Regulation of γ-glutamylcysteine synthetase by protein phosphorylation

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We previously reported that the activity of γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2), the rate-limiting enzyme in GSH synthesis, can be acutely inhibited ~ 20–40 % by agonists of various signal transduction pathways in rat hepatocytes [Lu, Kuhlenkamp, Garcia-Ruiz and Kaplowitz (1991) J. Clin. Invest. **88**, 260–269]. We have now examined the possibility that GCS is phosphorylated directly by activation of protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CMK). Phosphorylation of GCS was studied using both purified rat kidney GCS and cultured rat hepatocytes by immunoprecipitating the reaction product with specific rabbit anti-(rat GCS heavy subunit) (anti-GCS-HS) antibodies. All three kinases, PKA, PKC and CMK, phosphorylated rat kidney GCS-HS in a Mg^{2+}-concentration-dependent manner, with the highest degree of phosphorylation occurring at 20 mM Mg^{2+}. The maximum incorporation of phosphate in mol/mol of GCS was 1.17 for PKA, 0.70 for PKC and 0.62 for CMK. The degree of phosphorylation was correlated with the degree of loss of GCS activity, and no additional inhibition occurred when GCS was phosphorylated by all three kinases, suggesting that the kinases phosphorylated the same site(s). Phosphoamino analysis showed that all three kinases phosphorylated serine and threonine residues. Two-dimensional phosphopeptide mapping demonstrated that all three kinases phosphorylated the same five peptides, both PKA and PKC phosphorylated two other peptides, and only PKA phosphorylated one additional peptide. Phosphorylation of GCS decreased its V_{max} for cysteine and glutamate without changing its K_m. Finally, treatment of cultured rat hepatocytes with dibutyryl cAMP and phenylephrine significantly increased the phosphorylation of GCS, suggesting a potentially important physiological role. In summary, we have demonstrated that GCS is phosphorylated and suggest that phosphorylation/dephosphorylation may regulate GCS activity.

INTRODUCTION

Glutathione (GSH) is a tripeptide, γ-glutamylcysteinylglycine, that is synthesized by virtually all mammalian cells. GSH plays a vital role in defending organisms against toxins and free radicals, and in storing and transferring cysteine [1]. The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and the formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is rate-limiting and catalysed by γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2), which is regulated physiologically by feedback competitive inhibition by GSH and by the availability of cysteine [1,2]. The GCS enzyme is composed of a heavy (molecular mass ~ 73 kDa) and a light (molecular mass ~ 30 kDa) subunit, which are encoded by different genes and are dissociated under reducing conditions [3,4]. The GCS heavy subunit (GCS-HS) exhibits all of the catalytic activity of the isolated enzyme, as well as feedback inhibition by GSH [5]. The light subunit is enzymically inactive but plays an important regulatory function by lowering the K_m of GCS for glutamate and raising the K_i for GSH [4,6]. In many conditions (i.e. drug-resistant tumour cell lines, treatment with methyl mercury hydroxide) where GCS activity is increased, there is also an increase in the GCS-HS mRNA level [7–11]. We have also shown in primary cultures of rat hepatocytes that the activity of GCS can be induced by insulin (1 μg/ml) or cortisol (50 nM) treatment [12], and by lowering the initial plating cell density [13]. Recently we demonstrated that the mechanism of these effects was an increase in the transcription of GCS-HS [14]. Thus regulation of GCS-HS gene expression appears to be critical for GSH homoeostasis.

In addition to gene regulation, the activity of GCS is also under post-translational regulation. We showed previously that treatment of cultured hepatocytes and perfused rat livers with cAMP-, Ca^{2+}- and protein kinase C (PKC)-dependent hormones all lowered the capacity for GSH synthesis by inhibiting GCS activity [15]. We hypothesize that the mechanism of down-regulation of GCS by these different signal transduction mediators is via enzyme phosphorylation. This is supported by the fact that the inhibitory action occurred within minutes, was directed within the cytosol, and was blocked by a protein kinase A (PKA) inhibitor in the case of dibutyryl cAMP (DBcAMP), or by PKC and calmodulin inhibitors in the case of phenylephrine. In our current work we examined the possibility that GCS is phosphorylated directly by PKA, PKC and Ca^{2+}/calmodulin-dependent kinase II (CMK) and that phosphorylation alters GCS activity.

MATERIALS AND METHODS

Materials

PKA and PKC were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). CMK and calmodulin were obtained from Calbiochem (La Jolla, CA, U.S.A.). D,L-Buthionine-[S,R]-sulphoximine, α-aminobutyrate, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, cortisol, insulin, diolein, phosphatidylserine, phosphoserine, phosphothreonine, phosphotyrosine, collagenase (type IV), BSA, fetal bovine serum, dibutyryl cAMP (DBcAMP), phenylephrine, glutathione S-transferase, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (Tos-Phe-CH_2Cl;

Abbreviations used: GCS, γ-glutamylcysteine synthetase; GCS-HS, GCS heavy subunit; PKA, protein kinase A; PKC, protein kinase C; CMK, Ca^{2+}/calmodulin-dependent kinase II; DBcAMP, dibutyryl cAMP; mBCL, monochlorobimane; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'); DTT, dithiothreitol; TBS, 50 mM Tris/HCl, pH 8.0, 120 mM NaCl; TNB, 1 % Nonidet P40 and 1 mg/ml BSA in TBS.

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'TPCK')-treated trypsin, goat anti-rabbit IgG (whole molecule) antibody–agarose and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monochlorobimane (mBCl) was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Dulbecco's modified Eagle's medium/Ham's F12 medium was purchased from Irvine Scientific (Irvine, CA, U.S.A.). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{32}P]P_i (9000 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade and were obtained from commercial sources.

