Substrate Specificities of the Insulin and Insulin-like Growth Factor 1 Receptor Tyrosine Kinase Catalytic Domains*

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To compare the substrate specificities of the insulin and insulin-like growth factor 1 (IGF-1) receptor tyrosine kinases, the catalytic domains of the enzymes have been expressed in Escherichia coli as fusion proteins. The purified proteins have kinase activity, demonstrating that the catalytic domain of IGF-1 receptor, like that of insulin receptor, is active independent of its ligand-binding and transmembrane domains. The specificities of the two enzymes for the divalent cations Mg²⁺ and Mn²⁺ are indistinguishable. A series of peptides has been prepared that reproduces the major phosphorylation sites of insulin receptor substrate-1, a common substrate for the two receptor tyrosine kinases in vivo. Insulin and IGF-1 receptors show distinct preferences for these peptides; whereas insulin receptor prefers peptides based on Tyr-987 or Tyr-727 of insulin receptor substrate-1, the IGF-1 receptor preferentially recognizes Tyr-987. The latter site, when phosphorylated, is a binding site for the SH2 domain-containing adapter protein Grb2. The ability of the two receptor tyrosine kinases to be phosphorylated and activated by v-Src has also been examined. The catalytic activity of IGF-1 receptor is stimulated ~3.4-fold by treatment with purified v-Src, while insulin receptor shows very little effect of Src phosphorylation under these conditions. This observation is relevant to recent findings of IGF-1 receptor activation in Src-transformed cells, and may represent one method by which Src amplifies its mitogenic signal. Collectively the data suggest that the catalytic domains of the two receptor kinases possess inherently different substrate specificities and signaling potentials.

The polypeptide hormones insulin and insulin-like growth factor 1 (IGF-1) are closely related growth factors that regulate cell growth and metabolism. The two growth factors produce their biologic effects by binding to distinct receptors on the surface of target cells. Although insulin and IGF-1 receptors (IR and IGF-1R, respectively) have some functions in common, recent evidence suggests that the receptors play significantly different biological roles (for review, see Kahn (1985), Rechler and Nissey (1990), and Adamo et al. (1992)). While insulin primarily stimulates physiological processes such as glucose transport and biosynthesis of glycogen and fat (Kahn, 1985), IGF-1 has been shown to be more potent in stimulating cell growth by increasing DNA synthesis, and in promoting cell differentiation (Lammers et al., 1989; Rechler and Nissey, 1990). In keeping with its mitogenic role, IGF-1R has been shown to be important in the onset and maintenance of the transformed phenotype in vivo and in vitro (Kaikeo et al., 1990; Baserga, 1995).

The receptors for the two growth factors are highly homologous. They share the same digomeric structure: in each case the receptor is made up of two extracellular α subunits containing the ligand-binding domain and two transmembrane β subunits possessing tyrosine kinase activity (Yarden and Ullrich, 1988). The tyrosine kinase catalytic domains of the insulin and IGF-1 receptors, located in the cytoplasmic portions of the β subunits, possess ~84% sequence identity (Ullrich et al., 1986). The positions of N-linked glycosylation sites and cysteine residues in the extracellular domains are also highly conserved. Activation of the receptors is thought to occur in a similar manner. Binding of ligand to the α subunits activates IR or IGF-1R, leading to autophosphorylation of tyrosine residues in the β subunits (Yarden and Ullrich, 1988). Signaling via the IR and IGF-1R has been demonstrated to be dependent on their tyrosine kinase domains (Ebinu et al., 1987; Chou et al., 1987; Kato et al., 1993), which catalyze the phosphorylation of specific substrates, including the 185-kDa insulin receptor substrate-1 (IRS-1) protein (Sun et al., 1991). IRS-1 is a major substrate for both IR and IGF-1R in vivo (Sun et al., 1991; Myers et al., 1993), and it serves as an intermediate docking protein, providing binding sites for multiple downstream SH2 domain-containing proteins. For example, after IR and IGF-1R activation, the tyrosine-phosphorylated form of IRS-1 binds to the 85-kDa regulatory subunit of the phosphatidylinositol 3-kinase, and this interaction activates phosphatidylinositol 3-kinase (Backer et al., 1992; Giorgetti et al., 1993; Myers et al., 1993). In addition to phosphatidylinositol 3-kinase, IRS-1 can also interact with the growth factor receptor bound-2 (Grb2) protein and the SH2-containing tyrosine phosphatase Syp, in each case by SH2 domains in the downstream proteins binding to phosphorylated tyrosine residues within IRS-1 (Sun et al., 1993). Thus, insulin and IGF-1 appear to activate at least one common signaling pathway through IRS-1 phosphorylation and phosphatidylinositol 3-kinase activation. On the other hand, because the binding of insulin and IGF-1 to their respective receptors trigger distinct cellular responses, the signaling pathways emanating from the receptors are presumably different from each other, at least in part. At present the molecular basis for these differences in signal transduction remain unclear, particularly in light of the structural homology of the
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receptors. Effects specific for IGF-1 may be mediated by additional substrates that have not yet been identified, or by differential phosphorylation of sites on known substrates such as IRS-1.

