

# Insulin-like growth factor I binding and receptor kinase in red and white muscle

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IGF-I receptors were partially purified from red and white skeletal muscle by lectin-affinity chromatography and the resultant fraction was depleted of insulin receptors by insulin affinity chromatography. Equilibrium binding of <sup>125</sup>I-IGF-I to receptor preparations from red and white muscle yielded identical Scatchard plots. The integrity of the IGF-I receptor preparation in the two fiber types was identical as determined by affinity cross-linking. The tyrosine kinase activity of the receptor from red muscle was 2–3-fold more active towards exogenous substrates in both the basal and ligand-activated states as compared to white muscle. These data show that there is IGF-I-dependent kinase activity intrinsic to IGF-I receptors from skeletal muscle, and suggest that identical cellular factors may regulate the kinase activity of insulin and IGF-I receptors in a parallel manner in vivo.

IGF-I receptor; Tyrosine kinase; (Skeletal muscle, Rat)

## 1. INTRODUCTION

Insulin-like growth factor I (IGF-I) belongs to a family of peptides structurally related to insulin. In muscle and in cultured cell lines, IGF-I causes a variety of cellular metabolic changes including stimulation of glucose and amino acid uptake, and glycogen and protein synthesis [1–3]. These biological effects are initiated after binding to specific cell surface receptors. The receptor for IGF-I, like the closely related insulin receptor, is a tetrameric glycoprotein complex consisting of two ligand-binding  $\alpha$ -subunits and two  $\beta$ -subunits linked by disulfide bonds [4,5]. The  $\beta$ -subunits of the insulin [6] and IGF-I [7,8] receptors are ligand-

stimulatable, tyrosine-specific protein kinases. There is a considerable amount of indirect evidence to suggest that the intrinsic kinase activity of these receptors may be involved in signalling, however, the biochemical pathway of this process remains to be described.

We recently reported that quantitative differences in insulin action in red vs white muscle are mirrored by intrinsic differences in the kinase activity of the insulin receptor in the two muscle types [9]. Here, we have examined the kinase activity of the IGF-I receptor in red and white muscle.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of partially purified receptors

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 250–300 g and fed ad libitum were used for all studies. Hindlimb muscle was obtained from sodium pentobarbital anesthetized rats and frozen in liquid N<sub>2</sub> as in [9]. In experiments on red and white muscle, red muscle consisted of pooled soleus, red portions of the gastrocnemius and quadriceps muscles. White portions of the gastrocnemius and

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*Abbreviations:* IGF-I, insulin-like growth factor I; WGA, agarose-bound wheat germ agglutinin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin

quadriceps were pooled as the source of white muscle. Frozen muscle was powdered, homogenized and solubilized in 1% Triton X-100 as described [9]. The solubilized homogenate was centrifuged at  $150\,000 \times g$  for 90 min at  $4^{\circ}\text{C}$ . The supernatant of the centrifugation was applied to a column of wheat germ agglutinin bound to agarose (WGA) and receptors were eluted from the WGA column with a buffer containing 25 mM Hepes, 0.1% Triton X-100 and 0.3 M *N*-acetyl-D-glucosamine. In order to separate IGF-I receptors from insulin receptors present in the WGA eluate, samples were subjected to insulin-affinity

chromatography. Typically, a receptor preparation (2 ml) containing 0.5 M NaCl was recycled overnight at  $4^{\circ}\text{C}$ , through an insulin-affinity column (3 ml resin bed) at a rate of 30 ml/h and the flow-through was collected the next morning. The flow-through, which was depleted in insulin receptors, was then dialyzed extensively for 12 h at  $4^{\circ}\text{C}$  against 1000 vols of a buffer containing 25 mM Hepes, 0.1% Triton X-100, pH 7.4. The preparation was reconcentrated using WGA chromatography. Receptors were then eluted with a buffer containing 25 mM Hepes, 0.1% Triton X-100, 0.3 M *N*-acetyl-D-glucosamine.

