indiana University School of Medicine, Indianapolis, IN). Protein phosphate inhibitor 2 from rabbit skeletal muscle was a gift from Dr. B. S. Khatra (California State University, Long Beach, CA). Casein kinase II purified from bovine testis was a gift from Dr. D. Litchfield (University of Washington, Seattle, WA). Tyrosine hydroxylase peptide TH^{2-16} (PTPSAPSPQPKGFRR), $TH^{2-16/Ala3,5}$ (PAPAAPSPQPKGFRR), and RNA polymerase II peptide analogue (YSPTTPKKK) were gifts from Dr. F. L. Hall (Childrens Hospital of Los Angeles, Los Angeles, CA). [γ-^{32}P]ATP was purchased from ICN Radiochemicals. K2 TLC cellulose plates were purchased from Whatman. TPCK-treated trypsin and chymotrypsin were purchased from Worthington Biochemicals. Cyclic AMP dependent protein kinase inhibitor peptide PKI 5–24 (TTYADFIASGRTGRRNAIHD) was obtained from Peninsula Laboratories Inc. The sources of other chemicals were as described (Ramakrishna & Benjamin, 1985, 1988).

Phosphorylation Reactions. Phosphorylation reactions were performed as described previously (Ramakrishna & Benjamin, 1985, 1988). To determine the synergistic effect of phosphorylation with casein kinase II or cAMP-dependent protein kinase on phosphorylation by MFPK, the first phosphorylation was carried out for 2 h at 37 °C in 50 mM Tris-HCl, pH 7.5, 0.3 mM ATP, 8 mM $MgCl_2$, 100 mM NaCl, 0.3 μg/mL casein kinase II or 5 μg/mL catalytic subunit of cAMP-dependent protein kinase, and substrates (1 mg/mL ATP-citrate lyase, 1 mg/mL glycogen synthase, 0.1 mg/mL inhibitor 2, or 20 μM glycogen synthase synthetic peptide). The casein kinase II reaction was stopped by the addition of heparin to a final concentration of 8 μg/mL, and the cAMP-dependent protein kinase reaction was stopped by the addition of inhibitor peptide PKI 5–24. These inhibitors inhibited the respective kinase reactions over 96%. The second phosphorylation was initiated by the addition of MFPK or buffer and [γ-^{32}P]ATP and incubated for an additional 2 h. An aliquot of the reaction mixture was spotted on to P-81 disks, washed with 75 mM phosphoric acid containing 1 M HCl for 1 h, followed by three changes of phosphoric acid, and the radioactivity was counted in a liquid scintillation counter. Glycogen synthase synthetic peptide phosphorylation was determined as described (Fiol et al., 1987).

ATP-citrate lyase (5 mg) from rat adipose tissue was phosphorylated with purified MFPK and [γ-^{32}P]ATP as described (Ramakrishna & Benjamin, 1988). Saturated ammonium sulfate solution (pH 7.0) was added to the reaction mixture to obtain 40% saturation and centrifuged, and the precipitate was washed with 40% ammonium sulfate. The precipitate was dissolved in 300 μL of 100 mM ammonium bicarbonate and chromatographed on a Sephacryl S-200 column (1 × 24 cm) equilibrated in 100 mM ammonium bicarbonate. The [^{32}P]ATP-citrate lyase was dried in a Speed Vac concentrator (Savant). Inhibitor 2 was phosphorylated with [γ-^{32}P]ATP by MFPK as above, purified on a quick-spin Sephadex G-25 column (Boehringer) equilibrated in 25 mM Tris-HCl, pH 8.5, and dried.

Digestion with Trypsin. TPCK-treated trypsin was added to [^{32}P]ATP-citrate lyase (trypsin:lyase, 1:3 w/w) and incubated at 37 °C for 20 h. Perchloric acid was added to 5% final concentration, cooled on ice for 1 h, and centrifuged at 20000*g* for 30 min, and the precipitate was discarded. Purified [^{32}P]inhibitor 2 was digested with trypsin (trypsin:inhibitor 2; 1:20 w/w) at 37 °C for 20 h and dried.

Digestion with Chymotrypsin. ATP-citrate lyase was phosphorylated by MFPK plus cAMP-dependent protein kinase for 2 h as described and was purified on a quick-spin Sephadex G-25 column. ATP-citrate lyase labeled at both peptide A and peptide B or [^{32}P]inhibitor 2 peptide was digested with chymotrypsin in 100 mM *N*-ethylmorpholine, pH 8.5, for 4 h (chymotrypsin:substrate, 1:20 w/w) at 37 °C and fractionated on a C18 column.

Cleavage with Cyanogen Bromide. Peptide B of ATP-citrate lyase was isolated by digestion with trypsin (trypsin:lyase, 1:50 w/w) followed by HPLC on a C18 column. Peptide B was dissolved in 1 mL of 50% formic acid flushed with nitrogen, incubated at 25 °C for 1 h with CNBr (1 mg/mL), incubated at 5 °C overnight, and freeze-dried.

