Phosphorylation of hormone-sensitive lipase by cyclic GMP-dependent protein kinase

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In intact rat adipocytes hormone-sensitive lipase has been shown to be phosphorylated on serine residues in two different phosphorylation sites: a regulatory site phosphorylated by cyclic AMP-dependent protein kinase and a basal site, which does not directly affect the enzyme activity, phosphorylated by cyclic AMP-independent protein kinase(s) [(1984) Proc. Natl. Acad. Sci. USA 81, 3317–3321]. Cyclic GMP-dependent protein kinase catalyzed the phosphorylation of the same two phosphorylation sites on the isolated enzyme, at serine residues. Both sites were phosphorylated at about the same rate, with the hormone-sensitive lipase activity concomitantly enhanced.

Cyclic GMP-dependent protein kinase *Hormone-sensitive lipase* *Phosphorylation site*
Cyclic AMP-dependent protein kinase *Adipose tissue*

1. INTRODUCTION

Adipose tissue lipolysis is hormonally regulated through control of the activity of hormone-sensitive lipase by reversible phosphorylation [1]. Lipolytic hormones stimulate lipolysis through a cyclic AMP-dependent protein kinase catalyzed phosphorylation of a single serine residue in the hormone-sensitive lipase [2,3]. The same – regulatory – phosphorylation site is dephosphorylated and lipolysis arrested in response to insulin [3]. In addition, a second – basal – phosphorylation site rapidly equilibrates with cellular [^{32}P]ATP [3,4]. The extent of phosphorylation of this site is not influenced by insulin and it is only associated with a very low, basal enzyme activity [4].

In an approach to study the function of the basal phosphorylation we demonstrate here that cyclic GMP-dependent protein kinase, which previously has been shown to cause an activation of a lipase preparation from chicken adipose tissue [5], phosphorylates both the regulatory and the basal phosphorylation sites of hormone-sensitive lipase.

2. EXPERIMENTAL

2.1. *Materials*

[γ-^{32}P]ATP was synthesised as in [6]. Silicic acid thin-layer plates (0.25 mm) and HPTLC plates were obtained from Merck (FRG), cyclic GMP, phosphoserine, phosphothreonine, phosphotyrosine and trypsin treated with diphenylcarbamyl chloride from Sigma (USA), and *Staphylococcus aureus* V8 protease from Miles Laboratories (USA). Hormone-sensitive lipase was detergent solubilized and purified to approx. 50% protein purity [7]. Cyclic GMP-dependent protein kinase (cGMP-PrK), approx. 70% pure, was provided by Dr D. Aswad and Professor P. Greengard, New York, and the specific protein inhibitor of cyclic AMP-dependent protein kinase (cAMP-PrK) from Professor P. Cohen, Dundee, Scotland. The catalytic subunit of cAMP-PrK was purified to homogeneity from adipose tissue [8].

2.2. *Phosphorylation of hormone-sensitive lipase*

Hormone-sensitive lipase was incubated with

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cGMP-PrK, approx. 0.2 μg/ml, in 4 mM imidazole-HCl buffer, pH 7.0, containing 0.1 mM [γ-^{32}P]ATP, 1 μM cyclic GMP, 5 mM $MgCl_2$ and the specific inhibitor protein of cAMP-PrK for the indicated time periods. Phosphorylation with cAMP-PrK was carried out as in [2]. The extent of phosphorylation of hormone-sensitive lipase was determined by densitometric scanning of autoradiographs after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as in [2].

2.3. *Phosphopeptide mapping*

^{32}P-phosphorylated hormone-sensitive lipase was obtained in a homogeneous form by SDS-PAGE and electrophoretic elution of the protein from the gel [9]. The eluted enzyme protein was alkylated in 10 mM iodoacetic acid as in [2] and extensively dialysed against 0.5% (w/v) NH_4HCO_3 and lyophilized. The protein was redissolved in 0.5% (w/v) NH_4HCO_3 and digested with 100 μg/ml *S. aureus* V8 protease for 4 h at 37°C followed by 20 μg/ml trypsin and 2 mM $CaCl_2$ for 15 h at 37°C and then lyophilized.

The proteolytic digest was redissolved in 33% (v/v) pyridine and subjected to phosphopeptide mapping by two-dimensional electrophoresis–thin-layer chromatography (10 × 10 cm) on silicic acid as in [2]. The plates were autoradiographed at −80°C using Kodak X-Omat RP film with intensifying screen (DuPont) [10].

2.4. *Phosphoamino acid analysis*

^{32}P-phosphorylated hormone-sensitive lipase was isolated by SDS-PAGE [9], extensively dialysed against 0.5% (w/v) NH_4HCO_3, lyophilised and hydrolyzed in 6 M HCl under argon for 3 h at 110°C. The solution was then evaporated to dryness and redissolved in water containing phosphoserine, phosphothreonine and phosphotyrosine. The phosphorylated amino acids were separated by electrophoresis on silicic acid thin-layer plates (HPTLC, 10 × 10 cm) at pH 1.9 in 2.5% (v/v) formic acid and 15% acetic acid at 10 V/cm for 10 min and 55 V/cm for 140 min. The plates were dried, stained with ninhydrin and autoradiographed.