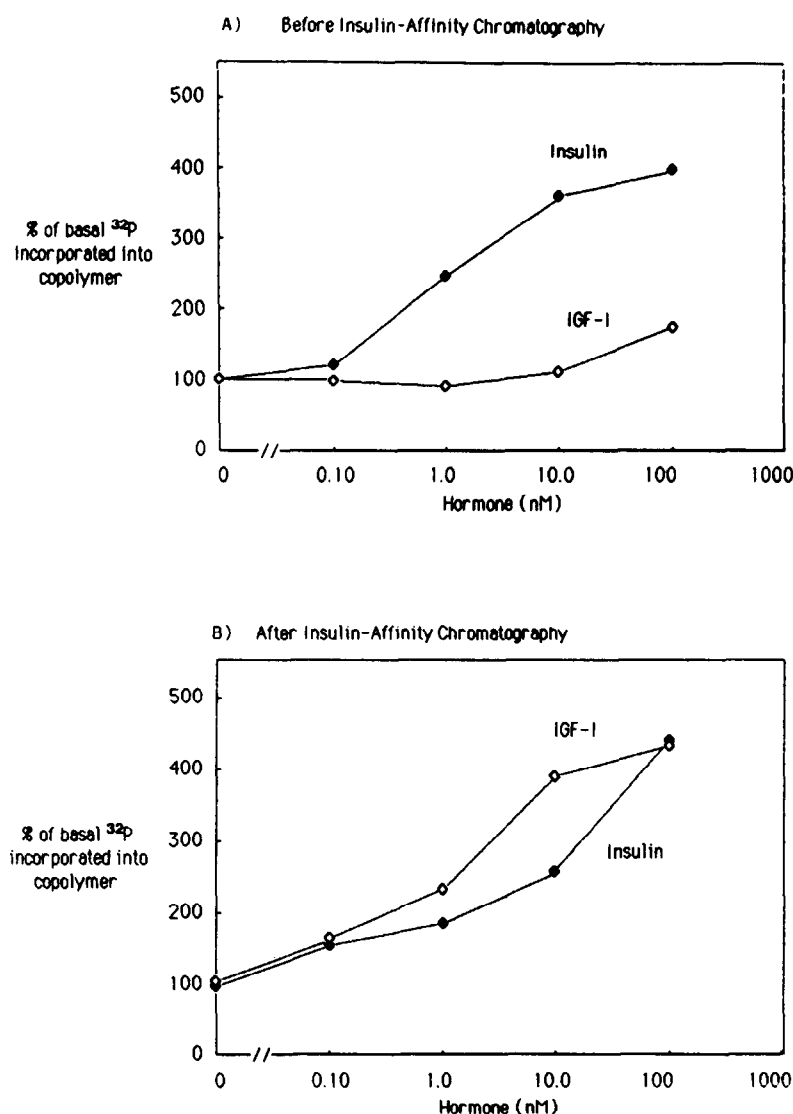


Fig.1. IGF-I- and insulin-stimulated phosphorylation of an exogenous substrate. Preparations obtained before (A) or after (B) partial depletion of insulin receptors by the insulin affinity step ( $30\ \mu\text{l}$ ) were incubated for 1 h at  $22^{\circ}\text{C}$  with varying concentrations of insulin ( $\blacklozenge$ ) or IGF-I ( $\diamond$ ) in 30 mM Hepes buffer, pH 7.6, and 10 mM  $\text{MgCl}_2$ . Samples were further incubated with  $50\ \mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  ( $5\text{--}10\ \mu\text{Ci}$ ) for 10 min and then the reaction was initiated by the addition of the substrate (copolymer of Glu/Tyr). Each data point is the mean of duplicate estimations and the results shown are representative of three different experiments.

### 2.2. Ligand binding and receptor cross-linking

Insulin and IGF-I binding was measured as in [9]. Sample eluate (20  $\mu$ l) was incubated in 50 mM Hepes containing 0.1% BSA and 100 U/ml bacitracin (pH 7.4, 1 h, 22°C, 200  $\mu$ l) and either  $^{125}$ I-Tyr A14 monoiodoinsulin (50 pM, 20000 cpm) and increasing concentrations of unlabelled insulin or  $^{125}$ I-IGF-I (50 pM, 35000 cpm) and increasing concentrations of unlabelled IGF-I. Non-specific binding was estimated as  $^{125}$ I-ligand bound in the presence of 1  $\mu$ M insulin or 0.25  $\mu$ M IGF-I (5–10% of total insulin binding and 12–18% of total IGF-I binding). Binding data were expressed per  $\mu$ g protein with the latter measured using the method of Bradford [10]. Receptor cross-linking protocols were carried out essentially as described by Pilch and Czech [11] using 0.5 nM  $^{125}$ I-IGF-I.