Purification of ^{32}P-Peptides. Peptide B of ATP-citrate lyase is a large hydrophobic peptide of ~6 kDa. To generate small peptides, ATP-citrate lyase phosphorylated by MFPK is digested with a high concentration of trypsin (trypsin:lyase, 1:3) because our earlier studies showed that trypsin at high concentration cleaved nonspecifically to give a series of less hydrophobic, small peptides. The tryptic digest was clarified by precipitation with $HClO_4$, and the soluble peptides were fractionated on a Ultrasphere-ODS C18 column using acetonitrile-0.2 M phosphate buffer, pH 3.5, gradient as described (Pucci et al., 1983; Ramakrishna et al., 1984) (Figure 1a). Peptides were desalted on Sep-pak C18 (Waters) and dried. The peptides were further purified by high-voltage electrophoresis (1500 V for 1 h) on K2 cellulose plates in 1.0% ammonium carbonate (pH 8.9) (Figure 1b). The radioactive peptide were eluted from the plates with 0.1% TFA and rechromatographed on a C18 column using acetonitrile–phosphate buffer (Figure 1c). Final purification was done by chromatography on a C18 column using 2-propanol–0.1% TFA (Figure 1d). The purified phosphopeptide fractions were dried.

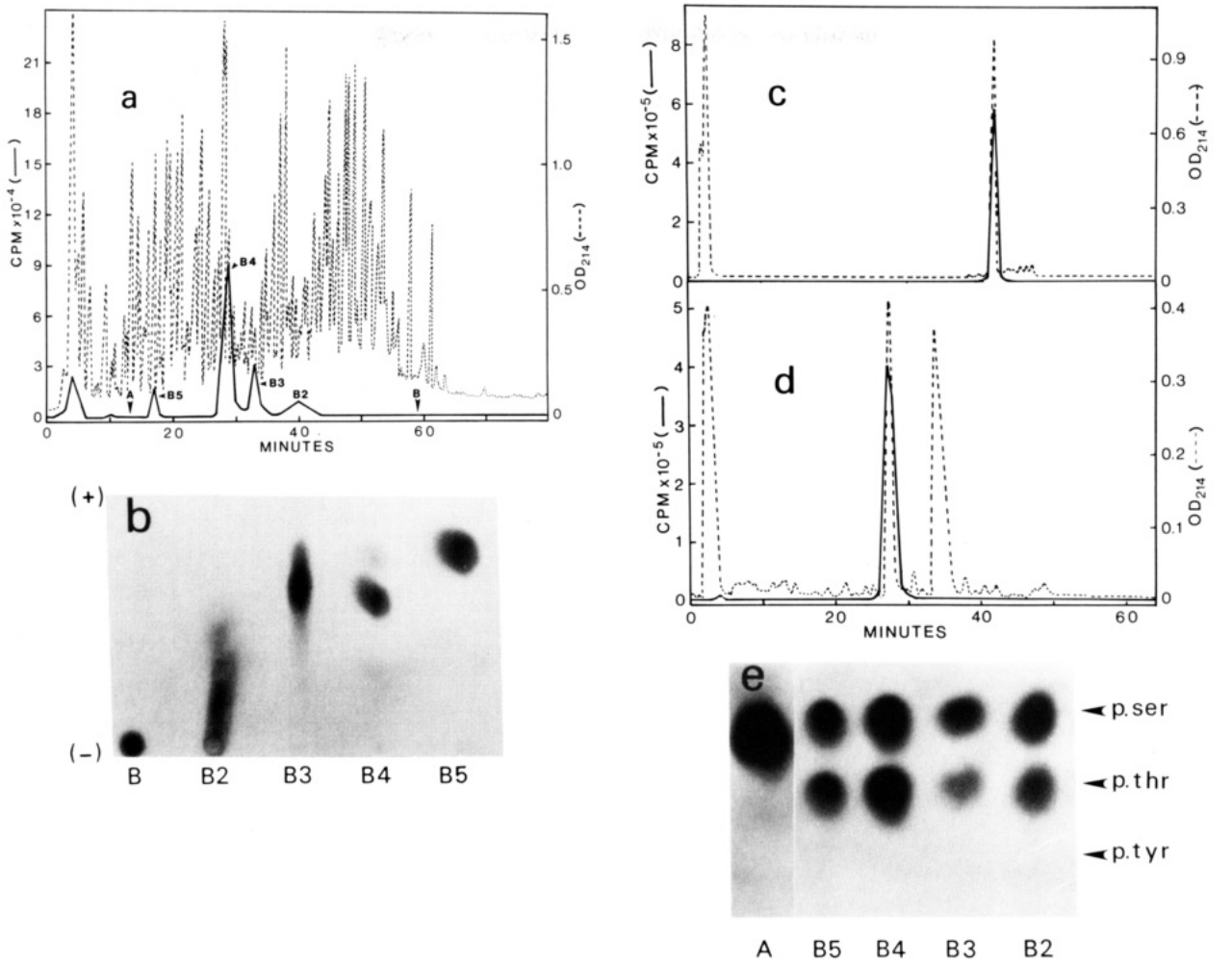

FIGURE 1: Purification of various ATP-citrate lyase peptides containing sites B_T and B_S. (a) HPLC of a tryptic digest of ATP-citrate lyase on a C18 reverse-phase column. ATP-citrate lyase phosphorylated by MFPK was digested with TPCK–trypsin (trypsin:lyase, 1:3) overnight, and the resulting phosphopeptides were applied to a C18 column equilibrated with 0.2 M phosphate buffer–5% acetonitrile. The column was eluted for 5 min with 5% acetonitrile followed by a linear gradient of 5–40% acetonitrile in phosphate buffer at a flow rate of 1 mL/min. One-milliliter fractions were collected, and the Cerenkov radiation in the fractions was determined in a liquid scintillation counter. Note that the UV tracing is from a UV monitor connected on line to the HPLC whereas the radioactivity plot is derived from a plot of 1-mL fractions. This leads to a decrease in the observed resoltuion of the radioactivity plot compared to the UV tracing. Because a high amount of trypsin was used for digestion of lyase and the sample was clarified with $HClO_4$, peptide B was not found. Instead, less hydrophobic peptides (B2, B3, B4, and B5) were obtained. The positions of peptides A and B which were not detected are indicated. (b) Thin-layer electrophoresis of phosphopeptides on K2 cellulose plates. The phosphopeptide peaks from four HPLC runs were pooled, desalted, dried, and spotted at the bottom of K2 cellulose plates and electrophoresed toward the cathode in 1% ammonium carbonate, pH 8.9. Phosphopeptides were located by