Some experimental systems for comparing the activities of IR and IGF-1R have been described. Using highly purified preparations of IGF-1R and IR from human placenta, Sahal et al. (1988) demonstrated intrinsic differences in substrate specificity between the two kinases toward polymeric substrates. Although (Glu4,Tyr1), (Glu4,Tyr3), and (Glu4,Ala3,Tyr1) served as substrates for both tyrosine kinases, IGF-1R phosphorylated the former polymer approximately four times more efficiently than the latter polymer. In contrast, insulin receptor phosphorylated the two polymeric substrates nearly equally. In addition, insulin receptor was at least 10-fold more sensitive to inhibition by polymers such as (Tyr,Ala,Glu), than (Tyr-Ala-Glu), than the IGF-1 receptor. The signaling potentials of insulin and IGF-1 receptors have also been compared in studies using chimeric receptors. A receptor consisting of the ligand-binding domain of IR and the cytoplasmic domain of IGF-1R is 10 times more active in stimulating DNA synthesis than the IR itself (Lammers et al., 1989), suggesting that dissection of the substrate specificities of IR and IGF-1R is possible. We hypothesize that a difference in substrate specificity between the two catalytic domains allows IGF-1R to transmit signals which are distinct from those mediated by IR. The experiments described in this paper attempt to identify “downstream” events which are specific for IGF-1 through an examination of substrate recognition by the IGF-1 receptor.

EXPERIMENTAL PROCEDURES

Materials—E. coli strain NB42 harboring each plasmid was inoculated into 1 liter of LB broth containing ampicillin (50 μg/ml). The cultures were incubated for 2 h at 37°C, at which time isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM. The cultures were grown for an additional 5 h at 30°C. E. coli cells expressing the GST fusion proteins were lysed in a French pressure cell. After centrifugation, the supernatant containing cell lysates (1200 × g, 30 min) suspended in 1.5% Na-lauroylsarcosine (Sigma), 25 mM triethanolamine, 1 mM EDTA, pH 8.0. This mixture was rocked at 4°C for 30 min, then centrifuged at 4°C (10,000 × g, 10 min). This supernatant was combined with that from the initial centrifugation and applied to glutathione-agarose (Molecular Probes, Inc.). After washing with 50 mM HEPES (pH 7.4), 100 mM EDTA, purification of the IR and IGF-1R constructs was carried out by elution with excess reduced glutathione (Smith and Johnson, 1988). In both cases, purification of the fusion proteins on glutathione-agarose yielded proteins which were >95% pure, as judged by SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining. Proteins were stored in 40% glycerol at −20°C. After purification, tyrosine kinase activity was demonstrated toward synthetic peptides using the phosphocellulose paper assay (Casnellie, 1991).

