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Discrete Region of the Insulin Receptor Carboxyl Terminus Plays Key Role in Insulin Action

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Abstract In the present study, we attempted to determine the importance of a 23–amino-acid sequence within the carboxyl terminus of the human insulin receptor (IR) molecule in modulating insulin action in Chinese hamster ovary cells. Stable expression of a minigene encoding the receptor fragment led to an increase in insulin-induced IR autophosphorylation that was 2.4-fold higher when compared to that of IR-expressing cells transfected with empty vector. Insulin-stimulated downstream signaling was also significantly elevated in cells expressing the minigene. It was found that expression of the minigene had no effect toward insulin-like growth factor I receptor kinase activity and function. These results indicate that the IR carboxyl terminus contains a motif that acts as a physiologic modulator of insulin signaling. J. Cell. Biochem. 78:160–169, 2000. Published 2000 Wiley-Liss, Inc.†

Key words: receptor tyrosine kinase; signal transduction; thymidine incorporation; phosphatidylinositol 3′-kinase; mitogen-activated protein kinase; CHO cells; transfection

The pleiotropic actions of insulin are mediated through activation of a membrane-bound heterotetrameric receptor. Following insulin binding, the intrinsic phosphotransferase function of the insulin receptor (IR) β subunit is activated, resulting in the tyrosine phosphorylation of a number of intracellular proteins, including insulin receptor substrate (IRS)-1 to 4, and a family of "src and collagen homology" adaptor proteins termed Shc [reviewed in Myers and White, 1996]. Insulin receptor substrate-1 is the most characterized substrate whose tyrosine phosphorylation leads to the recruitement and subsequent activation of a number of src-homology 2 (SH2) domain-containing proteins that include phosphatidylinositol 3′ kinase (PI-3′ kinase), adaptor molecules such as growth factor receptor binder-2 (GRB2) and Nck, and the Syp/SHP-2 tyrosine phosphatase [reviewed in Myers and White, 1996; Cheatham and Kahn, 1995]. The binding of GRB2 to tyrosine phosphorylated IRS-1 or Shc proteins induces its association with Sos, a Ras guanine nucleotide exchange factor, resulting in sequential activation of the Ras/Raf/mitogen-activated protein (MAP) kinase signal transduction cascade, and stimulation of DNA synthesis and gene transcription [Kyriakis et al., 1992; Rozakis-Adcock et al., 1993].

The carboxyl terminal domain is one of several structural regions that has been defined within the intracellular portion of the IR β subunit to contribute to the propagation of the insulin signal [Mothe et al., 1995]. This region (Leu^{1245}–Ser^{1343}),[1] which shows a high degree of divergence from the related insulin-like growth factor 1 (IGF-1) receptor [Ullrich et al., 1986], may allow the selective association of signaling proteins with one receptor but not the other [Maegawa et al., 1993; Levy-Toledano et al., 1994; Laviola et al., 1997;

Abbreviations used: IR, insulin receptor; IRS, insulin receptor substrate; PI-3′ kinase, phosphatidylinositol 3′-kinase; GRB2, growth factor receptor binder-2; MAP kinase, mitogen-activated protein kinase; IGF-1, insulin-like growth factor-1; CHO, Chinese hamster ovary; P5, peptide corresponding to receptor sequence 1292–1315; PVDF, poly(vinylidene) difluoride; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; PTPases, protein tyrosine phosphatases.

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[1]The numbering system used is the minus exon 11 variant of the IR [Ullrich et al., 1985].

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O'Neill et al., 1997] and/or promote phosphorylation of distinct substrates [Xu et al., 1995; Najjar et al., 1997], resulting in differential signaling [Kalloo-Hosein et al., 1997]. Indeed, swapping of the carboxyl terminus between the insulin and IGF-1 receptors significantly attenuates cellular responses elicited by insulin [Faria et al., 1994; Tartare et al., 1994]. Several studies from Van Obbergen's laboratory have revealed the importance of the carboxyl terminus in regulating IR kinase function using antipeptide antibodies directed against the receptor's acidic region (residues 1270–1280) [Baron et al., 1995], and the proreceptor sequences 1294–1317 [Gual et al., 1996] and 1309–1326 [Kaliman et al., 1993]. The purified 98–amino-acid carboxyl terminal domain of IR also stimulates the tyrosine kinase activity of the IR in a cell-free system [Kasuya et al., 1994].

Previously, we reported that a synthetic peptide corresponding to residues 1293–1307 within the carboxyl terminus of IR enhances insulin-induced autophosphorylation of partially purified insulin receptors [Kole et al., 1996b]. The introduction of an *N*-stearyl derivative of this peptide in IR-expressing Chinese hamster ovary (CHO) cells resulted in a significant increase in insulin-induced IR autophosphorylation and function [Kole et al., 1996b], suggesting that the entry of a fatty-acylated receptor fragment in cells can modulate intrinsic activity of the IR. However, peptide derivatization with stearate introduces several levels of complexity. First, the plasma membrane in which the IR and many signaling components are anchored provides a milieu for biochemical reactions where component access and component orientation relative to the membrane may enable a class of reactions that is quite distinct from that occurring in the cytoplasm [Tsunoda et al., 1998; Weng et al., 1999]. Second, the lipid environment at the plasma membrane may also introduce alterations in effective concentrations of the stearyl-conjugated peptide. Whether the separation of reactions in space allows the unmodified peptide to carry out comparable cellular actions is unknown. To clarify this issue, we attempted to express stably an IR fragment (residues 1292–1315), called P5, using a minigene approach and to assess its effect toward insulin and IGF-1 responses in intact cells.