autoradiography. Phosphopeptides were extracted from the cellulose with 0.1% TFA. (c) Chromatography of peptide B4 on a C18 column with an acetonitrile gradient. The phosphopeptides purified by electrophoresis are fractionated on a C18 column equilibrated in 5% acetonitrile–0.1% TFA. Phosphopeptide B4 was eluted with a linear gradient of 5–20% acetonitrile. Note that the gradient was different for different phosphopeptides. (d) Rechromatography of peptide B4 on a C18 column with a 2-propanol gradient. The phosphopeptide fraction from the previous step was dried, dissolved in 0.1% TFA, and resolved on a C18 column equilibrated with 3% 2-propanol. Phosphopeptide B4 was eluted with a 3–12% 2-propanol gradient. (e) Phosphoamino acid analysis of the purified phosphopeptides. Peptide A was purified from ATP-citrate lyase phosphorylated with cAMP-dependent protein kinase. The individual purified phosphopeptides containing B sites were hydrolized in 6 M HCl for 2 h, dried, and subjected to phosphoamino acid analyses as described under Materials and Methods. Peptide A contained only phosphoserine, and all B phosphopeptides contained both phosphoserine and phosphothreonine.

Phosphoamino acid analyses of peptide B and peptides B2, B3, B4, B5, and B-CNBr demonstrated that they all contain both phosphoserine and phosphothreonine (Figure 1e). The chymotrypsin digest of inhibitor 2 tryptic peptide was purified by a similar procedure with an additional purification on a C8 column using acetonitrile–0.1% TFA gradient. CNBr-digested peptide B (B-CNBr) was purified by HPLC on a C18 column using an acetonitrile–0.2 M phosphate buffer gradient followed by HPLC on a C18 column using an acetonitrile–0.1% TFA gradient.

Amino Acid Analysis. The purified radioactive peptides were hydrolized in 5 M HCl at 110 °C for 18 h in the vapor phase in vacuo. The samples were dried and analyzed for amino acid composition on a Waters Pico-Tag system. For phosphoamino acid analysis, purified phosphopeptides were hydrolyzed in 6 M HCl at 110 °C for 2 h, diluted 4-fold with water, and dried. The residue was dissolved in electrophoresis buffer, pH 2.8 (acetic acid/formic acid/pyridine/water, 10:2:1:187), spotted along with phosphoserine, phosphothreonine, and phosphotyrosine standards on a K2-cellulose plate, and electrophoresed at 1500 V for 1.5 h (Ramakrishna & Benjamin, 1988). The plates were dried and phosphoamino acids were visualized by ninhydrin staining. Radiolabeled phosphoamino acids were identified by autoradiography.

Peptide Sequencing. Purified phosphopeptide was dissolved in formic acid and sequenced on an Applied Biosystems ABI 470A protein sequencer connected on line to an ABI 120A PTH-amino acid analyzer. PTH-amino acid released at each

