Similar Control Mechanisms Regulate the Insulin and Type I Insulin-like Growth Factor Receptor Kinases

AFFINITY-PURIFIED INSULIN-LIKE GROWTH FACTOR I RECEPTOR KINASE IS ACTIVATED BY TYROSINE PHOSPHORYLATION OF ITS β SUBUNIT

Kin-Tak Yu, Mary A. Peters‡, and Michael P. Czech

From the Department of Biochemistry, University of Massachusetts Medical Center, Worcester, Massachusetts 01605 and ‡Amgen Development, Inc., Boulder, Colorado 80301

Insulin-like growth factor I (IGF-I) receptors are partially purified from human placenta by sequential affinity chromatography with wheat germ agglutinin-agarose and agarose derivatized with an IGF-I analog. Adsorption specificity to this affinity matrix demonstrates that low coupling ratios of IGF-I analog to agarose yield preparations that are highly selective in purifying IGF-I receptor with minimal cross-contamination by the insulin receptor present in the same placental extracts. Incubation of the immobilized IGF-I receptor preparation with [γ-32P]ATP results in a marked phosphorylation of the receptor β subunits, which appear as a doublet of Mr = 93,000 and 95,000 upon electrophoresis on dodecyl sulfate-polyacrylamide gels. The 32P-labeled receptor β subunit doublet contains predominantly phosphotyrosine and to a much lesser extent phosphoserine and phosphothreonine residues. The immobilized IGF-I receptor preparation exhibits tyrosine kinase activity toward exogenous histone.

The characteristics of the IGF-I receptor-associated tyrosine kinase are remarkably similar to those of the insulin receptor kinase. Thus, prior phosphorylation of the immobilized IGF-I receptor preparation with increasing concentrations of unlabeled ATP followed by washing to remove the unreacted ATP results in a progressive activation of the receptor-associated histone kinase activity. A maximal (10-fold) activation is achieved between 0.25 and 1 mM ATP. The concentration of ATP required for half-maximal (30 μM) activation of the IGF-I receptor kinase is similar to that of the insulin receptor kinase. Like the insulin receptor kinase, the elevated kinase activity of the phosphorylated IGF-I receptor is reversed following dephosphorylation of the receptor β subunit with alkaline phosphatase. Furthermore, the phosphorylation of the IGF-I receptor β subunit doublet is enhanced by 7-8-fold when reductant is included in the reaction medium, as is observed for the insulin receptor kinase. Significantly, the dose responses of both receptor types to reductant are identical. Both of the 32P-labeled IGF-I receptor β subunit bands are resolved into six matching phosphopeptide fractions when the corresponding tryptic hydrolysates are resolved by reverse phase high pressure liquid chromatography. Significantly, four out of the six phosphopeptide fractions derived from the trypsinized IGF-I receptor β subunits are chromatographically identical to those from the tryptic hydrolysates of 32P-labeled insulin receptor β subunit. These results suggest that the IGF-I receptor-associated and insulin receptor tyrosine kinases share common enzymatic and structural features.

Recent advances on the structure of the IGF-I receptor have been made by several laboratories using such independent approaches as affinity labeling of the receptor with 125I-IGF-I (1-5) and immunoprecipitation of 125I-labeled IGF-I receptor with monoclonal antibodies (6). It has been demonstrated that under reducing conditions two distinct receptor subunits of Mr = 130,000-140,000 and Mr = 90,000-94,000 can be identified on SDS-polyacrylamide gels, whereas under non-reducing conditions up to three labeled species of molecular weights ranging from 290,000 to 350,000 are detected (1, 3, 4). It has been postulated that the native IGF-I receptor is a disulfide-linked heterotetramer and that the subunit stoichiometries of the native unreduced IGF-I receptor are αββα (Mr ~ 350,000), αβββ (Mr ~ 320,000), and αββα (Mr ~ 290,000) (1). The α and β subunits correspond to the Mr = 130,000-140,000 and Mr = 90,000-94,000 reduced subunits, respectively, while the β1 subunit appears to be a proteolytic fragment of the β subunit (1). The IGF-I receptor exhibits high affinity for IGF-I and low affinity for insulin (7, 30-32). The general subunit composition and apparent molecular weights on SDS-polyacrylamide gels of the IGF-I receptor subunits closely resemble those of the insulin receptor (1). In addition, the functional properties of the IGF-I and insulin receptors are also very similar. Interaction of these receptors with their respective ligands stimulates both long term and acute biological responses such as cell proliferation (8-10), membrane transport processes (11), and enzymatic activities (12).

