The Insulin Receptor-related Receptor

TISSUE EXPRESSION, LIGAND BINDING SPECIFICITY, AND SIGNALING CAPABILITIES*

(Received for publication, May 4, 1992)

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In 1989, Shier and Watt identified a gene which was predicted to encode a new member of the insulin receptor (IR) family, and they called it the insulin receptor-related receptor (IRR) (Shier, P., and Watt, V. M. (1989) J. Biol. Chem. 264, 14605–14608). However, the tissues expressing this receptor, its ligand binding specificity and its signaling capability have remained unknown. In the present studies we report Northern blot analyses and polymerase chain reaction data, which indicate that the IRR mRNA is expressed in a variety of tissues, including the human kidney, heart, skeletal muscle, liver, and pancreas. In order to examine the ligand(s) recognized by IRR, we constructed a chimeric receptor with the extracellular domain of the IR replaced with that of IRR. This chimera was found not to bind radioactively labeled insulin, insulin-like growth factor I (IGF-I), or IGF-II. These ligands and relaxin, the only other known member of the mammalian insulin family, also failed to stimulate the tyrosine kinase activity of this chimeric receptor. A second chimeric receptor with the extracellular domain of IR and the kinase domain of IRR was also constructed and utilized to study the signaling capabilities of the kinase domain of IRR. This chimera exhibited high affinity insulin binding and insulin-stimulated tyrosine kinase activity. The kinase domains of the IR and IRR were found capable of phosphorylating the same spectrum of exogenous and endogenous substrates. However, Chinese hamster ovary (CHO) cells stably overexpressing the kinase domain of IRR exhibited elevated basal tyrosine incorporation and 2-deoxyglucose uptake compared with CHO cells and CHO cells overexpressing wild-type IR. We conclude that: 1) IRR is expressed in the human kidney, heart, skeletal muscle, liver, and pancreas, 2) IRR does not appear to be the receptor of any known member of the insulin family, and 3) the tyrosine kinase of IRR appears to be similar to that of IR in both the spectrum of substrates phosphorylated and the biological responses stimulated.

* These studies were supported by National Institutes of Health Grants DK 4175 and DK 34926 and a postdoctoral fellowship (to B. Z.) from the American Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Insulin receptor-related receptor (IRR) is a member of a family of receptors that includes the insulin receptor (IR) and the insulin-like growth factor I receptor (1–4). A genomic clone of IRR was initially obtained by Shier and Watt (1) by a low stringency screen of a genomic library using human insulin receptor cDNA as a probe. The nucleotide sequence of the IRR gene predicts that it encodes a novel receptor that is homologous to IR and IGF-1 receptor (1). All three receptors have similar predicted structures with a single transmembrane domain, an extracellular domain with a cysteine-rich region, and a cytoplasmic domain with an intrinsic tyrosine kinase activity. IRR, like the other two receptors, was also predicted to be synthesized as a single polypeptide, which is then cleaved to yield an α and β chain, with the α chain being completely extracellular and the β chain having both an extracellular and an intracellular domain. The predicted amino acid sequence of IRR is approximately 60% identical to that of IR and IGF-I receptor with the tyrosine kinase domains being most conserved and the carboxyl tails of the receptors showing the lowest sequence identity (1). The genes for all three receptors map to different chromosomes (5).

To begin to study the physiological role(s) for IRR, it is necessary to determine which tissues express this receptor, what its ligand specificity is, and what types of signals it is capable of mediating. In the present studies we have determined the expression of IRR mRNA in different tissues by Northern analyses and obtained IRR cDNA using PCR. In order to define the ligand specificity of IRR, we have constructed a chimeric receptor with the extracellular domain of IRR fused to the transmembrane and cytoplasmic domains of IR (IR/IRR). This construct was utilized to study the binding of insulin-like molecules to the chimera. We have also constructed a reciprocal chimeric receptor with the cytoplasmic domain of IR replaced with that of IRR (IR/IRK). This chimeric receptor was utilized to study the substrate specificity of the tyrosine kinase domain of IRR and to define its signaling capabilities. The present results indicate that IRR is expressed in several tissues, including human kidney, heart, skeletal muscle, pancreas, and liver, does not bind any of the known insulin-like molecules, and is similar to the IR in tyrosine kinase activity and ability to mediate various biological responses.

