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Phosphorylation of hepatic insulin receptor by casein kinase 2

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Casein kinase 2 was able to phosphorylate the β-subunit of hepatic insulin receptor in the presence of either ATP or GTP. Phosphorylation by casein kinase 2 was observed even in the absence of insulin, was inhibited by low heparin concentrations, and led to the incorporation of phosphate on serine and threonine residues. Casein kinase 2 phosphorylation of insulin receptor partially decreased its tyrosine kinase activity.

Casein kinase 2; Insulin receptor; Serine phosphorylation; Threonine phosphorylation; (Rat liver)

1. INTRODUCTION

One of the first events after insulin binding to its receptor is autophosphorylation of the receptor β-subunit on tyrosine residues [1]. Experiments in vivo have shown that insulin receptor is also phosphorylated on serine and threonine residues and that these phosphorylations may modulate the response of the cells to insulin [2]. Direct phosphorylation of insulin receptor by protein kinase C [3] and the cyclic AMP-dependent protein kinase [4] has been reported recently. The results reported here show that casein kinase 2 phosphorylates the β-subunit of insulin receptor on serine and threonine residues.

2. MATERIALS AND METHODS

2.1. *Isolation of insulin receptor and casein kinase 2*

Rat liver membranes were obtained by differential centrifugation [5], and the insulin receptor solubilized in 2% Triton X-100 was partially purified by affinity chromatography using wheat germ lectin-Sepharose [6]. Casein kinase 2 was purified from rat liver cytosol to apparent homogeneity as in [7].

2.2. *Phosphorylation assays*

Lectin-purified receptor (25 μl) was preincubated for 30 min at 30°C prior to phosphorylation in 50 μl of a solution containing 20 mM Hepes (pH 7.6), 4 mM $MgCl_2$ and 7.5 mg/ml bovine serum albumin with or without 75 nM insulin. Phosphorylation reactions were initiated by the addition of 25 μM [γ-^{32}P]ATP (30 μCi/nmol) and 2.5 mM $MnCl_2$ in a final volume of 65 μl. When indicated, casein kinase 2 was added to the reactions mixture. After 20–30 min incubation at 24°C, the reaction was terminated by adding 30 μl Laemmli sample buffer [8] containing 50 mM dithiothreitol (final concentration), followed by heating for 5 min at 95°C. Samples were subsequently analysed on one-dimensional 2% SDS/10% polyacrylamide gel. The gel was dried and then visualized by autoradiography. The molecular masses (in kDa) of the standards were: myosin, 205; β-galactosidase, 116; phosphorylase *b*, 97; bovine serum albumin, 66; ovalbumin, 45; carbonic anhydrase, 29. Phosphoamino acid analyses were performed at pH 3.5 as in [9].

Tyrosine kinase activity of insulin receptor was assayed using the Glu:Tyr (4:1) synthetic polymer [10].

2.3. *Source of materials*

Porcine insulin and wheat germ lectin-Sepharose were from Sigma. Acrylamide was obtained from Fluka. Other reagents for electrophoresis were from Bio-Rad. [γ-^{32}P]ATP was prepared from [^{32}P]orthophosphate (New England Nuclear) using a Gamma-prep kit from Promega Biotech. Other chemicals were as described [7].

3. RESULTS

3.1. *Evidence of phosphorylation of insulin receptor by casein kinase 2*

Insulin receptor isolated from rat liver plasma membranes by wheat germ lectin-Sepharose

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chromatography underwent insulin-dependent autophosphorylation on its β-subunit when incubated in the presence of [γ-^{32}P]ATP (fig.1). Inclusion of casein kinase 2 in the phosphorylation reaction led to levels of phosphorylation of the receptor higher than those achieved by autophosphorylation. Furthermore, casein kinase 2 was able to phosphorylate the receptor even in the absence of insulin. Control experiments with casein kinase 2 alone showed the presence of a phosphorylated polypeptide which corresponds to the β-subunit of this enzyme.

3.2. *Influence of GTP and heparin on the phosphorylation of insulin receptor by casein kinase 2*

The ability to use GTP as phospho donor and the high sensitivity to inhibition by heparin are two characteristics of casein kinase 2 [11]. As shown in fig.2, phosphorylation of the β-subunit of insulin receptor by casein kinase 2 also occurred when [γ-^{32}P]GTP was used as phospho donor. Under this condition, no autophosphorylation of the insulin receptor was observed even in the presence of insulin, in agreement with previous reports on the inability of the tyrosine kinase of insulin receptor to use GTP as substrate [12].