Kinetics of Peptide Phosphorylation—Phosphorylation assays were carried out in total volumes of 25 μl at 30°C. Each reaction contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, and 2 mM MnCl2 (except as noted). Bovine serum albumin, and 200–500,000 cpn/mg), with varying peptide concentrations. Reactions were initiated by the addition of 5 μl of GST-IR or GST-IGF-1R fusion protein (0.5 μg) and were terminated by the addition of 45 μl of cold 10% trichloroacetic acid. The reaction mixtures were centrifuged for 2 min in an Eppendorf microcentrifuge and 35 μl of the supernatants were spotted onto 2.1-cm diameter phosphocellulose paper circles, as described (Casnellie, 1991). The phosphocellulose pads were washed three times with cold 0.5% phosphoric acid and once with acetone, dried, and counted dry in a liquid scintillation counter to measure incorporation of 32P into peptide. Initial rates of reaction (5%) were measured in triplicate and kinetic constants were determined by weighted nonlinear least-squares fit to the hyperbolic velocity versus [substrate] plots using the iterative program NUFFIT (Island Products, Galveston, TX).

Phosphorylation by v-Src—GST fusions of IGF-1 or insulin receptor (2 μg) were incubated with purified v-Src (6 μg) in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM MgCl2, 1 mg/ml bovine serum albumin, and 200 μM unlabelled ATP for 20 min at 30°C. The GST fusions were resolated by rocking with 50 μl of a 1.2 slurry of glutathione-agarose for 25 min at 4°C. The glutathione-agarose beads were collected by brief centrifugation and washed with 500 μl of cold 50 mM Tris (pH 7.4). After centrifugation, the resin-bound receptor kinases were incubated with 800 μM Peptide Y727 and 200 μM [γ-32P]ATP under the conditions described above for peptide kinase measurements. Reactions proceeded for 20 min and were analyzed using the phosphocellulose paper assay (in this case the chloroacetic acid was not added). Reaction mixtures were terminated by centrifugation in ice, and the resin-bound bound to glutathione-agarose. In some experiments v-Src was added together with 1 μg of Yersinia tyrosine phosphatase YopSiA1* (Zhang et al., 1992) (kind gift of Dr. Zhong-Yin Zhang, Albert Einstein College of Medicine). The tyrosine phosphatase was not a GST fusion protein and consequently it was removed when the receptor tyrosine kinases were adsorbed onto glutathione-agarose.

RESULTS

Expression of Human IGF-1R and IR Catalytic Domains—The catalytic domains of IGF-1 and insulin receptors were expressed in Escherichia coli as GST fusion proteins. The expression vectors were assembled from polymerase chain reaction-amplified fragments of the catalytic domains from cDNAs provided by Dr. J. Whittaker, Div. of Endocrinology, SUNY at Stony Brook School of Medicine. For these experiments, the portion of tyrosine kinase sequences included is the region of high homology among all kinase catalytic domains (corresponding to residues 40–300 of the CAMP-dependent kinase protein) (Hanks et al., 1988). Pairs of oligonucleotides corresponding to the borders of catalytic domain sequences of insulin and IGF-1 receptors were used as polymerase chain reaction primers. The polymerase chain reaction primers had 25 nucleotides of complementarity with the template and encoded unique restriction sites (BamHI at the 5' end and EcoRI at the 3' end). Amplified fragments containing recombination regions for the IGF-1R kinase domain, amino acids 956 to 1246; for the IR kinase domain, amino acids 971 to 1262. Polymerase chain reaction products were digested with BamHI and EcoRI, purified from agarose gels, and subcloned into the EcoRI-BamHI sites of the expression vector pGEX 2T. The DNA sequences of the inserted regions were determined using the Sequenase kit (U. S. Biochemical Co.).