MATERIALS AND METHODS

Northern Blot Analysis—cDNAs encoding exons 2 or 3 of IRR were obtained as described (6, 7) and subcloned into BlueScript/KS

1 The abbreviations used are: IRR, insulin receptor-related receptor; IR, insulin receptor; IGF, insulin-like growth factor; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitriilo)] tetracetic acid; GAP, GTPase-activating protein; PtdIns, phosphatidylinositol; PLC, phospholipase C.
In Vivo Activation of Tyrosine Kinase Activities of Chimeric Receptors—Confluent CHO-IR/IRRK or CHO-IR/IRRK cells in 6-well dishes were washed and incubated with 0.5 ml serum-free medium with or without 0.5 mM sodium orthovanadate for 4 h at 37 °C. The CHO-IR/IRRK cells were then incubated with either HEPES-buff-fered insulin, 15% fetal calf serum, insulin, IGF-I, or monoclonal anti-IR antibody (5D9). The PtdIns 3-kinase activity was determined after immunoimmobilization of the receptor on microtiter wells precoated with monoclonal antibody 29B4. After an overnight incubation, the wells were washed three times with WGBP, and 20 μl of the kinase reaction mixture (50 mM HEPES, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 1 mg/ml poly(Glu:Tyr)(4:1), 2 μCi of carrier-free [γ-32P]ATP) was added to each well, and the incubation was continued at 25 °C for 30 min. The reaction was terminated by spotting 10 μl of the reaction mixture on a Whatman No. 3 MM filter strip. After air drying, the filter strips were soaked in ice-cold 10% trichloroacetic acid containing 10 mM sodium pyrophosphate (5 ml/1 x 3-cm strip) for 30 min, boiled in 5% trichloroacetic acid for 5 min, washed twice with 85% ethanol and once with acetone, and the radioactivity was determined by Cerenkov counting. For the activation of CHO-IR/IRRK, the cells were incubated with different concentrations of insulin at 37 °C for 10 min, lysates were prepared and tyrosine kinase activities were determined after immunomobilization of the receptor on microtiter wells precoated with monoclonal antibody 29B4. (12) As described above, CHO cells overexpressing IR/IRR (CHO-IR/IRR) were identified by 125I-insulin binding (see below). Cells overexpressing IR/IRR (CHO-IR/IRR) were identified by binding of 125I-labeled monoclonal antibody to the cytoplasmic domain of IR (17A3) (see below). 

125I-Labeled Ligand Binding Assay—125I-Labeled insulin, IGF-I, and monoclonal antibody 17A3 (9) were prepared by the IODO-GEN method (Pierce Chemical Co.) and their specific activities were 200, 210, 250, and 350 Ci/mg, respectively. 125I-Labeled Tyr A14)-insulin analog X92 (specific activity = 320 Ci/mg) (10) was a kind gift of Dr. Lauges Schaffer (Novo Nordisk). Transfected COS-7 cells or CHO-IRRE2/IR (7), CHO-IR/IRR, or CHO-IR/IRRK were lyased in a lysis buffer containing 50 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 150 mM NaCl, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 mM sodium fluoride, and 1 mg/ml bacitracin. Forty microliter samples were added to 96-well polyvinyl chloride plates previously coated with monoclonal antibody 29B4, 3/IR and IRR/IRK or 83-7 (for IR/IRRK) (a gift of Dr. K. Siddle). After an overnight incubation at 4 °C, the wells were washed three times with WGBP (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 0.1% bovine serum albumin). Different amounts of the radioactive ligands or antibodies were added to each well, and after 12-26 h at 4 °C, the wells were washed three times with WGBP and counted.

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(Ptrilins) 5-Kinase Assays—CHO, CHO-T, and CHO-IR/IRRK cells in 24-well plates were treated with 10 μM deoxyglucose in complete medium at 37 °C for 6 h to lower the basal level of glucose uptake (11). The cells were then washed twice with a buffer containing 140 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 1.47 mM potassium phosphate, 8.0 mM sodium phosphate, pH 7.4, 0.46 mM MgCl2, and 1 mg/ml bovine serum albumin. After incubation at 37 °C for 30 min in the presence or absence of 10−4 M insulin, the cells were incubated with 0.1 mM deoxyglucose and 0.2 μCi/ml of 2-deoxy-D-[1,2-3H]glucose for 10 min. Cells were then washed three times with the buffer containing 200 μM phloretin and lysed in 0.075% SDS. Aliquots of the lysates were counted and assayed for protein by the Coomassie dye binding assay (Bio-Rad). In each experiment, the amounts of deoxyglucose taken up by the different cell lines were normalized against their protein concentration as compared with CHO cells.

Endogenous Substrates of IR/IRR (CHO, CHO-T, and CHO-IR/IRRK cells) were incubated in serum-free medium in the presence or absence of 0.5 mM sodium orthovanadate at 37 °C for 4 h. The cells were then either treated with buffer or 10−7 M insulin for 10 min. After three washes with phosphate-buffered saline, the cells were lysed in the lysis buffer described above. The lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by Western transfer to nitrocellulose filters. The filters were incubated with either monoclonal anti-phosphotyrosine antibodies, or a monoclonal antibody directed against the a subunit of IR (3B11) (a gift of Dr. Kozui Shii). Bound antibody was detected using alkaline phosphatase-conjugated anti-immunoglobulin (Promega Biotech).