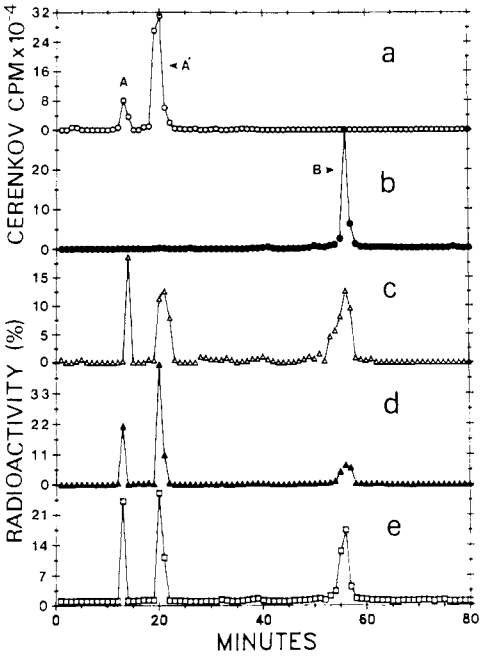

FIGURE 2: HPLC phosphopeptide maps of ATP-citrate lyase. ATP-citrate lyase phosphorylated with cAMP-dependent protein kinase (a) or MFPK (b), passed thorugh Sephacryl S-200, and digested with trypsin (trypsin:lyase, 1:50) at 37 °C for 20 h. The tryptic digest was fractionated on a C18 column using a 0.2 M phosphate, pH 3.5–acetonitrile gradient as described (Pucci et al., 1983). Radioactivity was detected by measuring the Cerenkov radiation of each 1-mL fraction. Because ATP-citrate lyase was treated with a limited amount of trypsin, the cAMP-dependent protein kinase phosphorylated lyase gave peptide A and its higher molecular weight fragment peptide A′ (due to incomplete digestion), and MFPK-phosphorylated lyase gave only peptide B. In vivo phosphorylated ATP-citrate lyase from control (c), 10 nM insulin (d), or 0.3 μM isoproterenol (e) treated rat fat pads was purified as described (Pucci et al., 1983; Ramakrishna et al., 1984) and digested with trypsin. The resulting tryptic peptides were fractionated by reverse-phase HPLC. Note that all three in vivo phosphorylated ATP-citrate lyase samples have peptide A, A′, and B but in differing amounts.

Edman sequencing cycle was counted for radioactivity. Peptide B4 was dissolved in 20% formic acid and applied to a Beckman 890M spinning cup sequencer, and radiochemical sequence analysis was performed as described (Lees-Miller & Anderson, 1989).

Sequence Determination of in Vivo Phosphorylated ATP-Citrate Lyase. Rat fat pads were incubated with [^{32}P]P_i for 60 min in modified Kreb's Ringer bicarbonate buffer without phosphate (Benjamin & Singer, 1975). ATP-citrate lyase was purified rapidly by chromatography on DE-52 and coenzyme A–agarose (Ramakrishna & Benjamin, 1983b) or immunoprecipitated with rabbit anti-ATP-citrate lyase serum and further purified by SDS–PAGE followed by electroelution (Pucci et al., 1983, Ramakrishna et al., 1984). Adipose tissue ATP-citrate lyase (2 mg) was added to the [^{32}P]ATP-citrate lyase purified by either method and was digested with trypsin (trypsin:lyase, 1:3). The phosphopeptides from the tryptic digest were purified by HPLC on a C18 column, thin-layer electrophoresis, and two additional HPLC steps on a C18 column. The purified peptides were analyzed for phosphoamino acids and sequenced.

Results and Discussion

ATP-citrate lyase, purified from fat pads of starved and refed rats, was phosphorylated in vitro on site A by cAMP-dependent protein kinase and on site B by MFPK. Typical HPLC patterns of the radioactive peptides generated by tryptic digestion of ATP-citrate lyase (trypsin:lyase, 1:50) are given in Figure 2a,b. In addition, immunoprecipitated ATP-citrate lyase from fat pads incubated with [^{32}P]P_i for 2 h followed by the addition of either insulin or isoproterenol for 20 min was digested with trypsin and chromatographed on a C18 column. The resultant radioactive peptide profiles are also shown in Figure 2c–e. Examination of the HPLC patterns suggests that peptides A and B phosphorylated by cAMP-dependent protein kinase and MFPK, respectively, are both distinct and phosphorylated in vivo. Furthermore, as had been suggested previously (Pucci et al., 1983; Ramakrishna et al., 1984) the percent radioactivity associated with peptide B decreases after insulin treatment in vivo (Figure 2d). This finding is consistent both with the observation that the activity of MFPK decreases in rat adipose tissue and chick hepatocytes[2] after insulin treatment (Ramakrishna & Benjamin, 1988) and with an insulin-stimulated dephosphorylation (Chan et al., 1988).

Table I: Summary of Phosphopeptide Sequences of ATP-Citrate Lyase and Inhibitor 2[a]

phosphopeptide	amino acid sequence
Tryptic peptide B-CNBr(CL)	AWAPAIPNQPPTAAHTANFLLNASGSTST*P
Tryptic peptide B-3(CL)	TANFLLNASGSTST*PAPS*
Tryptic peptide B-5(CL)	LNASGSTST*PAPS*
Chymotryptic peptide C (CL)	LLNASGSTST*PAPS*RTAS*F
Tryptic peptide A (CL)	TAS*FSESR
in vivo tryptic peptide A	TAS*FSESR
Tryptic peptide B-4(CL)	FLLNASGSTST*PAPS*R
in vivo tryptic peptide B-4	FLLNASGSTST*PAPS*R
Tryptic peptide I-T(I-2)	IDEPST*PYHSMIGDDDDAYSDTETTEA
Chymotryptic-tryptic peptide I-TC(I-2)	IDEPST*PY

[a] Phosphopeptides B3, B4, B5, andd B were derived from trypsin treatment of ATP-citrate lyase (CL) phosphorylated by MFPK. B-CNBr peptide was obtained by further digestion of peptide B with CNBr. Peptide A was derived from trypsin treatment of ATP-citrate lyase phosphorylated by cAMP-dependent protein kinase. Phosphopeptide C (CL) was obtained by digestion of ATP-citrate lyase with chymotrypsin. I-T was generated by digestion of inhibitor 2 (I-2) phosphorylated by MFPK with trypsin. I-TC was obtained by either the digestion of I-T with chymotrypsin or the simultaneous digestion of inhibitor 2 with chymotrypsin and trypsin. The peptide sequences are listed from amino terminal to carboxyl terminal. Asterisks denote the phosphorylated residues.