MATERIALS AND METHODS

Materials

Recombinant IGF-1, myelin basic protein, anti-IRS-1, anti-phosphotyrosine, and anti-rat MAP kinase (ERK1-CT) polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Hygromycin B and insulin were from Calbiochem (La Jolla, CA); Pefabloc SC was from Boehringer Mannheim (Indianapolis, IN); poly(vinylidene) difluoride (PVDF) membrane, precast 4%–12% and 4%–20% polyacrylamide gels, and 10%–20% Tricine gels were from Novex (San Diego, CA). Protein G-plus/protein A-agarose beads and monoclonal anti-insulin receptor antibody (α-IR, clone 29B4) were from Oncogene Sciences, Inc. (Uniondale, NY). Rabbit polyclonal anti-TFIIH p62 (sc-292) and anti-insulin receptor α subunit (sc-710) antibodies, and monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase (sc-508) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ham's F-12 medium was from Paragon Biotech., Inc. (Baltimore, MD), and fetal bovine serum was from Hyclone (Logan, UT). Rainbow prestained protein markers and [γ-^{32}P]ATP (~3,000 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL); [methyl-^{3}H]thymidine (70–90 Ci/mmol) was from DuPont, NEN.

Vector Construction

The expression plasmid containing the cDNA for the human insulin receptor, pCVSVHIRc (a gift from Dr. Peter A. Wilden, University of Missouri, Columbia, MO), was used as a template for amplification of receptor sequence P5 by polymerase chain reaction (PCR). The pSVP5 minigene contained an EcoRI site at the 5′ end for subcloning, followed by the ribosome-binding site consensus sequence GCCGCCACCATGG [Kosak, 1989], a methionine codon, and a glycine codon (GGA). The 3′ end of the minigene included a glycine codon (GGA) that was followed by a sequence confering stop codon in all three frames (TAGATAAATGA) and by an XbaI site for subcloning. The sequence of the 5′ primer was 5′CGGAATTCGCCGCCACCATGGGACGTTCCTCGCACTGTCAGA3′ and the sequence of 3′ primer was 5′GCTCTAGATCATTTATCTATCCGCTCCGCTTGAAACCCAGCG3′. The PCR product (100 bp) was subcloned in pSVK3 (Pharmacia Biotech Inc., Piscataway, NJ). The

presence of insert and its sequence was verified by dideoxynucleotide sequencing using Sequanase (United States Biochemical, Cleveland, OH).

Transfection and Cell Culture

Chinese hamster ovary cells overexpressing the human insulin receptors (CHO/HIRc cells) were a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA) whereas CHO/ER cells, which express undetectable levels of endogenous IRs, were from Dr. Roger J. Davis (University of Massachusetts, Worcester, MA). Cells (2×10^6 cells/ml) were cotransfected with 10 μg of *Xho*I-linearized pSVP5 plasmid DNA and 1 μg of *Bam*HI-linearized pSVHPH (American Type Culture Collection, Rockville, MD) by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) at 300 mV and 960 microfarads. Aliquots of the cells were then diluted and plated in multiwell culture dishes. After 24 h, cells were exposed to 300 μg/ml hygromycin, which has been tested to be 100% lethal after 5 days in nontransfected controls. Colonies resistant to hygromycin were identified after 5 days and allowed to grow for another 10 days in Ham's F-12 medium containing 10% fetal bovine serum in a humidified atmosphere composed of 95% air and 5% CO_2.

Polymerase Chain Reaction

Individual clones were passaged and tested for the presence of minigene P5 in the genomic DNA using PCR and promoter-specific primers. In brief, PCR amplification was performed [Saiki et al., 1988] using recombinant *Taq* DNA polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT) and two oligonucleotide primers: (5′-GAAGTAGTGAGGAGG-3′) was designed as an upstream primer according to the published pSVK3 sequence (Pharmacia), whereas (5′-TTGAAACCCAGCGAGGACC-3′) from the P5 sequence was used as a downstream primer. Thirty cycles of PCR were performed with each cycle consisting of denaturation (1 min at 94°C), annealing (1 min at 60°C), and extension (1 min at 72°C), except for the first cycle during which the denaturation time was increased to 5 min, and the last cycle, in which the extension time was increased to 10 min. Polymerase chain reaction products were subjected to electrophoresis on a composite gel consisting of 1% agarose (GIBCO BRL, Gaithersburg, MD) and 2% Nusieve (FMC Bioproducts, Rockland, ME). The gel was then stained with 0.2 μg/ml ethidium bromide and photographed to verify that the amplified product corresponded to the predicted size of 480 base pairs. Several positive clones were selected for further analysis.

Cell Treatments

Cells grown to confluency in 35-mm dishes were fed with serum-free Ham's F-12 medium containing 0.1% (wt/vol) bovine serum albumin for 3–4 h. CHO/HIRc cells were then stimulated with the indicated concentrations of insulin for up to 10 min at 37°C, whereas CHO/ER cells were induced with 25 nM IGF-1 for 5 min at 37°C. The fluid was removed and the dishes were rapidly immersed in liquid nitrogen. CHO/HIRc cells were lysed with an immune precipitation buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM Na_3VO_4, 100 mM NaF, 0.1% sodium dodecylsulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% NaN_3, 0.15 mM Pefabloc SC, 1 mM benzamidine, 8 μg/ml aprotinin, 2 μg/ml leupeptin), and the crude lysates, clarified at 12,000g for 20 min at 4°C, were incubated with α-IR monoclonal antibody in the presence of protein G-plus/protein A-agarose beads. After washing, the immunocomplexes were denatured in Laemmli gel sample buffer containing 5% 2-mercaptoethanol [Laemmli, 1970]. CHO/ER cells were lysed directly in Laemmli sample buffer containing 5% 2-mercaptoethanol.