Animals

Male Sprague–Dawley rats (Harlan Laboratory Animals, Inc., San Diego, CA, U.S.A.), weighing 260–320 g, were maintained on Purina rodent chow (Ralston Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum*. All animals received humane care in compliance with the National Research Council's criteria for humane care as outlined in 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23; revised 1985).

Rat hepatocyte culture preparation

Isolation of hepatocytes was done aseptically according to the method of Moldeus et al. [16]. Initial cell viability was $> 90\%$ as determined by exclusion of 0.2% Trypan Blue. The plating medium was Dulbecco's modified Eagle's medium/Ham's F12 with high glucose (3151 mg/l) supplemented with excess methionine (1 mM), 10% fetal bovine serum, insulin (1 μg/ml) and cortisol (50 nM). Aliquots of 2×10^6 cells in 5 ml of medium were plated on 60 mm $\times$ 15 mm dishes precoated with rat tail collagen and incubated at 37 °C in 5% CO_2/95% air. At 2 h after plating, the medium was changed to remove dead, unattached, cells; the fresh medium lacked fetal bovine serum. Cell attachment averaged $\sim 60\%$. Phosphorylation of cultured cells was carried out 18–20 h after plating.

Purification of GCS from rat kidney and measurement of GCS activity

GCS was purified from rat kidney exactly as described by Sekura and Meister [17]. GCS activity was determined as described by Seelig and Meister [18] with slight modifications. The assay measures the rate of oxidation of NADH (assumed to be equal to the rate of formation of ADP) from the change in absorbance at 340 nm in reaction mixtures (final volume 1 ml) containing the following: 0.1 M Tris/HCl, pH 8.0, 150 mM KCl, 5 mM Na_2ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM Na_2EDTA, 0.2 mM NADH, 17 μg of pyruvate kinase and 17 μg of lactate dehydrogenase. In the first ATP-requiring step, GCS catalyses the formation of α-aminobutyrylglutamate and ADP from L-α-aminobutyrate and glutamate; in the second step, ATP is regenerated as pyruvate kinase catalyses the dephosphorylation of phosphoenolpyruvate to pyruvate; and in the final step, lactate dehydrogenase catalyses the conversion of pyruvate into lactate in a reaction that oxidizes NADH to NAD^+. The assay was performed on each sample in the presence or absence of 0.25 mM D,L-buthionine-[S,R]-sulphoximine (an irreversible GCS inhibitor). The value obtained in the presence of this inhibitor was considered as the blank for the sample. The net value represents GCS activity, where 1 unit represents 1 nmol of ADP formed/min per mg of protein. Protein was measured by the method of Bradford [19]. The activity of purified GCS was typically 2000–2500 units. The enrichment achieved on purification was typically 1500–2000-fold. This degree of enrichment is in close agreement with that reported by Sekura and Meister [17].

Gel electrophoresis

PAGE was carried out under denaturing and non-denaturing conditions as described [20]. Briefly, for non-denaturing gel electrophoresis, purified GCS (12 μg in 10 μl) was treated with an equal volume of loading buffer (0.01 M Tris/glycine, pH 8.3, containing 4% SDS, 0.2% Bromophenol Blue, 20% glycerol) for 30 min at 65 °C and then transferred to 4 °C. This mixture was applied to a 7% acrylamide gel and electrophoresed using 0.01 M Tris/glycine, pH 8.3, buffer at 100 V. The effect of dithiothreitol (DTT) on the molecular mass of GCS was assessed by inclusion of DTT (200 mM) in the loading buffer. The effect of kinases on the molecular mass of purified GCS was assessed by first treating GCS with PKA, PKC or CMK as described below in the presence of 20 mM Mg^{2+} and non-radioactive ATP (0.1 mM), and then subjecting the mixture to gel electrophoresis as described above. In denaturing gel electrophoresis, GCS was mixed in Laemmli sample buffer (containing 100 mM DTT) and boiled for 3 min followed by SDS/10%-PAGE.

Phosphorylation of purified rat kidney GCS

Phosphorylation of purified GCS was carried out according to published protocols [21,22]. Purified GCS (7 μg) was suspended in 30–50 μl (30 μl for CMK; 50 μl for PKA and PKC) of phosphorylation buffer containing 20–50 mM (20 mM for PKA and PKC; 50 mM for CMK) Tris/HCl, pH 7.4, and 1–20 mM $MgCl_2$. The following were also added: for PKA, 0.05% Triton X-100, 1 mM EDTA and 0.2 m-units of enzyme; for PKC, 1 mM $CaCl_2$, 0.5 mg/ml phosphatidylserine, 10 μg/ml diolein and 0.03 m-unit of enzyme; for CMK, 1 mM $CaCl_2$, 50 μg/ml calmodulin and 20 ng of enzyme. The phosphorylation reaction was started by adding [γ-^{32}P]ATP (0.1 mM; 1–3 μCi/nmol). The reaction was stopped after 30 min at 30 °C by adding Laemmli sample buffer. Samples were boiled for 3 min and subjected to SDS/10%-PAGE. Gels were dried and subjected to autoradiography. Because all three kinases autophosphorylated their own subunits, which interfered with the recognition of GCS phosphorylation, we immunoprecipitated the phosphorylated product using specific rabbit anti-GCS-HS antibody. This antibody was raised against a synthetic GCS-HS peptide (TVEDNMRKRRKEA) which corresponds to amino acid residues 119–131 of rat kidney GCS-HS [3], and its specificity was demonstrated in Western blot analysis as recently described [14].

