Comparison of the Signaling Abilities of the Drosophila and Human Insulin Receptors in Mammalian Cells†

Takahide Yamaguchi, Rafael Fernandez,‡ and Richard A. Roth*

Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305, and Program in Molecular Biology, Memorial Sloan Kettering Cancer Center, New York, New York 10016

Received November 4, 1994; Revised Manuscript Received January 27, 1995®

ABSTRACT: Chimeric receptors encoding either the whole or a portion of the cytoplasmic domain of the drosophila insulin receptor (IR) with the extracellular domain of the human IR were expressed either transiently in COS cells or stably in Chinese hamster ovary cells and compared with the wild-type human IR. All three receptors bound insulin equally and exhibited an insulin-activated tyrosine kinase activity. The ability of the drosophila cytoplasmic domain to mediate the tyrosine phosphorylation of insulin receptor substrate 1, stimulate cell proliferation, and activate MAP kinase was found to be indistinguishable from that of the human IR. The chimeric drosophila receptors did not bind more phosphatidylinositol 3-kinase than the human IR, despite containing a C-terminal extension with potential tyrosine phosphorylation sites in the motif recognized by the SH2 domain of this enzyme. Thus, the essential signal-transducing abilities of the IR appear to have been conserved from invertebrates to mammals, despite the considerable differences in the sequences of these receptors.

The insulin receptor (IR)[1] is a member of a family of receptor tyrosine kinases that includes the insulin-like growth factor I (IGF-I) receptor and the insulin receptor-related receptor (Siddle, 1992; Seta et al., 1993; Lee & Pilch, 1994). All three receptors are synthesized as a single polypeptide, which is then cleaved to yield an α-chain that is completely extracellular (with an apparent molecular mass of approximately 130 kDa) and an 80−95 kDa β-chain, which contains an extracellular domain, a single membrane-spanning region, and a cytoplasmic domain. All three receptors exhibit considerable sequence identity throughout, although some regions appear to be more conserved than others (Ullrich et al., 1986; Shier & Watt, 1989). For example, the kinase domains of the three receptors appear to be most highly conserved (exhibiting 80% sequence identity), whereas the carboxy tails of the receptors appear to show the least conservation (exhibiting as little as 20% sequence identity). Different regions of the receptors have been identified as playing a role in ligand binding and signal transduction by the use of chimeric receptors containing portions of one receptor on the backbone of another member of the family. For example, the cysteine-rich region in the α-chain appears to be important in conferring ligand specificity in the IGF-I receptor, whereas the amino terminus of the α-chain and a region carboxy terminal to the cysteine-rich region appear to be important in the high-affinity binding of insulin to its receptor (Gustafson & Rutter, 1990; Zhang & Roth, 1991; Schaffer et al., 1993; Schumacher et al., 1993). The signal-transducing abilities of these three receptors have also been studied by the chimeric receptor approach. Although small differences in signaling have been observed in some studies, these three receptors appear to be quite similar in regard to the endogenous substrates phosphorylated, as well as the biological responses mediated via these molecules (Roth et al., 1988; Lammers et al., 1989; Siddle, 1992; Zhang & Roth, 1992; Myers et al., 1993; Seta et al., 1993; Faria et al., 1994; Lee & Pilch, 1994; Tartare et al., 1994).

Another potential member of this family is the putative drosophila IR. Although a drosophila insulin-like molecule has yet to be isolated, a receptor in *Drosophila melanogaster* was partially purified and shown to bind mammalian insulin, exhibit an insulin-activated tyrosine kinase activity, and react with antibodies to the mammalian receptor (Petruzzelli et al., 1985; Fernandez-Almonacid & Rosen, 1987). The ability of this receptor to stimulate various biological responses was not, however, reported. When cDNA clones encoding this receptor were isolated, their deduced sequence exhibited clear homologies to the mammalian IR family (Nishida et al., 1986; Petruzzelli et al., 1986; Fernandez-Almonacid, 1992). However, the sequence of this insect receptor diverges from that of the human IR to a greater degree than any other known member of this family. For example, the kinase and juxtamembrane domains of this receptor are only 64% and 29% identical, respectively, to the comparable regions of the human IR (Figure 1). The kinase domain of the drosophila IR does retain the three key tyrosines known to be required for activation of the mammalian IR tyrosine kinase (Avruch et al., 1990) in a 22 amino acid region that is 100% identical to that present in the human IR (Fernandez-Almonacid,

† This work was supported by NIH Grants DK 41765 and 34926.

* Please address all correspondence to Dr. Richard A. Roth, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.Telephone: 415-723-5933. FAX: 415-725-2952.

‡ Present address: Department of Pharmacology, New York University Medical Center, New York, NY 10016.

® Abstract published in *Advance ACS Abstracts,* March 15, 1995.

[1] Abbreviations: IR, insulin receptor; PI, phosphatidylinositol; PMSF, phenylmethanesulfonyl fluoride; dIR1, a chimeric receptor containing the extracellular transmembrane and juxtamembrane domains of the human insulin receptor and the remainder of the cytoplasmic domain of the drosophila receptor; dIR2, a chimeric receptor containing the extracellular domain of the human insulin receptor and the transmembrane and cytoplasmic domains of the drosophila receptor; PY20, anti-phosphotyrosine antibody; CHO, Chinese hamster ovary; Drk, drosophila Grb-2.

0006-2960/95/0434-4962$09.00/0

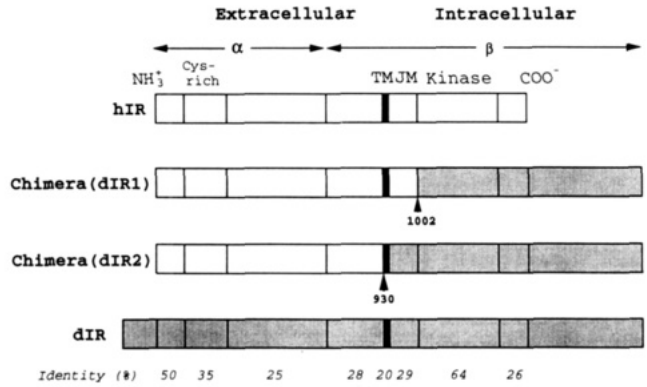

FIGURE 1: Schematic diagram of human (h) and drosophila (d) IR and the two chimeric receptors (dIR1 and dIR2). The transmembrane (TM), juxtamembrane (JM), cysteine-rich (Cys-rich), and kinase domains are indicated. The numbers of the last human amino acid in the chimeras are given. The % sequence identity of different regions of the drosophila IR is also given in comparison to the human IR [adapted from Fernandez-Almonacid (1992)].

