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Phosphorylation and dephosphorylation of hormone-sensitive lipase

Interactions between the regulatory and basal phosphorylation sites

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Phosphorylation of the basal site with glycogen synthase kinase-4 enhanced the rate of phosphorylation of the regulatory site by cyclic AMP-dependent protein kinase 1.7-fold. In contrast, the phosphorylation state of the regulatory site did not affect the rate of phosphorylation of the basal site with glycogen synthase kinase-4. The rate of dephosphorylation of either the regulatory or the basal phosphorylation site by protein phosphatase-1, 2A or 2C was independent of the phosphorylation state of the other site. These results suggest that the basal phosphorylation site could play an indirect role in the control of the hormone-sensitive lipase activity in the adipocyte by functioning as a recognition site for the cyclic AMP-dependent protein kinase in the phosphorylation of the activity-controlling regulatory phosphorylation site in response to lipolytic hormones.

Hormone-sensitive lipase; cyclic AMP-dependent protein kinase; Protein phosphatase; Glycogen synthase kinase-4; Phosphorylation site

1. INTRODUCTION

The rate-limiting step in adipose tissue lipolysis, the hydrolysis of the first ester bond of stored triacylglycerols, is catalyzed by hormone-sensitive lipase. The lipase activity, and thereby the lipolysis rate, is under hormonal and neural control through reversible phosphorylation. It has been demonstrated in intact adipocytes that lipolytic hormones, such as catecholamines, cause a rapid phosphorylation of the enzyme with concomitantly increased lipolysis rate (reviews [1–3]). The phosphorylation is catalyzed by cyclic AMP-dependent protein kinase [4], and the phosphate is incorporated into a single serine residue in a phosphorylation site termed regulatory [4,5]. After stimulation with catecholamines the extent of regulatory site phosphorylation, and consequently the lipolysis rate, is rapidly decreased by subsequent exposure of the fat cells to insulin [5,6].

Incubation of isolated fat cells with [^{32}P]phosphate in the absence of hormones ('basal' conditions) leads to the incorporation of radioactive phosphate into a second phosphorylation site in the lipase, termed basal [5]. The incorporation of [^{32}P]phosphate into this site closely parallels the increase in specific activity of intracellular [^{32}P]ATP [6], indicating a rapid turnover of the phosphate. In the isolated lipase, the basal site is selectively phosphorylated by glycogen synthase kinase-4 without any change in enzyme activity [7], whereas cyclic GMP-dependent protein kinase phosphorylates both the regulatory and the basal sites at the same rate, causing lipase activation as

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Abbreviations: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; $C_{12}E_8$, octaethylene glycol dodecylmonoether

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expected from the extent of phosphorylation of the regulatory site [8].

At present, there is no evidence for any direct effect of the phosphorylation state of the basal site on the lipase activity, and the extent of phosphorylation of this site is apparently unaltered in response to hormones [4]. However, since several reports on multi-site phosphorylation indicate that the phosphorylation state of one site can affect the phosphorylation or dephosphorylation of another [10–13], it was of interest to examine this question also for the hormone-sensitive lipase. By the selective phosphorylation of the two phosphorylation sites with cyclic AMP-dependent protein kinase and glycogen synthase kinase-4 it is demonstrated that phosphorylation of the basal site enhances the rate of phosphorylation of the regulatory site but does not affect its dephosphorylation by the three protein phosphatases active on hormone-sensitive lipase.

2. EXPERIMENTAL

2.1. *Materials*

Carrier-free [^{32}P]orthophosphate was from Amersham (England) and [γ-^{32}P]ATP was synthesized as in [14]. Hexokinase (yeast) was from Boehringer-Mannheim.

2.2. *Protein preparations*

Hormone-sensitive lipase was purified as described in [15] up to and including the high-performance liquid chromatography on a Mono Q anion-exchange column. The lipase constituted 5–10% of the protein in this preparation, but was the predominant phosphorylatable substrate for both glycogen synthase kinase-4 and cyclic AMP-dependent protein kinase (approx. 70–80% of the [^{32}P]phosphate incorporated into proteins).