### 2.3. Phosphorylation of an exogenous substrate

To assess phosphorylation of an exogenous substrate, the partially purified receptor preparation was preincubated for 1 h at 22°C in 30 mM Hepes buffer, pH 7.6, containing 10 mM  $\text{MgCl}_2$  and varying concentrations of insulin or IGF-I. The receptor kinase activity was initially activated by the addition of 50  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (5–10  $\mu$ Ci) for 10 min. The reaction was initiated by the addition of the exogenous substrate (copolymer of Glu/Tyr, 0.25 mg/ml). The reaction was stopped after 30 min by applying samples to filter paper squares (2  $\times$  2 cm, Whatmann 3 MM), which were immediately washed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. Papers were washed, dried and counted as described [9].

## 3. RESULTS

Scatchard analysis of  $^{125}$ I-IGF-I and  $^{125}$ I-insulin binding in partially purified receptor preparations from mixed skeletal muscle revealed the existence of 3-times more insulin than IGF-I receptors. The affinity of IGF-I for its receptor was in the nanomolar range ( $K_d = 1.6$  nM), similar to that of insulin for the insulin receptor ( $K_d = 0.9$  nM) (not shown). A partially purified receptor preparation obtained after lectin-affinity chromatography from skeletal muscle was used to assess exogenous kinase activity. Insulin stimulated  $^{32}$ P incorporation into the copolymer Glu/Tyr (4:1) (fig.1A) and the half-maximal effect of insulin was attained at 1 nM. IGF-I concentrations as high as 10 nM failed to show any significant increase in copolymer phosphorylation. At 100 nM IGF-I a 30% increase in  $^{32}$ P incorporation into copolymer was detected. It is likely that IGF-I-stimulated IGF-I receptor kinase activity is not demonstrable in the crude wheat germ agglutinin fraction due to masking by the more abundant insulin receptor kinase. Thus, insulin receptors were depleted from mixed receptor preparations by insulin-affinity

chromatography. This resulted in a 60% decrease of insulin binding while IGF-I binding increased somewhat (not shown). This step did not modify either the affinity of the ligands for their receptors or the structure of the IGF-I receptors as assessed by affinity cross-linking (not shown). After removal of insulin receptors, the basal copolymer phosphorylation was considerably reduced, as expected. Under these conditions IGF-I-dependent kinase activity was detectable at concentrations of IGF-I below the  $K_d$  for binding. The dose-response curve showed a half-maximal effect close to 1 nM, consistent with the  $K_d$  values for IGF-I binding (fig.1B).

This affinity-depletion technique was next employed to study the structure/function of the IGF-I receptor in red and white muscle. Equilibrium binding of  $^{125}$ I-IGF-I to partially purified IGF-I receptor preparations from red and white muscle yielded identical Scatchard plots for both muscle types (fig.2). IGF-I receptor number and affinity, calculated from three different Scatchard plots, were similar in red and white muscle. The molecular mass of the IGF-binding species present in the insulin receptor-depleted prepara-

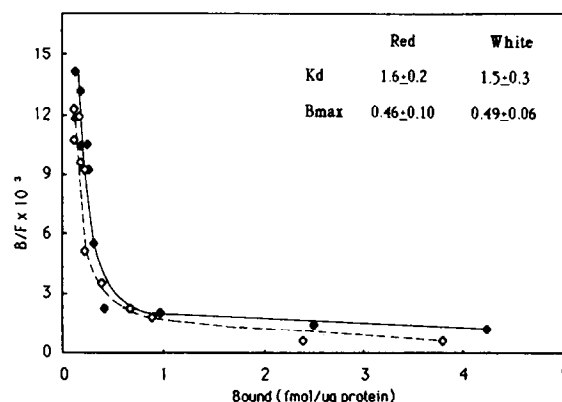


Fig.2. IGF-I binding in red and white muscle. IGF-I receptors were partially purified from red ( $\blacklozenge$ ) and white ( $\diamond$ ) skeletal muscle as described in section 2. Aliquots of purified extracts (20  $\mu$ l) were incubated for 1 h at 22°C in a buffer containing 25 mM Hepes, 0.1 mg/ml BSA, 100 U/ml bacitracin,  $^{125}$ I-IGF-I and varying concentrations of IGF-I. The data shown are from a representative Scatchard plot ( $n = 3$ ). Each point is the mean of duplicate determinations. The results of binding parameters are means  $\pm$  SE of three observations per group.  $K_d$  and  $B_{max}$  values are expressed as nM and fmol/ $\mu$ g protein, respectively.