To conclusively demonstrate phosphorylation of GCS, at the end of the phosphorylation reaction samples were immediately placed on ice and 10 μl of rabbit anti-(rat GCS-HS) antiserum (with or without pretreatment overnight at 4 °C with 10 μg of GCS-HS peptide), 250 μl of ice-cold TNB [1% Nonidet P40 and 1 mg/ml BSA in TBS (50 mM Tris/HCl, pH 8.0, 120 mM NaCl)] containing 2 mM PMSF was added and incubated at 4 °C overnight. To recover the antibody–antigen complex, 50 μl of goat anti-rabbit IgG antibody conjugated to agarose (Sigma, cat. no. A1027) was added and the mixture was incubated at 4 °C for 1 h on a rotator (Tube Rotator; Scientific Equipment Products Co., Baltimore, MD, U.S.A.). The mixture was centrifuged for 1 min at 2000 ***g***, the supernatant was discarded and the pellet was resuspended and washed with 2×1 ml of TNB, 2×1 ml of TBS and 1 ml of 50 mM Tris, pH 6.8. Each wash was followed by centrifuging for 1 min at 2000 ***g***. Prior to the last wash in Tris buffer, the pellet was transferred to a new tube. At the end of the washes, 20 μl of Laemmli sample buffer was added and the

mixture was placed in a water bath at 65 °C for 30 min, followed by centrifugation for 1 min in a Microfuge, and the supernatant was then subjected to SDS/PAGE (10 % acrylamide). Phosphorylated GCS-HS was visualized on dried gels by autoradiography (Kodak XRP-5 X-ray film).

Phosphoamino acid analysis

Gel slices containing ^{32}P-labelled GCS-HS were excised, rehydrated in 0.4 ml of 50 mM ammonium bicarbonate buffer, pH 8, homogenized and incubated with Tos-Phe-CH_2Cl-treated trypsin (final concentration 50 μg/ml) for 16 h at 37 °C on an Orbital Shaker (Bellco Biotechnology, Vineland, NJ, U.S.A.) at 120 rev./min. Another aliquot of Tos-Phe-CH_2Cl-treated trypsin (final concentration 50 μg/ml) was added, and the samples were incubated for another 5 h under the same conditions. At the end of the incubation the samples were spun (14000 ***g***, 5 min), and the supernatants were lyophilized to remove residual ammonium bicarbonate and then resuspended in 10 μl of deionized water. An aliquot (7 μl) was removed for tryptic peptide analysis, while the remainder was lyophilized and subjected to acid hydrolysis by heating at 110 °C in 6 M HCl (0.1 ml/tube) for 120 min. HCl was removed by repeated lyophilization, and the samples were dissolved in deionized water (5 μl/tube). Samples (5 μl) and a mixture of standards containing 7 μg each of phosphoserine, phosphothreonine and phosphotyrosine (total volume 1 μl) were spotted on to a 20 cm × 20 cm thin-layer cellulose chromatography plate (Analtech, Inc., Neward, DE, U.S.A.) followed by ascending chromatography in butan-1-ol/pyridine/acetic acid/water (6.5:5:1:4, by vol.). After drying, the ^{32}P-labelled phosphoamino acids were visualized by autoradiography and compared with ninhydrin-stained phosphoamino acid standards.

Two-dimensional phosphopeptide mapping

To perform two-dimensional peptide mapping, 400 μl of pH 1.9 electrophoresis buffer containing acetic acid/formic acid/water (3:1:16, by vol.) was added to samples saved for tryptic peptide analysis, which were then centrifuged in a Microfuge at 12000 ***g*** for 10 min; the clear supernatant was lyophilized. The pellets were dissolved in 5 μl of pH 1.9 electrophoresis buffer and spotted on to 20 cm × 20 cm thin-layer cellulose chromatography plates, and then moistened with the same buffer. The peptides were separated by electrophoresis with acetic/formic acid/water (3:1:16, by vol.) for 40 min at 1000 V (Bio-Rad Power PAC 3000 power supply), air-dried and then chromatographed in a second dimension at 25 °C with butan-1-ol/pyridine/acetic acid/water (6.5:5:1:4, by vol.). Plates were air-dried and then autoradiographed.

Stoichiometry of phosphorylation of GCS

To determine the stoichiometry of phosphorylation, purified GCS (1 μg or 10 pmol) in 40 or 50 μl volume (40 μl for CMK; 50 μl for PKA and PKC) was phosphorylated by incubating with PKA (0.114 μg or 2.78 pmol), PKC (0.15 μg or 1.88 pmol) or CMK (0.15 μg or 0.23 pmol) in buffers containing [γ-^{32}P]ATP (0.1 mM; 2–4 μCi/nmol) as described above for various durations. These amounts of kinases maximally phosphorylated GCS. The reactions were stopped by adding Laemmli sample buffer, boiled for 3 min and subjected to SDS/10 %-PAGE. The gels were stained with Coomassie Blue, and GCS bands were cut from the gel and added to 10 ml of ScintiVerse E for scintillation counting (Beckman model LS6000TA Liquid Scintillation Counter). For each experiment, blanks were obtained by cutting the gel at identical locations from lanes containing products phosphorylated in the presence of kinases alone. These blanks were subtracted from the above values.

Kinetic parameters of phosphorylation of GCS by PKA, PKC and CMK

To determine the kinetic parameters of phosphorylation of GCS, phosphorylation reactions were carried out using various amounts of GCS (1–15 μg in 40–50 μl, i.e. 0.20–3.65 nM) for 30 min. The rate of phosphate incorporation was linear up to 30 min for all three kinases. The amount of phosphate incorporated per μg of kinase used in these reactions was determined as described above.

Kinetic data were fitted with the Michaelis–Menten equation in the case of PKA and PKC, and with the Hill equation in the case of CMK, using SAAM II software (developed at the SAAM Institute, Redmond, WA, U.S.A.) on a Power MacIntosh 7100/66 computer, to obtain the V_{max} and K_m values.

Effect of phosphorylation on GCS activity

The effect of phosphorylation on GCS activity was examined by measuring the activity of GCS as described above after pretreating purified GCS (10 μg) in the presence or absence of the three kinases and various concentrations of Mg^{2+} (0–20 mM) in their respective phosphorylation buffers containing 0.1 mM ATP for 30 min.