1992). In addition, the juxtamembrane region of the insect receptor, although showing very little overall homology, does retain a tyrosine in the motif NPXYXS (Fernandez-Almonacid, 1992) that corresponds to a tyrosine present in the human IR, which has been shown to be important for signal transduction and the ability of this receptor to phosphorylate IRS-1, a major endogenous substrate of the IR (White & Kahn, 1994). Most surprisingly, the deduced sequence of the drosophila IR indicated that the β−subunit of this receptor contains a carboxy-terminal extension of 60 kDa (yielding a β-subunit of approximately 150−170 kDa) and that this extension had several potential tyrosine autophosphorylation sites, including several in the motif YXXM previously found to be recognized by the SH2 domains of the phosphatidylinositol (PI) 3-kinase (Fernandez-Almonacid, 1992). Thus, this carboxy-terminal extension could serve to bind different SH2-containing proteins, thereby playing an IRS-1-like function.

In the present study, we have prepared cDNAs encoding chimeric receptors containing the complete extracellular domain of the human IR and either the complete cytoplasmic domain of the drosophila IR or a portion of the cytoplasmic domain of this receptor. These chimeric receptors were expressed and their signal-transducing properties studied in mammalian cells. Despite the presence of the carboxy-terminal extension and great divergence in sequence, the drosophila IR appeared to signal in a manner indistinguishable from that of the human IR.

EXPERIMENTAL PROCEDURES

Construction of cDNAs Encoding Chimeric IR dIR1 or dIR2. To construct dIR1 and dIR2, cDNAs encoding the transmembrane and juxtamembrane regions were obtained by PCR using either the human or *Drosophila melanogaster* cDNA as template and cloned into pBluescript II KS (Promega). The forward and reverse primers were 5′-TCTTGGACGGAACCCACC-3′/5′-CGAAGGATCCCTGC-CCCAGCTCTC-3′ and 5′-GAATATTGCTAAGGTCTTT-TTCTGGCTACTG-3′/5′-GGACTTCAGGATACCCTCAT-ACAC-3′, respectively. *Bam*HI and *Ssp*I restriction sites (underlined) were introduced, and the PCR products were cut out with these enzymes. The 3 kb fragment of the drosophila IR cDNA encoding its kinase domain and carboxy tail extension and the PCR products were subcloned into the human IR cDNA (in the SRα expression vector) (Zhang et al., 1991) digested with *Ssp*I and *Xba*I to remove its transmembrane and cytoplasmic domain. The correct sequence of the junctions and PCR products was confirmed by DNA sequencing.

Transient and Stable Expression of Human and Chimeric IRs. COS-7 cells were transiently transfected with the cDNAs encoding human and chimeric IRs using the calcium phosphate method as described (Zhang et al., 1991). Stable transformants of CHO cells were established as described (Chin et al., 1993). In brief, 5×10^5 CHO cells were cotransfected with 20 μg of plasmid encoding IR and 1 μg of plasmid conferring puromycin resistance by calcium phosphate precipitation (de la Luna et al., 1988). After 48 h, 10 μg/mL puromycin was added to the culture medium. Two weeks later, colonies were picked and tested for [^{125}I]-insulin binding to select cells expressing high levels of receptors.

[^{125}I]Insulin and ^{125}I-Labeled Antibody Binding. Insulin and a monoclonal antibody to the α-subunit of the human IR (5D9) (Morgan & Roth, 1986) were labeled with ^{125}I by the IODOGEN method (Pierce Chemical Co.), and their specific activities were 200 and 400 Ci/g, respectively. Transfected COS-7 cells were lysed in a lysis buffer [50 mM HEPES (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mg/mL bacitracin]. Fifty microliters of lysates was added to 96-well poly(vinyl chloride) plates previously coated with a monoclonal antibody to the α-subunit of human IR (3D7) and incubated overnight at 4 °C. Wells were washed three times with WGBT [50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 0.1% Tween 20, and 0.1% bovine serum albumin] and 2×10^4 cpm of labeled insulin with various concentrations of unlabeled insulin or 1×10^5 cpm of labeled 5D9 was added to each well. After an overnight incubation at 4 °C, the wells were washed with WGBT three times, and the radioactivity of each well was counted. Binding of insulin to intact cells was also performed with transfected CHO cells grown in 24-well tissue culture plates. Cells were washed once with phosphate-buffered saline and incubated overnight at 4 °C with serum-free medium containing 4×10^4 cpm of labeled insulin. Cells were washed three times with ice-cold phosphate-buffered saline and lysed in 0.05% SDS, and the radioactivity in the lysates was measured.

In Vitro Activation of Tyrosine Kinase Activities of IRs. COS-7 cells were transiently transfected and IRs were adsorbed to 96-well plates as described earlier. The immunocaptured receptors were first treated to remove the variable levels of phosphotyrosine (Zhang et al., 1991) by incubation with 50 μL of 0.275 unit/mL alkaline phosphatase (EC 3.1.3.1, Type VII from bovine intestinal mucosa, Sigma) in 0.1 M Tris (pH 8.0) at 25 °C for 30 min, followed by three washes with WGBT. Dephosphorylated receptors were then activated by incubation with 50 μL of activation mixture [50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.1% bovine serum albumin, 5 mM $MnCl_2$, 10^{-6} M insulin, and 1 mM ATP] at 25 °C for 30 min. The wells were washed three times with WGBT, and 20 μL of kinase reaction mixture [50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/mL poly-(Glu,Tyr) (4:1), and 2 μCi of [γ-^{32}P]ATP] was added to each well and incubated at 25 °C. At the indicated times, 10 μL

of reaction mixture was spotted on a Whatman 3MM filter paper strip, and the strips were soaked in ice-cold 10% trichloroacetic acid/10 mM sodium phosphate for 10 min, boiled in 5% trichloroacetic acid/10 mM sodium phosphate for 30 min, washed twice in 95% ethanol and once in acetone, and then counted. The amount of receptors bound to parallel wells was measured by utilizing ^{125}I-labeled 5D9 as described earlier.

