Phosphorylation of the basal site of hormone-sensitive lipase by glycogen synthase kinase-4

Håkan Olsson, Peter Strålfors and Per Belfrage

Department of Physiological Chemistry 4, University of Lund, PO Box 94, S-221 00 Lund, Sweden

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In rat adipocytes hormone-sensitive lipase is phosphorylated at two sites termed 'regulatory' and 'basal', in the former case by cyclic AMP-dependent protein kinase causing an activation of the lipase [(1984) Proc. Natl. Acad. Sci. USA 81, 3317–3321]. Here, the basal phosphorylation site was found to be phosphorylated by glycogen synthase kinase-4 without any effects on lipase activity, or on the extent of its activation subsequent to phosphorylation of the regulatory site. Glycogen synthase kinase-3, casein kinase-I, and casein kinase-II did not phosphorylate the lipase. Phosphorylase kinase phosphorylated it to a very low extent at a third phosphorylation site not phosphorylated in the fat cell.

Hormone sensitivity *Lipase* *Glycogen synthase kinase-4* *Phosphorylation site* *Adipose tissue*

1. INTRODUCTION

Hormone-sensitive lipase catalyses the rate-limiting step in adipose tissue lipolysis, the process whereby free fatty acids, the quantitatively most important energy source in mammals, are released from the stored triacylglycerols in this organ. The lipase is tightly controlled, primarily by the sympathetic nervous system, circulating catecholamines and insulin. The activation of the enzyme is mediated by cyclic AMP and cyclic AMP-dependent protein kinase through its reversible phosphorylation [1–3]. Cyclic AMP-dependent protein kinase phosphorylates a single serine residue in a phosphorylation site termed 'regulatory' [4,5]. Addition of insulin to noradrenaline-stimulated fat cells causes a rapid net dephosphorylation of the regulatory phosphorylation site and inhibition of the lipolysis [4,6].

Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis

In the absence of hormonal stimulation of the adipocytes, hormone-sensitive lipase incorporates [^{32}P]phosphate from cellular [γ-^{32}P]ATP into a serine residue in a second phosphorylation site, termed 'basal' [4]. The phosphate at this site turns over rapidley [3,6] and it appears to be highly phosphorylated in unstimulated adipocytes [3]. The function of this phosphorylation is not known.

The protein kinase(s) which phosphorylates the basal phosphorylation site in vivo is unknown. Cyclic GMP-dependent protein kinase has been demonstrated to phosphorylate both the regulatory and the basal phosphorylation sites of the isolated lipase at about the same rate [7], but this reaction is not believed to be important in the fat cell [7]. In this work we have examined the phosphorylation of isolated hormone-sensitive lipase by glycogen synthase kinase-3, glycogen synthase kinase-4, phosphorylase kinase, casein kinase-I and casein kinase-II. We found that glycogen synthase kinase-4 is the only one of these five protein kinases that specifically phosphorylates the basal phosphorylation site.

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2. EXPERIMENTAL

2.1. *Materials*

[^{32}P]Orthophosphate was from Amersham (England) and [γ-^{32}P]ATP synthesized as in [8]. ATP, cyclic AMP, phosphoserine, phosphothreonine, phosphotyrosine and dithioerythritol were from Sigma (USA). The non-ionic homogeneous detergent dodecyloctaoxyethylene, referred to as $C_{12}E_8$ in the following, was from Nikko Chemicals (Tokyo, Japan). SDS was from Merck (FRG), acrylamide from Serva (FRG) and bis-acrylamide from Bio-Rad (USA).

2.2. *Enzyme preparations*

Hormone-sensitive lipase was purified as described in [9] up to and including the MonoQ chromatography step. Glycogen synthase kinase-4 was purified from rabbit skeletal muscle as in [10] up to the gel filtration step. The catalytic subunit of cyclic AMP-dependent protein kinase was purified from rat adipose tissue [11]. Glycogen synthase, the specific inhibitor protein of the cyclic AMP-dependent protein kinase, glycogen synthase kinase-3, phosphorylase kinase, casein kinase-I and casein kinase-II (all purified from rabbit skeletal muscle) were generous gifts from Professor Philip Cohen's laboratory (Dundee, Scotland). Trypsin treated with diphenylcarbamyl chloride was from Sigma (USA) and *Staphylococcus aureus* V8 protease from Miles Laboratories (USA).