To determine the sequence of phosphorylation sites of peptide B, phosphopeptides B3, B4, and B5 were isolated as described earlier. The amino acid composition of peptides B3, B4, and B5 was determined on the Pico-Tag system. All the three peptides contained 4 seryl residues, peptide B3 contained 3 threonyl residues, and peptides B4 and B5 contained 2 threonyl residues, which is consistent with the sequence determinations (Table I). The purified peptides were sequenced on an Applied Biosystems 470 A gas-phase sequencer. The amino acid sequence of peptide B4 was determined to be FLLNASGSTSTPAPSR (Ramakrishna & Benjamin, 1990). The primary amino acid sequence of the various phosphopeptides of ATP-citrate lyase from multiple runs is given in Table I. As there are multiple threonyl and seryl residues, the PTH-amino acid released at each Edman sequencing cycle was counted for radioactivity to identify the residue phosphorylated. From the analysis of radioactivity recovered in sequencing of peptide B4, position 11 (threonine) and position

[2] S. Ramakrishna, K. S. Murthy, R. K. Seethala, and W. B. Benjamin, unpublished observations.

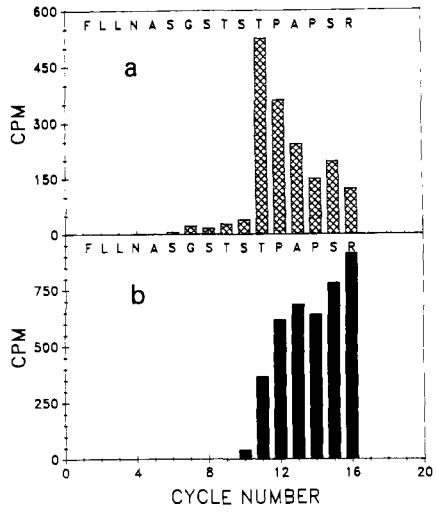

FIGURE 3: Radiochemical sequence analysis of phosphopeptide B4. (a) Peptide B4 purified (560 000 cpm, Cerenkov radiation) by HPLC and electrophoresis was dissolved in 22 μL of 88% formic acid, and 20 μL was loaded on the disk and sequenced on an ABI 470A sequencer connected on line to an ABI 120A PTH-amino acid analyzer. A portion of PTH-amino acid released in each Edman cycle was mixed with Ecolume (ICN Radiochemicals), and the radioactivity was measured. Note that most of the released ^{32}P-labeled inorganic phosphate is associated with the filter, and a very small percent (<0.2%) was eluted with the amino acid. (b) Purified peptide B4 (81 000 cpm, Cerenkov radiation) applied to a Beckman spinning cup sequenator. A portion of the residue from each degradation cycle was analyzed for Cerenkov radiation. A broad release of ^{32}P beginning with the 11th residue with a maximum at the 16th cycle was observed. Note that residues distal to the phosphorylation site usually contain substantial amounts of radioactivity (Lees-Miller & Anderson, 1989). The sudden increase in released radioactivity is indicative of a phosphorylation residue at that position in the Edman degradation. Threonine and serine at positions 11 and 15, respectively, are identified as the phosphorylated residues because at the 11th and 15th cycles there was a sudden rise in radioactivity.

15 (serine) were assigned as the radioactive phosphoamino acid residues (Figure 3a). These phosphorylation positions were confirmed by radiochemical sequence analysis of peptide B4 in a Beckman 890M spinning cup sequencer (Figure 3b).

To confirm that peptides B3, B4, and B5 are part of peptide B, peptide B was isolated and cleaved with CNBr. The cleaved peptides were fractionated on a C18 column and sequenced. Note the overlapping sequences of B-CNBr and B3, B4, and B5, suggesting that these peptides are fragments of peptide B. Our peptide sequence of peptide A of ATP-citrate lyase phosphorylated by cAMP-dependent protein kinase (site A) agrees with that reported previously (Pierce et al., 1982; Swergold et al., 1982) (Table I).

To determine whether the phosphorylated tryptic peptides generated from in vivo phosphorylated ATP-citrate lyase are the same as that generated from ATP-citrate lyase phosphorylated in vitro by MFPK and cAMP-dependent protein kinase, fat pads were incubated with [^{32}P]P_i for 2 h as described. ATP-citrate lyase was purified and digested with trypsin (trypsin:lyase, 1:3), and the radiolabeled phosphopeptides were purified. The radioactivity profile of these tryptic peptides (Figure 4) was strikingly similar to those produced by in vitro phosphorylation of ATP-citrate lyase with the appropriate kinases. The sequences of in vivo phosphorylated peptides with similar retention times to B4 and A were found to be identical with those of the in vitro phosphorylated phosphopeptides (Table I). Phosphoamino acid analysis of these peptides demonstrated both phosphoserine and phosphothreonine in in vivo peptide "B4" and only phosphoserine in peptide "A" (data not shown). Therefore, it is suggested that the in vivo phosphorylated peptides are the same as those phosphorylated in vitro by MFPK and cAMP-dependent protein kinase.

