Novel heparin-activated protein kinase activity in rabbit skeletal muscle

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A heparin-activated protein kinase has been identified in rabbit skeletal muscle. The enzyme, which had a native molecular mass of 70 kDa as judged by gel filtration, was stimulated 3- to 5-fold by heparin, half-maximally at 3 μg/ml heparin. The stimulation by heparin was not reproduced by other polyanions such as polyaspartate and polyglutamate. The protein kinase was detected by its ability to phosphorylate glycogen synthase; it was ineffective in phosphorylating caseins, phosvitin, histone, or phosphorylase. Glycogen synthase was phosphorylated to a stoichiometry of 0.7–0.8 phosphates/subunit, exclusively at serine residues located in the COOH-terminal CNBr-fragment of the subunit, with a corresponding reduction in the −/+ glucose-6P activity ratio from 0.96 to 0.43. The activity of the protein kinase was unaffected by the presence of Ca^{2+} and/or phospholipid, cyclic AMP or heat-stable inhibitor protein of cyclic AMP-dependent protein kinase. The enzyme was inhibited about 60% by the presence of glycogen, half-maximal effect at 25 μg/ml. The heparin-activated protein kinase is clearly distinguishable from other known glycogen synthase kinases.

Protein kinase *Heparin* *Glycogen synthase* *Rabbit muscle*

1. INTRODUCTION

Covalent modification is one of the basic mechanisms that have evolved for the control of protein function (e.g., see [1–3]). During the last few years, it has become evident that cells contain numerous protein kinases (review, [4]). Previous studies in this laboratory have confronted the question of multiple protein kinases in an investigation of glycogen synthase, an enzyme shown to be phosphorylated in vitro by no fewer than eight different protein kinases [1–6]. Selective inhibitors and activators have proven useful in discriminating among protein kinases (e.g., cyclic nucleotides, Ca^{2+}, calmodulin). The glucosaminoglycan heparin, for reasons as yet obscure, has also found application in this context. Heparin is a potent inhibitor of enzymes of the casein kinase II class [7] and in [5] was also able to inhibit rabbit liver casein kinase I. Phosphorylase kinase was shown to be activated by heparin [8,9] and recent work has also described a stimulation of cyclic AMP-dependent protein kinase [10]. We report here the identification in rabbit muscle of a novel heparin-activated glycogen synthase kinase activity.

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2. EXPERIMENTAL

2.1. *Isolation of heparin-activated protein kinase*

The enzyme was identified during attempts to purify another protein kinase, designated F_A/GSK-3 (see [5,11]), from rabbit skeletal muscle using the method of [11]. At the stage of chromatography on phosphocellulose, elution was effected using a gradient of NaCl: the heparin-activated protein kinase eluted at 0.12 M NaCl and was separated from F_A/GSK-3 which eluted at 0.3 M NaCl. The heparin-activated enzyme was concentrated and subjected to chromatography on Sephadex G150, CM-Sephadex and finally Affigel blue. The specific activity of the enzyme in the presence of heparin was 10–12 nmol phosphate

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transferred/min per mg. The preparation contained undetectable phosphorylase phosphatase activity. A native molecular mass of 70 kDa was estimated from gel filtration on Sephadex G200. The preparation was stored at −70°C.

2.2. *Other materials and methods*

Protein kinase activity was measured using published methods [5] with 0.2 mg/ml rabbit skeletal muscle glycogen synthase as substrate and [γ-^{32}P]ATP at a specific activity of 1000 cpm/pmol. For routine assays, heparin was present at 10 μg/ml. Variations in conditions are noted in the text. Heparin, average molecular mass 12 kDa, was the generous gift of Drs P. Allison and N. Bang, Lilly Laboratory for Clinical Research, Indianapolis. Polyglutamate and polyaspartate were from Sigma, St. Louis. Other materials and methods are described in [5,12].