Phosphatidylinositol (Pasinae) 5-Kinase Assays—CHO, CHO-T, and CHO-IR/IRRK cells were grown in serum-free medium with increasing concentrations of insulin and lysed in the lysis buffer. The lysates were subjected to immunoprecipitation with either normal mouse IgG, monoclonal anti-phosphotyrosine antibody (PY20) (12) (a gift of Dr. John Glenney), or monoclonal anti-IR antibody (5D9). The Py20s 5-kinase activity was measured directly in the immunoprecipitates as described previously (13). Immunoprecipitation and Immunoblotting of p85, GAP,
**RESULTS**

Tissue Expression of IRR—In order to identify tissues expressing IRR, poly(A)-selected mRNAs from human heart, liver, skeletal muscle, kidney, pancreas, brain, lung, and placenta were probed in a Northern blot with an antisense riboprobe directed against exon 3 of human IRR (Fig. 1). Kidney, heart, skeletal muscle, liver, and pancreas were all found to give a positive signal with a band at 5.8 kb. Similar results were obtained with an antisense riboprobe to exon 2 of human IRR (data not shown). To further test whether IRR was expressed in kidney, PCR was performed on cDNA obtained from reverse-transcribed human fetal kidney mRNA and a pool of cDNA generated from an adult human kidney cDNA library. PCR products of the appropriate size for the primers used were generated from both sources. The cDNA encoding the extracellular domain of IRR was cloned from the PCR products with the fetal cDNA, and the cDNA encoding the intracellular domain of IRR was obtained from the PCR products with the kidney library. The nucleotide sequences of these cloned PCR products were identical to those of IRR (1).

Ligand Binding Specificity of the Extracellular Domain of IRR—In order to identify the ligand recognized by IRR, we constructed a cDNA that encodes a chimeric receptor, IRR/IRK, which contains the extracellular domain of IRR (residues 7–892) on the backbone of IR (Fig. 2). We first determined the ability of the expressed chimeric receptor to bind to several 125I-labeled ligands. Lysates of COS-7 cells transfected with vector or the cDNAs encoding either wild-type IR or IRR/IRK were incubated in microtiter wells coated with a monoclonal antibody to the cytoplasmic domain of IR (29B4). After washing the wells, either 125I-labeled insulin, IGF-I, IGF-II, or a monoclonal antibody to a distinct epitope on the cytoplasmic domain of IR (17A3) was added, and the radioactivity bound by the immobilized receptors was determined. As indicated by 125I-17A3 binding, similar amounts of wild-type IR and IRR/IRK were expressed in COS-7 cells and bound to the wells (Fig. 3). However, IRR/IRK did not bind significantly greater amounts of either insulin, IGF-I, or IGF-II than the vector control (Fig. 3), suggesting that IRR is not the receptor for any of these ligands. The control transfectants with the native IR bound, as expected, much greater amounts of insulin than the control transfectants. To further test the ability of these as well as other ligands to bind to IRR/IRK, we examined the ability of various ligands to stimulate the intrinsic tyrosine kinase activity of the chimeric receptor in CHO cells stably overexpressing it. This cell line was demonstrated to overexpress the processed chimeric receptor by immunoblotting in amounts comparable to the overexpressed human IR in CHO.T cells (data not shown). Insulin, IGF-I, and IGF-II (all at 100 nM) were all found incapable of activating the intrinsic tyrosine kinase activity of the chimera (Fig. 4). The only other known member of the vertebrate insulin family, relaxin (14), was also incapable of activating the kinase activity of the chimera (Fig. 4). Several invertebrate insulin-like molecules, including bombxin II and IV (15) and molluscan insulin-like peptide (16), as well as an unrelated hormone, human growth hormone, were similarly found to be incapable of activating the tyrosine kinase activity of IRR/IRK (Fig. 4). To test for the intactness of this chimera, we examined whether vanadate and wheat germ agglutinin, activators of the insulin receptor kinase (17, 18), could stimulate its activity. Both of these agents were
found to significantly stimulate the kinase activity of the chimera (Fig. 4 and data not shown). In our prior studies, we had observed a small but significant amount of labeled insulin binding to a chimeric receptor (IRRE2,3/IR) that only had the amino-terminal 450 amino acid residues of IRR on the backbone of the insulin receptor. We therefore compared the binding of labeled insulin to the two chimeric receptors, IRRE2,3/IR and IRR-IRRE2,3/IR. 125I-Labeled insulin was incubated in the presence or absence of a large excess of unlabeled insulin with similar amounts of the two chimeric receptors (as assessed by antibody binding) captured on microtiter wells. IR/IRR exhibited no significant increase in specific insulin binding in comparison to that for an untransfected control (Fig. 5). However, IRRE2,3/IR was able to bind significantly more insulin than this control (approximately 7–8 times the amount specifically bound by the control lysates) (Fig. 5). The specific binding of insulin to IRRE2,3/IR was inhibited by a human specific monoclonal antibody to the insulin receptor (5D9) (Fig. 5). This monoclonal antibody has previously been shown to recognize an epitope present in the chimera IRRE2,3/IR (7) but does not recognize the endogenous insulin and IGF-I receptors in CHO cells (19). Furthermore, a labeled analog of insulin (called X92), which has a much higher affinity for the IR than native insulin (10), was also found to bind to IRRE2,3/IR in amounts approximately 5–6 times greater than that observed bound to the control lysates. This analog dissociated much more rapidly from IRRE2,3/IR than from native IR, indicating that the chimeric receptor IRRE2,3/IR can bind insulin although with a much weaker affinity than the native IR.