Phosphorylation of Endogenous Substrates. Parental CHO cells or CHO cells overexpressing IRs were incubated in serum-free medium for 30 min and treated with various concentrations of insulin for the indicated times. The cells were lysed in a lysis buffer [50 mM HEPES (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mg/mL bacitracin, 1 mM sodium orthovanadate, and 50 mM β-glycerophosphate]. The lysates were electrophoresed on 7.5% SDS−PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-phosphotyrosine antibody RC20 (Transduction Laboratories), and bound antibody was visualized by chemiluminescence (ECL, Amersham).

Immunoprecipitation and Immunoblotting of IRS-1 and IRs. CHO cells or CHO cells overexpressing IRs were serum-starved for 30 min and treated with 10^{-9} M insulin for 10 min. Cells were lysed as before and centrifuged. The supernatants were incubated with anti-IRS-1 serum (a gift of Dr. Alan Saltiel) (Ohmichi et al., 1993) or 5D9 and 3D7 previously bound to protein G−Sepharose. The immunoprecipitates were electrophoresed as before and analyzed by immunoblotting with either RC20, anti-drosophila IR β-subunit antiserum, or anti-human IR monoclonal antibodies (3B11+2H2) (a gift of Dr. Kozui Shii) (Hagino et al., 1994).

Phosphatidylinositol 3-Kinase Activity. CHO cells or CHO cells overexpressing IRs were serum-starved for 30 min and treated with 10^{-9} M insulin for 10 min. Cells were lysed as before, and the lysates were immunoprecipitated with either normal mouse IgG or anti-phosphotyrosine (PY20) antibody. The PI 3-kinase activity in the precipitates was determined as described previously (Chin et al., 1993).

Proliferation Assay. Five thousand parental CHO cells or CHO cells overexpressing IRs were incubated in 96-well tissue culture plates with serum-free medium containing 0.1% bovine serum albumin, 20 mM HEPES (pH 7.4), and various concentrations of insulin for 48 h at 37 °C. After this period, 20 μL of MTS/PMS solution (Promega) was added to each well and incubated for 2 h at 37 °C. The absorbance at 490 nm was measured in a microtiter plate reader (Bio-Rad).

MAP Kinase Assay. CHO cells were serum-starved for 18 h and stimulated with the indicated concentration of insulin for 5 min. The cells were washed with ice-cold phosphate-buffered saline and lysed with 400 μL of lysis buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 μM aprotinin, 1 mM vanadate, 50 mM NaF, and 0.5 mM EGTA and centrifuged. The supernatants were incubated for 4 h at 4 °C with anti-MAP kinase antibody (a gift of Dr. John Blenis) (Chen & Blenis, 1990) previously bound to protein A−Sepharose. The immunoprecipitates were washed and incubated in 40 μL of kinase reaction mixture [20 mM Tris-HCl (pH 7.6), 13 mM $MgCl_2$, 20 μM ATP, 1.5 mM EGTA, 1 mg/mL myelin basic protein, and 0.22 μCi of [γ^{32}P]ATP] for 15 min at 25 °C. The reaction was stopped by adding 20 μL of 3× Laemmli's sample buffer and boiling. The samples were electrophoresed on, 15% SDS−PAGE, and the gel was stained with Coomassie Brilliant Blue, dried, and autoradiographed. Myelin basic protein was excised from the gel and counted.

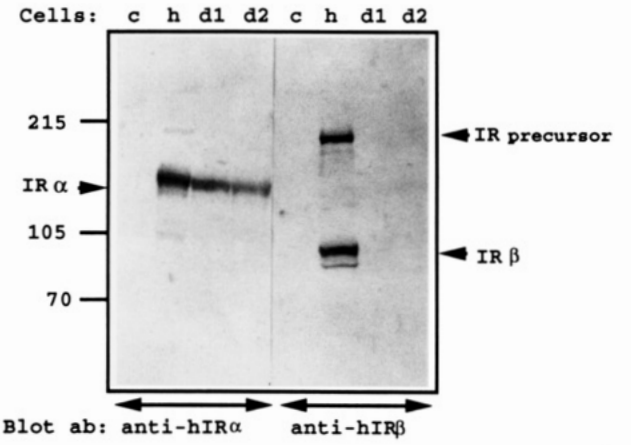

FIGURE 2: Expression of chimeric IRs in COS-7 cells. COS-7 cells were transiently transfected with either the vector alone (c), a plasmid encoding human IR (h), or chimeric receptors dIR1 (d1) or dIR2 (d2). Cells were lysed, and the lysates were analyzed by immunoblotting with either anti-human IR α-subunit or anti-human IR β-subunit, as indicated.

RESULTS AND DISCUSSION

Two chimeric drosophila IRs were analyzed: one contained the complete extracellular domain of the human IR with the transmembrane and cytoplasmic domains of the drosophila receptor (called dIR2), whereas the other contained the extracellular domain, the transmembrane domain, and the juxtamembrane domain of the human IR with the remainder of the cytoplasmic domain of the drosophila receptor (called dIR1) (Figure 1). The cDNAs encoding either the wild-type human IR or the two chimeric drosophila IRs were transiently transfected in COS cells, and the resulting proteins were analyzed. All three receptors were comparably expressed and processed to give the same human α-chain (Figure 2) and bound insulin with comparable affinity (Figure 3). The tyrosine kinase activities of these three receptors were also assessed in vitro after immunocapture on microtiter plates. All three receptors exhibited dramatic (approximately 100-fold) activation after pretreatment with insulin in the presence of 1 mM ATP (data not shown). For comparable amounts of receptor, dIR1 and dIR2 were approximately 70% and 50% as active as human IR, respectively, at phosphorylating poly(Glu,Tyr) (Figure 4).