Glycogen synthase kinase-4 was prepared from skeletal muscle as in [16] with the modifications in [17], and the catalytic subunit of cyclic AMP-dependent protein kinase was purified to apparent homogeneity from rat adipose tissue [18]. The catalytic subunit of protein phosphatase-1 and 2A [19], protein phosphatase-2C [20] and the heat-stable protein kinase inhibitor [21] (all purified from rabbit skeletal muscle) were generous gifts from Professor P. Cohen, Dundee.

2.3. *Phosphorylation of hormone-sensitive lipase*

Hormone-sensitive lipase was selectively phosphorylated at the regulatory phosphorylation site by cyclic AMP-dependent protein kinase or at the basal phosphorylation site by glycogen synthase kinase-4 [7]. Prior to phosphorylation, hormone-sensitive lipase was transferred to 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 $C_{12}E_8$. The phosphorylation incubations (0.06 ml) were carried out at 15 μg/ml hormone-sensitive lipase (determined from its specific activity [22]), 0.1 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$ and 1 mM dithioerythritol. Incubations with glycogen synthase kinase-4 also contained 10 mM sodium glycerol-1-phosphate, pH 7.0, 0.4 mM EDTA and 0.2 mM EGTA. The reactions were initiated with cyclic AMP-dependent protein kinase (1 μg/ml) or glycogen synthase kinase-4 (0.15 U/ml [7]) and terminated and analyzed as in [7].

Both sites were phosphorylated to approximately the same extent (0.4–0.6 mol/mol [7]) by incubating the lipase with the two protein kinases in sequence. The lipase was either incubated with glycogen synthase kinase-4 and unlabelled ATP for 100 min, followed by incubation with the catalytic subunit of cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP for 20 min or, alternatively, it was incubated with cyclic AMP-dependent protein kinase and unlabelled ATP for 20 min, with the subsequent addition of glycogen synthase kinase-4, [γ-^{32}P]ATP and the inhibitor protein of cyclic AMP-dependent protein kinase. The former procedure ^{32}P-labelled the regulatory site while the basal site was phosphorylated with unlabelled phosphate, and the latter procedure ^{32}P-labelled the basal site while the regulatory site was phosphorylated with unlabelled phosphate [7].

2.4. *Dephosphorylation of the lipase with protein phosphatases*

Hormone-sensitive lipase (25 nM) phosphorylated at either the basal or the regulatory phosphorylation site, or both, were incubated with protein phosphatases in 35 mM Tris-HCl, pH 7.0, 1 mM $MnCl_2$, 1 mM dithioerythritol, 0.1 mM EDTA and 0.2 mg/ml bovine serum albumin [17]. The assays (0.06 ml) were initiated with hormone-sensitive lipase and terminated by the addition of 0.02 ml twice-concentrated SDS-PAGE sample buffer [23] and boiling for 3 min. Proteins were separated in 7% slab gels according to [23], and after staining, the gels were dried and autoradiographed. The ^{32}P content of the 84 kDa lipase polypeptide was determined densitometrically (ChromoScan 3, Joyce Loebl, England) or by liquid scintillation counting of the gel slice containing the lipase protein [17].

3. RESULTS

3.1. *Effect of basal site phosphorylation on the rate of phosphorylation of the regulatory site*

Preincubation of hormone-sensitive lipase with glycogen synthase kinase-4 prior to phosphorylation with cyclic AMP-dependent protein kinase enhanced the rate of phosphate incorporation into the regulatory site of the lipase (fig.1). The initial rate of phosphorylation when the basal site was phosphorylated was 170 ± 10% (mean of 5 independent experiments ± SE) of the rate in the absence of basal site phosphorylation.

The interdependence between the sites in the phosphorylation reactions was further investigated by reversing the phosphorylation sequence. Prior phosphorylation of the regulatory site with cyclic AMP-dependent protein kinase did not, however, significantly affect the rate of phosphorylation of the basal phosphorylation site (not shown).