Tyrosine Kinase Activity of the Cytoplasmic Domain of IRR—Since the ligand that activates the intrinsic kinase activity of IRR was not available, we constructed a cDNA that encodes a chimera, IR/IRRK, with the extracellular and transmembrane domains of IR and the intracellular domain of IRR (Fig. 2). Stable transfectants of CHO cells that overexpress this chimera were selected. These cells were shown to produce the mature chimera by Western blotting in amounts comparable with CHO.T cells that overexpress native IR (see below). This chimera was also able to bind to insulin with an affinity indistinguishable from that for the wild-type IR (Fig. 6). CHO, CHO.T, and CHO-IR/IRRK cells were treated with different concentrations of insulin and the tyrosine kinase activities of the receptors from these cells towards exogenous substrate (poly(Glu:Tyr) (4:1)) were determined. As shown in Fig. 7, in vitro insulin treatment resulted in a dose-dependent activation of the tyrosine kinase activity of IR/IRRK, which closely followed the activation observed with native IR and was clearly above that observed in the parental CHO cells. However, the maximum kinase activity for IR/IRRK was about one-tenth that observed for wild-type IR, although approximately the same amount of the two receptors were adsorbed to the wells (Fig. 7). Similar results were obtained with a second independent clone of CHO cells expressing the chimera (Fig. 7B). This lower kinase activity with the chimera was also observed in vitro with two other substrates (poly(Glu:Tyr) (1:1) and histone) (data not shown). The activation of the tyrosine kinase activity of the chimeras, like the wild-type IR, could also be accomplished in vitro by autophosphorylation (data not shown). As with the in vivo activated receptor, the in vitro activated chimera had approximately one-tenth the kinase activity of the native IR.

To assess the ability of the IRR kinase domain to phosphorylate endogenous substrates, two lines of CHO-IR/IRRK, CHO, and CHO.T cells were treated with or without insulin in the presence or absence of vanadate, and their lysates were examined for tyrosine phosphorylated proteins by immunoblotting with antibodies to phosphotyrosine (Fig. 8A). In the

**Fig. 4. Lack of activation of the intrinsic tyrosine kinase activity of IRR/IRR by insulin-like molecules.** CHO cells over-expressing the chimeric receptor were incubated with either buffer (1), 0.5 mM vanadate (2), or 100 nM insulin (3), IGF-I (4), IGF-II (5), relaxin (6), bombyxin-II (7), bombyxin-IV (8), molluscan insulin-related peptide (9), or human growth hormone (10). The cells were then lysed, the receptor immobilized on microtiter wells and then tested for the ability to phosphorylate poly(Glu:Tyr). Shown are the number of counts incorporated into poly(Glu:Tyr). Results shown are representative of three independent experiments.

**Fig. 5. Binding of 125I-labeled insulin to IRR/IRR and IRRE2,3/IR.** Parental CHO cells CHO-IRR/IRR and CHO-IRRE2,3/IR were lysed, and the receptors present were immunoadsorbed on microtiter wells. The immobilized receptors were tested for their ability to bind labeled insulin either in the presence of buffer (0), 100 nM unlabeled insulin (Ins) or 100 nM of a monoclonal antibody to the human IR (Ab). Results shown are representative of three independent experiments.