Chinese hamster ovary (CHO) cells stably expressing the two chimeric drosophila IRs were produced and characterized. Insulin, in a dose (Figure 5) and time (Figure 6) dependent manner, clearly stimulated a greater increase in the tyrosine phosphorylation of high molecular weight proteins in cells expressing the two chimeric receptors than in the parental CHO cells, comparable to what was observed in the CHO cells overexpressing the human IR. Both chimeric receptors also did not contain the major 95 kDa tyrosine phosphorylated protein observed in CHO cells overexpressing the human IR (Figures 5 and 6), which is consistent with the predicted larger size of the drosophila IR β-chain. In cells expressing dIR1, the major bands exhibiting increased tyrosine phosphorylation migrated at positions of approximately 170 and 150 kDa, whereas in dIR2 a single insulin-stimulated phosphotyrosine-containing

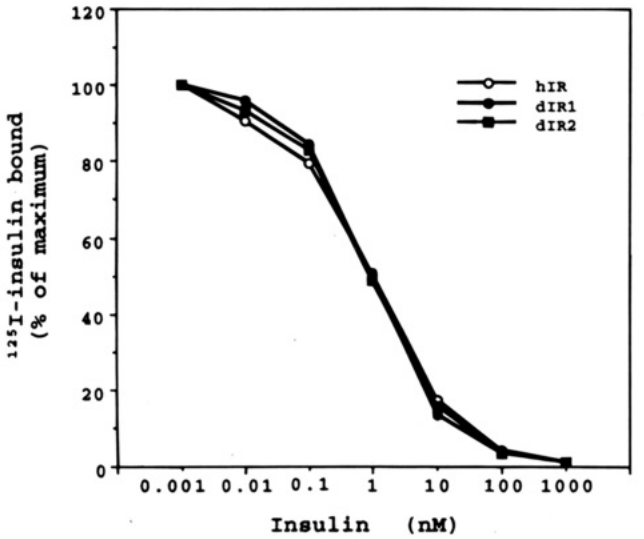

FIGURE 3: ^{125}I-labeled insulin binding to human IR and chimeric receptors. Human IR and chimeric receptors dIR1 and dIR2 were transiently expressed in COS-7 cells. Receptors were adsorbed to microtiter wells coated with an anti-IR monoclonal antibody and then incubated with [^{125}I]insulin in the presence of the indicated concentrations of unlabeled insulin. Results are presented as the percentage of binding in the absence of a competing ligand (the maximum binding). These values were 8830, 8094, and 9354 cpm for hIR, dIR1, and dIR2, respectively. Results shown are means of triplicate determinations.

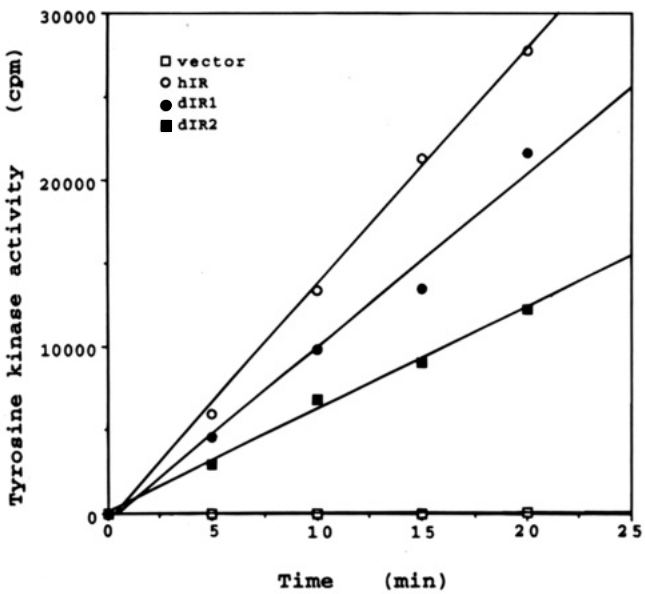

FIGURE 4: Tyrosine kinase activities of human IR and chimeric IRs. COS-7 cells were transiently transfected with either vector alone, cDNAs encoding the human IR or chimeric receptors dIR1 or dIR2. The expressed receptors were adsorbed to microtiter wells and activated as described in the Experimental Procedures. Tyrosine kinase activity was measured as the incorporation of radioactivity into the exogenous substrate poly(Glu,Tyr) (4:1). In parallel wells, the amount of captured receptor was assessed by labeled antibody binding. Antibody binding was 9, 607, 712, and 706 for vector alone, hIR, dIR1, and dIR2, respectively. Results are means of triplicate determinations.

band of 170 kDa was observed (Figures 5 and 6). To further identify these high molecular weight tyrosine phosphorylated proteins, extracts of insulin-treated cells were immunoprecipitated with either an antibody to the extracellular domain of the human IR (this antibody reacts with both chimeric receptors as well as the human IR), an antibody to IRS-1, or control Ig. The precipitates were then electrophoresed,