Similar to a number of hormone and growth factor receptors such as insulin (13-15), epidermal growth factor (16, 17), and platelet-derived growth factor (18-20) receptors, the IGF-I receptor undergoes phosphorylation on its β subunit upon binding of IGF-I in both intact and broken cell preparations (21, 22). The phosphorylation of the IGF-I receptor occurs exclusively on tyrosine residues under in vitro conditions (22). However, it is not clear whether the ligand-induced IGF-I

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1The abbreviations used are: IGF-I, insulin-like growth factor I; PMSF, phenylmethylsulfonyl fluoride; TPCK-treated trypsin, N-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
 Insulin and Type I Insulin-like Growth Factor Kinases

Receptor phosphorylation is an autocatalytic event as in the case of insulin and EGF receptors or whether it is actually mediated through the action of other exogenous kinases.

Using affinity chromatography methods, several laboratories have purified insulin and EGF receptors to apparent homogeneity and have demonstrated intrinsic kinase activities of these receptors (17, 23, 24). Similar approaches to purifying the IGF-I receptor have been hampered by the scarcity of IGF-I for preparation of affinity columns. We have, therefore, linked an analog of IGF-I, produced by recombinant DNA, to agarose for affinity chromatography of the receptor. This analog contains an 8-amino acid leader peptide (Met-Lys-Lys-Tyr-Trp-Ile-Pro-Met) and a threonine substitution for methionine at position 59. Its affinity for human placental membranes is similar to that of human-derived IGF-I (25). This analog will be referred to as IGF-I*, and the derivatized agarose will be referred to as IGF-I*-agarose.

In this report, we document the partial purification of the human placental IGF-I receptor by sequential adsorption to immobilized wheat germ agglutinin and IGF-I*-agarose preparations. This IGF-I receptor preparation immobilized on IGF-I*-agarose exhibits tyrosine kinase activity toward both the receptor β subunit and exogenous histone. Furthermore, we demonstrate that like the insulin receptor kinase (26-28) the IGF-I receptor-associated kinase activity is dramatically activated by prior phosphorylation of its β subunit on tyrosine residues2 or by treatment with reductant. In addition, a subset of the phosphopeptides generated by tryptic digestion of the IGF-I receptor β subunit appears to be similar to those of the insulin receptor in respect to their high pressure liquid chromatographic behavior and phosphoamino acid contents.

**Experimental Procedures**

**Materials**—Leupeptin, N-acetylglucosamine, FMSF, phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma. TPCK-treated trypsin and histone H2B were from Worthington, and γ[32P]ATP was obtained from Amersham Corp. Wheat germ agglutinin immobilized on agarose was obtained from Pharmacia P-L Biochemicals. Precoated cellulose plastic sheets were purchased from Erkraum Instruments. Porcine insulin was a gift from Dr. Ronald Chance of Lilly. Affi-Gel 10 and molecular weight standard resin was mixed with 2 ml of ice-cold 0.1 M Hepes (pH 7.6) containing 0.4 M urea, 80 mM CaCl2, 130 μg of IGF-I*, and a trace amount of [γ-32P]ATP (specific activity, 100 μCi/nmol). After the indicated period of incubation, the reaction was terminated by adding electrophoresis sample buffer containing 0.17 M Tris, 10% SDS, and 100 mM dithiothreitol. The 32P-labeled samples were electrophoresed on 7% SDS-polyacrylamide gels or 6-16% SDS-polyacrylamide gradient gels. After electrophoresis, the gel was stained, destained, dried, and autoradiographed with Kodak X-Omat film. Phosphoamino acid analysis was determined either by autoradiography or by Cerenkov counting of the excised spots.

**HGPI Tryptic Peptide Mapping**—The preparation of [32P]-labeled IGF-I and insulin receptor samples for HGPI tryptic peptide mapping was the same as previously described (20). Briefly, after phosphorylation of [32P]ATP (specific activity, 2000 cpm/pmol) and receptor preparation, washed free of unreacted ATP, was reduced and alkylated with [32P]-labeled samples were electrophoresed on 7% SDS-polyacrylamide gels. [32P]-Labeled bands were located by autoradiography, excised from the gel, washed, and finally suspended in 50 mM NH4HCO3 buffer containing 50 μg/ml TPCK-treated trypsin. Tryptic digestion was performed at 37°C for 20 h. After digestion, the samples were lyophilized, dissolved in 0.2 ml of 1% trifluoroacetic acid, and then digested with 0.02% TFA. The digests were evaporated to dryness and dissolved in 3 ml of a 0.5% acetonitrile gradient over a period of 45 min at a flow rate of 1 ml/min. 1-min fractions were collected, and radioactivity was determined by Cerenkov counting. The peak fractions were then pooled, lyophilized, and hydrolyzed in 6 M HCl at 110°C for 1

1 During the course of submitting this manuscript for publication, an article by Sasaki and co-workers (35) appeared reporting the characteristics of the IGF-I-stimulated tyrosine kinase activity in the wheat germ agglutinin-agarose-enriched receptor preparation from BRL-3A2 rat liver cells. Their results suggest that the IGF-I receptor-associated tyrosine kinase activity toward exogenous substrates is activated following tyrosine phosphorylation of the receptor β subunit.