tion of red and white muscle was determined on SDS-PAGE following affinity cross-linking of  $^{125}\text{I}$ -IGF-I to the receptor (fig.3). Under non-reducing conditions two bands, specifically labelled with  $^{125}\text{I}$ -IGF-I were detected. The 300 kDa band corresponds to the reported IGF-I receptor [5], whereas that of 220 kDa has been identified as the IGF-II receptor [3]. Under reducing conditions three different bands, that were specifically labelled with  $^{125}\text{I}$ -IGF-I, were evident. Unlabelled IGF-I competed with all three bands. We interpret these data in the light of previous studies [11,12] to mean that the high molecular mass species that is not competed for by insulin is probably the IGF-II receptor and the other band is an  $\alpha$ - $\alpha$  cross-linked complex of the IGF-I receptor, often observed after  $^{125}\text{I}$ -insulin and  $^{125}\text{I}$ -IGF-I affinity cross-linking to their respective receptors [11,12]. Under all conditions, the bands specifically labelled with

$^{125}\text{I}$ -IGF-I did not differ between red and white muscle, showing that there are no gross structural differences in the integrity of IGF-I receptors partially purified from both fiber types. The dose-response relationship between IGF-I and the  $^{32}\text{P}$  incorporation into a copolymer of Glu/Tyr, in the presence of partially purified IGF-I receptor (depleted of insulin receptors) is shown in fig.4. Equal amounts of high-affinity IGF-I receptors, as estimated by equilibrium binding, were used for red and white muscle. IGF-I stimulated the exogenous kinase activity of the IGF-I receptor, purified from red and white muscle, at all concentrations examined (fig.4). These effects were specific for the IGF-I receptor, since increased exogenous kinase activity was demonstrable using concentrations of IGF-I (1 nM) which did not cross-react with the insulin receptor [9]. The receptor from red muscle was 2–3-fold more active in

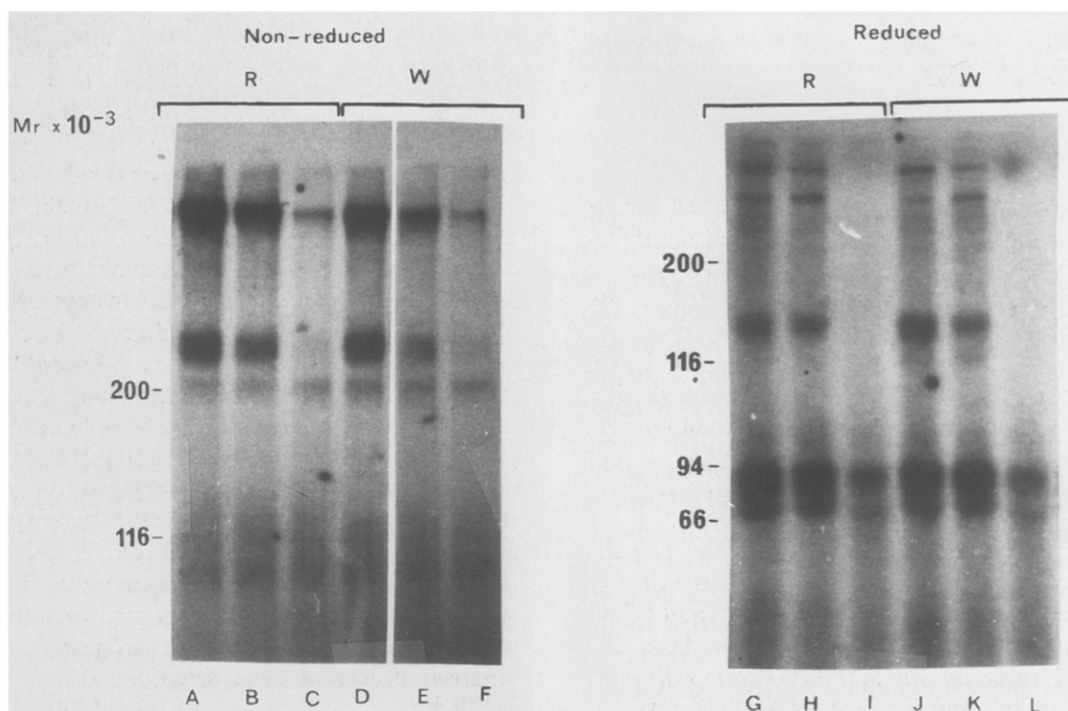


Fig.3. Affinity cross-linking of  $^{125}\text{I}$ -IGF-I to the IGF receptors in red and white muscle. IGF receptors purified were obtained from red and white muscle as described in section 2. Partially purified IGF receptors were incubated with 0.5 nM  $^{125}\text{I}$ -IGF-I in the absence of unlabelled ligands (lanes A,D,G,J) or in the presence of 1  $\mu\text{M}$  insulin (lanes B,E,H,K) or 0.25  $\mu\text{M}$  IGF-I (lanes C,F,I,L) at 22°C for 30 min. After 5 min incubation at 0°C, disuccinimidyl suberate was added at a final concentration of 1 mM, and samples were incubated for a further 15 min at 0°C. Affinity cross-linking reactions were terminated by addition of Laemmli sample buffer without (lanes A–F) or with (lanes G–L) 0.1 M dithiothreitol.