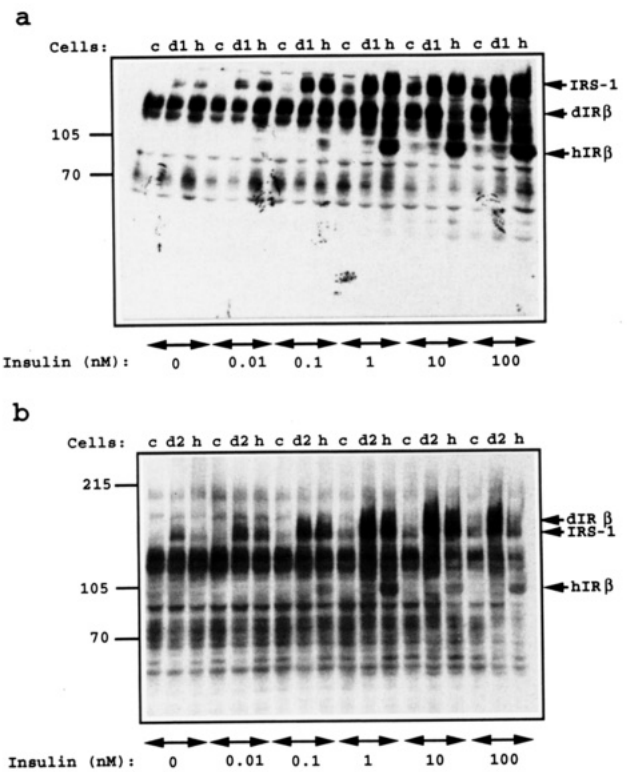

FIGURE 5: Insulin dependent stimulation of the tyrosine phosphorylation of endogenous substrates by the chimeric IRs. Either parental CHO cells (c) or cells overexpressing the human IR (h), the chimeric dIR1 (d1), or the chimeric dIR2 (d2) were serum-starved for 30 min and then treated with the indicated concentration of insulin at 37 °C for 10 min. Cells were lysed, and the lysates were analyzed by immunoblotting with anti-phosphotyrosine antibody (RC20).

transferred to nitrocellulose, and blotted with either antibodies to phosphotyrosine, the β-subunit of the drosophila IR, or a mix of monoclonal antibodies to the α- and β chains of the human IR. The anti-IR precipitates from the cells expressing dIR1 clearly exhibited a single band of 150 kDa, which reacted with both the antibody to the β-subunit of drosophila IR and anti-phosphotyrosine antibodies, indicating that this was the β-subunit of this chimeric IR (Figure 7).

In addition, the anti-IRS-1 precipitates of the extracts of these cells contained a 170 kDa band that reacted with the antibodies to phosphotyrosine but not the antibodies to drosophila IR, identifying this band as IRS-1 (Figure 7). The tyrosine phosphorylation of the IRS-1 band was clearly increased in these cells in comparison to that in the parental CHO cells (Figure 7). In dIR2 cells, the increase in tyrosine phosphorylation of the IRS-1 band over that in CHO cells was also observed; however, the β-subunit of this chimeric receptor appeared to migrate at a slightly higher position than that of dIR1, close to that of IRS-1 (Figure 7). Anti-IR precipitates of both cells also appeared to contain a precursor form of the chimeric receptors (corresponding to the band above the 215 kDa marker protein), which was not tyrosine phosphorylated, as well as a protein of the appropriate molecular weight and immunoreactivity of the α-chain of the human receptor. In cells overexpressing the human IR, the anti-IR precipitates contained a tyrosine phosphorylated band at 95 kDa that comigrated with the band that interacted with the monoclonal antibody to IR (Figure 7). This precipitate also contained a tyrosine phosphorylated protein that migrated at a position close to IRS-1. However, this

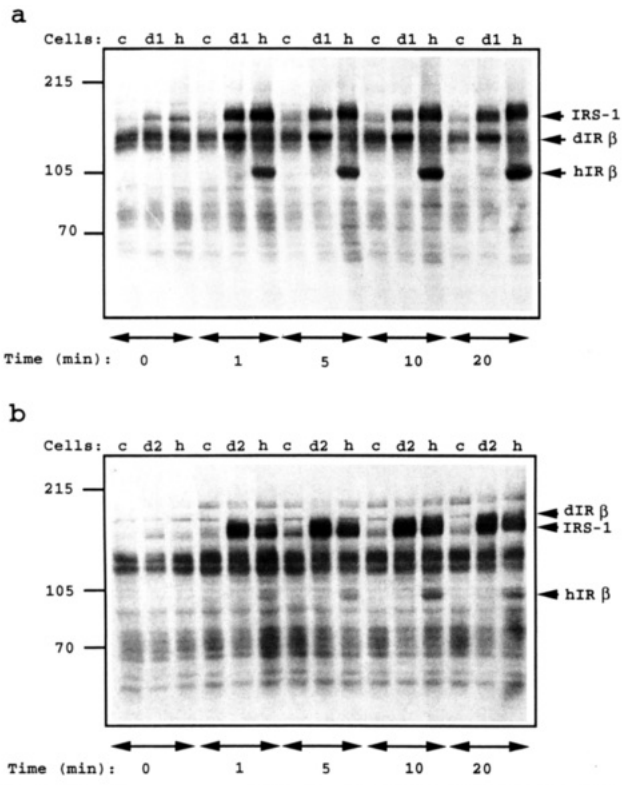

FIGURE 6: Time course of the insulin-stimulated tyrosine phosphorylation of endogenous substrates by the chimeric IRs. Either parental CHO cells (c) or cells overexpressing the human IR (h), the chimeric dIR1 (d1), or the chimeric dIR2 (d2) were treated with 10^{-9} M insulin at 37 °C for the indicated times. Cells were lysed, and the lysates were analyzed by immunoblotting with anti-phosphotyrosine antibody (RC20).

band migrated to a position slightly different from that of immunoprecipitated IRS-1 and instead comigrated with a band that was recognized by the monoclonal anti-IR antibodies (Figure 7), indicating that this was probably a precursor form of the IR and not IRS-1. Thus, under the conditions of these studies, no association of IRS-1 was observed with either the human or drosophila IR.

The preceding studies indicated that the chimeric drosophila IRs could mediate the tyrosine phosphorylation of IRS-1 in the intact cell. Additional studies have shown that IRS-1 could also be phosphorylated in vitro with these chimeric receptors (data not shown). Insulin treatment of the CHO cells expressing the chimeric drosophila IRs was found not to stimulate the tyrosine phosphorylation of either GAP or the PI 3-kinase. Insulin treatment of these cells did result in an increase in the tyrosine phosphorylation of the GAP-associated p60 and Shc proteins (data not shown). These results are identical to what has been observed previously with the human IR (Zhang & Roth, 1992; Seta et al., 1993). After insulin-stimulated tyrosine phosphorylation by the human IR, IRS-1 has been shown to be bound by the PI 3-kinase (White & Kahn, 1994). The chimeric receptors therefore were tested to determine whether they could mediate this response. Cells expressing the different receptors were treated with or without insulin and lysed, the lysates were precipitated with anti-phosphotyrosine antibodies, and the amount of PI 3-kinase activity in the precipitates was measured. Both chimeric receptors were found to mediate an insulin-induced increase in anti-phosphotyrosine precipitable PI 3-kinase activity comparable to that observed in cells expressing the human IR and much greater than that