Phosphorylation of the insulin receptor by casein kinase 2, was strongly inhibited by heparin (fig.2). In a separate experiment it was determined that 50% inhibition of receptor phosphorylation occurred at 0.3 μg/ml heparin (not shown). As expected from a previous report [13], autophosphorylation of the β-subunit of casein kinase 2 was also strongly inhibited by heparin.

3.3. *Analysis of phosphoamino acids present in phosphorylated insulin receptor*

Autophosphorylation of insulin receptor is known to occur on tyrosine residues [1,2]. Proteins

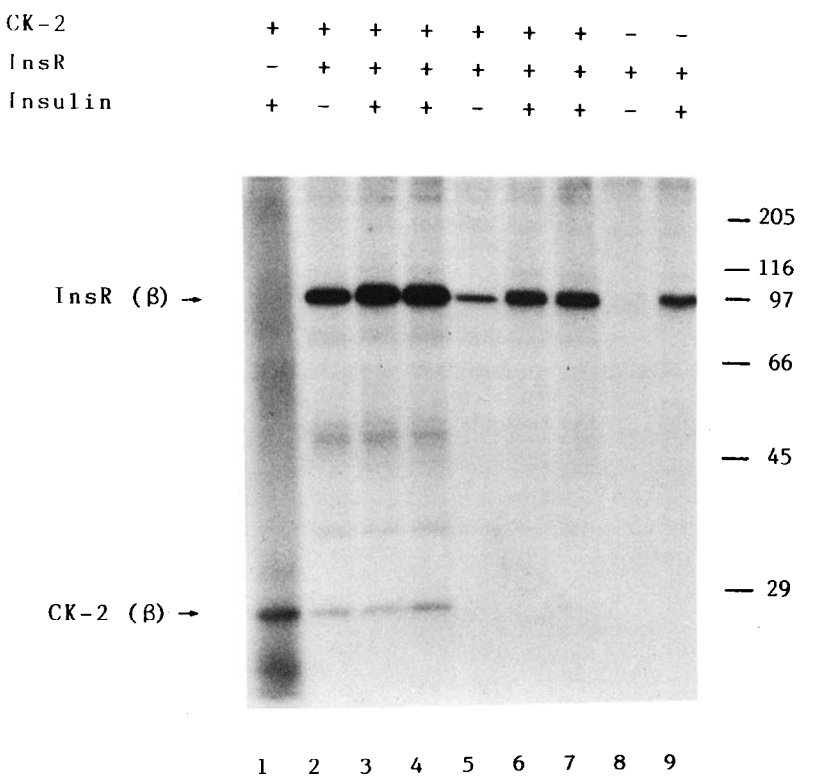


Fig.1. Phosphorylation of the insulin receptor (InsR) by casein kinase 2 (CK-2). InsR, preincubated without (lanes 2,5,8) or with (lanes 3,4,6,7,9) 75 nM insulin was phosphorylated by either 0.5 U/ml (lanes 2–4) or 0.2 U/ml (lanes 5–7) of CK-2. Controls with either InsR alone (lanes 8,9) or CK-2 alone (lane 1) were performed. Samples were electrophoresed, dried and autoradiographed.