Phosphorylation of GCS in cultured rat hepatocytes

After overnight culture, rat hepatocytes were incubated for 1 h in phosphate-free medium [1 litre: 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 116 mM NaCl and 5.5 mM D-glucose, containing 10 ml of 100 × vitamin mix, 10 ml each of the amino acids and 29.3 ml of 7.5 % $NaHCO_3$ provided in the Selectamine kit (Gibco-BRL, Grand Island, NY, U.S.A.)]. Medium was changed and the cells were then labelled with [^{32}P]P_i (1 mCi/ml) in the same medium for 5 h in the presence of DBcAMP (0.2 mM), phenylephrine (0.2 mM) or vehicle (control) in the incubator (5 % CO_2/95 % air) at 37 °C. The incubation was terminated by washing the cells with 2 × 2 ml of ice-cold PBS. The cells were scraped off the plate with a rubber policeman into 0.2 ml of TNB containing 2 mM PMSF and 100 mM NaF (stop buffer), homogenized and centrifuged at 12000 ***g*** for 30 min. Supernatants were saved for immunoprecipitation as described above with slight modifications. Samples were placed on ice and 10 μl of rabbit pre- or post-immune anti-(rat GCS-HS) serum and 250 μl of ice-cold TNB containing 2 mM PMSF were added, followed by incubation at 4 °C overnight. Subsequent steps were exactly the same as described above. To quantify differences in GCS phosphorylation, autoradiograms were analysed by densitometry (model SLR-2D Soft Laser Scanning Densitometer; Biomed Instruments, Fullerton, CA, U.S.A.).

Effect of phosphorylation on the kinetic parameters of GCS

We examined the effects of adding DBcAMP and PKA to liver cytosol on the kinetics of GCS using mBCl, as described previously [12,15]. This approach is not only more physiological, but also allows measurement of GSH synthesis directly from its substrates, i.e. cysteine, glutamate and glycine. Since the first step, catalysed by GCS, is rate-limiting, this is a convenient measure of GCS activity.

Liver cytosol was prepared as described [12,15] and dialysed overnight using molecular porous membrane tubing (cut-off 12–14 kDa; Spectrum Medical Industries, Inc., Los Angeles,

CA, U.S.A.) in a 100× volume of 0.01 M sodium phosphate, pH 7.4, in order to avoid high background with mBCl and to eliminate feedback inhibition exerted by pre-existing GSH. After overnight dialysis, the cytosolic GSH concentration was decreased by 99–99.5%.

The rate of GSH synthesis was measured by addition of dialysed liver cytosol (2.0–3.5 mg of protein) to a cuvette containing 100 mM Tris/HCl, 150 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, pH 7.3, glutamate (0.1–10 mM), glycine (10 mM), ATP (3 mM), cysteine + DTT (1:1) (0.025–0.5 mM) and 100 μM mBCl in a final volume of 2.5 ml at 37 °C. The difference in the initial rate of linear increase in fluorescence over 6–8 min with and without pretreatment with buthionine sulphoximine (5 mM for 5 min at 37 °C) is equivalent to the rate of GSH synthesis. The change in fluorescence was converted to GSH concentration units by using standard curves. Specifically, the formation of fluorescent adducts was monitored by adding mBCl (100 μM) and glutathione S-transferase (0.1 unit/ml) to the cuvette containing GSH standards. Standard curves were then generated by applying linear regression analysis to the relationship between fluorescence and GSH concentration.

The effects of DBcAMP (0.2 mM) and PKA (200 μg/ml) on the rate of GSH synthesis were studied by adding these agents directly to the cytosol. The kinetic parameters of GCS for cysteine and glutamate were obtained by keeping glutamate constant at 10 mM while varying the cysteine concentration, or by keeping cysteine constant at 0.1 mM while varying the glutamate concentration, respectively. Kinetic data were fitted with the Michaelis–Menten equation using the SAAM program to extract the V_{max} and K_m values.

Statistical analysis

The effect of varying the concentration of Mg^{2+} on the phosphorylation-mediated inhibition of GCS activity was analysed by ANOVA followed by Fisher's test. Significance was defined as $P < 0.05$.

In the kinetic fitting of data, the S.E.M.s at each concentration of substrate were submitted as statistical weights (proportional to the inverse of standard errors). The significance of the differences in kinetics parameters were determined using the paired Student's t test.

RESULTS

Phosphorylation of purified rat kidney GCS

Using purified rat kidney GCS, we first demonstrated phosphorylation of GCS *in vitro*. Figure 1 shows that PKA, PKC and CMK all phosphorylated GCS-HS in a Mg^{2+}-concentration-dependent manner, with the highest degree of phosphorylation achieved with 20 mM Mg^{2+}. The identity of the phosphorylated protein was confirmed by immunoprecipitation using specific anti-GCS-HS antibody. No band was seen if the phosphorylated product was immunoprecipitated by anti-GCS-HS antibody that had been pretreated with GCS-HS peptide (Figure 1, lanes E) or if phosphorylation was performed in the presence of the kinase alone (lanes F). The band at 73 kDa represents GCS-HS, and a minor band at 63 kDa was also sometimes noted (Figure 1, PKC). This minor band was also sometimes present in the purified GCS protein when analysed by Western blot analysis (results not shown), and was previously described by Yan and Meister [3] to be related antigenically to GCS-HS.