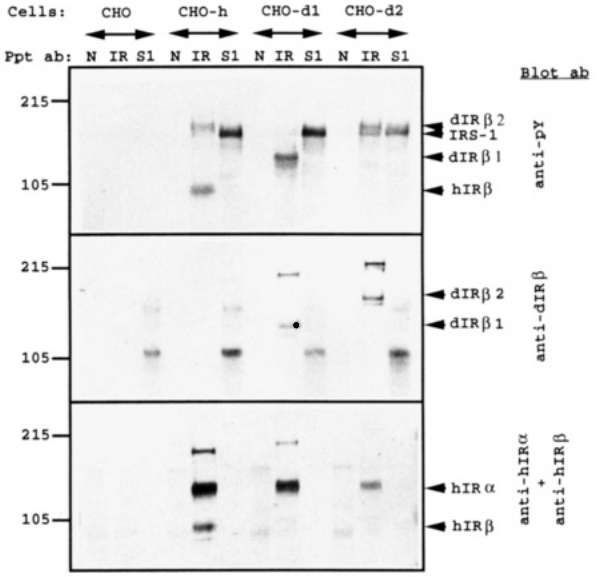

FIGURE 7: Identification of the tyrosine phosphorylated proteins by immunoprecipitation. Either parental CHO cells or CHO cells overexpressing IRs were treated with 10^{-9} M insulin at 37 °C for 10 min and lysed, and the lysates were immunoprecipitated with normal mouse IgG (N), monoclonal anti-IR, or rabbit polyclonal anti-IRS1 (S1) antibodies. The immunoprecipitates were analyzed by immunoblotting with either an anti-phosphotyrosine antibody (anti-pY), an anti-drosophila IR β-subunit antibody (anti-dIRβ), or a mixture of anti-human IR antibodies (anti-hIRα+anti-hIRβ). The nonspecific bands at 105 kDa in the blot with anti-dIRβ represent a reaction of the secondary antibody used to develop this Western blot with the rabbit anti-IRS-1 antibodies used to precipitate this protein.

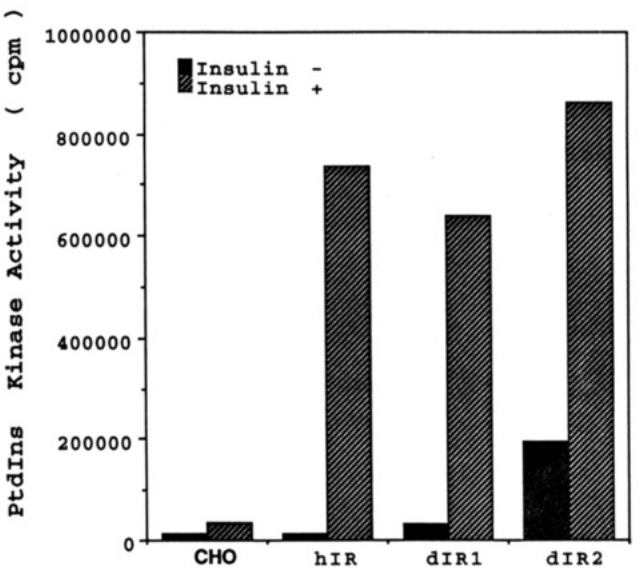

FIGURE 8: Insulin-stimulated PI 3-kinase activity. Either parental CHO cells or cells overexpressing IRs were treated with or without 10^{-9} M insulin at 37 °C for 10 min and lysed, and the lysates were immunoprecipitated with anti-phosphotyrosine antibody (PY20). PI 3-kinase activity was measured by adding PI and [γ-^{32}P]ATP as described in the Experimental Procedures.

observed in the parental CHO cells (Figure 8). To determine whether the PI 3-kinase activity was bound to the receptors, the extracts were also precipitated with antibodies to IR. The two chimeric receptors did not exhibit any significant increase in PI 3-kinase activity in the anti-IR precipitates over that observed with the human IR (Figure 9). Additional experiments studying the association in vitro of the PI 3-kinase from either mammalian cells or drosophila cells

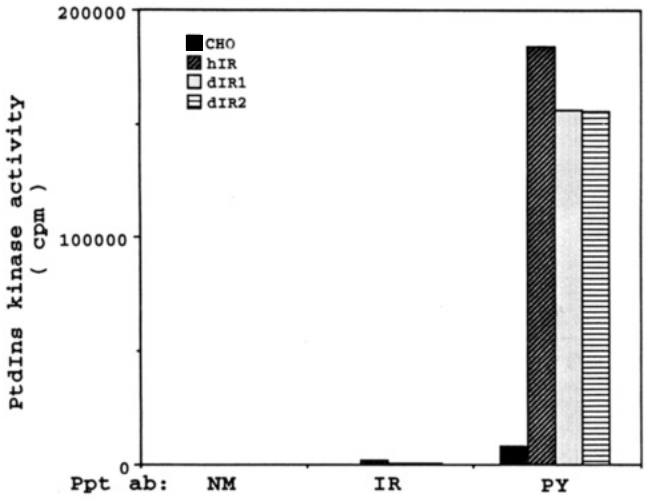

FIGURE 9: Lack of association of PI 3-kinase activity with human and chimeric IRs. Either parental CHO cells or cells overexpressing IRs were serum-starved for 30 min, treated with 10^{-9} M insulin at 37 °C for 10 min, and lysed, and the lysates were immunoprecipitated with either normal mouse IgG (NM), anti-IR antibodies (IR), or anti-phosphotyrosine antibody (PY). PI 3-kinase activity in the precipitates was measured as described.

(Schneider cells) with the different autophosphorylated receptors also did not indicate that the drosophila IR bound PI 3-kinase with any higher affinity than the human IR. Attempts to measure in vitro a specific binding of the drosophila chimeric IRs with recombinantly produced drosophila Grb-2 (called Drk) (Simon et al., 1993) or the drosophila tyrosine phosphatase corkscrew (Perkins et al., 1992) were also unsuccesful.