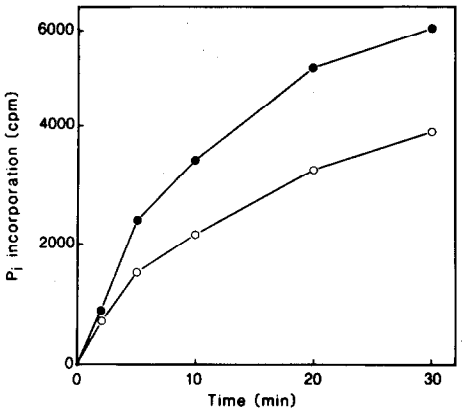

Fig.1. Time course of phosphorylation of hormone-sensitive lipase regulatory and basal sites by cyclic AMP-dependent protein kinase and glycogen synthase kinase-4. (A) Hormone-sensitive lipase was incubated with 0.1 mM unlabelled ATP and 0.5 mM Mg^{2+} in the presence (●) or absence (○) of 0.15 U/ml glycogen synthase kinase-4 for 100 min at 37°C. 1 μg/ml cyclic AMP-dependent protein kinase and 0.1 mM [γ-^{32}P]ATP were then added and at the indicated time points aliquots were withdrawn, the reaction arrested with SDS-PAGE sample buffer and the extent of ^{32}P phosphorylation of the lipase determined. The figure shows a representative experiment, each point representing the mean of duplicates.

3.2. *Effects of phosphorylation of either site on the dephosphorylation of the other*

We have recently presented results showing that protein phosphatase-1, 2A and 2C are able to dephosphorylate both sites of the lipase, though at different rates [17]. Moreover, results in our laboratory have demonstrated that protein phosphatase-2A is the major hormone-sensitive lipase phosphatase in the fat cell, while protein phosphatase-1 and 2C appear to be less important (Olsson, H. and Belfrage, P., unpublished). The dephosphorylation of the regulatory and basal sites of the lipase by protein phosphatase-1, 2A and 2C was therefore investigated with or without prior phosphorylation of the other site. The previous phosphorylation of the basal site with glycogen synthase kinase-4 did not affect the rate of dephosphorylation of the regulatory site by any of these protein phosphatases (fig.2). Also, prior phosphorylation of the regulatory site by cyclic AMP-dependent protein kinase did not affect the rate of dephosphorylation of the basal site (fig.2). In accordance with previous results [17], the basal site was dephosphorylated at approximately twice the rate of the regulatory site by protein phosphatase-2A and 2C, and approx. 15% faster than the regulatory site by protein phosphatase-1 (fig.2).

4. DISCUSSION

Hormone-sensitive lipase is phosphorylated at two distinct sites in the intact adipocyte, the basal