Phosphoamino analysis of phosphorylated GCS-HS revealed that all three kinases phosphorylated threonine and serine residues (Figure 2). Two-dimensional phosphopeptide mapping

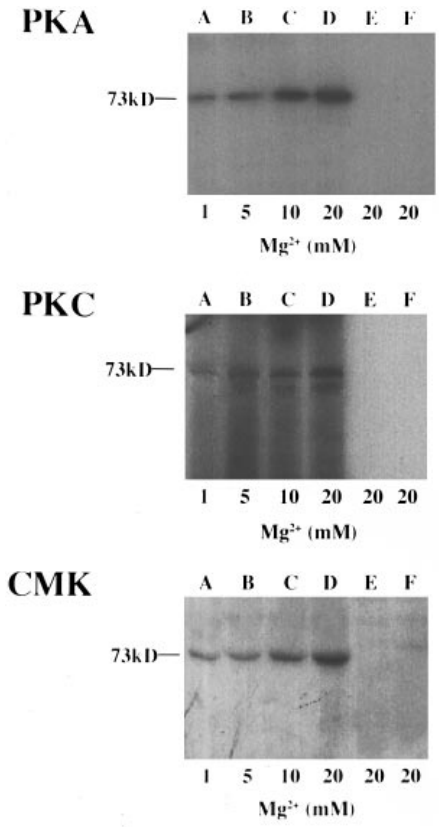

Figure 1 Phosphorylation of GCS-HS by PKA, PKC and CMK

Representative autoradiograms from three separate experiments with each of the kinases are shown. Purified rat kidney GCS was phosphorylated, immunoprecipitated by specific anti-(rat GCS-HS) antibodies, and subjected to SDS/PAGE and autoradiography as described in the Materials and methods section. Lanes A–D represent phosphorylated GCS-HS in the presence of various Mg^{2+} concentrations. In lanes E, GCS-HS was immunoprecipitated using antibodies that had been pretreated with excess GCS-HS peptide to which the antibodies were raised; in lanes F, only phosphorylated kinases were immunoprecipitated. The bands at 73 kDa (kD) represent GCS-HS. Sometimes a minor band at 63 kDa also appeared, as previously described [3]. The autoradiograms for PKA and PKC were exposed for 4 days, and that for CMK was exposed for 5 days.

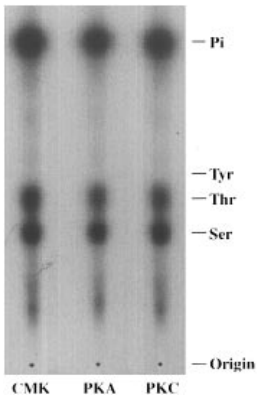

Figure 2 Phosphoamino analysis of phosphorylated GCS-HS

After phosphorylation of GCS-HS with each of the three kinases at 20 mM Mg^{2+}, gel slices containing the phosphorylated GCS-HS were excised and processed for phosphoamino analysis as described in the Materials and methods section. A representative autoradiogram from three separate experiments is shown. The positions of the phosphoamino acids were confirmed by ninhydrin staining of standards.

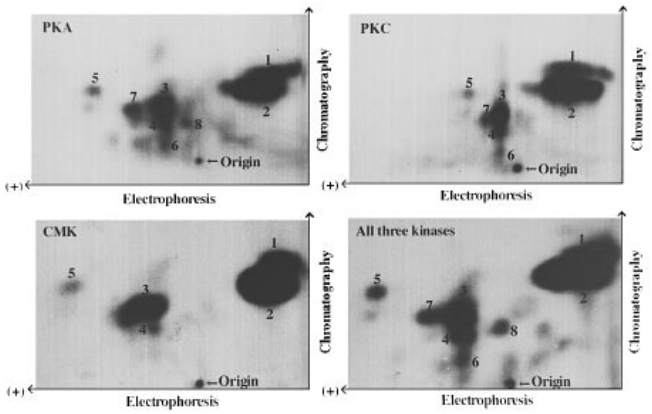

Figure 3 Two-dimensional phosphopeptide mapping

After phosphorylation of GCS-HS with each of the three kinases at 20 mM Mg^{2+}, separately and then together, gel slices containing the phosphorylated GCS-HS were excised and processed for two-dimensional peptide mapping as described in the Materials and methods section. A representative autoradiogram from three separate experiments is shown. Peptide numbering is discussed in the text.

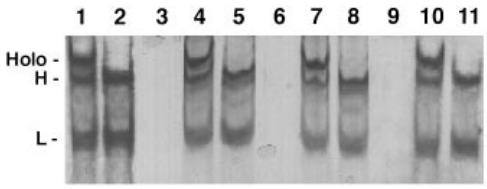

Figure 4 Effect of DTT and protein kinases on gel electrophoresis of purified GCS under non-denaturing conditions

Purified GCS was analysed by gel electrophoresis under non-denaturing conditions as described in the Materials and methods section. Lanes 1 and 2 represent purified GCS (12 μg per lane) in the absence and presence of 100 mM DTT respectively. No bands are seen in the presence of PKA (lane 3), PKC (lane 6) or CMK (lane 9) alone. After treating purified GCS (8 μg) with each of the kinases, the products in the absence (even lanes) or presence (odd lanes) of 100 mM DTT are shown for PKA (lanes 4 and 5), PKC (lanes 7 and 8) and CMK (lanes 10 and 11). The positions of the holoenzyme (Holo), heavy subunit (H) and light subunit (L) of GCS are marked.

using electrophoresis in the first dimension and TLC in the second dimension (Figure 3) showed that the same five peptides (nos. 1–5 in Figure 3) were phosphorylated by all three kinases, two other peptides (nos. 6 and 7) were phosphorylated by both PKA and PKC, and one additional peptide (no. 8) was phosphorylated by PKA only. The same number of phosphopeptides was observed even after an additional 21 h of treatment of phosphorylated GCS with Tos-Phe-CH_2Cl-treated trypsin as described in the Materials and methods section, excluding the possibility of partial digestion (results not shown).

Effect of phosphorylation on GCS subunit structure

Figure 4 shows that purified rat kidney GCS is made up of both holoenzyme with molecular mass of $\sim$ 100 kDa and individual heavy and light subunits with molecular masses of 73 kDa and 30 kDa respectively when analysed by non-denaturing PAGE (lane 1). In the presence of 100 mM DTT, the holoenzyme was dissociated completely into its subunits (lane 2). Treatment with PKA (lane 4), PKC (lane 7) or CMK (lane 10) did not cause disappearance of the holoenzyme. Addition of 100 mM DTT then dissociated the holoenzyme into its subunits (lanes 5, 8 and 11). This suggests that phosphorylation of GCS does not cause dissociation of the subunits.