Expression of the GST fusion proteins was carried out in bacterial strain B424 as described previously (Smith and Johnson, 1988; Garcia et al., 1993), with the following modifications. Overnight cultures (50 ml) of E. coli strain NB42 harboring each plasmid were inoculated into 1 liter of LB broth containing ampicillin (50 μg/ml). The cultures were incubated for 2 h at 37°C, at which time isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.2 mM. The cultures were grown for an additional 5 h at 30°C. E. coli cells expressing the GST fusion proteins were lysed in a French pressure cell. After centrifugation, the supernatant containing cell lysates (1200 × g, 30 min) suspended in 1.5% Na-lauroylsarcosine (Sigma), 25 mM triethanolamine, 1 mM EDTA, pH 8.0. This mixture was rocked at 4°C for 30 min, then centrifuged at 4°C (10,000 × g, 10 min). This supernatant was combined with that from the initial centrifugation and applied to glutathione-agarose (Molecular Probes, Inc.). After washing with 50 mM HEPES (pH 7.4), 100 mM EDTA, purification of the IR and IGF-1R constructs was carried out by elution with excess reduced glutathione (Smith and Johnson, 1988). In both cases, purification of the fusion proteins on glutathione-agarose yielded proteins which were >95% pure, as judged by SDS-polyacrylamide gel electrophoresis with Coomassie Blue staining. Proteins were stored in 40% glycerol at −20°C. After purification, tyrosine kinase activity was demonstrated toward synthetic peptides using the phosphocellulose paper assay (Casnellie, 1991).

Expression of Human IGF-1R and IR Catalytic Domains—The sources of enzyme for these studies were E. coli expression vectors encoding fusion proteins between the kinase catalytic domains and glutathione S-transferase (GST). This expression system allows purification of the protein under nondenaturing conditions by absorption to glutathione-agarose, followed by elution with excess reduced glutathione (Smith and Johnson, 1988). Our laboratory has recently described the construction and characterization of such vectors for the nonreceptor tyrosine kinases v-Src and v-Abl (Garcia et al., 1993). The specific activities of these fusion proteins are comparable to that of the kinases purified from transformed cells (Garcia et al., 1993). In the present study the catalytic domains of IR and IGF-1R were purified to homogeneity using this system. The procaryotic

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expression system for the receptor tyrosine kinase domains developed here will permit more facile structure-function studies of the enzymes (for example, by the use of site-directed mutagenesis to probe substrate specificity).

Although the GST-IR and GST-IGF-1R fusion proteins were isolated in a soluble form, a substantial portion (∼75%) of the protein was lost as a pellet in the clarifying centrifugation step after the bacterial cells were sonicated. This loss might be due to the insolubility of the expressed protein or to coaggregation with bacterial membranes. Because of the low yield of soluble fusion protein (∼100 µg/liter of cells), we were unable to carry out the thrombin cleavage reaction (Smith and Johnson, 1988) to produce the catalytic domain in sufficient quantities for kinetic analyses. Consequently, we used the intact GST fusion proteins to test for phosphorylation of the IRS-1 peptide substrates. Tyrosine kinase activity was tested using the phosphocellulose paper assay. These results (Tables II and III) indicated that insulin and IGF-1 receptor catalytic domains, expressed in E. coli as described above, possess functional tyrosine kinase activity. The specific activities of the fusion proteins were similar to those reported for other recombinant tyrosine kinase catalytic domains (e.g., Herrera et al., 1988; Morgan et al., 1991; Garcia et al., 1993), and the specificities appear to be unaltered, as judged from comparison of the GST-IR protein to a partially purified preparation of IR holoreceptors (Mothe et al., 1988; Cobb et al., 1989). In particular, at either 10 or 50 mM MgCl2, the ligand-stimulated phosphorylation of an exogenous substrate by IR was increased by the addition of MnCl2. In contrast, the addition of Mn2+ decreased the stimulatory effect of IGF-1 on IGF-1R kinase activity (Mothe et al., 1995). In those studies, the chimeric receptor resembled the IR at low concentrations of Mg2+ and the IGF-1R at high concentrations of Mg2+ (50 mM).