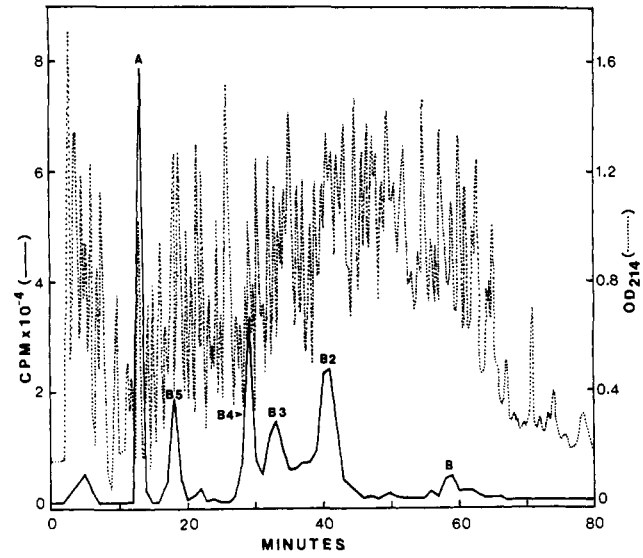

FIGURE 4: Reverse-phase chromatography of tryptic digest of in vivo phosphorylated ATP-citrate lyase. To [^{32}P]ATP-citrate lyase (in vivo labeled) purified from rat adipose tissue was added purified ATP-citrate lyase (2 mg) was added, and the mixture was digested with trypsin (trypsin:lyase, 1:3 w/w). The reaction mixture was diluted 4-fold and acidified with 0.1% TFA. The tryptic digest was fractionated on a C18 column as described in the legend to Figure 1a.

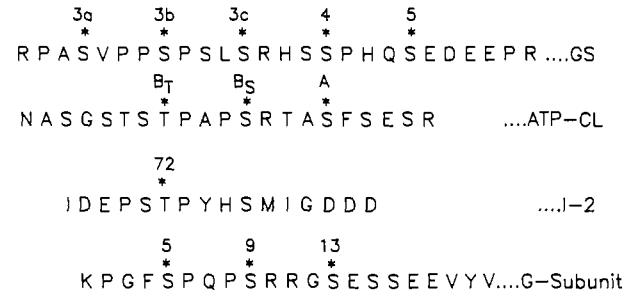

FIGURE 5: Comparison of the MFPK-phosphorylated sequences of rat ATP-citrate lyase (ATP-CL), rabbit glycogen synthase (GS), and rabbit phosphatase inhibitor 2 (I-2). For comparison, the sequence containing the glycogen synthase kinase 3 phosphorylation site of the G-subunit of protein phosphatase 1 is shown.

To study the structural requirements for MFPK phosphorylations, the sequence about the site phosphorylated by MFPK on inhibitor 2 was determined. Inhibitor 2 was phosphorylated with MFPK and digested with trypsin and/or chymotrypsin. Phosphoamino acid analysis of the radiolabeled phosphopeptide showed only phosphothreonine (data not shown). The sequence of the tryptic phosphopeptide is IDEPST*PYHSMIGDDDDAYSD, and the chymotryptic phosphopeptide is IDEPST*PY. This sequence is precisely the same as that phosphorylated at Thr 72 by glycogen synthase kinase $3/F_A$ (Holmes et al., 1986). As the MFPK-phosphorylated tryptic and chymotryptic phosphopeptide of inhibitor 2 contains only one threonine, it is suggested that MFPK phosphorylates Thr 72 of inhibitor 2.

The substrate recognition site for many protein kinases has been determined (Edelman et al., 1987). To identify common structural elements that MFPK recognizes, the amino acid sequences of the MFPK-directed phosphorylation sites of ATP-citrate lyase, inhibitor 2, glycogen synthase sites 3a, 3b, 3c, 4, and 5, and G-subunit of protein phosphatase 1 were compared. The comparison suggests that among the sites phosphorylated by MFPK is a Thr/Ser flanked by an immediate caboxyl-terminal proline (Figure 5).

Table II: Synergistic Effect of Prior Phosphorylation on MFPK Phosphorylation[a]

first incubation	second incubation	pmol of phosphate: ATP-citrate lyase	glycogen synthase	glycogen synthase synthetic peptide	inhibitor 2
casein kinase II	buffer		0	3	0
cAMP-dependent protein kinase	buffer	0			
buffer	MFPK	40	64	0	5.7
cAMP-dependent protein kinase	MFPK	332			
casein kinase II	MFPK		358	1403	14.3

[a] The first phosphorylation was carried as described under Materials and Methods with casein kinase II or cAMP-dependent protein kinase for 2 h without [γ-^{32}P]ATP, and the reaction was stopped with the addition of either heparin or PKI 5-24 peptide, respectively. Buffer or MFPK was added after stopping the first kinase activity, and the second incubation (1 h) was started by the addition of [γ-^{32}P]ATP. Phosphate incorporation was measured as described. Values are the averages of duplicate samples from two independent experiments.

The minimal recognition sequence of -X-Ser/Thr-Pro-X was found not be sufficient for phosphorylation by MFPK because a series of synthetic peptides of tyrosine hydroxylase and RNA polymerase II with this consensus sequence was not phosphorylated by MFPK (data not shown). These peptides, however, are good substrates for phosphorylation by a novel proline-directed Ser/Thr protein kinase isolated from rat pheochromocytoma (Vulliet et al., 1988).