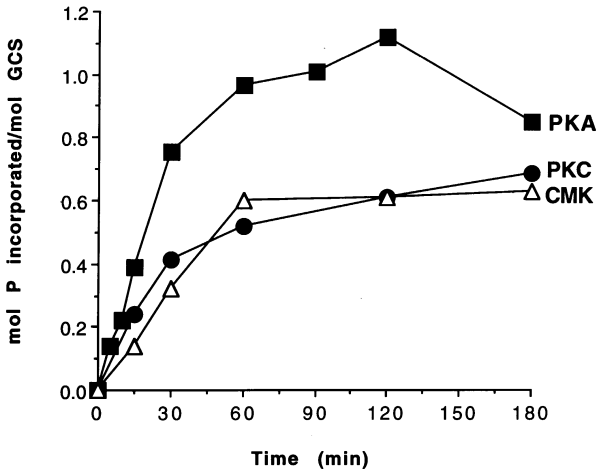

Figure 5 Time course of phosphorylation of GCS by PKA, PKC and CMK

Purified GCS (1 μg) was phosphorylated by incubating with PKA (0.114 μg), PKC (0.15 μg) or CMK (0.15 μg) in buffer containing [γ-^{32}P]ATP (0.1 mM, 2–4 μCi/nmol) as described in the Materials and methods section for up to 3 h. Results represent mean values from two separate experiments which agreed closely.

Table 1 Kinetic parameters of PKA, PKC and CMK for the phosphorylation of GCS

Results are expressed as means $\pm$ S.D. Incoporation of phosphate (P) was measured using various amounts of GCS (1–15 μg in 40–50 μl; 0.20–3.65 nM). Mean values from three seperate experiments for each kinase were analysed for kinetic fitting by the SAAM program as described in the Materials and methods section.

Kinase	K_m (nM)	V_{max} (nmol of P incorporated/30 min per μg)
PKA	0.49 ± 0.13	0.24 ± 0.02
PKC	0.64 ± 0.04	0.11 ± 0.004
CMK	0.93 ± 0.22	0.21 ± 0.03

Stoichiometry of phosphorylation of GCS

Figure 5 shows the time courses of phosphorylation of GCS by PKA, PKC and CMK. The rate of phosphate incorporation remained linear up to 30 min with all three kinases. Maximum phosphate incorporation occurred after 60 min for CMK, 120 min for PKA and 180 min for PKC. The stoichiometry (mol of phosphate/mol of GCS) as determined under these optimal conditions was 1.17 ± 0.04 for PKA, 0.70 ± 0.09 for PKC and 0.62 ± 0.04 for CMK (results represent means $\pm$ S.E.M. from 5–6 separate determinations for each kinase).

Kinetic parameters of phosphorylation of GCS by PKA, PKC and CMK

Table 1 describes the kinetic parameters of phosphorylation of GCS by these kinases. The K_m value was lowest for PKA and highest for CMK.

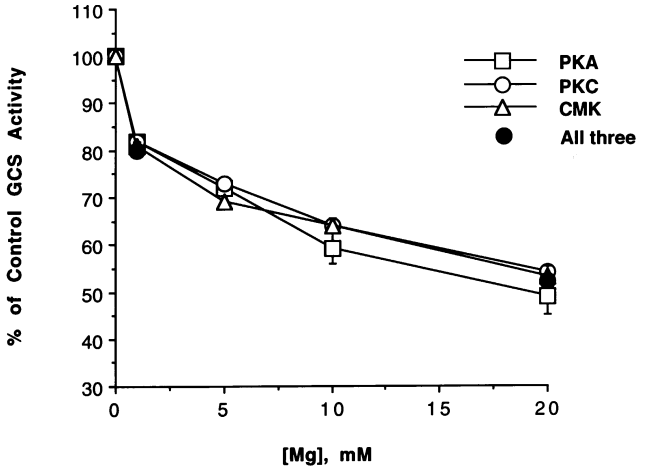

Figure 6 Effect of phosphorylation on GCS activity

After phosphorylation of purified GCS (10 μg) with each of the three kinases separately, and then together, in the presence of different Mg^{2+} concentrations, GCS activity was measured as described in the Materials and methods section. The Mg^{2+} concentration was varied only during phosphorylation; it was 20 mM in the GCS activity assay. Controls were treated with the same phosphorylation buffers containing 20 mM Mg^{2+} but without kinases. In the absence of kinases, the concentration of Mg^{2+} during this pretreatment period did not influence the subsequently measured GCS activity. Results represent means $\pm$ S.E.M. from five to seven experiments using three different preparations of purified GCS. GCS activity (units) for PKA: control, 2115 $\pm$ 18; 1 mM Mg^{2+}, 1742 $\pm$ 10; 5 mM Mg^{2+}, 1528 $\pm$ 42; 10 mM Mg^{2+}, 1238 $\pm$ 78; 20 mM Mg^{2+}, 1031 $\pm$ 92; for PKC: control, 2118 $\pm$ 25; 1 mM Mg^{2+}, 1732 $\pm$ 20; 5 mM Mg^{2+}, 1542 $\pm$ 14; 10 mM Mg^{2+}, 1348 $\pm$ 32; 20 mM Mg^{2+}, 1139 $\pm$ 23; for CMK: control, 2060 $\pm$ 46; 1 mM Mg^{2+}, 1662 $\pm$ 23; 5 mM Mg^{2+}, 1417 $\pm$ 28; 10 mM Mg^{2+}, 1298 $\pm$ 34; 20 mM Mg^{2+}, 1087 $\pm$ 25. All activities in the presence of kinases were significantly different from their respective controls by ANOVA followed by Fisher's *t* test.