3. RESULTS

3.1. *Dependence of protein kinase activity on heparin*

As shown in fig.1, the enzyme was stimulated by heparin, with half-maximal activation at approximately 3 μg/ml. Maximal activation in the range 3–5-fold was observed in this study. As a preliminary test of whether the stimulation was related solely to the polyanionic nature of heparin, polyglutamate (average M_r 14000 and 20000) and polyaspartate (average M_r 6000 and 32000) were tested as effectors of the enzyme. Neither polyamino acid, up to concentrations of 100 μg/ml, caused significant stimulation of the protein kinase. Features other than negative charge are thus involved in the activation.

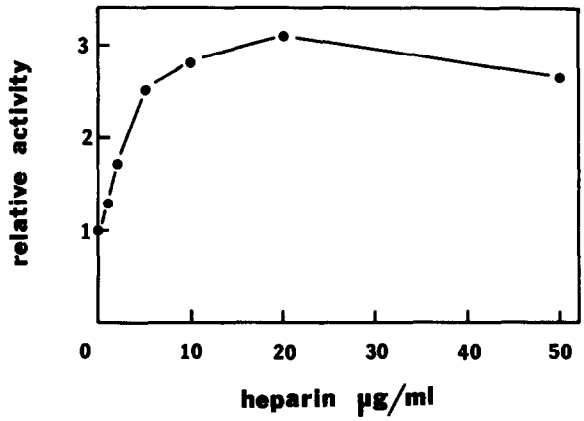

Fig.1. Heparin activation of the protein kinase. The rate of glycogen synthase phosphorylation was determined as a function of heparin concentration and is referred to the activity in the absence of heparin.

3.2. *Substrate specificity and comparison with other protein kinases*

The activity of the protein kinase was tested with a number of potential substrates that we have used in characterizing other glycogen synthase kinases.

Table 1

Effect of heat stable inhibitor on heparin-activated protein kinase and cyclic AMP-dependent protein kinase

Enzyme	Heparin	Activity (nmol/min per assay)[a]	
		−inhibitor[b]	+inhibitor
Heparin-activated kinase	−	0.76	0.84
	+	2.72	3.0
Cyclic AMP-dependent protein kinase (catalytic subunit)	−	1.23	0.13
	+	3.59	0.38

[a] Glycogen synthase phosphorylation was assayed as in [5]
[b] 'Inhibitor' refers to the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase [13]

These were phosvitin, β-casein A and B, α_{S1}-casein, κ-casein, histone (Sigma type IIA and type IIIS), and glycogen phosphorylase from muscle. None of these proteins underwent detectable phosphorylation by the heparin-stimulated protein kinase. The enzyme did, however, phosphorylate rat liver glycogen synthase (not shown).

The protein kinase was not inhibited by the heat-stable inhibitor [13] of cyclic AMP-dependent protein kinase (table 1), and in fact slight activation was observed. In parallel assays, the cyclic AMP-dependent protein kinase was inhibited 90% by the inhibitor protein, whether or not heparin was present. The results of table 1 are also consistent with the report of [10] that cyclic AMP-dependent protein kinase was stimulated by heparin. In other experiments, it was established that the activity of the heparin-activated protein kinase was unaffected by cyclic AMP, Ca^{2+}, or Ca^{2+} plus phosphatidyl serine and diacylglycerol (not shown).

3.3. *Phosphorylation of glycogen synthase*

The heparin-activated protein kinase phosphorylated glycogen synthase to a stoichiometry of 0.7–0.8, with a corresponding reduction in the $-/+$ glucose-6-P activity ratio from 0.96 to 0.43. The phosphate was associated exclusively with the glycogen synthase subunit (fig.2). Polyacrylamide gel electrophoresis of CNBr-fragments of the substrate indicated that the phosphate was contained in a single fragment of apparent molecular mass 26 kDa (fig.2). From previous studies, this fragment, usually designated CB-2, is known to represent the COOH-terminal region of the glycogen synthase subunit [3,12]. Only phosphoserine was detected in acid hydrolysates of glycogen synthase phosphorylated by the heparin activated protein kinase (not shown).