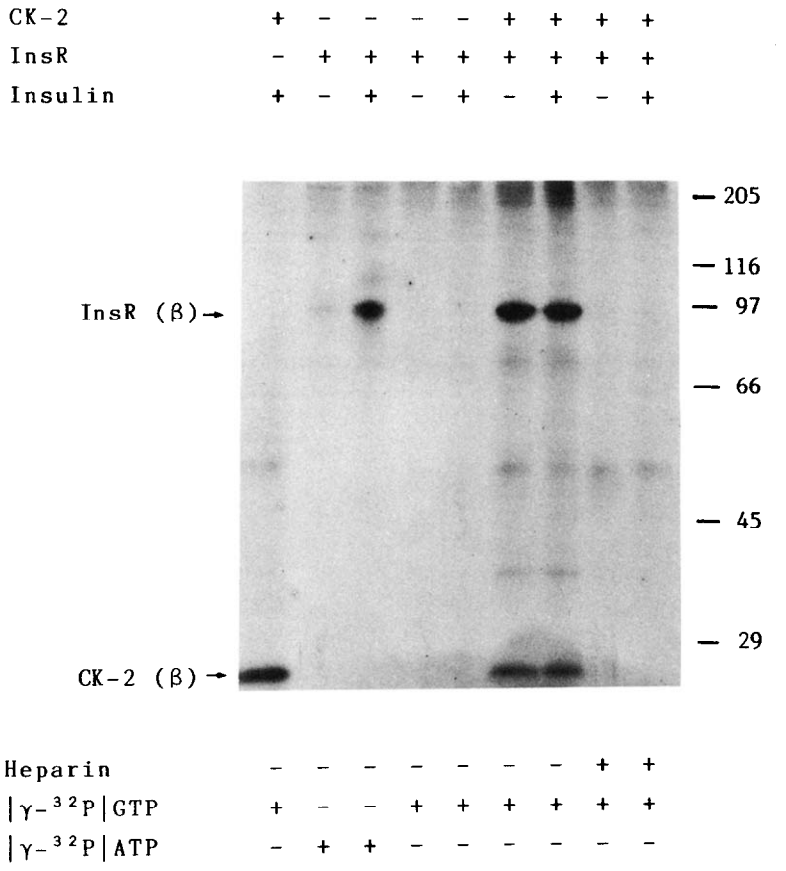


Fig.2. Phosphorylation of the insulin receptor (InsR) by casein kinase 2 (CK-2) with [γ-^{32}P]GTP as phospho donor. When indicated, insulin (75 nM), heparin (1.2 μg/ml), and CK-2 (0.5 U/ml) were present. [γ-^{32}P]ATP was used for detecting autophosphorylation of InsR. Autoradiograph of gel is shown.

phosphorylated on tyrosine can be distinguished from those labelled on serine and threonine by heating for 3 h at 60°C with 1 M KOH since only phosphotyrosine is resistant to this treatment [14]. When the gels from electrophoresis of insulin receptor phosphorylated by casein kinase 2 in the absence of insulin were subjected to this treatment all radioactivity was removed (fig.3A). In contrast, the presence of ^{32}P was still observed in the gels from samples where autophosphorylation was favored by the presence of insulin. These results indicated that phosphorylation of the β-subunit of insulin receptor by casein kinase 2 did not take place on tyrosines but, probably, on serine or threonine residues.

Phosphoamino acid analysis of the phosphorylated β-subunit of insulin receptor extracted from the gels revealed the presence of phosphoserine and phosphothreonine after phosphorylation by casein kinase 2, whereas only phosphotyrosine was observed in the autophosphorylated receptor (fig.3B).

3.4. *Effect of insulin receptor phosphorylation by casein kinase 2 on its tyrosine kinase activity*

Phosphorylation of the insulin receptor by ca-

Fig.3. Analysis of phosphoamino acids present in phosphorylated insulin receptor. (A) Gel corresponding to lanes 2–4 of fig.1 was treated with 1 M KOH for 3 h at 60°C, dried and autoradiographed. (B) The insulin receptor bands from lanes 5, 7 and 9 of the gel shown in fig.1 were excised and treated for phosphoamino acid analysis.

Table 1

Influence of phosphorylation by casein kinase 2 on insulin receptor tyrosine kinase activity

	Tyrosine kinase activity	
	− insulin	+ insulin
Control	30.9 ± 2.6	100
Casein kinase 2	24.1 ± 2.5	76.9 ± 6.1

Insulin receptor was preincubated at 24°C for 25 min with 125 μM GTP in either the absence or presence of 1.5 U/ml of casein kinase 2. Samples were removed and incubated with 1.2 mg/ml Glu:Tyr and 30 μM [γ-^{32}P]ATP for 30 min at 24°C in the absence or presence of 75 nM insulin. Data are given as percentage of the activity of control insulin receptor in the presence of insulin. Values are means ± SE of three different experiments carried out in duplicate