**Fig. 6. Binding of 125I-labeled insulin to wild-type IR and chimeric IR/IRRK.** Wild-type IR and the IR/IRR chimera were transiently expressed in COS-7 cells. Receptors were adsorbed to microtiter wells and then incubated with 125I-insulin in the presence of increasing amounts of unlabeled insulin. Results are shown as percentage of maximal binding. Maximal binding was 7015 and 6381 cpm for IR and IR/IRRK, respectively. Results shown are representative of three independent experiments.
CHO-IR/IRRK cells, insulin alone stimulated the tyrosine phosphorylation of a protein of 85 kDa, a value consistent with that expected for the β subunit of this receptor, and one of approximately 170 kDa, a value consistent with that expected of a substrate called IRS-1 (20). In the presence of insulin and vanadate, a large number of tyrosine phosphorylated proteins were observed in these transfected cells that were not observed in the control parental CHO cells. The pattern and amount of tyrosine-phosphorylated proteins were similar to those observed in the CHO.T cells overexpressing native IR with the exception of a more prominent 95-kDa band present in the CHO.T cells. This 95-kDa band presumably represents the β subunit of the IR (the β subunit of IR is predicted from its sequence to be larger than that of IRR). A control immunoblot with a monoclonal antibody to the α subunit of the insulin receptor demonstrated that comparable levels of receptor were expressed in the different transfected cells (Fig. 8B).

One of the apparent endogenous substrates for the insulin and IGF-I receptors is the PtdIns 3-kinase in that insulin and IGF-I stimulate an increase in the amount of anti-phosphotyrosine-precipitable PtdIns 3-kinase activity (13, 21–23). We therefore tested whether insulin could also stimulate an increase in anti-phosphotyrosine-precipitable PtdIns 3-kinase activity in CHO-IR/IRRK cells. CHO, CHO.T, and CHO-IR/IRRK cells were treated in vivo with insulin and lysed, and the PtdIns 3-kinase activities were determined in anti-phosphotyrosine immunoprecipitates prepared from these cells. As shown in Fig. 9, insulin was able to stimulate the amount of PtdIns 3-kinase activity in anti-phosphotyrosine precipitates from CHO-IR/IRRK cells in a dose-dependent manner. The maximal activity precipitated from these cells was 15-fold above that observed in CHO cells, and it was about 50% of that observed in CHO.T cells.

To determine whether the PtdIns 3-kinase was immuno-precipitated with the anti-phosphotyrosine antibodies because of a stable association with the overexpressed receptors, the cell lysates of insulin-treated cells were precipitated with

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**Fig. 7.** Activation of the tyrosine kinase activities of wild-type and chimeric IR/IRRK. CHO, CHO-T, or CHO-IR/IRRK were treated with the indicated concentrations of insulin for 10 min at 37 °C. Cells were then lysed in the presence of phosphatase inhibitors and receptors were captured on microtiter wells coated with monoclonal antibody against IR. Receptor tyrosine kinase activities were determined as the radioactivity incorporated into the substrate poly(Glu,Tyr). In parallel wells, the amount of receptor captured was assessed by labeled antibody binding. In the experiment shown, 6652, 6800, and 6932 cpm of labeled antibody were bound for IR, IR/IRRK-1, and IR/IRRK-2, respectively. Results shown are representative of three independent experiments.

**Fig. 8.** Endogenous substrates of the tyrosine kinases of IR and IRR in CHO cell lines. CHO, CHO-T, or CHO-IR/IRRK cells were preincubated with or without 0.5 mM vanadate for 4 h and then incubated for 10 min at 37 °C with either buffer or 10^{-7} M insulin, as indicated. Cells were lysed, and the lysates were analyzed by Western blotting with either polyclonal anti-phosphotyrosine antibodies (A) or monoclonal antibody against the α subunit of IR (B). Molecular masses (in kDa) of marker proteins are indicated.

**Fig. 9.** Insulin-stimulated anti-phosphotyrosine-precipitatable PtdIns kinase activity in CHO, CHO-T, or CHO-IR/IRRK cells. The cells were treated with the indicated concentrations of insulin for 10 min at 37 °C. PtdIns kinase activity was measured in anti-phosphotyrosine immunoprecipitates using PtdIns as a substrate. Shown is a representative of three independent experiments each performed in triplicates.
either control IgG, anti-IR antibody 5D9, or the anti-phosphotyrosine antibody PY20. PtdIns 3-kinase activities in the immunoprecipitates were assayed. In both CHO-IR/IRRK and CHO.T cells, less than 2% of the total PtdIns 3-kinase activity (determined in the PY20 immunoprecipitates) is associated with the receptors (determined in the 5D9 immunoprecipitates) (Fig. 10). Immunoblots demonstrated that similar amounts of receptor were precipitated with the anti-IR and anti-phosphotyrosine antibodies (data not shown).