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The ability of IGF-I*-agarose to adsorb the IGF-I receptor and its associated kinase from wheat germ agglutinin agarose-enriched human placental detergent extracts is shown in Fig. 1. Incubation of such a preparation of receptor-bearing IGF-I*-agarose in the presence of 10 μM [γ-32P]ATP at 22°C results in the phosphorylation of a relatively broad band on SDS-polyacrylamide gels with an average Mr = 94,000 (Fig. 1A, lanes 1 and 2). When the gel is electrophoresed longer, this broad 32P-labeled band is resolved into a doublet of species (lane 3). The 32P-labeled phosphoamino acids in the hydrolysates were obtained after incubation with placental extracts in the presence of 10 and 100 nM free IGF-I* (Fig. 1A, lanes 3 and 4). This inhibition is specific for IGF-I* because free insulin at 100 nM (lane 5) is less effective (50% decrease), and at 10 nM (lane 6) insulin has essentially no effect in displacing the Mr = 94,000 32P-labeled species.

Silver staining of the same polyacrylamide gel (Fig. 1B) reveals that adsorption to the IGF-I*-agarose matrix of a prominently stained band of Mr = 130,000 and a less obvious band of Mr = 94,000 exhibits graded sensitivity to inhibition by free IGF-I* and insulin. Interestingly, the Mr = 130,000 silver stained band also appears to be broad and possibly consists of a doublet of slightly different molecular weight species. The highly specific adsorption of the Mr = 130,000 silver stained band and the Mr = 94,000 32P-labeled species to the IGF-I*-agarose matrix and their similarity in apparent molecular weights to that of the human placental IGF-I receptor α and β subunits (1, 6) strongly suggest that the Mr = 130,000 and 94,000 species specifically adsorbed by the IGF-I*-agarose preparation represent the α and β subunits, respectively, of the IGF-I receptor.

The presence of other silver stained bands in Fig. 1B indicates that our IGF-I receptor preparations are not contaminated. However, it should be pointed out that most of the silver stained species with Mr < 60,000 are not contaminants associated with the immobilized IGF-I receptor preparation because they are also present when a buffer blank is analyzed (data not shown). Thus, the extent of contamination is restricted to 3–4 protein species in our receptor preparation. Apart from a major protein of Mr = 100,000, the amounts of these contaminants as estimated by the intensity of silver staining are relatively minor when compared to that of the IGF-I receptor subunits. Furthermore, these contaminants appear to adsorb to the IGF-I*-agarose matrix nonspecifically, and their adsorption is not dependent on the presence of the IGF-I receptor. Thus, the degrees of contamination by these protein species as evidenced by the silver staining patterns are not diminished when the IGF-I receptor is prevented from binding to the IGF-I*-agarose in the presence of excess free IGF-I* (compare lanes 1, 2, 3, and 4 in Fig. 1B).

Activation of IGF-I Receptor Kinase by Prior Phosphorylation—Findings in this (27) and another laboratory (26) indicate that the tyrosine kinase activity of the insulin receptor toward exogenous substrates is activated when the receptor β subunit is phosphorylated on tyrosine residues. In view of the general similarities in subunit structures (1) between the insulin and IGF-I receptors and the ability of the IGF-I receptor to undergo phosphorylation on tyrosine residues in the receptor β subunit upon ligand binding (21, 22), we examined whether the immobilized IGF-I receptor would exhibit tyrosine kinase activity toward exogenous substrates and, if so, whether this receptor kinase activity would be enhanced following tyrosine phosphorylation of the receptor β subunit. In order to ensure that the IGF-I receptor preparation used for the following studies were not contaminated by the insulin receptor, the adsorption of the IGF-I receptor to IGF-I*-agarose was performed in the presence of a saturating concentration (100 nM) of free insulin so that the insulin receptor is competitively inhibited from binding to the IGF-I*-agarose matrix. As shown in Fig. 2, the immobilized IGF-I receptor exhibits kinase activity using histone as exogenous substrate. Significantly, when such a preparation of immobilized IGF-I receptor is first incubated in the presence of increasing concentrations of ATP for 15 min at 22°C followed by extensive washing to remove the unreacted ATP, the histone kinase activity of the IGF-I receptor preparation from the ATP-treated group is progressively increased (Fig. 2).
IGF-I receptor preparations were incubated with different concentrations of unlabeled or [γ-32P]ATP for 15 min at 22 °C. The preparations were washed extensively to remove the unreacted ATP. Samples incubated with [γ-32P]ATP were boiled in 0.2 ml of electrophoresis buffer containing SDS and dithiothreitol and electrophoresed on 7% gel. The 32P-labeled band corresponding to the β subunit of the IGF-I receptor was excised, and radioactivity was determined by Cerenkov counting. Activity in the receptor-bearing preparation relative to the receptor-deficient preparation at 1 mM ATP is between 15 and 20% relative to the receptor-bearing preparation.