To test whether the catalytic domains of IR and IGF-1R themselves possess intrinsic specificities for divergent cations, we tested the phosphorylation of Peptide Y727 (Table I) by IR and IGF-1R under a variety of ionic conditions (Fig. 1). In all cases (for IR and for IGF-1R, and at low and high Mg2+ concentrations), addition of 2 mM Mn2+ was found to increase the phosphorylation of the exogenous peptide by the tyrosine kinases. Both receptors were found to be slightly more responsive to Mn2+ at a lower concentration of Mg2+ (Fig. 1). Additional Mn2+ (4 mM) appeared to increase the catalytic efficiencies of both receptor kinases (data not shown). Thus, the two catalytic domains do not appear to have any intrinsic differences in their divergent cation preferences. The results for ligand-induced stimulation of holoreceptors (Mothe et al., 1995) may have been due to the presence of additional regions of IR and IGF-1R not included in this study, such as the ligand-binding domain or the C-terminal amino acids, which have been shown to play an important role in signal transmission (Mothe et al., 1995). The similarity in cation specificity observed here is consistent with results reported previously for partially purified preparations of IR and IGF-1R from rat liver cells (Sasaki et al., 1985).

Phosphorylation of IRS-1 Peptides—Experiments with chimeric receptors in vivo (Lammers et al., 1989) and phosphorylation of synthetic tyrosine-containing polymers in vitro (Sahal et al., 1988) demonstrate a difference in substrate specificity between the insulin and IGF-1 receptors. Detailed knowledge about the relative substrate specificities of the two receptor kinases would be an important first step in the identification of their natural substrates. Both IR and IGF-1R are known to phosphorylate IRS-1 (Sun et al., 1991; Myers et al., 1993). Several tyrosine phosphorylation sites in IRS-1 are predicted to be within Tyr-Met-X-Met (YMXXM) motifs, and synthetic peptides corresponding to these sequences are excellent substrates for IR in vitro (Shoelson et al., 1992). When phosphorylated, several of these motifs become specific binding sites for SH2-containing signaling molecules (Sun et al., 1993). Thus, differential phosphorylation of tyrosines within IRS-1 could account for the differences in signaling observed in intact cells. To test this notion, the substrate specificity of IGF-1R and IR was measured with a series of peptides derived from IRS-1, as

| Peptide name | Sequence | Binding site |
|-------------|----------|-------------|
| Y628        | RKGNGDGYMPMSPKSV | Grb2 |
| Y658        | KKRVDPNGMMMSPSGV | Phosphatidylinositol 3-kinase p85 |
| Y727        | KKKLPATGDYMNMSGPVD | Syp |
| Y895        | KKKSPGEYVRLEFG | |
| Y939        | KKGSSEYRNMDLGPR | |
| Y987        | KKRSGDYTMROIIG | |
| Y1172       | KKKNGLNTIDDL | |

The synthetic peptides are named according to the position of tyrosine in the sequence of IRS-1. Tyrosine residues are underlined, along with amino acids at the +1 to +3 positions that are important for SH2 domain binding. For those sites which have been shown to bind specific SH2-containing proteins (Sun et al., 1993), the name of the protein is given in the right-hand column.
shown in Table I. These sequences correspond to major sites for IR phosphorylation in vivo, and include those sites which have been shown to bind SH2-containing proteins after phosphorylation. For each synthetic peptide, if sufficient basic residues were not present within the native sequence for absorption to phosphocellulose paper under the acidic conditions of the assay (Casnellie, 1991), additional lysine residues were added to the N terminus.