Effect of phosphorylation on GCS activity

We next determined the effect of phosphorylation on the activity of GCS. The degree of inhibition of GCS activity was correlated with the degree of phosphorylation (Figure 6). At 1 mM Mg^{2+}, each of the three kinases inhibited GCS activity by $\sim$ 20 %. This increased to $\sim$ 50 % at 20 mM Mg^{2+}. The degree of inhibition at low Mg^{2+} concentrations (1 and 5 mM) varied slightly (up to a maximum of 10 %) depending on the GCS preparation. At 1 mM Mg^{2+}, each of the three kinases resulted in inhibition of 15–25 % (average 20 %). No further inhibition of GCS activity was observed in the presence of all three kinases at either 1 or 20 mM Mg^{2+}.

Phosphorylation of GCS *in vivo*

To investigate whether phosphorylation of GCS is physiologically relevant, we next examined phosphorylation of GCS in cultured rat hepatocytes. In this model we previously showed that agonists of signal transduction pathways, such as DBcAMP and phenylephrine, inhibited GSH synthesis by 25–30 % [13]. Figure 7 shows that DBcAMP and phenylephrine significantly increased the phosphorylation of GCS-HS, by 91 and 210 % respectively over the control basal level, as determined by densitometry. Specificity was confirmed by the use of preimmune serum. Phosphoamino analysis and peptide mapping could not be performed because of the limited amount of phosphorylated GCS. GCS levels in hepatocytes are very low relative to those in kidney [3] and, in fact, phosphorylation of hepatic GCS was only

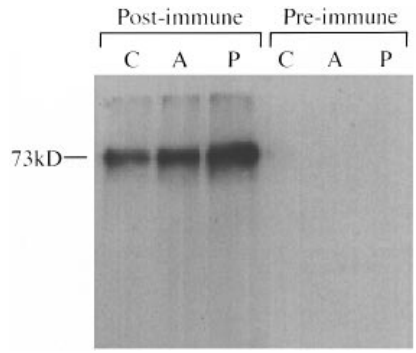

Figure 7 Phosphorylation of GCS-HS in cultured rat hepatocytes

Cultured rat hepatocytes were phosphorylated in the presence of 0.2 mM DBcAMP (lanes A), 0.2 mM phenylephrine (lanes P) or vehicle control (lanes C) as described in the Materials and methods section. Cells were then scraped off and subjected to immunoprecipitation with pre- or post-immune anti-(rat GCS-HS) serum. The band at 73 kDa (kD) represents GCS-HS. An autoradiogram that was exposed for 5 days, representative of three separate experiments, is shown.

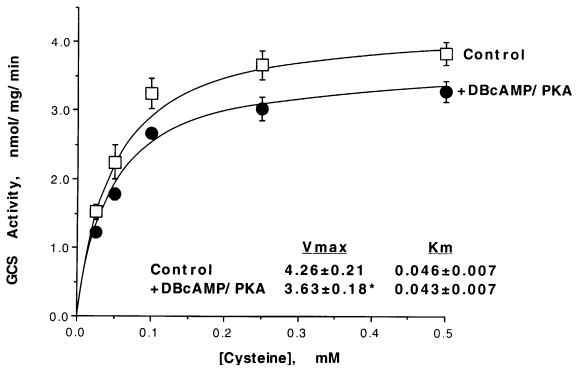

Figure 8 Effects of DBcAMP (0.2 mM) and PKA (200 μg/ml) on the kinetics of GCS with cysteine in rat liver cytosol

GSH synthesis rates in liver cytosol were measured while increasing the concentration of cysteine from 0.025 to 0.5 mM. V_{max} and K_m values were estimated by fitting the Michaelis–Menten equation to the data using the SAAM program. Mean data were weighted with their variances. Values represent means $\pm$ S.D. Data were fitted using mean values from five different liver cytosol samples. $^*P < 0.05$ compared with control (paired *t* test).

demonstrated after using a large amount of label and exposing the X-ray film for > 3 days.

Effect of phosphorylation on the kinetic parameters of GCS

Figure 8 shows the effect of phosphorylation on the kinetic parameters of GCS. GCS activity was measured using liver cytosol and the effect of PKA was studied by adding DBcAMP and PKA to the cytosol. Phosphorylation caused a significant decrease in the V_{max} of GCS for cysteine without changing the K_m. This is consistent with our previous findings of significant inhibition of GCS activity by DBcAMP + PKA at both 0.1 and 1 mM cysteine [15]. Similar results were also obtained for the kinetic parameters of GCS with glutamate [V_{max} (nmol/min per mg of protein): control, 3.45 $\pm$ 0.17; + DBcAMP and PKA, 2.81 $\pm$ 0.08; K_m (mM): control, 0.38 $\pm$ 0.04, + DBcAMP and PKA, 0.37 $\pm$ 0.03; means $\pm$ S.D.]. Data were fitted using mean

values from five different liver cytosol samples; the two V_{max} values are significantly different ($P < 0.05$ by paired Student's t test).

DISCUSSION

The intracellular GSH content is influenced by several factors: (1) the availability of cysteine, (2) the activity of GCS, (3) the rate of GSH efflux, and (4) intracellular utilization of GSH. Many recent studies have emphasized the importance of GCS in the overall regulation of GSH homoeostasis. In particular, drug resistance and oxidative stress are associated with increased cell GSH and, in both cases, increased GCS activity and steady-state GCS-HS mRNA levels have been found [7–9,11]. We recently showed increased GCS-HS gene transcription to be responsible for the observed increase in cellular GSH levels when cultured rat hepatocytes are treated with insulin or glucocorticoids, or are plated under low cell density [14]. Thus transcriptional regulation of GCS-HS clearly is important in determining GCS activity. We also suggested that GCS activity is modulated by protein phosphorylation, since we found a 20–40 % decrease in GCS activity in response to agonists of PKA, PKC and CMK [15]. There are several consensus phosphorylation site motifs for PKA, PKC and CMK in the published amino acid sequence of rat GCS-HS [3,23]. Thus we hypothesized that the change in GCS activity is a result of the direct phosphorylation of GCS-HS.