To study the ability of the cytoplasmic domain of the drosophila receptor to mediate various biological responses, the different CHO cells were treated with various concentrations of insulin and then examined for either a proliferative response or the activation of MAP kinase. Insulin caused a dose dependent proliferative response and an increase in MAP kinase activity in cells expressing the two chimeric receptors, which was shifted to the left (i.e., more sensitive to low concentrations of insulin) in comparison to the parental CHO cells (Figures 10 and 11). The shift in this response was comparable to that observed in cells expressing the human IR (Figures 10 and 11).

SUMMARY

The ability of two chimeric receptors containing either the entire cytoplasmic domain of the drosophila IR or only a portion of it to signal in CHO cells was found to be indistinguishable from that of the human IR expressed in the same cells, despite the considerable differences in the sequences of these two receptors as well as the presence of a cytoplasmic extension on the drosophila receptor (Fernandez-Almonacid, 1992). Thus, the drosophila insulin receptor is the most distantly related member of the insulin receptor family so far that has been shown to have this property. In contrast, a chimeric receptor containing the extracellular domain of the human insulin receptor and the cytoplasmic domain of the chicken sarcoma virus UR2 protein tyrosine kinase called *ros* (which shares 50% sequence identity with the human insulin receptor kinase domain) was previously found to be incapable of mimicking the human insulin receptor's signaling ability (Ellis et al., 1987).

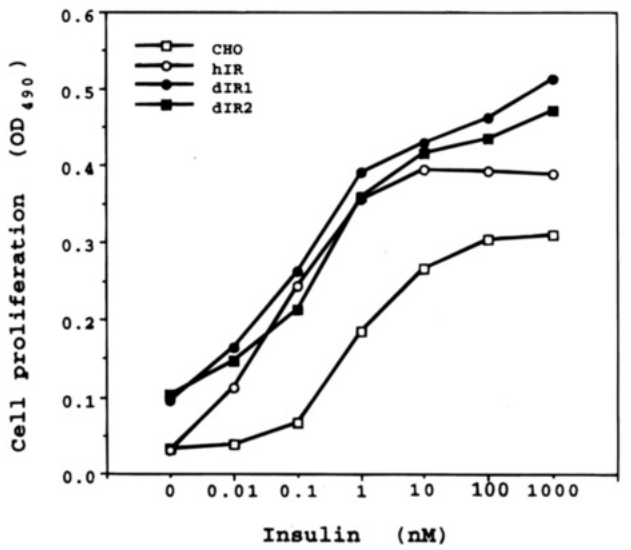

FIGURE 10: Insulin-stimulated proliferation of CHO cells expressing the different receptors. The indicated cells were cultured for 48 h in 96-well dishes with serum-free medium containing the indicated amounts of insulin. Cells were incubated with MTS/PMS, and the absorbance at 490 nm was measured. Results are means of quadruplicate determinations.

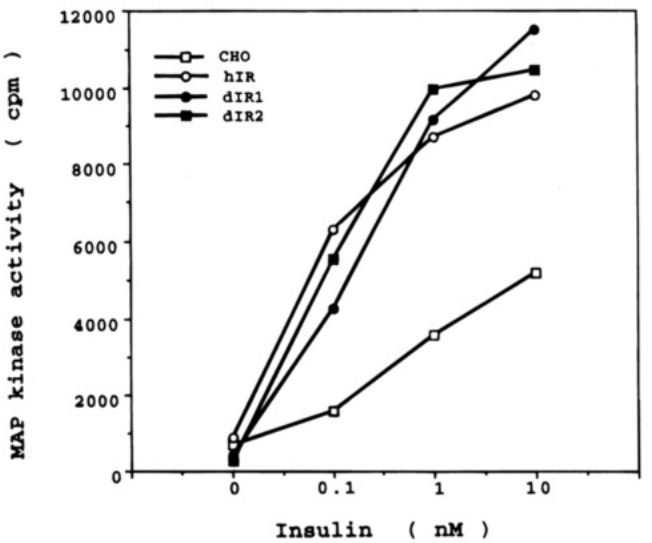

FIGURE 11: Insulin-stimulated MAP kinase activity in CHO cells expressing the different receptors. The indicated cells were serum-starved for 18 h, treated with the indicated concentrations of insulin for 5 min at 37 °C, and lysed, and the lysates were immunoprecipitated with an anti-MAP kinase antibody. Kinase activity was measured in the precipitates by using the exogenous substrate myelin basic protein as described in the Experimental Procedures.

The cytoplasmic domains of the human and drosophila insulin receptors appeared to have comparable abilities to phosphorylate three different substrates, IRS-1 (Figures 5−7), Shc, and the 60 kDa GAP-associated protein (data not shown), and to stimulate cellular growth (Figure 10) and MAP kinase (Figure 11). These results are consistent with the high degree of sequence conservation in regions of the kinase domains important for the signal transduction of these two IRs, as well as the retention of a critical tyrosine in the NPXYXS motif in the juxtamembrane region of the drosophila IR (Fernandez-Almonacid, 1992). The finding that both receptors directly bound comparable low levels of PI 3-kinase in the CHO cells was surprising, considering the presence of three tyrosines in the YXXM motif in the carboxy tail extension of the drosophila IR. However, these

tyrosines are not preceded by the negatively charged residues generally preferred by the IR tyrosine kinase (Avruch et al., 1990). Thus, in the context of the CHO cells in which IRS-1 is present, the drosophila receptor may preferentially phosphorylate the tyrosines present in IRS-1 since these residues are preceded by negatively charged amino acids (White & Kahn, 1994). In contrast, in cells lacking IRS-1 (i.e., drosophila cells), the phosphorylation of the tyrosines in this carboxy tail extension may be more predominant. The phosphorylation of these tyrosines may create binding sites for SH2-containing proteins. However, two such known proteins, drosophila Grb-2 (Simon et al., 1993) and the drosophila SH2 containing tyrosine phosphatase called corkscrew (Perkins et al., 1992), did not appear to bind to the autophosphorylated Drosophila IR (data not shown).