Immunoblotting

Unless otherwise indicated, total cell lysates or IR immunoprecipitates were subjected to one-dimensional 4%–12% SDS-polyacrylamide gel electrophoresis along with prestained protein markers, and electrotransferred onto PVDF membrane (30 V for 90 min) in 12.5 mM Tris-base, 96 mM glycine, 0.05% (wt/vol) SDS, 10% (vol/vol) MeOH, pH 8.3 [Towbin et al., 1979]. Blots were probed with specified antibodies in TBS-T buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% (wt/vol) Tween-20] containing 5% (wt/vol) nonfat dried milk, and then incubated with horseradish peroxidase-conjugated secondary antibody. Positive signals were visualized by chemiluminescence detection system (Amersham), and quantified by laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Measurement of PI-3′ Kinase Activity

PI-3′ kinase activity was measured as described previously [Kole et al., 1996a] with some minor modifications. Serum-deprived cells grown in 35-mm dishes were stimulated with a range of insulin concentrations (0.1–10 nM) for 1 min at 37°C, and the IRS-1-associated PI-3′ kinase activity present in the clarified lysates was precipitated with an anti-IRS-1 antibody and protein G-plus/protein A-agarose. L-α-phosphatidylinositol (Sigma Chemical, St-Louis, MO) was used as substrate in the kinase assay.

Measurement of MAP Kinase Activity

Cells grown in 35-mm dishes were maintained in serum-free medium for 5 h and then treated with 10 or 25 nM insulin for 5 min at 37°C. The cells were washed and solubilized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM NaF, 10 mM sodium pyrophosphate, 0.15 mM Pefabloc SC, 1 mM Na_3VO_4, 20 μg/ml aprotinin, and 10 μg/ml leupeptin. Insoluble material was removed by centrifugation, and aliquots of the lysates (containing equal amounts of protein) were incubated with a polyclonal anti-MAP kinase antibody and protein G-plus/protein A-agarose. After a series of washes, the immunocomplexes were incubated with 10 μg/ml myelin basic protein and 20 μM [γ-^{32}P]ATP for 15 min at 22°C [Kole et al., 1996a]. The reaction was stopped by addition of Laemmli gel sample buffer containing 5% 2-mercaptoethanol, and the samples were analyzed by one-dimensional 4%–20% SDS-polyacrylamide gel electrophoresis and autoradiography.

Measurement of Thymidine Incorporation

Subconfluent cells grown in 24-multiwell plates were incubated in serum-free Ham's F-12 medium supplemented with 0.5% fetal bovine serum. After 24 h, the medium was changed and the indicated concentrations of insulin were added for an additional 16 h. The medium was removed and cells were incubated in fresh serum-free medium containing 0.1% bovine serum albumin supplemented with [^{3}H]thymidine (1 μCi/well) for 90 min at 37°C. After three washes with ice-cold phosphate-buffered saline, the cells were lysed and DNA was precipitated with 10% (wt/vol) trichloroacetic acid. The wells were rinsed once, and the precipitated material was solubilized in 1 N NaOH and quantitated by liquid scintillation counting.

Statistical Analysis

Data are presented as the mean ± the standard error of the mean (mean ± SEM) where appropriate. Comparison between groups was made by analysis of variance coupled to Fisher's protected least significance differences post-hoc test using STATVIEW II software for Apple Macintosh computers (Abascus Concepts, Berkeley, CA).

RESULTS

Cellular Expression of the P5 Minigene

After cotransfection of CHO/HIRc cells with the pSVHPH and pSVP5 vectors, cell lines from the control "pSVP5-null" vector transfection and those expressing the minigene were selected by propagation of isolated colonies resulting from the antibiotic selection. Polymerase chain reaction amplification of genomic DNA from cells transfected with the minigene demonstrated integration of the P5 construct in several cell clones. The migration of the 480 bp PCR transcript was as expected from amplification of the 5′ region of pSVK3 promoter sequence and 3′-coding region of P5 (Fig. 1A). Accordingly, Western blot analysis using cell lysates probed with an antibody specific for P5 sequence showed that expression level of the peptide dramatically increased in two independent P5-transfected clones as compared with clones transfected with an empty vector (Fig. 1B).

Effect of P5 Expression on IR Autophosphorylation

To investigate whether expression of the minigene P5 has any effect on IR autophosphorylation, serum-starved cells were first incubated in the absence or the presence of 25 nM insulin for 5 min. Western blot analysis using IR immunoprecipitates probed with antiphosphotyrosine antibody revealed that P5-transfected cells were more responsive to insulin, as evidenced by a larger increase in the phosphorylation levels of the IR β subunit (Fig. 2A). The expression of the minigene had no effect on the basal receptor tyrosine phosphorylation level. Reprobing the membrane with IR α-subunit antibodies indicated no difference in