As shown in Table I, all of the IRS-1 peptides served as good substrates for the catalytic domain of the IGF-1R kinase. Experiments were performed at saturating concentrations of ATP (200 μM) and Mg2+ (10 mM) to arrive at values of K_m and V_max for the peptides. The IGF-1R catalytic domain exhibited distinct preferences for the IRS-1 peptides; these preferences tended to be dominated by K_m rather than by V_max. K_m values ranged from a low of 26 μM (Y895) to a high of 249 μM (Y628), whereas V_max values fell between 0.5 and 1.6 nmol/min/mg (Table II). These peptides are among the best reported in vitro substrates for the IGF-1R tyrosine kinase to date. In terms of K_cat/K_m, the most meaningful parameter for substrate specificity comparison (Fersht, 1985), the best substrate was Y628 (Table II). When phosphorylated, the site surrounding Y895 in IRS-1 has been shown to be a binding site for Grb2 (Sun et al., 1993), a small adapter molecule that contains one SH2 domain and two SH3 domains. The poorest substrate of this series was Peptide Y987, with K_cat/K_m reduced ~14-fold from Peptide Y895.

With the exception of Y895 and Y1172, all of the peptides listed in Table I have been tested as substrates for the insulin receptor tyrosine kinase (Shoelson et al., 1992). These results showed that the preferred sequence for phosphorylation was Y895, although for IR the peptides were all excellent substrates and only displayed a 2-fold range of K_cat/K_m (Shoelson et al., 1992). Peptides Y895, Y987, and Y1172 were therefore compared as substrates for the catalytic domain of the IR (Table III). Peptide Y987 had the lowest value of K_m (30 μM) and the highest value of K_cat/K_m (2.6 × 10^3 M^{-1} min^{-1}) of these three peptides. These results contrasted with those for IGF-1R (Table II), in which Y895 was preferred over Y987 and Y1172. The results obtained for IGF-1R and IR catalytic domains, along with those obtained previously for IR, are compared directly in terms of K_cat/K_m in Fig. 2. It can be seen from these data that the IGF-1R has a stricter substrate specificity for the IRS-1 phosphorylation sites, with Y895 being the best and Y628, Y987, and Y1172 being more poorly recognized. In contrast, the insulin receptor kinase preferentially phosphorylates peptides corresponding to IRS-1 sites Y727 and Y987. These differences in YMMK substrate specificity may also represent a difference in signaling potential between IR and IGF-1R.

Phosphorylation by v-Src. It has recently been reported that IGF-1R exhibits an elevated level of tyrosine phosphorylation in cells expressing v-Src, even in the absence of stimulation by IGF-1 (Peterson et al., 1994). In these cells, phosphorylation of IGF-1R was correlated with increased tyrosine kinase activity of the receptor, as measured both by autophosphorylation and by phosphorylation of exogenous substrates. These studies suggest that IGF-1R is an in vivo substrate for v-Src. We tested whether the catalytic domains of IR and IGF-1R would be activated by treatment with purified v-Src. For these studies, IR and IGF-1R were incubated with Src and unlabeled ATP for 20 min and then resolated by adsorption onto glutathione-agarose. Because the baculovirus vector used to express v-Src does not encode a GST fusion protein, glutathione-agarose adsorption served to remove Src from the reactions. Recovery of the IR and IGF-1R was quantitative under these conditions, as judged by comparisons of enzymatic activity with equivalent amounts of receptors which were not adsorbed onto glutathione-agarose (data not shown). The tyrosine kinase activities of the IR and IGF-1R were then measured by addition of kinase assay buffer, Peptide Y727, and [γ-32P]ATP directly to the resin-bound enzymes. In these experiments, the activity of IGF-1R was elevated approximately 3.4-fold after treatment with v-Src, relative to the untreated control (Fig. 3). In contrast, the activity of the GST-IR protein was increased only by a factor of 1.2 (Fig. 3). In a control reaction with Src alone, no Src activity remained bound to the glutathione-agarose beads after washing (Fig. 3).