When phosphoamino acid contents of the 32P-labeled histone were analyzed, the increase by IGF-I receptor-mediated phosphorylation of other phosphate acceptor sites on the receptor subunit. It should be noted that the ATP-activated kinase activity is dependent on the presence of the IGF-I receptor because no such enhancement in kinase activity is observed when IGF-I receptor-deficient agarose preparations are used (data not shown).

When the phosphoamino acid contents of the 32P-labeled histone are analyzed, the increase by IGF-I receptor-mediated phosphorylation occurs primarily on tyrosine residues (Fig. 3). Interestingly, relatively minor increases in serine and threonine phosphorylation of histone are also detected after ATP treatment. The basis for these increased phosphorylations is unclear. These results, together with those in Fig. 9 showing the predominant tyrosine phosphorylation of the IGF-I receptor β subunit, indicate that the IGF-I receptor-associated kinase is like the insulin receptor kinase in that it is also activated by prior tyrosine phosphorylation of the receptor β subunit.

Fig. 4 depicts the specificity of activation of the IGF-I receptor kinase by ATP. Among the nucleosides and nucleotide tested, only ATP is effective in activating the receptor kinase. The small degree of activation of the IGF-I receptor kinase by ADP is probably due to the presence of contaminating ATP in the ADP sample. Alternately, it may be related to the ability of ADP to serve as phosphate donor. It should be noted that the nucleotide preference of kinase activation exhibited by the IGF-I receptor is identical to that for the insulin receptor (27).

The notion that the IGF-I receptor kinase is activated following tyrosine phosphorylation of the receptor β subunit is further evaluated by examining the reversibility of the ATP-activated receptor kinase activity following dephosphorylation of the receptor by treatment with alkaline phosphatase. It is clear in Fig. 5 that the ATP-activated IGF-I receptor histone kinase activity is markedly reduced (60%) when the phosphorylated receptor preparation is first incubated with 12 units/ml bovine alkaline phosphatase for 3 h at 8 °C. Accompanying this diminution in receptor kinase activity, there is a similar magnitude of decrease in the phosphate content of the IGF-I receptor β subunit (data not shown). Significantly, the phosphatase-mediated decrease in receptor kinase activity is completely blocked when a specific tyrosine

**Fig. 2.** Relationship between the phosphorylation of the IGF-I receptor β subunit and its associated histone kinase activity. IGF-I receptor-bearing and -deficient IGF-I*-agarose preparations were incubated with different concentrations of unlabeled or [γ-32P]ATP for 15 min at 22 °C. The preparations were washed extensively to remove the unreacted ATP. Samples incubated with [γ-32P]ATP were boiled in 0.2 ml of electrophoresis buffer containing SDS and dithiothreitol and electrophoresed on 7% gel. The 32P-labeled band corresponding to the β subunit of the IGF-I receptor was excised, and radioactivity was determined by Cerenkov counting. Activity in the receptor-bearing preparation relative to the receptor-deficient preparation at 1 mM ATP is between 15 and 20% relative to the receptor-bearing preparation.

**Fig. 3.** Phosphoamino acid analysis of IGF-I receptor-mediated histone phosphorylation. Gel pieces containing the 32P-labeled histone band were dried in air. The dried gel pieces were soaked in 2 ml of 50 mM NH4HCO3 containing 0.1% SDS and 100 mM mercaptoethanol and were then homogenized. Extraction of phosphorylated histone was affected by incubating the gel homogenate at 37 °C for 16 h. After incubation, the homogenates were centrifuged. The extracted 32P-labeled protein in the supernatant was precipitated, partially hydrolyzed in 6 M HCl and analyzed for phosphoamino acid contents as described under "Experimental Procedures."

**Fig. 4.** Specificity of activation of IGF-I receptor kinase by ATP. IGF-I receptor-bearing and -deficient IGF-I*-agarose preparations were incubated at 22 °C for 15 min in the presence and absence of 200 μM of the indicated nucleosides and nucleotides. After incubation, the resin was washed extensively to remove the unreacted nucleosides or nucleotides. The preparations were then assayed for their kinase activities using histone as substrate as described under "Experimental Procedures."

**Fig. 5.** This figure depicts the specificity of activation of the IGF-I receptor kinase by ATP. Among the nucleosides and nucleotide tested, only ATP is effective in activating the receptor kinase. The small degree of activation of the IGF-I receptor kinase by ADP is probably due to the presence of contaminating ATP in the ADP sample. Alternately, it may be related to the ability of ADP to serve as phosphate donor. It should be noted that the nucleotide preference of kinase activation exhibited by the IGF-I receptor is identical to that for the insulin receptor (27).