Low levels of mammalian PI 3-kinase binding were detected in vitro with the autophosphorylated chimeric receptors, comparable to what was observed with the human IR. It should be noted that, at the present time, a PI 3-kinase in drosophila has not been identified. Thus, it is possible that the drosophila PI 3-kinase binds to the phosphorylated tyrosines in the drosophila receptor with much higher affinity. Alternatively, it is possible that a distinct drosophila protein binds to these tyrosines. The chimeric drosophila IR expressed in CHO cells also differed from the previously described receptor in drosophila cells, since no processing of this receptor to yield a 90 kDa fragment of the β-subunit was observed in the CHO cells as was previously noted in drosophila cells (Petruzzelli et al., 1985; Fernandez-Almonacid & Rosen, 1987). Thus, the processing of the drosophila receptor may occur by a species or tissue specific process.

ACKNOWLEDGMENT

The authors are grateful to Drs. Kozui Shii, John Blenis, Mike Simon, and Alan Saltiel for generous gifts of the antibodies to IR, MAP kinase, drosophila Grb-2, and IRS-1, respectively. The authors also thank Dr. Mike Simon for the gift of recombinant drosophila Grb-2 and Dr. Lizabeth Perkins for the construct encoding corkscrew.

REFERENCES

Avruch, J., Tornqvist, H. E., Gunsalus, J. R., Yurkow, E. J., Kyriakis, J. M., & Price, D. J. (1990) in *Handbook of Experimental Pharmacology: Insulin*, pp 313−366, Springer-Verlag, Berlin/Heidelberg.

Chen, R.-H., & Blenis, J. (1990) *Mol. Cell. Biol. 10*, 3204−3215.

Chin, J. E., Dickens, M., Tavare, J. M., & Roth, R. A. (1993) *J. Biol. Chem. 268*, 6338−6347.

Ellis, L., Morgan, D. O., Jong, S., Wang, L., Roth, R. A., & Rutter, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5101−5105.

de la Luna, S., Soria, I., Pulido, D., Ortin, J., & Jimenez, A. (1988) *Gene 62*, 121−126.

Faria, T. N., Blakesley, V. A., Kato, H., Stannard, B., LeRoith, D., & Roberts, C. T., Jr. (1994) *J. Biol. Chem. 269*, 13922−13928.

Fernandez-Almonacid, R. (1992) Ph.D. Thesis, Cornell University, Ithaca, NY.

Fernandez-Almonacid, R., & Rosen, O. M. (1987) *Mol. Cell. Biol. 7*, 2718−2727.

Gustafson, T. A., & Rutter, W. J. (1990) *J. Biol. Chem. 265*, 18663−18667.

Hagino, H., Shii, K., Yokono, K., Matsuba, H., Yoshida, M., Hosomi, Y., Okada, Y., Kishimoto, M., Hozumi, T., Ishida, Y., Nishimura, R., Kasuba, M., & Baba, S. (1994) *Diabetes 43*, 274−280.

Lammers, R., Gray, A., Schlessinger, J., & Ullrich, A. (1989) *EMBO J. 8*, 1369−1375.

Lee, J., & Pilch, P. F. (1994) *Am. J. Physiol. 266*, C319−334.

Morgan, D. O., & Roth, R. A. (1986) *Biochemistry 25*, 1364−1371.

Myers, M. G., Jr., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., & White, M. F. (1993) *Endocrinology 132*, 1421−1430.

Nishida, Y., Hata, M., Nishizuka, Y., Rutter, W. J., & Ebina, Y. (1986) *Biochem. Biophys. Res. Commun. 141*, 474−481.

Ohmichi, M., Pang, L., Ribon, V., & Saltiel, A. R. (1993) *Endocrinology 133*, 46−56.

Perkins, L. A., Larsen, I., & Perrimon, N. (1992) *Cell 70*, 225−236.

Petruzzelli, L., Herrera, R., Garcia-Arenas, R., & Rosen, O. M. (1985) *J. Biol. Chem. 260*, 16072−16075.

Petruzzelli, L., Herrera, R., Arenas-Garcia, R., Fernandez, R., Birnbaum, M. J., & Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4710−4714.

Roth, R. A., Steele-Perkins, G., Hari, J., Stover, C., Pierce, S., Turner, J., Edman, J. C., & Rutter, W. J. (1988) *Insulin and insulin-like growth factor receptors and responses*, Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schaffer, L., Kjeldsen, T., Andersen, A. S., Wiberg, F. C., Larsen, U. D., Cara, J. F., Mirmira, R. G., Nakagawa, S. H., & Tager, H. S. (1993) *J. Biol. Chem. 268*, 3044−3047.

Schumacher, R., Soos, J., Schlessinger, J., Brandenburg, D., Siddle, K., & Ullrich, A. (1993) *J. Biol. Chem. 268*, 1087−1094.

Seta, K. A., Kovacina, K. S., & Roth, R. A. (1993) in *Current Directions in Insulin-like Growth Factor Research (Advances in Experimental Medicine and Biology, Vol. 343)* pp 113−132, Plenum Press, New York.

Shier, P., & Watt, V. M. (1989) *J. Biol. Chem. 264*, 14605−14608.

Siddle, K. (1992) *Prog. Growth Factor Res. 4*, 301−320.

Simon, M. A., Dodson, G. S., & Rubin, G. M. (1993) *Cell 73*, 169−177.

Tartare, S., Mothe, I., Kowalski-Chauvel, A., Breitmayer, J.-P., Ballotti, R., & Van Obberghen, E. (1994) *J. Biol. Chem. 269*, 11449−11455.

Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Hanzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., & Fujita-Yamaguchi, Y. (1986) *EMBO J. 5*, 2503−2512.

White, M. F., & Kahn, C. R. (1994) *J. Biol. Chem. 269*, 1−4.

Zhang, B., & Roth, R. A. (1991) *Biochemistry 30*, 5113−5117.

Zhang, B., & Roth, R. A. (1992) *J. Biol. Chem. 267*, 18320−18328.

Zhang, B., Tavare, J. M., Ellis, L., & Roth, R. A. (1991) *J. Biol. Chem. 266*, 990−996.

BI9425821