To confirm that activation of IGF-1R in the presence of Src depended on the tyrosine phosphorylation of the receptor, the reactions were also carried out in the presence of the Yersinia tyrosine phosphatase. After exposure to Src and the Yersinia phosphatase, IGF-1R and IR were isolated by adsorption onto glutathione-agarose and tested for tyrosine kinase activity toward the synthetic peptide. As shown in Fig. 3, treatment with the tyrosine-specific phosphatase reversed the activation by Src, indicating that the increased IGF-1R activity was due to increased tyrosine phosphorylation of the receptor. Thus, in an

### Table II

| Peptide | K_m (μM) | V_max (nmol/min/mg) | K_cat/K_m (M⁻¹ min⁻¹) |
|---------|----------|---------------------|------------------------|
| Y628    | 249 ± 37 | 1.6 ± 0.23          | 4.0 × 10²               |
| Y658    | 79 ± 12  | 1.5 ± 0.25          | 1.2 × 10³               |
| Y727    | 50 ± 5.1 | 1.5 ± 0.06          | 1.9 × 10³               |
| Y895    | 26 ± 3.9 | 1.1 ± 0.05          | 2.6 × 10⁴               |
| Y939    | 73 ± 12  | 0.9 ± 0.18          | 7.8 × 10⁴               |
| Y987    | 178 ± 20 | 0.5 ± 0.09          | 1.9 × 10⁴               |
| Y1172   | 143 ± 9.8| 1.2 ± 0.19          | 5.2 × 10⁴               |

### Table III

| Peptide | K_m (μM) | V_max (nmol/min/mg) | k_cat/K_m (min⁻¹) |
|---------|----------|---------------------|-------------------|
| Y895    | 52.9 ± 5.6| 1.0 ± 0.11          | 1.2 × 10³          |
| Y987    | 30.0 ± 2.4| 1.2 ± 0.13          | 2.6 × 10³          |
| Y1172   | 43.9 ± 6.6| 1.2 ± 0.22          | 1.7 × 10³          |
The insulin and IGF-1 receptors exhibit a large degree of similarity, both with respect to their enzymatic properties and to their structural organization. On the other hand, the biological responses elicited by the two receptors are different, suggesting that specific signaling pathways must exist. The IGF-1 receptor appears to be intrinsically more effective at stimulating DNA synthesis than the insulin receptor (Kahn, 1985; Rechler and Nissey, 1990; Adamo et al., 1993). Because of the large number of potential sites for tyrosine phosphorylation in IRS-1 (Sun et al., 1991), the possibility exists that different sites (or combinations of sites) are recognized and phosphorylated by the insulin and IGF-1 receptors, giving rise to different docking sites for downstream signaling molecules containing SH2 domains. Additionally or alternatively, individual SH2 domain-containing proteins might be expressed preferentially with one or the other receptor tyrosine kinase.

To test whether the sites of IRS-1 are recognized differentially by the two enzymes, we have tested a series of synthetic peptides which reproduce the major tyrosine phosphorylation sites on IRS-1. Interestingly, we observe differences in substrate specificity between the two enzymes in these studies (Tables II and III; summarized in Fig. 2). Whereas the insulin receptor displays a rather broad specificity, with Peptides Y727 and Y987 being the best substrates, IGF-1 receptor has a more restricted specificity and prefers Peptide Y895. While we cannot identify the precise amino acid determinants for phosphorylation by the two enzymes, it is interesting to note that substitutions at either methionine residue in YMXX motifs invariably leads to decreased catalytic efficiency for the insulin receptor, suggesting that the Met$^1$- and Met$^3$-residues play important roles in enzyme-substrate recognition (Shoelson et al., 1992). The IGF-1 receptor kinase appears to be less dependent on C-terminal methionine residues; Peptide Y895, which contains the sequence Tyr-Val-Asn-Ile, is the preferred site. These experiments demonstrate for the first time distinctions between the substrate specificities of the insulin and IGF-1 receptors using amino acid sequences that are physiologically relevant. The results are consistent with earlier studies on random copolymers of amino acids, which also showed different preferences for the two receptor kinases (Sahal et al., 1988).