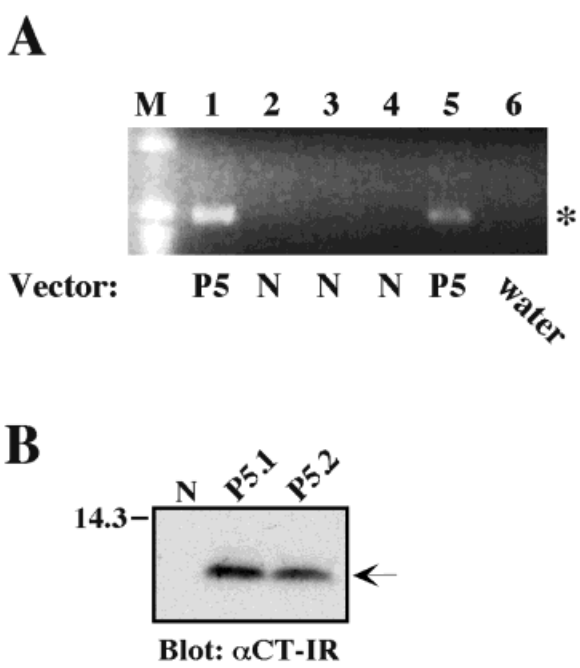

Fig. 1. Minigene expression in the transfected CHO/HIRc cells. **A:** Genomic DNA from cells transfected with either the null vector or the minigene was used to amplify P5 by polymerase chain reaction (PCR). The upstream primer corresponded to a promoter-specific sequence, whereas the downstream primer was designed from the 3′-coding region of P5 sequence. After 30 cycles PCR, a band with the predicted size of 480 bp was observed in independent clones (P5), but was absent in the null-transfected cells (N) and in the water control. **B:** Comparable amounts of total lysates from null-transfected cells or cells transfected with the minigene (clones P5.1 and P5.2) were loaded on a gradient 4%–20% gel, and the proteins were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotted with an antibody specific for residues 1293–1306 of IR (αCT-IR) [Kole et al., 1996b].

receptor expression between the two cell lines. These experiments were repeated three times and the results quantitated (Fig. 2B). Insulin's ability to induce IR autophosphorylation was approximately twofold higher in minigene-transfected cells than in control cells ($P < 0.01$). In a second set of experiments, cells were incubated with 10 nM insulin for periods of time up to 10 min. As shown in Fig. 2C and 2D, the rate of IR autophosphorylation was greater in two independent P5 clones than in cells transfected with the empty vector ($P < 0.01$).

Effect of P5 Expression on Insulin-Stimulated Cellular Responses

The results described above indicated that the minigene has positive effects toward insulin-induced IR kinase activity. To determine whether downstream signaling events are also influenced, we selected key components of the insulin-signaling cascade, including PI-3′ kinase activity, MAP kinase activation, and thymidine incorporation. Incubation of the cells with insulin (0.1–10 nM) led to a dose-dependent increase in PI-3′ kinase activity measured in IRS-1 immunoprecipitates (Fig. 3). In the cells transfected with the empty expression vector, insulin (5 nM) stimulation resulted in a sevenfold increase in PI-3′ kinase activity over basal. Expression of the minigene further enhanced insulin's ability to stimulate PI-3′ kinase up to 12-fold over basal while having minimal effect in the unstimulated transfected cells (Fig. 3).

An immunoprecipitation-based kinase assay of ERK1/ERK2 was carried out with myelin basic protein as the substrate to establish whether P5 expression can enhance this response of insulin. In control cells, 10 nM insulin caused a 3.2-fold increase in ERK activity above the basal level, and stimulation with 25 nM insulin augmented this activity 5.4-fold (Fig. 4A). Expression of the minigene had no effect on basal ERK activity level; however, increases 10.4- and 16.8-fold above the basal level were observed in response to 10 nM and 25 nM insulin, respectively ($P < 0.001$; Fig. 4A). Similar effects of P5 expression were noticed when assessing DNA synthesis (Fig. 4B). In both cell lines, treatment with insulin resulted in a dose-dependent increase in thymidine incorporation. In control cells, insulin (10 nM) increased this response 5.6-fold over basal, whereas a 9.2-fold increase over basal was observed in P5-transfected cells stimulated with insulin ($P = 0.001$). These results have been reproduced with another independent P5-transfected clone (data not shown).

Effect of P5 Expression on Ligand-Induced IGF-1 Receptor Function

The ability of P5, or its lack thereof, to enhance the activation of related growth-factor receptors was studied by introducing the minigene into CHO/ER cells. Chinese hamster ovary cells express very low levels of endogenous IR but contain a significant number of endogenous IGF-1 receptors that exhibit high levels of phosphorylation upon ligand stimulation [Kato et al., 1993]. Amplification of a 480-bp PCR transcript from genomic DNA was observed in a cell clone expressing the mini-