To determine whether insulin stimulated the tyrosine phosphorylation of the PtdIns 3-kinase, cells were treated with insulin in the presence of vanadate (to maximize the extent of substrate phosphorylation), the lysates were immunoprecipitated with an antibody to one of the subunits of the PtdIns 3-kinase (p85) (24–27) and the precipitates were analyzed by blotting with anti-phosphotyrosine antibodies. No significant increase in the level of tyrosine phosphorylation of the 85-kDa subunit of PtdIns 3-kinase was observed in either CHO.T or CHO-IR/IRRK cells even in the presence of the tyrosine phosphatase inhibitor vanadate (Fig. 11). A control blot that was probed with the anti-p85 antibody demonstrated that this antibody was capable of precipitating p85 from these cells (Fig. 11).

We also compared the ability of IR and IRR to tyrosine-phosphorylate two other substrates of tyrosine kinases, phospholipase Cγ (PLCγ) and the GTPase-activating protein of Ras, called GAP (28). Cells were treated with insulin, vanadate, or both, the lysates were immunoprecipitated with anti-PLCγ or anti-GAP, and the precipitated proteins subjected to anti-phosphotyrosine immunoblotting. The controls were conducted by probing the same immunoblots with antibodies to either PLCγ or GAP, respectively. In both CHO.T and CHO-IR/IRRK cells, insulin plus vanadate treatment resulted in a small amount of tyrosine phosphorylation of a 145-kDa protein that comigrated with PLCγ (Fig. 12). This increase in PLCγ phosphorylation was specifically mediated by the overexpressed receptors, since no increase was observed in the parental CHO cells. An even smaller increase in the tyrosine phosphorylation of GAP p120 was observed in CHO.T and CHO-IR/IRRK cells (Fig. 13). However, insulin stimulated an increase in tyrosine phosphorylation of one of the GAP-associated proteins, p60, in the transfected cells but not in the control cells (Fig. 13) (28).

**Biological Responses Mediated by the Intracellular Domain**

**FIG. 10. Test for association of PtdIns kinase activity with the wild-type IR and chimeric IR/IRRK.** CHO cells overexpressing IR or IR/IRRK were treated with 10−7 M insulin at 37 °C for 10 min. The cells were then lysed, and the extracts were absorbed with protein G-agarose beads coated with either normal mouse IgG (Nlg) or antibodies to p85 (α p85) and the precipitates were analyzed by SDS-gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibodies. The positive control blot was probed with antibody to p85, and the position corresponding to p85 is indicated.

**FIG. 11. Lack of detectable tyrosine phosphorylation of the p85 subunit of the PtdIns 3-kinase.** CHO, CHO.T, and CHO-IR/IRRK cells were treated with or without insulin plus vanadate. The lysates were subjected to immunoprecipitation with either normal rabbit IgG (Nlg) or antibodies to p85 (α p85) and the precipitates were analyzed by SDS-gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibodies. The positive control blot was probed with antibody to p85, and the position corresponding to p85 is indicated.

**FIG. 12. Tyrosine phosphorylation of PLCγ in CHO, CHO.T, and CHO-IR/IRRK cells.** The cells were treated with or without insulin plus vanadate, the lysates were immunoprecipitated with either normal mouse IgG (Nlg) or anti-PLCγ antibody (α PLCγ), and the precipitated proteins were subjected to anti-phosphotyrosine immunoblotting. For the positive control, the blot was probed with anti-PLCγ. The protein band corresponding to PLCγ is indicated.

of IRR—CHO cells stably overexpressing IR/IRRK were also utilized to see if the intracellular domain of IRR was capable of mediating stimulation of thymidine incorporation and glucose uptake. Two independent cell lines of CHO-IRR/IRRK exhibited a higher basal level of thymidine incorporation than
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Vanadate cells overexpressing IR/IRRK (Fig. 14) stimulate the parental CHO cells (Fig. 14) more than in either the parental CHO or CHOT cells (Fig. 14). Insulin also stimulated the parental CHO cells to a greater extent as that observed in the cells overexpressing IR/IRRK (Fig. 15). Maximal insulin stimulation of deoxyglucose uptake was only approximately 20–30% above these elevated basal levels of uptake for the transfected cell lines expressing these different receptors, although the absolute increment in deoxyglucose uptake with insulin treatment is greater than that observed in the parental CHO cells (Fig. 15). This low level of stimulation precluded the performance of quantitative dose response curves. However, it appeared that insulin at concentrations of less than 1 nM could stimulate glucose uptake in the transfected cells without stimulating glucose uptake in the parental CHO cells (data not shown). These results indicate that IR/IRRK, like the IR, can mediate the stimulation of glucose uptake.