2.3. *Phosphorylation of hormone-sensitive lipase*

Prior to phosphorylation, the lipase was desalted and transferred to a buffer (termed lipase buffer), containing 5 mM imidazole/HCl, pH 7.0, 1 mM dithioerythritol, 50 mM NaCl, 50% (w/v) glycerol, 0.04% bovine serum albumin and 2 mM $C_{12}E_8$, by centrifugation through 1 ml Sephadex G-25 superfine (Pharmacia, Sweden) [12]. The phosphorylation incubations with glycogen synthase kinase-4 (0.15 U/ml), casein kinase-I (1.8 U/ml) and casein kinase-II (8.8 U/ml) were carried out in a mixture of 10 mM sodium glycerol-1-phosphate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTE, 0.2 mM EGTA, and 0.4 mM EDTA, and the incubations with glycogen synthase kinase-3 (9 U/ml) in 5 mM imidazole, pH 7.0, 1 mM DTE, 3 mM magnesium acetate and 0.1 mM EGTA. One unit of activity (U) is that amount of enzyme which incorporates 1 nmol of phosphate/min into glycogen synthase and casein, respectively. The incubations with phosphorylase kinase (1 μg/ml) contained 50 mM Tris/glycerol-1-phosphate, pH 8.3, 10 mM magnesium acetate, pH 8.3, and 0.1 mM $CaCl_2$, and phosphorylations with cyclic AMP-dependent protein kinase were performed in the lipase buffer containing 5 mM $MgCl_2$ and 1 mM DTE. All phosphorylation incubations contained 5 μg/ml hormone-sensitive lipase (as determined from its specific activity), 0.1 mM [γ-^{32}P]ATP and, except for the incubation with cyclic AMP-dependent protein kinase, the specific inhibitor protein of cyclic AMP-dependent protein kinase. Controls with the protein kinases omitted were run in parallel. The phosphorylation reactions were terminated by precipitation in 9% (w/v) trichloroacetic acid in 30% acetone (to avoid coprecipitation of the non-ionic detergent) with 30 μg calf thymus DNA (Sigma; fragmented by sonication) as carrier. The proteins were then solubilized in SDS-PAGE sample buffer containing 5% (w/v) SDS and 1.25 M β-mercaptoethanol, and separated by SDS-PAGE (T = 8%, C = 2.4%) according to Laemmli [13]. The extent of phosphorylation of hormone-sensitive lipase was determined by liquid scintillation counting of gel slices containing the lipase protein, dissolved in H_2O_2.

2.4. *Measurement of hormone-sensitive lipase activation*

The extent of activation of the lipase due to phosphorylation was determined after incubation with the respective phosphorylation mixtures described above, except that unlabeled ATP was used. The activation was terminated by the addition of 3 vols of an ice-cold solution of 10 mM EDTA, 1 mM dithioerythritol and 0.1 mg/ml bovine serum albumin (crystallised, Sigma) and the lipase was then immediately assayed for triacylglycerol hydrolase activity against 0.75 mM trioleoylglycerol as described in [5].

2.5. *Phosphopeptide mapping*

^{32}P-phosphorylated lipase was isolated by SDS-PAGE and obtained in a homogeneous form by electrophoretic elution of the enzyme protein from

gel slices [14]. The eluted enzyme protein was alkylated in 10 mM iodoacetic acid as in [5], extensively dialysed against 0.5% (w/v) NH_4HCO_3 and lyophilised. The protein was then redissolved in 50 μl of 0.5% NH_4HCO_3, 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 lyophilised.

The proteolytic digest was redissolved in 33% (v/v) pyridine and subjected to phosphopeptide mapping by two-dimensional electrophoresis–thin layer chromatography on silicic acid plates (10 × 10 cm) [5]. The plates were autoradiographed at −80°C using a pre-flashed Kodak X-Omat RP film with intensifying screen (DuPont).