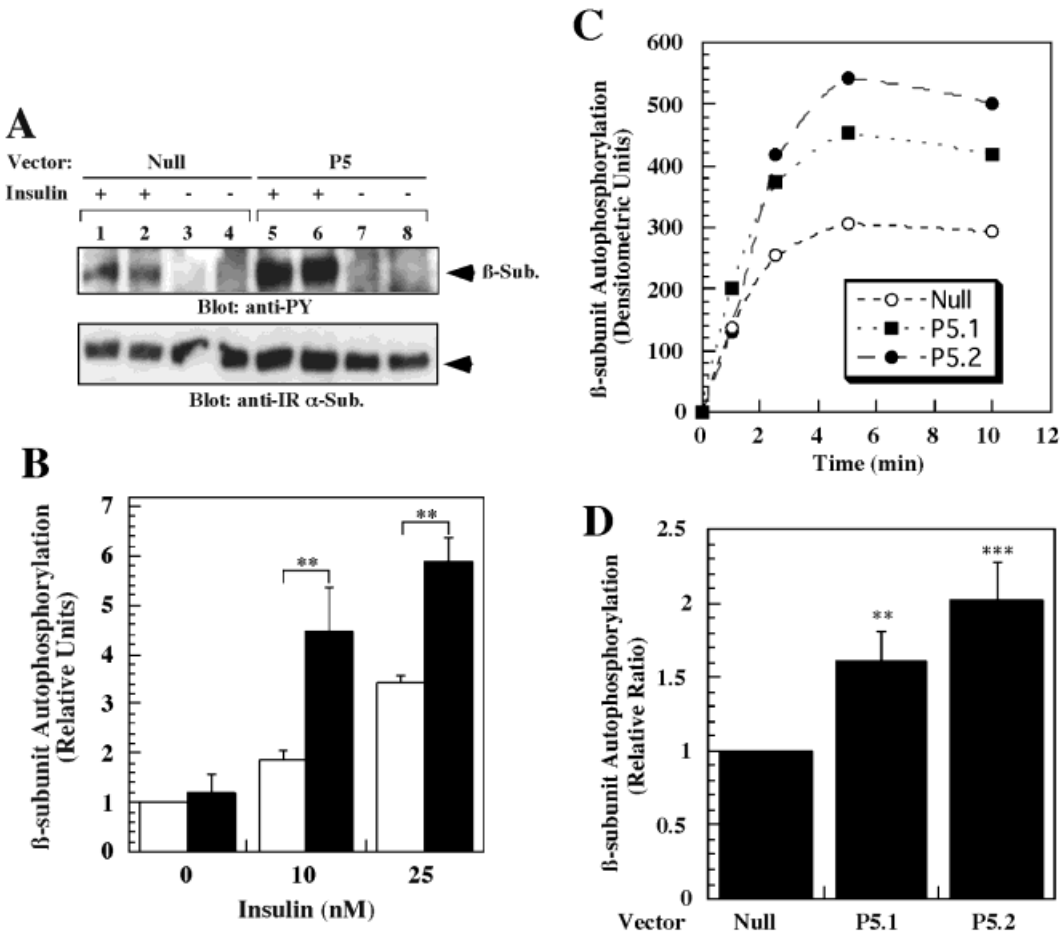

Fig. 2. Insulin-induced insulin receptor (IR) autophosphorylation in intact cells. **A:** Serum-starved CHO/HIRc cells transfected with the null expression vector (**lanes 1–4**) or with the minigene (**lanes 5–8**) were incubated in the absence (−) or presence (+) of 25 nM insulin for 5 min at 37°C. Insulin receptor immunoprecipitates were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-phosphotyrosine (α-PY) antibody (upper panel). The same membrane was then reprobed with the polyclonal IR α subunit antibody (lower panel). **B:** The signals associated with tyrosine-phosphorylated IR β subunit present in null vector transfected cells (open bars) and P5-transfected cells (filled bars) were normalized with the relative levels of expression of the IR α subunit. **C:** Serum-starved cells were stimulated with 10 nM insulin for the indicated times. Insulin receptor immunoprecipitates were analyzed as in Panel A. **D:** Results from the 5-min time point are shown. Bars represent the means ± SEM of three independent experiments.
$^{**}P < 0.01$
$^{***}P < 0.001$ compared with null vector transfected cells.

gene (Fig. 5A). Cells transfected with either the null vector or the minigene were treated with IGF-1 (25 nM), and the phosphorylation state of the IGF-1 receptor was assessed by immunoblotting with anti-phosphotyrosine antibodies. To control for protein loading, the same membranes were then reprobed with a polyclonal anti-TFIIH p62 antibody. As indicated in Fig. 5B, expression of the minigene had no effect on ligand-induced IGF-1 receptor autophosphorylation. Consistent with this finding, it was found that the IGF-1–mediated increase in thymidine incorporation was identical in both control and P5-transfected cells (data not shown).

DISCUSSION

Using a peptide-based approach, we previously reported that a synthetic peptide corresponding to a region within the IR carboxyl terminus (residues 1293–1307) enhances ligand-induced IR autophosphorylation in a semipurified preparation of receptors in vitro [Kole et al., 1996b]. In contrast, the addition of truncated or unrelated sequences, including a peptide from the major autophosphorylation domain within the IR β

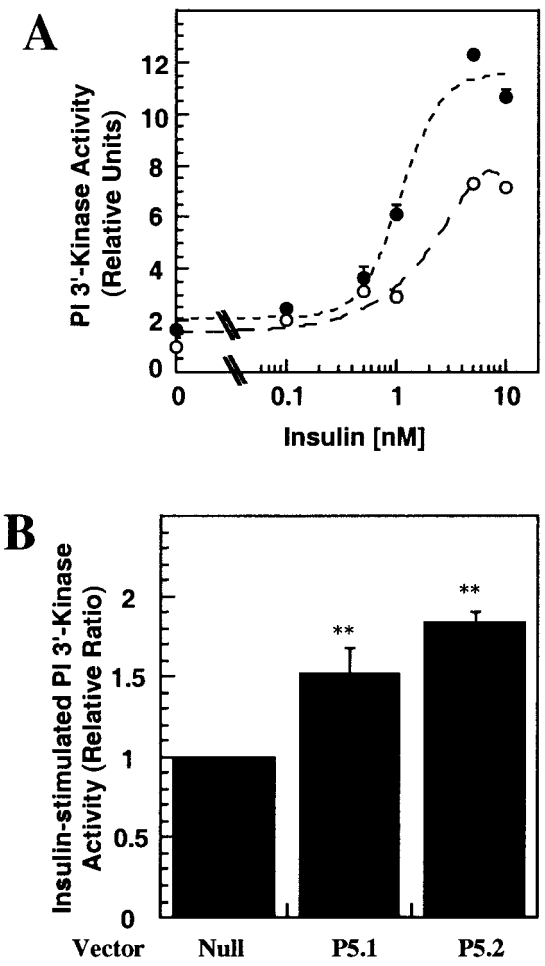

Fig. 3. Insulin-stimulated PI-3′ kinase activity. **A:** CHO/HIRc cells transfected with either the null vector (open symbols) or the minigene (filled symbols) were serum-starved for 4 h and then stimulated with the indicated concentrations of insulin for 1 min at 37°C. The activity of PI-3′ kinase present in IRS-1 immunoprecipitates was measured as described in Materials and Methods. The data are means ± range of two independent experiments. **B:** Cells were stimulated with 25 nM insulin for 1 min. The signal from null vector transfected cells was arbitrarily set at 1.0. Results are the means ± SEM of four experiments. $^{**}P < 0.01$ compared with null vector transfected cells.