**DISCUSSION**

In the present studies the mRNA for IRR has been found by Northern blot analyses to be expressed in a variety of human tissues including kidney, heart, skeletal muscle, pancreas, and liver (Fig. 1). It is likely that other tissues that have not been tested will also express the IRR mRNA. The expression of IRR mRNA in kidney was further confirmed, since we were able to obtain properly processed cDNA for IRR by PCR from both fetal and adult kidney. Our success in detecting IRR mRNA in some of the same tissues that were previously considered negative (1) may be due to the enhanced sensitivity of the antisense riboprobes used in the Northern analyses in the present work. It is also possible that the different results in the two studies arise from species specific differences, since the prior work was with rat tissues whereas the present studies were performed with human tissues. Of course, additional studies are required to demonstrate expression of the IRR protein in these tissues and to determine which cell types express IRR within these tissues.

In the present work we have utilized chimeric receptors constructed from insulin receptor and IRR to examine the ligand specificity of the extracellular domain of IRR and the signal transduction capacity of the cytoplasmic domain of IRR (Fig. 2). Extensive prior studies have shown that chimeric receptors can be constructed between the different domains of the IR and other molecules and that these two domains can mediate their ligand binding and signaling abilities independently (29–34). Thus it is likely that the extracellular domain of IRR can also fold independently and should have been capable of binding its proper ligand. The inability of the chimeric receptor with the extracellular domain of IRR to either bind labeled insulin, IGF-I or IGF-II (Fig. 3) or to...
exhibit ligand-stimulated kinase activity (Fig. 4) with any of the insulin-like molecules tested (which included all of the known vertebrate insulin-like molecules as well as several invertebrate insulin-like molecules) indicates that the ligand recognized by IRR is either a presently unknown insulin-like molecule or a known molecule that has not been previously considered insulin-like. With the chimeric IRR/IRK and the assay procedures described in this study, it is now possible to screen for the presence of a ligand for IRR in various biological fluids and in the supernatants of various cell lines.

The inability of the chimera IRR/IRK to bind significantly any insulin differs from the low level of insulin binding observed with the chimera IRRE23/IR, which has had only the amino-terminal 450 residues of the IR replaced with those of IRR (Fig. 5). The binding of insulin to the latter chimera was inhibited by insulin as well as by a monoclonal antibody that is specific for the human IR (Fig. 5). The inhibition by this antibody demonstrates that the binding of insulin by this chimera is not due to endogenous insulin or IGF-I receptors, since this antibody does not recognize either of these receptors (19). These results are consistent with the hypothesis that regions of the IR carboxyl to the cysteine-rich region also can contribute to ligand binding (7, 35, 36).

The tyrosine kinase domain of IRR shares a high degree of sequence identity (~80%) to that of both the IR and IGF-I receptor (1-4). The ATP binding site and the regulatory tyrosine autophosphorylation residues (corresponding to residues 960, 1158, 1162, and 1163 of IR) are all conserved, suggesting that the IRR tyrosine kinase could mediate signal transduction via mechanisms similar to those for IR and IGF-I receptor (1-4). However, a much lower level of sequence homology was found in the COOH-terminal region of the β subunit of IRR (only 19% identical to IR) and the intracellular domain of IRR is 48 residues shorter than that of the insulin receptor (1-4). Truncations of the β subunit of the IR have in some studies been found to affect the signaling capabilities of the IR, although such differences were not found in other studies (37-39). Therefore, it was possible that IRR tyrosine kinase could differ from the IR in its signaling capabilities.

Since the ligand for IRR is unidentified, it is impossible to study the signaling abilities of IRR using the native receptor. The strategy we used to overcome this limitation was to construct a chimeric receptor (IR/IRK) composed of the extracellular and the transmembrane domains of the insulin receptor and the intracellular domain of IRR. This approach allowed us to study the signaling potential of IRR following the stimulation of the chimeric receptor with insulin. Such chimeric receptors have also been utilized to study the signaling capabilities of other orphan receptors (40, 41).

As expected, the IR/IRK chimera is functional when transiently expressed in COS-7 cells and stably expressed in CHO cells. The chimeric receptor was able to bind insulin with an affinity equivalent to that of the wild-type insulin receptor (Fig. 6). In vivo stimulation of CHO-IR/IRK cells with insulin resulted in a dose-dependent activation of the tyrosine kinase activity of IR/IRK toward exogenous substrate (poly(Glu:Tyr) (4:1)) (Fig. 7). The in vitro assays of the tyrosine kinase activities were conducted using substrates and conditions optimized for IR, which may partially account for the approximate 10-fold lower kinase activity of IR/IRK compared to the native IR (Fig. 7). The kinase activities of the two receptors appeared more comparable in the intact cells. By immunoblotting with anti-phosphotyrosine antibodies, insulin was found to stimulate the tyrosine phosphorylation of approximately the same spectrum of proteins to approximately the same levels in cells overexpressing the native IR and IR/IRK (Fig. 8). The only major difference in the patterns observed were in the phosphorylation of the β subunits of the receptors, with the IR β subunit having a higher molecular weight and a greater extent of phosphorylation than that of IRR. Both of these differences can be accounted for by the lack of 48 amino acids in the COOH terminus of the β subunit of IRR, which contains two tyrosine autophosphorylation sites in the IR (1-4).