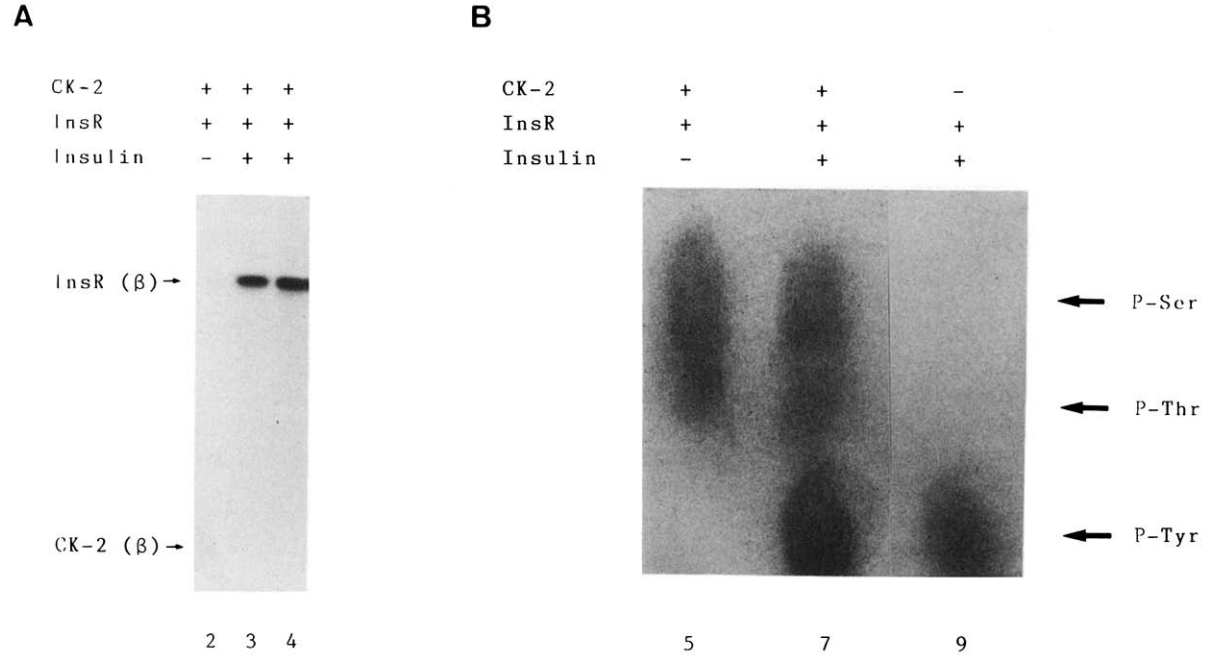
sein kinase 2 caused a decrease in the tyrosine kinase activity of the receptor towards the Glu:Tyr (4:1) synthetic polymer assayed either in the absence or in the presence of insulin (table 1).

4. DISCUSSION

Our results indicate that the β-subunit of the insulin receptor is a substrate for casein kinase 2. This contrasts with a previous report in which no phosphorylation of the insulin receptor by casein kinase 2 was observed [15]. The reason for this discrepancy could reside in the level of endogenous phosphorylation of the receptor preparations in the presence of ATP observed in that report. The use of [γ-^{32}P]GTP has allowed us to detect phosphorylation of the receptor by casein kinase 2 under conditions where no autophosphorylation of the receptor occurred. The strong inhibition of the phosphorylation reaction by low concentrations of heparin confirms the involvement of casein kinase 2 in this process.

Phosphorylation of the receptor by casein kinase 2 decreases its tyrosine kinase activity, and the extent of inhibition observed is similar to that reported for phosphorylation by the cyclic AMP-dependent protein kinase [4].

In contrast with other protein kinases [3,4], receptor phosphorylation by casein kinase 2 occurred not only on serine but also on threonine residues. It is interesting to point out that both serine and threonine residues on the insulin receptor are phosphorylated in vivo in unstimulated cells [16]. Furthermore, besides a direct influence on the insulin receptor activity, phosphorylation by casein kinase 2 may also affect the ability of the receptor to be phosphorylated by other kinases as



observed previously with other protein substrates of this enzyme [17–19].

In summary, our data show that, in addition to protein kinase C [3] and cyclic AMP-dependent protein kinase [4], the potential involvement of casein kinase 2 in phosphorylation of the insulin receptor should also be taken into consideration.

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