2.6. *Phosphoamino acid analysis*

Homogeneous ^{32}P-phosphorylated hormone-sensitive lipase was isolated as described above, extensively dialysed against 0.5% NH_4HCO_3, lyophilised and hydrolysed in 6 M HCl under argon for 3 h at 110°C. The solution was then evaporated to dryness and redissolved in an aqueous solution of phosphoserine, phosphothreonine and phosphotyrosine. The phosphorylated amino acids were separated by electrophoresis on silicic acid thin layer plates (HPTLC, 10 × 10 cm) [5]. The plates were stained with ninhydrin and autoradiographed as above.

3. RESULTS

Of the five protein kinases tested, glycogen synthase kinase-3, casein kinase-I and casein kinase-II did not phosphorylate hormone-sensitive lipase to any measurable extent under conditions that phosphorylated glycogen synthase, and are therefore not further commented on.

3.1. *Phosphorylation by glycogen synthase kinase-4*

Glycogen synthase kinase-4 catalysed the incorporation of [^{32}P]phosphate into the lipase molecule in a time-dependent manner to a maximal level of about 0.4 mol/mol of 84 kDa lipase protein (fig.1). The kinase activity was totally dependent on Mg^{2+} with maximal activity at 10 mM and half-maximal activity at approx. 3 mM $MgCl_2$ (not shown). Addition of cyclic AMP-dependent protein kinase after maximal phosphorylation by glycogen synthase kinase-4 approximately doubled the extent of phosphorylation of the lipase to 0.9 mol/mol 84 kDa lipase protein (fig.1).

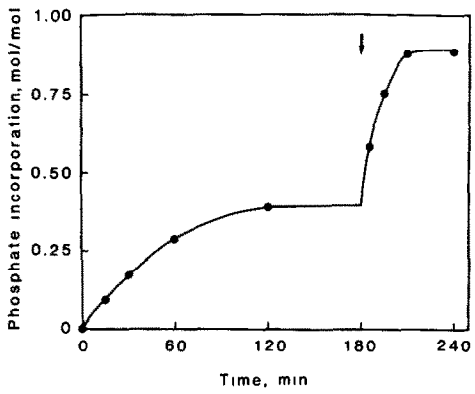

Fig.1. Time course of sequential phosphorylation of hormone-sensitive lipase with glycogen synthase kinase-4 and cyclic AMP-dependent protein kinase. Hormone-sensitive lipase (5 μg/ml) was incubated with 0.15 U/ml glycogen synthase kinase-4 and [γ-^{32}P]ATP as described in section 2. After 120 min, 1 μg/ml of cyclic AMP-dependent protein kinase was added as indicated by the arrow. At indicated time points aliquots were withdrawn and the extent of phosphorylation determined by liquid scintillation counting of gel slices from SDS-PAGE containing the phosphorylated hormone-sensitive lipase.

3.2. *Identification of the phosphorylated site*

Phosphopeptide mapping of the hormone-sensitive lipase by two-dimensional thin layer electrophoresis-chromatography showed a single phosphopeptide after phosphorylation by glycogen synthase kinase-4 (fig.2A). This phosphopeptide was identified with that carrying the basal phosphorylation site by its relative mobility in comparison with the phosphopeptide containing the regulatory phosphorylation site in experiments in which the lipase was phosphorylated with a mixture of glycogen synthase kinase-4 and cyclic AMP-dependent protein kinase (fig.2B). Phosphoserine was the only phosphorylated amino acid detectable after high voltage electrophoresis of a partial acid hydrolysate (not shown).