These distinctions in specificity toward IRS-1 peptides in vitro may parallel a difference in signaling by IGF-1 and insulin receptors through IRS-1. Recently it has been shown that a 262-amino acid portion of IRS-1 (residues 516–777), which contains five potential YMXX or YYXX phosphorylation sites, is recognized similarly by insulin and IGF-1 receptors ($K_m = 6.8$ and $9.9 \mu M$, respectively) (Siemeister et al., 1995). However, Tyr-895, which lies outside of this region of IRS-1, may be a determinant for downstream signaling that is favored by IGF-1 receptor. After phosphorylation, the Tyr-895 site in IRS-1 constitutes a binding site for the Grb2 adapter protein (Sun et al., 1993). Genetic and biochemical evidence suggests that Grb2 is an upstream regulator of the GTP exchange protein mSOS, which stimulates the formation of an active p21ras-GTP complex (Egan et al., 1993; Gale et al., 1993; Olivier et al., 1993; Rozakis-Adcock et al., 1993). Thus, promotion of stable binding between IRS-1 and Grb2 may play a role in the mitogenic signaling of IGF-1. On the other hand, alternate pathways to IRS-1 may exist for insulin or IGF-1 signaling, as suggested by experiments with transgenic IRS-1 knockout mice (Araki et al., 1994; Tamemoto et al., 1994). In the case of insulin receptor, it has recently been shown that Grb2 associates rapidly and transiently with IRS-1 after insulin treatment, but that Shc plays a more important role than IRS-1 in the binding of Grb2 and formation of p21ras-GTP (Sasaoka et al., 1994).

We also show that the insulin and IGF-1 receptors differ with regard to their potential for activation by v-Src (Fig. 3). It has been shown recently that the IGF-1 receptor becomes tyrosine phosphorylated in cells expressing v-Src, and that the in vivo increase in phosphorylation parallels an increase in vitro tyrosine kinase activity of the IGF-1 receptor (Peterson et al., 1994). In these studies it was not shown whether IGF-1 receptor acted as a direct substrate for v-Src in vitro, or where on the IGF-1 receptor the site(s) for Src phosphorylation occurred. Under the in vitro conditions reported here, the kinase activity...
of the IGF-1 receptor catalytic domain was stimulated approximately 3.4-fold by v-Src, whereas the activity of insulin receptor showed only a modest increase after treatment with v-Src under identical conditions. Activation was due to increased tyrosine phosphorylation, as demonstrated by reversal of the effect by a tyrosine-specific phosphatase. In these experiments Src may catalyze the direct phosphorylation of IGF-1R catalytic domain, or act indirectly, by promoting receptor autophosphorylation. We favor the former explanation since it has been shown that v-Src can cause tyrosine phosphorylation of an inactive mutant of the IGF-1 receptor in vitro (Peterson et al., 1994). Our in vitro experiments indicate that the IGF-1 receptor has the potential to act as a direct substrate for v-Src, and that at least one target for Src phosphorylation may be present in the catalytic domain of IGF-1R itself. The level of activation observed (≈3.4-fold) is similar to the level of increased IGF-1 receptor kinase activity seen in vivo (≈4-fold). Activation of IGF-1 receptor (or other receptor tyrosine kinases) could be a regulatory mechanism by which v-Src amplifies its signaling potential. In the case of IGF-1 receptor, this may be particularly important because of the role of the receptor in stimulating mitogenesis and because of its oncogenic potential (Kaleyko et al., 1990). Overexpression of IGF-1 receptor in NIH3T3 cells leads to ligand-dependent morphological transformation and colony growth in soft agar, and cells overexpressing IGF-1R cause the formation of tumors when introduced into nude mice (Kaleyko et al., 1990).

In conclusion, we have demonstrated distinctions between the tyrosine kinase catalytic domains of the IGF-1 receptor and insulin receptor. Studies with chimeric receptors indicate that the biological specificity of these two polypeptide hormones can be attributed to the cytoplasmic portions of their β subunits. For example, a chimeric receptor composed of the extracellular domain of the insulin receptor and the cytoplasmic portion of the IGF-1 receptor behaves similarly to the wild-type IGF-1 receptor (Lammers et al., 1989). This implies that a detailed knowledge of receptor kinase substrate specificity may shed light on the different downstream signaling pathways triggered by insulin and IGF-1.

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