subunit (residues 1142–1153), fails to exert similar response [Liotta et al., 1994]. It is noteworthy that the derivatization of 1142–1153 peptide with nonhydrolyzable phosphotyrosyl residues markedly inhibits the dephosphorylation of preactivated IR by protein tyrosine phosphatases (PTPases) [Kole et al., 1998]. Our group also provided evidence that the conjugation of 1293–1307 peptide and tris-sulfotyrosyl 1142–1153 peptide (3S-peptide-I) with the fatty acid stearate causes the potentiation of insulin-induced responses in intact cells via distinct mechanisms [Kole et al., 1996a, b]. However, without techniques for measuring rates of incorporation and concentrations of stearylated peptides in the plasma membrane, it is not possible to perform meaningful kinetic analysis at this level. Furthermore, the hydrophobic interaction between stearyl-conjugated peptides and the membrane lipid bilayer introduces a two-dimensional reaction environment, thus enabling efficiency and specificity that may be orders of magnitude lower (or higher) than would be possible with freely diffusing peptide. An example of such principle has been obtained with *N*-stearyl-3S-peptide-I and its effect on PTPases acting on the IR. These enzymes have been shown to dephosphorylate phosphotyrosyl residues within the IR kinase domain, thereby resulting in the termination of insulin action [Goldstein et al., 1998; Kellerer et al., 1999]. Despite its broad reactivity toward several protein tyrosine phosphatases that is apparent in the test tube, introduction of the lipophilic derivative of 3S-peptide-I in intact cells leads to a selective increase in insulin-induced IR phosphorylation while having no effect on ligand-mediated EGF receptor function [Liotta et al., 1994; Kole et al., 1996a]. Thus, it appears that compartmentalization and microcellular environment may play an important role in defining the degree of specificity exerted by *N*-stearyl-3S-peptide-I toward PTPases acting on the IR. Unfortunately, the inability of cells to express a minigene encoding 1142–1153 peptide with nonhydrolyzable phosphotyrosyl residues prevented us to investigate whether freely diffusible peptide-I can exert similar effect.

With respect to the 1293–1307 peptide, our previous results with its *N*-stearylated analog does not exclude the possibility that the nonconjugated peptide may have little role, if any, in facilitating IR signaling in intact cells. An important observation in our studies is that the biologic outcomes of insulin were potentiated in CHO/HIRc cells stably transfected with a minigene encoding a similar portion of the IR C-terminal domain, the 1292–1315 peptide. This peptide does not contain tyrosine residues and, thus, cannot be tyrosyl phosphorylated. A limitation of this study is that no comparison in the transfection of wild type and mutated or unrelated sequences has been carried out. Nevertheless, the effect of insulin on the stimulation of IR tyrosine phosphorylation, PI-3′ kinase

and MAP kinase activation, and thymidine incorporation into DNA was increased in minigene-expressing cells. Because constitutive expression of the minigene has not altered the level of basal activities, it suggests that freely diffusing 1292–1315 peptide causes stimulation of IR-mediated signaling events subsequent to the addition of insulin. The level of stimulation induced by the minigene was found to be similar in several independent clones when compared to cells transfected with the empty vector, indicating that the effect of the 1292–1315 peptide is not merely the result of clonal variation within this population of cells. Additionally, our results are suggestive that the maximal effect of insulin is altered, not the EC50. Upon addition of insulin, the extent of IR tyrosine phosphorylation reflects the balance between the competing activities of the receptor autokinase and the cellular PTPases that act on the insulin receptor. Our earlier work has clearly shown that a peptide related to the minigene sequence failed to inhibit IR dephosphorylation while potentiating insulin-mediated receptor autophosphorylation in semi-permeabilized cells [Kole et al., 1996b]. Thus, it is unlikely that the increased amount of IR autophosphorylation seen in the minigene-expressing cells be the result of PTPase inhibition. Two-dimensional phosphopeptide mapping analysis will be required to ascertain whether additional tyrosine residues of the IR are phosphorylated upon addition of insulin in CHO/HIRc cells transfected with the minigene.

Although the exact mechanism of IR activation in minigene-transfected cells has not been identified, our data show a selective potentiation of insulin-induced responses because the minigene expression exerts no effect on the regulation of IGF-1 signaling. Upon autophosphorylation in the presence of insulin, there is rearrangement of the IR activation loop that facilitates a reorientation of the N- and C-terminal lobes of the kinase, thereby allowing productive ATP binding and, consequently, substrate phosphorylation [Hubbard, 1997]. Thus, binding of the 1292–1315