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activity by alkaline phosphatase. IGF-I receptor-bearing and -deficient IGF-1*-agarose preparations were phosphorylated with 1 mM unlabeled ATP at 22 °C for 15 min. The resin was then extensively washed and incubated in the presence and absence of 12 units/ml alkaline phosphatase in 200 mM Tris-HCl, pH 8, containing 0 or 1 mM sodium vanadate at 8 °C for 3 h. The agarose preparations were washed and then suspended in 50 mM Hepes buffer, pH 7.4, containing 0.1% Triton X-100, 10 mM MgCl₂, 3 mM MnCl₂, 1 mM PMSF, 0.4 mg/ml histone H2B, and 100 mM sodium vanadate. The phosphorylation reaction was initiated by the addition of [γ-32P]ATP to a final concentration of 0.3 μM and continued for 10 min at 22 °C. The values of histone phosphorylation shown in the figure represent the IGF-I receptor-dependent kinase activity calculated by subtracting the histone-associated 32P radioactivity in the receptor-deficient preparation from that in the receptor-bearing preparation. The amount of histone-associated 32P radioactivity in the IGF-I receptor-deficient agarose preparation is approximately 20% of that in the receptor-bearing agarose preparation and is the same among the various groups shown in the figure.

phosphatase inhibitor, vanadate, is included in the dephosphorylation buffer (Fig. 5).

Taken together, the data presented above indicate that the IGF-I receptor-associated tyrosine kinase, like the insulin receptor kinase, is activated following tyrosine phosphorylation of the receptor β subunit.

Reductant-stimulated IGF-I Receptor Phosphorylation—Previous studies (28, 30) showed that under in vitro conditions the kinase activity of the insulin receptor is stimulated by the presence of reductant such as diithiothreitol in the reaction mixture. We have been able to confirm this finding using insulin receptor preparations devoid of IGF-I receptor (Fig. 6, lanes 3 and 4). To investigate whether the IGF-I receptor exhibits a similar increase in kinase activity in the presence of reductant, immobilized IGF-I receptor preparations devoid of insulin receptor are incubated with 10 μM [γ-32P]ATP in the presence and absence of 5 mM diithiothreitol. It is clear in Fig. 6 (lanes 1 and 2) that the 32P labeling of the β subunit doublet of the IGF-I receptor is increased by approximately 7–8-fold when 5 mM diithiothreitol is included in the phosphorylation buffer.

In order to investigate and compare the nature of the stimulating effects of diithiothreitol on the phosphorylation of IGF-I and insulin receptors, the dose-response relationships of the two receptor systems to the reductant are examined (Fig. 7). Immobilized IGF-I and insulin receptor preparations are 32P labeled in the presence of increasing concentrations (0–9 mM) of diithiothreitol at 22 °C for 30 min. The phosphorylation reaction is stopped by the addition of electrophoresis sample buffer containing excess N-ethylmaleimide to neutralize the reductant. The samples are then electrophoresed under nonreducing conditions to preserve the different oligomeric forms of the two receptor types. In the absence of diithiothreitol, a 32P-labeled band of Mr = 350,000 is detected in both the IGF-I and insulin receptor preparations. The Mr = 350,000 phosphoproteins represent the native disulfide-linked heterotetramers (αβ₂) of the IGF-I and insulin receptors (1). Reduction of the Mr = 350,000 32P-labeled protein species with diithiothreitol leads to the generation of the Mr, = 93,000–95,000 32P-labeled β subunits of both receptors (data not shown).

The phosphorylation of the αβ₂ heterotetramers of the IGF-I and insulin receptors is markedly enhanced in the presence of diithiothreitol. The effects of the reductant are already evident at 0.1 mM and maximal at 1 mM. When the concentration of diithiothreitol is raised to 3 mM, the stimulatory action of the reductant on the phosphorylation of the native αβ₂ IGF-I and insulin receptors tends to diminish slightly. Beyond this critical concentration of reductant, the 32P labeling of the αβ₂ forms of both receptor types declines.
sharply and is barely detectable at 9 mM. Interestingly, the decline in phosphorylation of the αβ2 receptor forms at dithiothreitol concentration >1 mM is accompanied by the appearance of 32P-labeled bands of M, = 200,000 (Fig. 7). The M, = 200,000 phosphoproteins represent the disulfide-linked αβ monomer of the IGF-I and insulin receptor structures. A second electrophoresis of the excised M, = 200,000 species from either receptor preparation under reducing conditions results in the appearance of the 32P-labeled M, = 93,000–95,000 β subunits of the corresponding receptor type (data not shown).

An important point to note in Fig. 7 is that the dose-response relationships of the IGF-I and insulin receptor kinases to dithiothreitol are identical. In addition, the sensitivities to reduction of the heterotetrameric structures (αβ2) of both receptor types are also indistinguishable. These results further illustrate the similarity between the kinase characteristics of the insulin and IGF-I receptors.