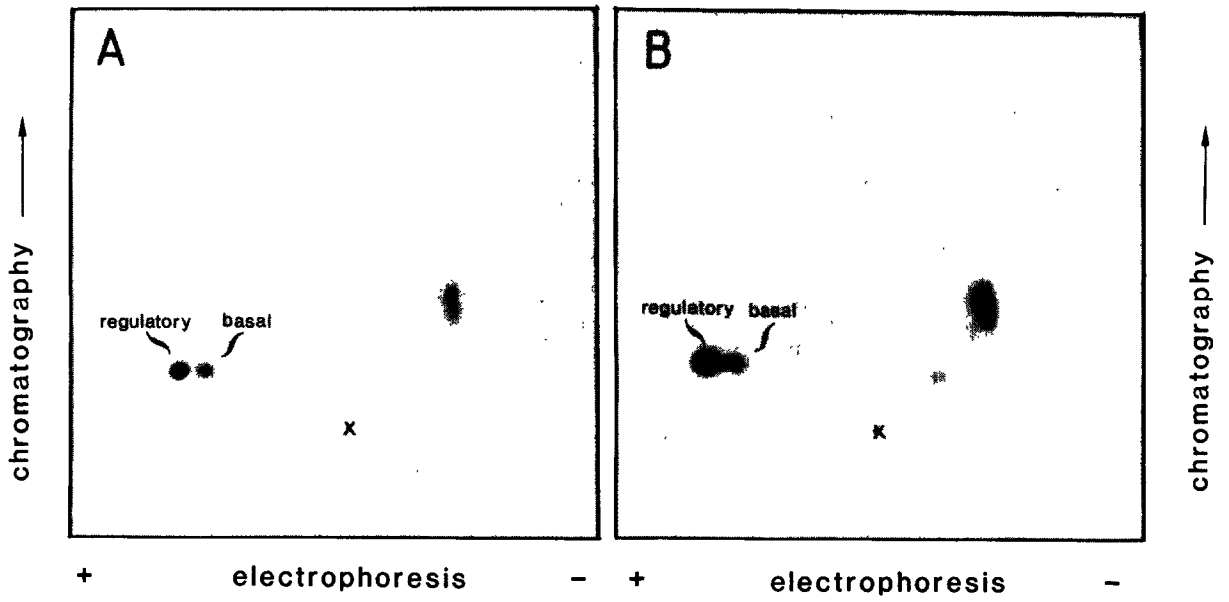

Fig.1. Phosphorylation sites. (A) Hormone-sensitive lipase phosphorylated with cGMP-PrK and [γ-^{32}P]ATP was isolated by SDS-PAGE, electrophoretically eluted from the polyacrylamide gel, and digested with *S. aureus* V8 protease and trypsin before phosphopeptide mapping (section 2). For explanation of 'regulatory' and 'basal' see text. (B) Hormone-sensitive lipase phosphorylated by cGMP-PrK was mixed with lipase phosphorylated by cAMP-PrK and treated as in A. The asymmetric spot to the left of the centre on the map represents partially digested material. The cross indicates the point of application.

2.5. *Other methods*

Hormone-sensitive lipase was activated by phosphorylation as above, except that unlabeled ATP was used; in controls ATP was omitted. Incubations were stopped by addition of 3 vols of an ice-cold solution of 10 mM EDTA, 1 mM dithioerythritol and 0.1 mg/ml bovine serum albumin (crystallized, Sigma) and immediately assayed against 0.75 mM trioleoylglycerol as in [2]. The amount of cAMP-PrK and of cGMP-PrK was determined from the specific activity of the enzymes as in [8] and [11], respectively.

3. RESULTS

3.1. *Identification of phosphorylation sites*

cGMP-PrK catalyzed the incorporation of [^{32}P]phosphate into two phosphopeptides (fig. 1A) which have been previously identified as corresponding to a regulatory and a basal phosphorylation site in intact adipocytes [3]. Addition of hormone-sensitive lipase phosphorylated in the regulatory site with cAMP-PrK [2] to lipase phosphorylated with cGMP-PrK and phosphopeptide mapping selectively increased radioactivity in the 'regulatory' phosphopeptide labeled in fig. 1B (the asymmetric spot just to the right of the center of the chromatogram represents partially digested material; cf. fig. 2). This identifies one of the phosphopeptides that cGMP-PrK phosphorylates with the regulatory site which is phosphorylated by cAMP-PrK in vitro [2] and in intact fat cells [3]. The migration of the second phosphopeptide on the phosphopeptide map identifies it with the basal phosphorylation site previously demonstrated in intact fat cells [3].

After electrophoresis of a partial acid hydrolysate of hormone-sensitive lipase phosphorylated with cGMP-PrK (see section 2) phosphoserine was the only phosphorylated amino acid residue detected in either phosphopeptide (not shown), in accordance with the phosphorylation of the same phosphopeptides in intact adipocytes [3].