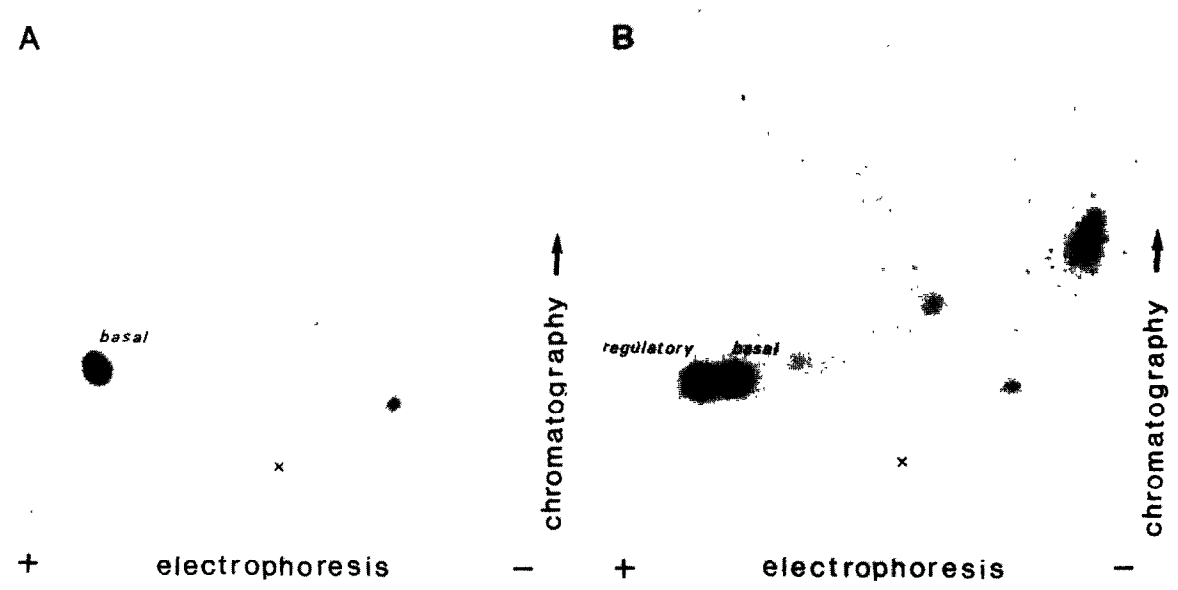

Fig.2. Peptide mapping of phosphorylated sites. Hormone-sensitive lipase phosphorylated with (A) glycogen synthase kinase-4 or (B) glycogen synthase kinase-4 plus cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP was isolated by SDS-PAGE, eluted from the polyacrylamide gel [14] and digested with *S. aureus* V8 protease and trypsin before phosphopeptide mapping (see section 2). For explanation of 'regulatory' and 'basal' see text. The asymmetric spot to the right of the centre on the map represents partially digested material. The cross indicates the point of application.

3.3. *Effect of phosphorylation by glycogen synthase kinase-4 on the hormone-sensitive lipase activity*

Phosphorylation of the lipase by glycogen synthase kinase-4 was not accompanied by any measurable effect on the lipase activity (fig.3). Subsequent incubation of the lipase with cyclic AMP-dependent protein kinase after the phosphorylation of the basal site had reached its maximal level (fig.1) increased the lipase activity by approximately 85% (fig.3). The extent of lipase activation by phosphorylation of the regulatory site by cyclic AMP-dependent protein kinase was not affected by the prior phosphorylation of the basal site by glycogen synthase kinase-4 (fig.3).

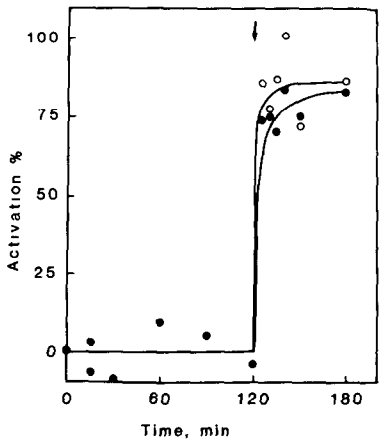

Fig.3. Time course of activation of hormone-sensitive lipase. Hormone-sensitive lipase (5 μg/ml) was incubated with 0.15 U/ml glycogen synthase kinase-4 and ATP as described in section 2. After 120 min, 1 μg/ml of cyclic AMP-dependent protein kinase was added as indicated by the arrow. A control incubation (○) with glycogen synthase kinase-4 omitted was run in parallel. At indicated time points aliquots were withdrawn and assayed for lipase activity (see section 2).