**HPLC Tryptic Peptide Mapping of 32P-Labeled β Subunit Doublet of the IGF-I Receptor**—In order to investigate the number and nature of the phosphorylation sites on the β subunit of the IGF-I receptor, immobilized receptor on IGF-I-agarose was intensely phosphorylated with 5 μM [γ-32P]ATP of very high specific activity in the presence of 5 mM dithiothreitol (Fig. 8). The sample was then extensively electrophoresed on an SDS-polyacrylamide gel to achieve better separation of the high and low molecular weight species of the IGF-I receptor β subunit doublet (Fig. 8, panel A). The 32P-labeled IGF-I receptor β subunits were excised and exhaustively digested with trypsin. The tryptic hydrolysates were resolved on a reverse phase HPLC column using a 0–45% acetonitrile gradient. It is clear in Fig. 8 (panel B) that the 32P-labeled tryptic hydrolysates derived from the high molecular weight species of the IGF-I receptor β subunit are resolved into six phosphopeptide fractions designated peaks 1H–6H with retention times of 22, 25, 28, 30, 34, and 41 min, respectively. Interestingly, when the trypsinized low molecular weight form of the IGF-I receptor β subunit is subjected to the same HPLC phosphopeptide-mapping analysis (Fig. 8, panel C), the elution profile of the 32P-labeled peptides closely resembles that of the high-molecular weight form. Thus, a minimum of five labeled phosphopeptide peaks derived from the low molecular weight form of the IGF-I receptor β subunit doublet can be readily identified. These five phosphopeptide fractions exhibit similar retention times on the HPLC column to the corresponding peaks derived from the high molecular weight form. The broad peak eluted between 27 and 30 min may contain a mixture of unresolved phosphopeptides with retention times similar to those of peaks 3H and 4H, respectively. For comparison, the phosphopeptide fractions of the hydrolyzed lower molecular weight form of the IGF-I receptor β subunit doublet are labeled 1L–6L.

In order to compare the tryptic phosphopeptide maps of the IGF-I receptor β subunit to that of the insulin receptor, insulin receptor was prepared by mixing wheat germ agglutinin-agarose-enriched placental extracts with insulin-agarose in the presence of 100 nM free IGF-I to eliminate possible contamination with IGF-I receptor. The immobilized insulin receptor was then [32P]labeled with high specific activity [γ-32P]ATP in the presence of dithiothreitol, exhaustively trypsinized, and then resolved on the reverse phase HPLC column using the same elution conditions as for the IGF-I receptor. As shown in Fig. 8 (panel D), the tryptic digest of the 32P-labeled β subunit of the insulin receptor is resolved into four phosphopeptide fractions with retention times on the column of 25, 28, 35, and 42 min, respectively. The retention times of these four peaks are essentially the same as those corresponding to peaks 2, 3, 5, and 6 of the IGF-I receptor β subunit doublet. However, the 32P-labeled insulin receptor β subunit lacks the two IGF-I receptor phosphopeptide peaks designated peaks 1 and 4 (compare panel D to panels B and C in Fig. 8). These results suggest that the autophosphorylation sites in the IGF-I receptor and insulin receptor β subunits may have some structural features in common and that the IGF-I receptor β subunit may contain additional phosphorylation sites.

In order to further characterize the phosphorylation sites on the β subunit doublet of the IGF-I receptor, the phosphoamino acid contents in each of the HPLC phosphopeptide
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Experimental Procedures. After electrophoresis, the cellulose sheets at 110 °C for 1 h. The samples were then spotted on precoated cellulose plastic sheets and electrophoresed as described under "Exfractions of the tryptic hydrolysates derived from the "P-labeled IGF- were dried and autoradiographed.

and the "P-labeled phosphoamino acids were released by hydrolysis lyophilized. The dried materials were dissolved in 0.5 ml of 6 M HCl, and the "P-labeled phosphoamino acids were released by hydrolysis at 110 °C for 1 h. The samples were then spotted on precoated cellulose plastic sheets and electrophoresed as described under "Experi PEAK

Fig. 9. Analysis of the phosphoamino acid contents of HPLC-resolved tryptic phosphopeptide fractions of "P-la beled β subunits of the IGF-I and insulin receptors. The peak fractions of the tryptic hydrolysates derived from the "P-labeled IGF-I and insulin receptor subunits shown in Fig. 8 were pooled and lyophilized. The dried materials were dissolved in 0.5 ml of 6 M HCl, and the "P-labeled phosphoamino acids were released by hydrolysis at 110 °C for 1 h. The samples were then spotted on precoated cellulose plastic sheets and electrophoresed as described under "Experimental Procedures." After electrophoresis, the cellulose sheets were dried and autoradiographed. HMW, high molecular weight; LMW, low molecular weight.

fractions were analyzed (Fig. 9, upper and middle panels). Phosphotyrosine is found to be the predominant species in all the HPLC peak fractions. In addition, significant amounts of phosphoserine residues are also present in peaks 3-6, and trace amounts of phosphothreonine residues are detected in peak 4. The source of the serine and threonine phosphorylating activities is not known and may be due to some contaminating serine/threonine kinases adsorbed nonspecifically to the IGF-I*-agarose matrix. A comparison of the phosphoamino acid specificity of the corresponding phosphorylation site in the β subunits of the insulin and IGF-I receptor reveals that the matching phosphopeptide fractions from the two receptor types contain similar phosphoamino acid residues and that the ratio of phosphotyrosine to phosphoserine also appears to be similar (Fig. 9, lower panel).