3.2. *Rate of phosphorylation and activation of hormone-sensitive lipase activity*

As expected from phosphorylation of the regulatory site the enzyme activity was enhanced by its phosphorylation with cGMP-PrK (fig. 2A).

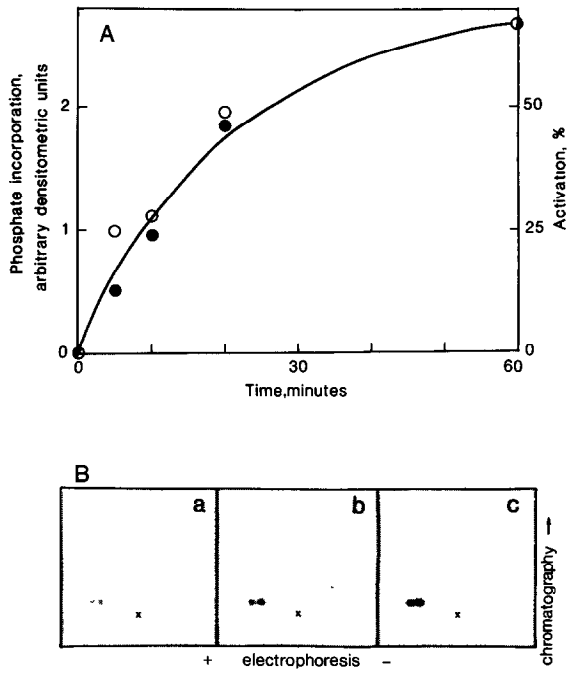

Fig.2. Phosphorylation and activation of hormone-sensitive lipase by cyclic GMP-dependent protein kinase. Hormone-sensitive lipase was incubated with cGMP-PrK and [γ-^{32}P]ATP-Mg as in section 2. (A) At the indicated time points aliquots were analyzed for ^{32}P incorporation into hormone-sensitive lipase and for extent of lipase activation (section 2). (B) At 5 min (a), 20 min (b) and 60 min (c) samples were analyzed for relative extent of phosphorylation of the two phosphorylation sites: the lipase protein was isolated by SDS-PAGE, digested with *S. aureus* V8 protease and trypsin, and subjected to phosphopeptide mapping (section 2).

Both sites were phosphorylated at about the same rate as demonstrated by phosphopeptide mapping at different time points (fig. 2B).

The rate of phosphorylation was compared with that obtained with cAMP-PrK. Using the same amounts of cGMP-PrK and cAMP-PrK on a weight-basis the initial rate of phosphorylation with cGMP-PrK was about 30% of the phosphorylation with cAMP-PrK. This indicates that the cGMP-PrK subunit phosphorylates the regulatory and the basal site at about 25% of the rate obtained with the cAMP-PrK catalytic subunit.

4. DISCUSSION

Hormone-sensitive lipase is phosphorylated on two discrete sites in the intact adipocyte, one of which is identical to that phosphorylated by cAMP-PrK [2,3]. The findings here that cGMP-PrK can catalyze the phosphorylation of both sites in vitro could suggest a function for cGMP-PrK in the regulation of the lipase.

Cyclic GMP-PrK and cAMP-PrK have very similar, but not identical substrate specificities [12,13]. One of the problems in defining any physiological function of cyclic GMP and its protein kinase has been to identify substrates that are specifically phosphorylated by the cGMP-PrK. The in vitro phosphorylation of the basal site of hormone-sensitive lipase is apparently one such specific phosphorylation by cGMP-PrK, which, moreover, occurs at a reasonably high rate. Although this site is phosphorylated and turns over rapidly in intact adipocytes [1,3,4], we have not been able to detect any direct changes in the extent of phosphorylation of this site in response to hormones.

The phosphorylation of hormone-sensitive lipase on its regulatory site by cGMP-PrK is not likely to challenge the importance of cyclic AMP in the hormonal control of adipose tissue lipolysis since cGMP-PrK is present at a very low level in adipose tissue [14] and there is no evidence for a role of cyclic GMP in the regulation of adipose tissue lipolysis [15]. The recent findings indicating that hormone-sensitive lipase may be a widely distributed hormonally controlled tissue lipase [1,16,17] hydrolyzing acylglycerols and cholesteryl esters open the possibility that regulatory pathways different from those regulating the hormone-sensitive lipase activity in adipose tissue operate in the control of its activity in other tissues. In this context, it is interesting that cyclic GMP and cGMP-PrK have been implicated in the regulation of adrenal steroidogenesis [18,19] and that hormone-sensitive lipase catalyzes one of the rate-limiting steps in this process [1,16].

Regardless of its possible physiological relevance the ability of cGMP-PrK to catalyze the phosphorylation of both phosphorylation sites in hormone-sensitive lipase at about the same rate makes cGMP-PrK a valuable tool for examination of the function of the basal phosphorylation of this enzyme in the fat cell.

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