3.4. *Inhibition of heparin-activated protein kinase by glycogen*

Glycogen affects the activities of several glycogen synthase kinases (for example, [5]). It was found that glycogen also inhibited the phosphorylation of glycogen synthase by the heparin-activated protein kinase (fig.3). The inhibition was not complete and was maximally 60–65%. Half-maximal effect was obtained at approximately 25 μg/ml glycogen. These inhibition parameters were almost identical whether or not heparin was present, arguing that the unstimulated activity was associated with the same enzyme and not a contaminant.

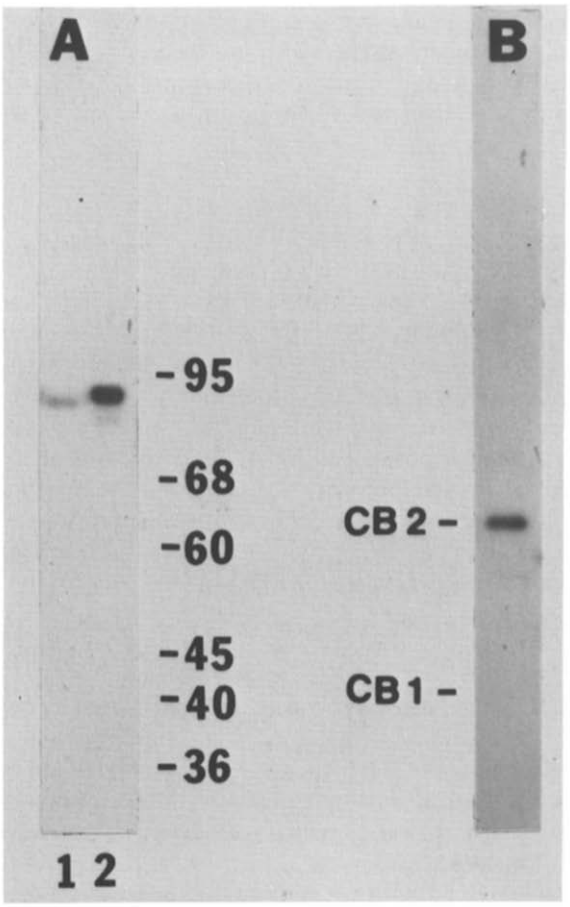

Fig.2. Electrophoretic analysis of phosphorylated glycogen synthase. Glycogen synthase was phosphorylated by the heparin-activated protein kinase. The phosphorylated enzyme was analyzed by PAGE in the presence of SDS (7% acrylamide) and an autoradiogram prepared (panel A). Tracks 1 and 2 correspond to enzyme phosphorylated in the absence (0.23) and presence (0.65) of heparin, respectively. The numbers in parentheses represent the degree of phosphorylation as phosphates/subunit. The two-digit numbers in the figure indicate the molecular masses ($\times 10^{-3}$) of standard proteins. Panel B shows an autoradiogram of an electrophoretic analysis of CNBr-fragments prepared from glycogen synthase phosphorylated in the presence of heparin; note that in this case a 6–20% acrylamide gradient gel was used. The migration of the two phosphorylatable CNBr fragments of the enzyme are indicated by 'CB-1' and 'CB-2'.

Table 2

Comparison of glycogen synthase kinases[a]

Protein kinase	Effect of		Phosphorylation of muscle glycogen synthase in[b]	
	Heparin	Glycogen	CB-1	CB-2
Cyclic AMP-dependent protein kinase	+	0	Yes	Yes
Phosphorylase kinase	+	+	Yes	No
Protein kinase C	0	–	Yes	Yes
Calmodulin-dependent protein kinase	0	0	Yes	Yes
Casein kinase I	–[c]	–	Yes	Yes
Casein kinase II	–	–	No	Yes
$PC_{0.4}$	0	0	Yes	No
F_A/GSK-3	0	0	Yes[d]	Yes
Heparin-activated protein kinase	+	–	No	Yes

[a] Original citations can be obtained through [3–6]

[b] The multiple phosphorylation sites of muscle glycogen synthase are mainly grouped in two CNBr-fragments, a smaller NH_2-terminal fragment (CB-1) and a larger COOH-terminal fragment (CB-2) (see [3])

[c] At least, for the rabbit liver enzyme [5]