The present studies clearly demonstrate the phosphorylation of GCS-HS using both purified rat kidney GCS and cultured rat hepatocytes. The stoichiometry of phosphorylation was significant with all three kinases; that with PKA was greatest at 1.2, and those with PKC and CMK were similar at ~ 0.6–0.7. There are six potential CMK phosphorylation sites in GCS-HS: Thr^{132}, Thr^{188}, Ser^{331}, Thr^{506}, Ser^{567} and Ser^{591}. All of these are also potential PKA and PKC sites. At least five other potential PKA and PKC phosphorylation sites are also present in the GCS-HS sequence. GCS phosphorylation *in vitro* was correlated with loss of activity and, since no additional loss of activity was observed when GCS was phosphorylated by all three kinases, one or more of the phosphorylation sites shared by all three kinases is likely to be the target of regulation of GCS activity by phosphorylation/dephosphorylation. Additional studies involving site-directed mutagenesis will be required to elucidate the exact site(s) of phosphorylation that result in the inhibition of GCS activity.

We next examined the mechanism of the phosphorylation-mediated loss of GCS activity. There are several ways in which this can occur. Although the heavy subunit alone is catalytically active, the light subunit has an important regulatory function [4,6]. The heavy subunit is much less efficient enzymically than the holoenzyme, exhibiting a higher K_m for glutamate and a lower K_i for GSH [4,6]. We already know that the heavy subunit is phosphorylated. In addition, it would be important to examine (1) if phosphorylation leads to dissociation of the two GCS subunits, and (2) if the light subunit is also phosphorylated, which could then alter its regulatory function. Our data show that neither of these possibilities played a significant role in the phosphorylation-mediated inhibition of GCS activity. As demonstrated in Figure 4, the purified holoenzyme remained in the holoenzyme form after treatment with PKA, PKC or CMK. No phosphorylated GCS light subunits were detected by autoradiography after immunoprecipitation of phosphorylated GCS either using the purified enzyme or in cultured hepatocytes (Figures 1 and 7). Treatment of liver cytosol with DBcAMP and PKC lowered the V_{max} values of GCS for cysteine and glutamate without changing the K_m values. This is consistent with the notion that the light subunit is not involved in the phosphorylation-mediated inhibition of GCS activity, since the light subunit mainly affects the affinity or K_m value of the enzyme [4,6]. Thus the most likely scenario is that the PKA-, PKC- and CMK-mediated phosphorylation of GCS occur predominantly on the heavy subunit, which then leads to inactivation of the enzyme by some unknown mechanism. The degree of inhibition of activity may simply reflect the proportion of GCS that is phosphorylated at the critical site(s) by each of the kinases.

The demonstration of phosphorylation of GCS using cultured hepatocytes suggests that phosphorylation/dephosphorylation may be an important physiological regulatory mechanism for GCS. The inhibition of GCS activity was 20 % at 1 mM Mg^{2+}, a physiologically relevant concentration, similar to what we previously observed when GCS activity was measured in rat liver cytosol treated acutely with DBcAMP [15]. This degree of inhibition was again observed in the current study over a physiological range of intracellular cysteine concentrations (0.1–0.2 mM) (Figure 8). Although the degree of inhibition of GCS activity is small, the impact on GSH synthesis and hepatic GSH turnover may be significant. The turnover of normal hepatocyte GSH is estimated at 20 %/h [24], or 12 nmol/h per 10^6 cells for a repleted cell which has 60 nmol of GSH/10^6 cells. This is the amount of GSH that is lost per h, which is normally balanced by synthesis of GSH. Efflux of GSH from an adult rat liver occurs at a rate of 12 μmol of GSH/h (assuming 10^8 cells/g and a liver weight of 10 g). Thus even a 20 % inhibition of GCS activity would translate into a significant decrease in GSH synthesis by the liver (2.4 μmol/h). Since the liver GSH level is normally very high (5–10 mM), a slight fall would not jeopardize its defence capacity. The same signal transduction agonists that inhibit GCS also stimulate sinusoidal GSH efflux [25,26], so that delivery of GSH and hence cysteine to systemic tissues is maintained. Many of the hormones that are released during stress activate these signal transduction pathways. Down-regulation of GCS may be part of the liver's stress response by channelling cysteine away from GSH synthesis into synthesis of stress proteins, many of which are rich in cysteine.

Another potentially important pathophysiological consequence of GCS phosphorylation relates to cell injury and death. A sustained increase in the cytosolic free Ca^{2+} concentration has been implicated in mediating or potentiating cell death in a variety of toxicity models [27–31]. Exposure of cultured cells to cytotoxic compounds such as calcium ionophores and CCl_4 ultimately leads to increased intracellular free Ca^{2+}, followed by cell death [27–30]. Lauterburg and Mitchell [32] reported that toxic doses of acetaminophen administered *in vivo* suppressed hepatic GSH synthesis in rats. No mechanism was provided in their work, however. The inhibition of GSH synthesis may be due to activation of various signal transduction pathways during acute stress, or may be an epiphenomenon due to changes in cytosolic Ca^{2+} during cell injury. Therefore it is possible that inhibition of GCS further contributes to toxicity in these models. This warrants further study.

Finally, the occurrence of phosphorylated GCS-HS under basal control conditions (Figure 7) suggests that GCS activity may be normally under an inhibitory tone. Unfortunately we were not able to measure GCS activity after treatment of purified GCS with alkaline phosphatase due to interference by this treatment with the activity assay. The physiological significance of this observation will need to be explored further.

In summary, we have shown for the first time that GCS is phosphorylated by PKA, PKC and CMK. Phosphorylation/dephosphorylation may represent an important regulatory mechanism of GCS activity.

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