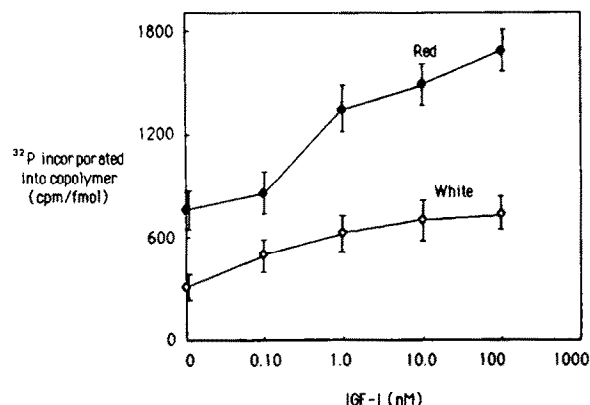


Fig.4. Kinase activity of IGF-I receptors from red and white muscle towards an exogenous substrate. Partially purified IGF-I receptors from red or white muscle (40  $\mu$ l) were incubated at 22°C in 30 mM Hepes buffer, pH 7.6, containing 10 mM  $MgCl_2$  and varying concentrations of IGF-I. See details in legend to fig.1 and section 2. Each data point is the mean  $\pm$  SE of triplicate estimations and the results shown are representative of four different experiments.

both the basal and ligand-activated states as compared to white muscle.

#### 4. DISCUSSION

In this report, we provide evidence for an IGF-I-stimulated tyrosine kinase activity of the IGF-I receptor in muscle. Half-maximal phosphorylation of an exogenous substrate was observed in the vicinity of 1 nM IGF-I, which is essentially identical to the  $K_d$  for IGF-I binding to the IGF-I receptor. Furthermore, we have also shown that IGF-I receptor kinase activity is differentially expressed in red and white muscle. Thus, low concentrations of IGF-I, such as 1 nM, caused a much greater stimulation of the exogenous tyrosine kinase activity in red than in white muscle. The differences were not due to alterations in the quality of the receptors, because studies of equilibrium binding and affinity cross-linking showed that the structural integrity of the IGF-I receptors was identical in red and white muscle. These results, together with the previous report from our laboratory describing the existence of similar differences in the activation of the insulin receptor kinase in red and white muscle [9], support the existence of common regulatory mechanisms for both insulin and IGF-I receptors in muscle.

However, the molecular mechanisms that are responsible for these differences and the cellular factors involved remain to be determined.

There is a growing body of evidence indicating that activation of insulin receptor kinase is the initial signal that regulates some or all of the biological responses to this hormone. The insulin and IGF-I receptors have many similarities in the expression and regulation of their intrinsic kinase activities [13,14]. In view of this and of the marked similarities in the biological effects of insulin and IGF-I [1-3], it seems quite likely that as with insulin, the intrinsic kinase activity of its receptor will be crucial in initiating the cellular action of IGF-I. However, definite proof is still lacking.

Both insulin and IGF-I receptor kinases showed the same pattern of differences when comparing red vs white muscle. Thus, the existence of common, as yet unknown, regulatory mechanisms, could be envisaged. These receptors are expressed from different genes on different chromosomes [15], therefore the most likely regulatory mechanism is one involving a cellular component that would effect both receptors in a similar fashion, e.g. a covalent modification. In this regard, it is noteworthy that the insulin receptor kinase can be modulated by several other protein kinases. Cyclic AMP-dependent protein kinase phosphorylates the insulin receptor and decreases insulin-stimulated tyrosine receptor kinase [16]. Similarly, phorbol esters cause insulin receptor phosphorylation [17-19] and decrease insulin action in some cells [17]. The phorbol esters stimulate phosphorylation of the insulin and IGF-I receptors at sites different from those phosphorylated upon ligand binding [18,19]. Protein kinase C directly phosphorylates the insulin receptor in vitro, causing a decrease in its tyrosine kinase activity [20]. Thus, a counter-regulatory relationship may exist between activation of the insulin and IGF-I receptor kinases and the activity of protein kinase C or another unknown protein kinase that acts on these receptors.

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