The phosphorylation of specific sequences by MFPK should be dependent on other structural requirements. The MFPK-directed phosphorylations of ATP-citrate lyase are dependent on phosphorylation at another site by cAMP-dependent protein kinase (Ramakrishna et al., 1983). This is similar to the observation that the phosphorylation of G-subunit of protein phosphatase 1 by glycogen synthase kinase 3/F_A is dependent on the phosphorylation at another site by cAMP-dependent protein kinase (Fiol et al., 1988). In addition, glycogen synthase and inhibitor 2 phosphorylation by glycogen synthase kinase 3/F_A are dependent on phosphorylation by casein kinase II (Picton et al., 1982, DePaoli-Roach et al., 1983; DePaoli-Roach, 1984). To evaluate the regulation of the phosphorylation of glycogen synthase and inhibitor 2 by MFPK, native glycogen synthase, inhibitor 2, and glycogen synthase synthetic peptide were first phosphorylated by casein kinase II. ATP-citrate lyase was first phosphorylated by cAMP-dependent protein kinase. The ability of MFPK to phosphorylate these substrates with and without a prior phosphorylation by the other protein kinase was studied (Table II). In all cases, when these protein and synthetic substrates were first phosphorylated by casein kinase II or cAMP-dependent protein kinase, the ability of MFPK to phosphorylate its specific sites was enhanced. The synthetic peptide was not phosphorylated by MFPK unless it was first phosphorylated by casein kinase II. The order and rate of phosphorylation of glycogen synthase sites 3a, 3b, 3c, and 4 and ATP-citrate lyase sites B_T and B_S by MFPK have not been determined.

The ability of MFPK to phosphorylate its specific sites on glycogen synthase and glycogen synthase synthetic peptide is dependent on the phosphorylation of site 5, four amino acids carboxyl terminal to site 4. Therefore, the controlling site for the phosphorylation of ATP-citrate lyase peptide B by MFPK could be four amino acids carboxyl terminal to the phosphoserine of site B_S. When peptide A is placed carboxyl to the arginine residue of peptide B4, the sequence containing B_T and B_S is similar to glycogen synthase sites 3b and 3c, and the third phosphorylated residue (site A) resembles glycogen synthase site 4 (Figure 5). To look for this postulated single peptide containing the three phosphorylation residues, ATP-citrate lyase phosphorylated by both MFPK and cAMP-dependent protein kinase was digested with chymotrypsin (enzyme:lyase, 1:20) for 4 h. The radiolabeled phosphopeptide was purified by HPLC, thin-layer electrophoresis, and two HPLC runs and sequenced. The sequence of the chymotryptic peptide (Table I) shows that peptide A is carboxyl terminal to the arginine of peptide B. Thus, the phosphorylation sites of MFPK and cAMP-dependent protein kinase on ATP-citrate lyase are similar in structure to that of the glycogen synthase sites phosphorylated by MFPK or glycogen synthase kinase 3/F_A (Figure 5).

After we had demonstrated the phosphorylation sequence of ATP-citrate lyase, the nucleotide sequence of rat liver ATP-citrate lyase cDNA and the derived amino acid sequence were published (Elshoubagy et al., 1990). The sequence from amino acids 437 to 459 was found to be precisely the same as that of the chymotryptic peptide containing sites B_T, B_S, and A. Therefore, the peptide sequence containing the three phosphorylated sites of ATP-citrate lyase is as given in Figure 5 with the phosphorylated residues being Threonine 446, Serine 450, and serine 454. The CNBr-cleaved peptide B (B-CNBr), amino acids 418–447, matched exactly with the sequence predicted from nucleotide analysis of the cDNA clone, lending credence to the correctness of this sequence.

The data presented in this paper suggest that phosphorylation of ATP-citrate lyase by MFPK is associated with those Ser/Thr when there is a prior phosphorylation of Ser/Thr (permissive) four amino acids carboxyl terminal [-S/T-X-X-X-S(p)-X-]. This conclusion is similar to that proposed for glycogen synthase (Fiol et al., 1987) with the following addition. In the case of ATP-citrate lyase a dephosphorylation of sites B_T and B_S increases the ability of site A to be phosphorylated by cAMP-dependent protein kinase (Ramakrishna et al., 1983, 1989). Whether a decrease in the phosphate content of glycogen synthase sites 3a, 3b, 3c, and 4 makes site 5 a better substrate for phosphorylation by casein kinase II has not been shown.

The finding that the phosphate content of a cAMP-dependent protein kinase phosphorylation site regulates the subsequent phosphorylation of other sites by MFPK is similar to the observation that phosphorylation at the cAMP-dependent protein kinase phosphorylation site of a synthetic peptide based on the G-component of protein phosphatase 1 (Ser 13) permits the recognition of serine residues (Ser 9 and Ser 5) at the requisite four amino acid spacings, amino terminal to the permissive site by glycogen syntahse kinase 3/F_A (Fiol et al., 1988). The findings presented in this paper and other observations (Fiol et al., 1987, 1988; Ramakrishna et al., 1983, 1989) provide examples where the presence of phosphate in the substrate and not a requisite change in the activity of any particular kinase determines the phosphate content of specific sites.

Recently, it has been suggested that the insulin-induced increase in ATP-citrate lyase, site A phosphorylation (Ser 454), is due in part to an insulin-mediated increase in the activity of another cAMP-independent protein kinase (Yu et al., 1990). As there is a glutamic acid at position 457 of ATP-citrate lyase, a configuration that promotes phosphorylation of a serine three positions amino terminal by casein kinase II (Litchfield

et al., 1990), and as evidence indicates that insulin increases casein kinase II activity (Sommercorn et al., 1987), it is suggested that in adipose tissue insulin increases the activity of a casein kinase II like kinase with specificity for Ser 454 (site A).