DISCUSSION

In the present study, we have used IGF-I*-agarose to partially purify the IGF-I receptor for investigating the tyrosine kinase characteristics of this receptor type. The success of this approach is based on utilizing an agarose matrix containing a low density of coupled IGF-I*-agarose matrix. Thus, when the IGF-I receptor is being adsorbed to the IGF-I*-agarose beads, the effective concentration of IGF-I* is expected to be approximately 100 nM. This consideration is important in view of the potential contamination by the insulin receptor present in the same wheat germ agglutinin agarose-enriched placental extracts. Previous studies (31, 32) have indicated that minimal occupancy of the insulin receptor by IGF-I* occurs when the ligand concentrations are below 50 nM. Hence, the conditions used in this study to immobilize IGF-I receptor preparations are expected to minimize contamination by the insulin receptor.

Another line of evidence that the M, = 130,000 and 94,000 doublet species adsorbed by the IGF-I*-agarose represent the respective α and β subunits of the IGF-I receptor is provided by the differential abilities of IGF-I* and insulin to inhibit the adsorption of these two protein bands to the IGF-I*-agarose matrix. At a concentration of 10 nM free IGF-I*, the adsorption of the M, = 130,000 and 94,000 species to the agarose beads is completely eliminated, whereas free insulin at the same concentration has little or no effect (Fig. 1). When the concentration of free insulin is raised to 100 nM, significant inhibition of adsorption of these two protein species becomes evident. This observation is consistent with the previous findings that insulin at higher concentrations is capable of binding to the IGF-I receptor (32, 33). Further support for the identity of the IGF-I receptor in our preparation is that both the M, = 130,000 and 94,000 receptor species appear to be doublets. This finding is in agreement with that by Jacobs and co-workers (6, 21). In addition, a recent report (33) that the IGF-I receptor exists as two immunologically distinct populations in human placental membranes is consistent with our finding.

Significantly, it should be emphasized that the experiments presented in this report are performed under extremely stringent conditions in order to eliminate the possibility of contamination by the insulin receptor in our IGF-I receptor preparation (experiments shown in Figs. 2-9). This degree of stringency is useful in that the insulin receptor exhibits high affinity for insulin and at least 2 orders of magnitude lower affinity for IGF-I. Thus, when excess free insulin (100 nM) is added to saturate the insulin receptors in the wheat germ-agarose-enriched placental extracts, the insulin receptor, but not the IGF-I receptor, will be completely inhibited from binding to the IGF-I* agarose. Although the inclusion of excess free insulin in the placental extracts lowers to some degree the yield of IGF-I receptors adsorbed to the IGF-I*-agarose due to partial occupancy of the IGF-I receptor by insulin, this measure ensures exclusion of the IGF-I receptor preparation from cross-contamination by insulin receptors. Based on a similar strategy, insulin receptor preparations used for comparative studies (Figs. 6-9) were obtained by incubating lectin-enriched placental extracts with insulin-agarose in the presence of a saturating concentration of free IGF-I* (100 nM) to prevent adsorption to the insulin-agarose matrix. Therefore, any resemblance in enzymatic property between the insulin and IGF-I receptor kinases reflects the true commonality shared by these two receptor species rather than the cross-contamination of one receptor type by another.

Unlike the insulin receptor which has been purified to near homogeneity and shown to retain tyrosine kinase activity (23, 24), the IGF-I receptor has not yet been highly purified and shown to possess intrinsic kinase activity. However, it has been suspected that the IGF-I receptor is itself a kinase. Our preparations of the IGF-I receptor are not entirely pure in that they still contain 3-4 contaminating protein bands on SDS-polyacrylamide gels visualized by silver staining (Fig. 1, panel B). However, the fact that the IGF-I receptor-bearing preparations but not the receptor-deficient ones exhibit tyrosine kinase activity toward the receptor β subunit and added...
histone strongly supports the hypothesis that the IGF-I receptor is a tyrosine kinase. It is unlikely that the tyrosine kinase activity of the IGF-I receptor preparation is due to the presence of the contaminating protein bands visualized by silver staining. This is because the contaminating species are present in both receptor-bearing and -deficient preparations in similar amounts, and only the receptor-bearing IGF-I*-agarose exhibits tyrosine kinase activity. Nevertheless, the possibility still remains that the IGF-I receptor lacks intrinsic tyrosine kinase activity and that the phosphorylation of the receptor ϒ subunit is mediated by other tyrosine kinases adsorbed to the IGF-I-agarose matrix.