3.4. *Phosphorylation by phosphorylase kinase*

Hormone-sensitive lipase was phosphorylated also by phosphorylase kinase but at a very low rate (<1% of the phosphorylation rate of glycogen phosphorylase). The phosphorylation was not accompanied by any change in the enzyme activity. Phosphopeptide mapping showed that the phosphate was incorporated into a third

phosphorylation site distinct from both the regulatory and the basal sites (not shown). The only amino acid residue found to be phosphorylated at this site was phosphoserine.

4. DISCUSSION

In the study presented here we found that glycogen synthase kinase-4 can catalyse the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP selectively at the basal phosphorylation site of the hormone-sensitive lipase. The only other protein kinase that has previously been found to phosphorylate the basal phosphorylation site of the lipase is cyclic GMP-dependent protein kinase [7]. However, since this kinase phosphorylated both the basal and the regulatory phosphorylation sites at about the same rate in vitro [7], and since it is present in very low amounts in adipose tissue [15], it is not likely that this kinase is responsible for the phosphorylation of the basal site in the adipocytes.

As expected from previous findings in intact adipocytes [2–4], the phosphorylation of the basal phosphorylation site was not accompanied by any increase in lipase activity (fig.3). Moreover, the phosphorylation state did not influence the extent of activation due to phosphorylation of the regulatory site by cyclic AMP-dependent protein kinase (fig.3). This is consistent with the finding in [7] that cyclic GMP-dependent protein kinase phosphorylated the lipase to the same level at both sites without any additional effects on the activity. Taken together with previous findings [5,7] it also demonstrates that phosphorylation of the basal phosphorylation site is not a prerequisite for the phosphorylation of the regulatory site in vitro, a kind of sequential phosphorylation that has previously been reported to be necessary for, or strongly potentiate, the phosphorylation of several substrates by glycogen synthase kinase-3 [16–18] and of ATP-citrate lyase site B by ATP-citrate lyase kinase [19].

At steady state, a stable phosphorylation level of the basal phosphorylation site has been calculated to be 0.8 mol/mol 84 kDa lipase protein [3]. Since [^{32}P]phosphate is incorporated into the basal phosphorylation site in parallel with the increase of the specific activity of the intracellular [^{32}P]ATP [2,3,6], this indicates a rapid turnover of the phosphate at this site. The incorporation of phosphate into the respective sites of the lipase protein was somewhat lower in the present work, for unknown reasons. One possibility is that the lipase contained some phosphate since the improved purification protocol [9] decreases the time during which the lipase is exposed to protein phosphatases in the crude tissue extract.

The finding that glycogen synthase kinase-4 selectively phosphorylated the basal phosphorylation site of the isolated lipase could indicate that this is a function of the kinase also in the fat cell. Supporting this, in preliminary experiments we have found a cyclic nucleotide-independent protein kinase in adipose tissue which copurifies with and phosphorylates hormone-sensitive lipase. The pattern of phosphorylated cyanogen bromide fragments of glycogen synthase phosphorylated by this kinase indicates that it may in fact be glycogen synthase kinase-4 (Olsson, H., unpublished).

The phosphorylation of the lipase by phosphorylase kinase occurred at a third site which is not phosphorylated in vivo in intact adipocytes. Taken together with the fact that the lipase was phosphorylated to a very low extent and at a slow rate, it could suggest that it does not have any physiological relevance.

The lack of direct effects from the basal site phosphorylation on the lipase activity raises the question of whether it serves to control some long-term process which is important for the regulation of hormone-sensitive lipase activity. A possibility is that it is involved in the control of the degradation of the lipase, similar to the case with pyruvate kinase in which phosphorylation of the enzyme makes it more readily available to proteolytic degradation [17]. As hormone-sensitive lipase may be a widespread tissue lipase/cholesterol ester hydrolase [1–3], it is also possible that the phosphorylation of the basal site plays a role in the control of the lipase activity in other tissues than adipose tissue.

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