[d] A lesser phosphorylation of CB-1 was observed in [12]

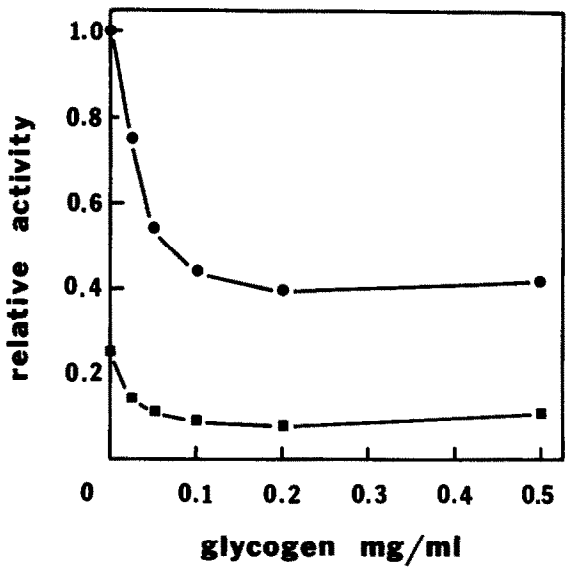

Fig.3. Glycogen inhibition of the heparin-activated protein kinase. The protein kinase activity towards glycogen synthase was measured in the absence (squares) or presence (circles) of 10 μg/ml heparin and the indicated concentration of rabbit liver glycogen.

4. DISCUSSION

The results presented demonstrate the existence, in rabbit skeletal muscle, of a protein kinase activated 3–5-fold by heparin. The enzyme, of apparent native molecular mass 70 kDa, is specific for glycogen synthase among the substrates tested so far. The principal question then is whether this enzyme represents a novel protein kinase. It can be distinguished by several criteria from the eight other protein kinases known to phosphorylate glycogen synthase in vitro. First, it is the only glycogen synthase kinase that is activated by heparin but inhibited by glycogen (table 2). The specificity of the heparin-activated protein kinase in phosphorylating glycogen synthase only in the larger CNBr-fragment CB-2 distinguishes it from all the other enzymes except casein kinase II and possibly F_A/GSK-3 (table 2). The lack of recognition of any casein variant as a substrate makes the enzyme different from all of the other enzymes except $PC_{0.4}$. The insensitivity to cAMP, the Walsh inhibitor, Ca^{2+} and phospholipid indicates that the

enzyme is distinct from cAMP-dependent protein kinase, holoenzyme or catalytic subunit, phosphorylase kinase or protein kinase C. On the basis of a native molecular mass of 70 kDa, the protein kinase is clearly distinguished from phosphorylase kinase, the calmodulin-dependent protein kinase, casein kinase II and possibly some of the other enzymes. Therefore, the heparin-activated protein kinase is not related in any simple fashion to the known glycogen synthase kinases. For it to be derived from any of these enzymes, such as by proteolysis, would require not only a change in at least one effector interaction but also significant modification in substrate specificity. Nonetheless, definitive structural comparison of the enzyme with other protein kinases will require its purification to homogeneity. Efforts to achieve this are under way.

The heparin-activated protein kinase thus represents a novel enzyme activity and the next question is to consider its possible physiological function. Glycogen synthase is phosphorylated by several protein kinases in vitro and the relative importance of these different enzymes in vivo is still a matter of active investigation. A priori, there is no reason yet to exclude the heparin-activated enzyme as a physiologically relevant glycogen synthase kinase. Also, it may well be that further screening will uncover other important substrates for this protein kinase. Although heparin is not a strong candidate to be a physiological regulator, it may be mimicking some other relevant effector. From a practical standpoint, the present report is of special significance. In studies of glycogen synthase phosphorylation, it is necessary to recognize the existence of the heparin-activated protein kinase and to note that the inclusion of heparin in assays of Ca^{2+}- and cyclic AMP-independent glycogen synthase kinases may not only inhibit casein kinases I and II but could also activate this novel enzyme. Future studies must address a more detailed characterization of the heparin-activated protein kinase and its phosphorylation of glycogen synthase.

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