To address this issue, attempts were made to purify the IGF-I receptor further by acid elution (1 M NaCl, pH 5) of the adsorbed receptor from the IGF-I*-agarose matrix according to procedures described for the insulin receptor (34). However, these experiments were unsuccessful due to the poor recovery and lack of enrichment of the IGF-I receptor and the loss of the receptor kinase activity after elution. The lack of success of this approach may be partly related to the relatively lower density of IGF-1* coupled to agarose beads (50 μg/ml resin) in comparison to that utilized for insulin-agarose (250 μg/ml) (34). Furthermore, the yield of IGF-I receptor may also be compromised by the limited quantity of IGF-I*-agarose (1 ml) used for affinity purification and the inclusion of free insulin in the adsorption buffer to ensure an IGF-I receptor preparation free from contamination by insulin receptor. Thus, unequivocal resolution of this issue awaits the purification of the IGF-I receptor to homogeneity, perhaps with alternate approaches.

It has been well established that the IGF-I receptor and the insulin receptor share similar physical characteristics as well as biological activities. Both receptors exhibit similar tetrameric subunit composition of αβ, and similar subunit molecular weights on SDS-gel electrophoresis (1). Protease-generated peptide maps of the insulin and IGF-I receptor α subunits labeled by affinity cross-linking with 125I-labeled ligands revealed further resemblance between the two receptor subunit structures (4). Functionally, the two receptor systems elicit similar long term effects such as growth promotion (8–10) and short term responses such as stimulation of hexose and amino acid transport (11) and activation of glycogen synthetase (12). The physiological significance of the presence of these two very similar receptor systems in the same tissue type is not yet completely clear.

The present results strongly reinforce the concept that the immobilized IGF-I receptor is similar to the insulin receptor in the following aspects. 1) The ϒ subunit of the partially purified IGF-I receptor is phosphorylated predominately on tyrosine residues upon incubation with [γ-32P]ATP (Figs. 1 and 9). 2) The receptor ϒ subunit phosphorylation is stimulated by dithiothreitol (Fig. 6). The dose-response curves to the reductant for the IGF-I and insulin receptor phosphorylation are identical (Fig. 7). Furthermore, the subunit composition of both receptor types exhibits similar sensitivity to reduction by dithiothreitol (Fig. 7). 3) The phosphorylation of the IGF-I receptor like the insulin receptor (27) is associated with activation of its tyrosine kinase activity toward exogenous substrate (Figs. 2, 4, and 5). Significantly, both receptor kinases exhibit identical concentration dependence and specificity to activation by ATP (Figs. 2 and 4 and Ref. 27). The list of similarities between the IGF-I and insulin receptors even extends to the phosphopeptide maps of the two receptor ϒ subunits. Thus, four out of the six phosphopeptide fractions derived from the trypsin-treated IGF-I receptor ϒ subunit doublet are similar chromatographically to those from the insulin receptor ϒ subunit tryptic hydrolysates on reverse phase HPLC (Fig. 8). They are also similar in phosphoamino acid composition to the corresponding phosphopeptides in the insulin receptor ϒ subunit.4 Taken together, the present study indicates that in addition to the resemblance in structure and biological functions between the IGF-I and insulin receptors the enzymatic properties of these two receptor systems are also very similar. However, the presence of two additional phosphorylation sites in the IGF-I receptor ϒ subunit doublet when compared to the insulin receptor phosphopeptides suggest that the cytoplasmic domains of these receptors are not identical (Fig. 8).

The presence of two molecular weight forms of the IGF-I receptor raises questions about the nature of the differences between these two receptor species. It is possible that the lower molecular weight form is a proteolytic product of the higher molecular weight receptor species. Alternatively, the different apparent masses may be the result of differential glycosylation of the same protein structure. The similarity in tryptic phosphopeptide maps between the two IGF-I receptor ϒ subunits in the doublet band suggests that both receptor species exhibit tyrosine kinase activity. Whether there are any subtle differences in tyrosine kinase activities between these two forms of IGF-I receptor remains to be determined. It will be of interest to investigate whether the two immunologically distinct IGF-I receptors (33) reported in a recent study actually represent the two different receptor forms identified in this and one other laboratory (6).

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4 During the course of preparing this manuscript, we received a manuscript from Dr. Steven Jacobs of the Wellcome Research Laboratories documenting the phosphorylation characteristics of the insulin and IGF-I receptors in intact cells in response to their respective ligands. Their findings also indicate that there is a striking similarity between phosphopeptide maps of the insulin and the IGF-I receptors (36).
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