The abilities of IR and IR/IRK to phosphorylate several specific endogenous proteins were also analyzed. Insulin treatment of CHO-IR/IRK, as previously shown for cells overexpressing IR (13, 21), resulted in a large increase in anti-phosphotyrosine-precipitable PtdIns 3-kinase activity (Fig. 9). Most (greater than 98%) of this PtdIns 3-kinase activity did not appear to be associated with the chimeric receptor, since it was not precipitated with antibodies to the receptor (Fig. 10). This low level of association of the kinase domain of IRR with the PtdIns 3-kinase is identical to the results with the native IR (Ref. 13 and Fig. 10) and the receptor for IGF-I (23, 42). Surprisingly, the immunoprecipitation of the 85-kDa subunit of PtdIns 3-kinase did not reveal any insulin-stimulated increase in tyrosine phosphorylation of this subunit by immunoblotting in cells overexpressing either the native IR or IR/IRK (Fig. 11). These results suggest that this protein does not get tyrosine-phosphorylated by the IR and IRR kinase domains. In a recent study, the platelet-derived growth factor receptor tyrosine kinase was also reported not to mediate the tyrosine phosphorylation of this protein (43). An explanation for the ability of anti-phosphotyrosine antibodies to precipitate the PtdIns 3-kinase in insulin-treated cells may be that this enzyme becomes associated with another tyrosine-phosphorylated protein. One such candidate would be IRS-1, a 170-180-kDa protein that appears to associate with PtdIns 3-kinase after becoming tyrosine-phosphorylated by the IR (20).

Two other identified substrates for tyrosine kinases are PLCγ and GAP (24). Insulin treatment of CHO-IR/IRK and CHO.T in the presence of vanadate resulted in the same low level of tyrosine phosphorylation of PLCγ (Fig. 12). These results are consistent with prior studies indicating that PLCγ is not a very good substrate for the IR tyrosine kinase (44). Insulin treatment of both of these cells also resulted in a large amount of tyrosine phosphorylation of a GAP-associated protein of 60 kDa (Fig. 13). This protein has previously been found to be tyrosine-phosphorylated in cells treated with platelet-derived growth factor (28). The important finding in the present studies is that the ability of the kinase domains of IR and IRR to phosphorylate various specific endogenous proteins are quite similar.

The CHO cells overexpressing IR/IRK exhibited a much higher basal level of thymidine incorporation and 2-deoxyglucose uptake than either the parental CHO cells or the CHO cells overexpressing the native IR (Figs. 14 and 15). Insulin was also found to stimulate thymidine incorporation and glucose uptake in CHO-IR/IRK at concentrations that do not significantly stimulate responses in the parental CHO cells (Fig. 14 and data not shown). These results indicate that the kinase domain of IRR, like that of the IR, can mediate both of these biological responses. Moreover, the absolute increment in thymidine and glucose uptake in cells overexpressing the kinase domain of IRR is greater than in the cells overexpressing the native IR. However, because of the higher basal levels of thymidine incorporation and glucose uptake in the CHO-IR/IRK, it is difficult to conclude whether the kinase domain of IRR is more potent than that of IR in stimulating these responses.
In conclusion, the mRNA for IRR appears to be expressed in a variety of human tissues including human heart, skeletal muscle, kidney, liver, and pancreas. IRR also does not appear to be the receptor for any known member of the insulin family, either in vertebrates or invertebrates. Finally, the tyrosine kinase of IRR is similar to that of IR in that: 1) it is activated by autophosphorylation, 2) phosphorylates the same spectrum of exogenous and endogenous substrates, 3) does not tightly associate with PtdIns 3-kinase, and 4) mediates both thymidine incorporation and glucose uptake.

Acknowledgments—We are grateful to Dr. Gneeme Bell for the gift of the kidney cDNA library, Dr. A Suzuki for the bombyxin II and IV, Dr. A. B. Smit for the molluscan insulin-related peptide, Dr. Ken Siddle for monoclonal antibody 83,2, Dr. J. Rayment for insulin analog X92, Dr. K. Shii for monoclonal antibody 3Bl1, Dr. M. Smith for IGF-1, Dr. A. Perlman for relaxin, Dr. L. Shaffer for PY20, Dr. J. Merryweather for IGF-I, Dr. M. Smith for IGF-II, Dr. A. Perlman for relaxin, Dr. L. Shaffer for insulin analog X92, Dr. K. Shii for monoclonal antibody 3Bl1, Dr. J. Berry Gibbs for a polyclonal antibody to GAP, and Dr. J. Gieney for PY20.

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