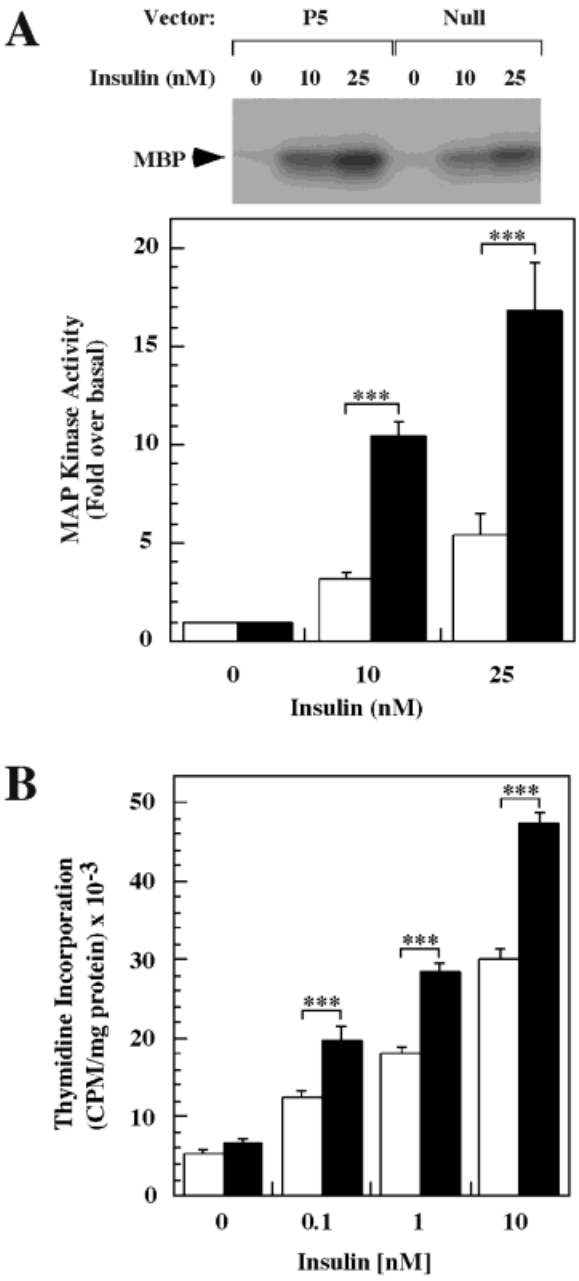

Fig. 4. Effect of P5 expression on insulin-stimulated MAP kinase activity and thymidine incorporation. **A:** Serum-starved cells were stimulated with the indicated concentrations of insulin for 5 min at 37°C. ERK1/ERK2 activity was determined in an in vitro immunoprecipitation-based kinase assay with [γ-^{32}P]ATP in the presence of myelin basic protein as the substrate. Autoradiogram of a representative experiment is shown (upper panel). In the lower panel, the signals from cells incubated without insulin were arbitrarily set at 1.0. **B:** Subconfluent cells were made quiescent in serum-free media for 24 h and then incubated with the indicated concentrations of insulin for 16 h. [^{3}H]Thymidine was added, and tritium incorporation into DNA was determined after 90 min as described in Materials and Methods. Results are the means ± SEM of three experiments performed in duplicate dishes (**A**) or of two independent experiments performed in triplicate dishes (**B**). Open and filled bars, null vector transfected and P5-transfected cells, respectively.
***P = 0.001.

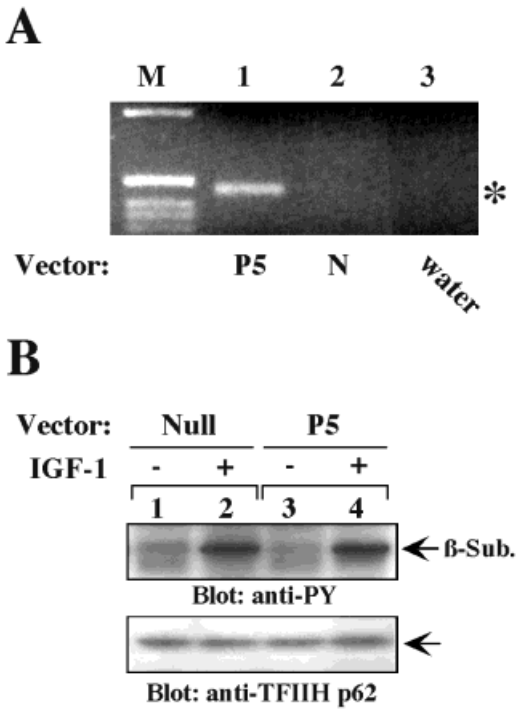

Fig. 5. Effect of P5 minigene expression on IGF-1 receptor activation. **A:** Genomic DNA from CHO/ER cells transfected with either the null vector (N) or the P5 minigene (P5) was subjected to polymerase chain reaction (PCR) amplification with the same set of primers as described in the legend of Fig. 1. After 30 cycles PCR, a band with the predicted size of 480 bp was observed in P5-transfected cells, but was absent in the water control. **B:** Cells transfected with either the null vector or the P5 minigene were stimulated with 25 nM IGF-1 for 5 min at 37°C. Cell lysates (30 μg) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotted using antiphosphotyrosine antibody (upper panel) and anti-TFIIH p62 antibody (lower panel). The figure is representative of two independent experiments. The migration positions of the β subunit of the IGF-1 receptor (β-Sub.) and TFIIH p62 are shown.

sequence with another noncatalytic region of the receptor β subunit may facilitate lobe rotation and efficient binding of ATP by the receptor kinase upon insulin addition both in vitro and in intact cells. Our data showing specific association of a related sequence (1293–1307 sequence) with the IR β subunit supports this hypothesis [Kole et al., 1996b]. Taken together, these results indicate that the freely diffusing 1292–1315 peptide specifically potentiates insulin-stimulated responses and also indicate that the site of action of the minigene is proximal to the IR. Clearly, further studies to precisely elucidate the mechanism of this potentiation are required.

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REFERENCES

Baron V, Kaliman P, Alengrin F, Van Obberghen E. 1995. Interaction of the C-terminal acidic domain of the insulin receptor with histone modulates the receptor kinase activity. Eur J Biochem 229:27–34.

Cheatham B, Kahn CR. 1995. Insulin action and the insulin signaling network. Endocr Rev 16:117–142.

Faria TN, Blakesley VA, Kato H, Stannard B, LeRoith D, Roberts CT Jr. 1994. Role of the carboxyl-terminal domains of the insulin and insulin-like growth factor I receptors in receptor function. J Biol Chem 269:13922–13928.

Goldstein BJ, Ahmad F, Ding W, Li PM, Zhang WR. 1998. Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. Mol Cell Biochem 182: 91–99.

Gual P, Baron V, Alengrin F, Mothe I, Van Obberghen E. 1996. Insulin receptor-induced phosphorylation of cellular and synthetic substrates is regulated by the receptor beta-subunit C-terminus. Endocrinology 137:3416–3423.

Hubbard SR. 1997. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J 16:5572–5581.