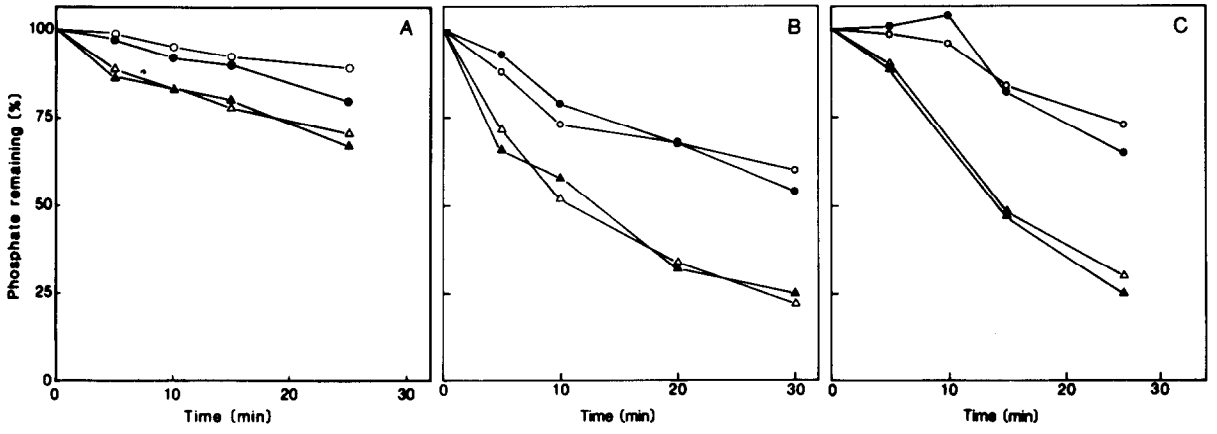

Fig.2. Time course of the dephosphorylation of hormone-sensitive lipase by protein phosphatase-1, 2A and 2C. Hormone-sensitive lipase was phosphorylated with [γ-^{32}P]ATP at either the regulatory site with (●) or without (○) phosphorylation of the basal site with unlabelled ATP, or at the basal site with (▲) or without (△) phosphorylation of the regulatory site with unlabelled ATP as described in fig.1. The lipase was then incubated with protein phosphatase-1 (A), 2A (B) or 2C (C) at 30°C. At the indicated time points aliquots were withdrawn, the reaction arrested by mixing with SDS-PAGE sample buffer and the extent of ^{32}P phosphorylation of the lipase determined. The figure shows a representative experiment, each point representing the mean of duplicates.

site, which appears to be phosphorylated irrespective of hormonal stimulation, and the regulatory site, phosphorylated only in response to adenylate cyclase stimulators. It has previously been demonstrated that phosphorylation of the basal site is not a prerequisite for the phosphorylation of the activity-controlling regulatory site, at least not in vitro [7], and it has so far not been possible to assign any function to the basal site. However, the demonstration that the rate of phosphorylation of the regulatory phosphorylation site was dependent on the phosphorylation state of the basal site suggests a role for this site in the regulation of hormone-sensitive lipase activity. The basal site may take part in the control of lipolysis in an indirect way by increasing the rate of phosphorylation of the regulatory site and thereby activation of the lipase. This type of site-site interaction has previously been demonstrated in the phosphorylation of glycogen synthase [10], inhibitor-2 [11], ATP-citrate lyase [12] and the type-II regulatory subunit of cyclic AMP-dependent protein kinase [13], which are all phosphorylated at a faster rate at a second site if a first site is already phosphorylated.

In contrast, the rate of phosphorylation of the basal site was not affected by the phosphorylation state of the regulatory site. This is comparable to the findings in [10–12] in which the rate of phosphorylation of the first site was independent of the phosphorylation state of the second site. Although the physiological responses to the phosphorylations are somewhat different, it therefore would appear that a common mechanism is responsible for the effects. The finding that the rate of phosphorylation but not dephosphorylation of the regulatory site of the lipase is affected by the phosphorylation state of the basal site suggests that this site, when phosphorylated, may serve as a recognition site and/or induce a conformational change of the lipase, which improves the availability of the regulatory site to the cyclic AMP-dependent protein kinase. This hypothesis is strengthened by recent results indicating that the basal phosphorylation site is located close to the regulatory phosphorylation site [24].

The observation that the rate of dephosphorylation of the respective phosphorylation sites of the lipase is independent of the phosphorylation state of the other site indicates that the basal site is not involved in the control of the dephosphorylation of the regulatory site and therefore does not participate in the insulin-induced net dephosphorylation of the regulatory site (cf. [3,25]). However, the possibility of a rapid transient dephosphorylation of the basal site in response to insulin, causing a decreased rate of phosphorylation of the regulatory site, cannot be entirely excluded. This would be accomplished by activation of one or more of the lipase phosphatases, which would increase the rate of dephosphorylation of both the regulatory and basal sites of hormone-sensitive lipase, and also, as a consequence of the decreased basal site phosphorylation, decrease the rate of phosphorylation of the regulatory site. Studies on the hormonal effects on the lipase protein phosphatases and the basal phosphorylation site and site-site interactions are in progress to test this hypothesis.

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