Glycogen synthase activity is regulated by a phosphorylation/dephosphorylation mechanism. The phosphoenzyme has little activity without glucose 6-phosphate, whereas the dephospho form is almost fully active in the absence of this allosteric modifier. Though no physiological change in ATP-citrate lyase activity as a function of its phosphorylation has as yet been found, ATP-citrate lyase activity (as a function of specific seryl and threonyl phosphorylations) may vary when assayed in the presence of proper allosteric modifiers.

MFPK differs from the rabbit muscle glycogen synthase kinase 3 as MFPK phosphorylates ATP-citrate lyase, acetyl-CoA carboxylase, and site 2 of glycogen synthase (Ramakrishna & Benjamin, 1985; Sheorain et al., 1985b) which are not phosphorylated by glycogen synthase kinase 3 (Cohen et al., 1985). In addition, glycogen synthase kinase 3 used GTP as substrate with at least 65% of the efficiency of ATP whereas GTP was found to be a very poor substrate for MFPK. MFPK has many similar properties to glycogen synthase kinase $3/F_A$ such as its behavior on various columns during purification (Cohen et al., 1985; Ramakrishna & Benjamin, 1985; Fiol et al., 1990) and its ability to phosphorylate glycogen synthase, glycogen synthase synthetic peptide, and inhibitor 2. These similarities suggest that MFPK may be a physiological isozyme of glycogen synthase kinase 3 in rat adipose and liver tissues.

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Registry No. MFPK, 9026-43-1; ATP-citrate lyase, 9027-95-6; glycogen synthase kinase, 9059-09-0; phosphoprotein phosphatase, 9025-75-6.

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Protein Kinase C Interaction with Calcium: A Phospholipid-Dependent Process†

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ABSTRACT: The calcium-binding properties of calcium- and phospholipid-dependent protein kinase C (PKC) were investigated by equilibrium dialysis in the presence and the absence of phospholipids. Calcium binding to PKC displayed striking and unexpected behavior; the free proteins bound virtually no calcium at intracellular calcium concentrations and bound limited calcium (about 1 mol/mol of PKC) at 200 μM calcium. However, in the presence of membranes containing acidic phospholipids, PKC bound at least eight calcium ions per protein. The presence of 1 μM phorbol dibutyrate (PDBu) in the dialysis buffer had little effect on these calcium-binding properties. Analysis of PKC–calcium binding by gel filtration under equilibrium conditions gave similar results; only membrane-associated PKC bound significant amounts of calcium. Consequently, PKC is a member of what may be a large group of proteins that bind calcium in a phospholipid-dependent manner. The calcium concentrations needed to induce PKC–membrane binding were similar to those needed for calcium binding (about 40 μM calcium at the midpoint). However, the calcium concentration required for PKC–membrane binding was strongly influenced by the phosphatidylserine composition of the membranes. Membranes with higher percentages of phosphatidylserine required lower concentrations of calcium. These properties suggested that the calcium sites may be generated at the interface between PKC and the membrane. Calcium may function as a bridge between PKC and phospholipids. These studies also suggested that calcium-dependent PKC–membrane binding and PKC function could be regulated by a number of factors in addition to calcium levels and diacylglycerol content of the membrane.

The calcium- and phospholipid-dependent protein kinase C (PKC)[1] is an important regulatory enzyme believed to be critically situated in the signal transduction cascade (Nishizuka, 1986; Nikkawa & Nishizuka, 1986). The activation of PKC by diacylglycerols links this enzyme to the regulatory scheme known as the phosphatidylinositol cycle. PKC is also a phorbol ester receptor (Castagna et al., 1982), and many phorbol ester effects are attributed to PKC. Recently, PKC has been shown to consist of a family of closely related isozymes [for a review, see Nishizuka (1989)]. Despite some molecular heterogeneity, most PKC isozymes exhibit similar biochemical properties including activation by calcium and phospholipids.

Association of PKC with membranes showed a calcium requirement that was distinct from that of activation (Bazzi & Nelsestuen, 1987; Wolf et al., 1985). In addition, calcium alters the phorbol ester binding properties of membrane-associated PKC. Thus, calcium appears to play multiple roles in the various functions of PKC. While this evidence suggests that PKC is a calcium-binding protein, we are not aware of reports of direct PKC–calcium-binding measurements. The primary sequence of PKC did not reveal the presence of known calcium sites (Parker et al., 1986; Ohno et al., 1987), and a unique structure may be responsible for calcium binding. Since PKC is an intracellular protein, it would be expected to bind calcium with high affinity.

This study was initiated to examine the calcium-binding properties of protein kinase C. A surprising result was that free PKC bound virtually no calcium at intracellular calcium concentrations and bound very little calcium at much higher calcium concentrations. However, PKC bound a large number of calcium ions (at least 8) when acidic phospholipids were present. The presence of phorbol esters did not significantly influence the the calcium-binding properties of PKC.

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), and dansyl-PE were purchased from the Sigma Chemical Co. [γ-^{32}P]ATP (3 Ci/mmol) and 1,2-dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline (73 Ci/mmol) were purchased from Amersham Corp. $^{45}CaCl_2$ (32.89

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PKC, phospholipid- and calcium-dependent protein kinase C.

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