Kaliman P, Baron V, Alengrin F, Takata Y, Webster NJG, Olefsky JM, Van Obberghen E. 1993. The insulin receptor C-terminus is involved in regulation of the receptor kinase activity. Biochemistry 32:9539–9544.

Kalloo-Hosein HE, Whitehead JP, Soos M, Tavare JM, Siddle K, O'Rahilly S. 1997. Differential signaling to glycogen synthesis by the intracellular domain of the insulin versus the insulin-like growth factor-1 receptor. Evidence from studies of TrkC-chimeras. J Biol Chem 272:24325–24332.

Kasuya J, Li S-L, Orr S, Siddle K, Fujita-Yamaguchi Y. 1994. The purified COOH-terminal domain of the insulin receptor carries activity to stimulate protein kinase activity or autophosphorylation of the β subunit domain of the insulin receptor. Biochem Biophys Res Commun 200: 777–783.

Kato H, Faria TN, Stannard B, Levy-Toledano R, Taylor SI, Roberts CT Jr, LeRoith D. 1993. Paradoxical biological effects of overexpressed insulin-like growth factor-1 receptors in Chinese hamster ovary cells. J Cell Physiol 156:145–152.

Kellerer M, Lammers R, Haring HU. 1999. Insulin signal transduction: possible mechanisms for insulin resistance. Exp Clin Endocrinol Diabetes 107:97–106.

Kole HK, Garant MJ, Kole S, Bernier M. 1996a. A peptide-based protein-tyrosine phosphatase inhibitor specifically enhances insulin receptor function in intact cells. J Biol Chem 271:14302–14307.

Kole HK, Liotta AS, Kole S, Roth J, Montrose-Rafizadeh C, Bernier M. 1996b. A synthetic peptide derived from a COOH-terminal domain of the insulin receptor specifically enhances insulin receptor signaling. J Biol Chem 271:31619–31626.

Kole HK, Kole S, Mallory BP, Li PM, Goldstein BJ, Bernier M. 1998. Inhibition of the transmembrane protein tyrosine phosphatase lar by 3S-peptide-I enhances insulin receptor phosphorylation in intact cells. J Basic Clin Physiol Pharmacol 9:111–126.

Kosak M. 1989. The scanning model for translation: an update. J Cell Biol 108:229–241.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, Avruch J. 1992. Raf-1 activates MAP kinase-kinase. Nature 358:417–421.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Laviola L, Giorgino F, Chow JC, Baquero JA, Hansen H, Ooi J, Zhu J, Riedel H, Smith RJ. 1997. The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. J Clin Invest 99:830–837.

Levy-Toledano R, Taouis M, Blaettler DH, Gorden P, Taylor SI. 1994. Insulin-induced activation of phosphatidyl inositol 3-kinase. Demonstration that the p85 subunit binds directly to the COOH terminus of the insulin receptor in intact cells. J Biol Chem 269:31178–31182.

Liotta AS, Kole HK, Fales HM, Roth J, Bernier M. 1994. A synthetic tris-sulfotyrosyl dodecapeptide analogue of the insulin receptor 1146-kinase domain inhibits tyrosine dephosphorylation of the insulin receptor in situ. J Biol Chem 269:22996–23001.

Maegawa H, Ugi S, Ishibashi O, Tachikawa-Ide R, Takahara N, Tanaka Y, Takagi Y, Kikkawa R, Shigeta Y, Kashiwagi A. 1993. Src homology 2 domains of protein tyrosine phosphatase are phosphorylated by insulin receptor kinase and bind to the COOH-terminus of insulin receptors in vitro. Biochem Biophys Res Commun 194:208–214.

Mothe I, Tartare S, Kowalski-Chauvel A, Kaliman P, Van Obberghen E, Ballotti R. 1995. Tyrosine kinase activity of a chimeric insulin-like-growth-factor-1 receptor containing the insulin receptor C-terminal domain. Comparison with the tyrosine kinase activities of the insulin and insulin-like-growth-factor-1 receptors using a cell-free system. Eur J Biochem 228:842–848.

Myers MG Jr, White MF. 1996. Insulin signal transduction and the IRS proteins. Annu Rev Pharmacol Toxicol 36: 615–638.

Najjar SM, Blakesley VA, Li Calzi S, Kato H, LeRoith D, Choice CV. 1997. Differential phosphorylation of pp120 by insulin and insulin-like growth factor-1 receptors: role for the C-terminal domain of the beta-subunit. Biochemistry 36:6827–6834.

O'Neill TJ, Zhu Y, Gustafson TA. 1997. Interaction of MAD2 with the carboxyl terminus of the insulin receptor but not with the IGFIR. Evidence for release from the insulin receptor after activation. J Biol Chem 272: 10035–10040.

Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363:83–85.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn G, Mullis KB, Erlich HA. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491.

Tartare S, Mothe I, Kowalski-Chauvel A, Breittmayer J-P, Ballotti R, Van Obberghen E. 1994. Signal transduction by a chimeric insulin-like growth factor-1 (IGF-1) receptor having the carboxyl-terminal domain of the insulin receptor. J Biol Chem 269:11449–11455.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Tsunoda S, Sierralta J, Zuker CS. 1998. Specificity in signaling pathways: assembly into multimolecular signaling complexes. Curr Opin Genet Dev 8:419–422.

Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, Ramachandran J. 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 313:756–761.

Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, Jacobs S, Francke U, Ramachandran J, Fujita-Yamaguchi Y. 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J 5:2503–2512.

Weng G, Bhalla US, Iyengar R. 1999. Complexity in biological signaling systems. Science 284:92–96.

Xu B, Bird VG, Miller WT. 1995. Substrate specificities of the insulin and insulin-like growth factor 1 receptor tyrosine kinase catalytic domains. J Biol Chem 270: 29825–29830.