Differential phosphorylation of IRS-1 by insulin and insulin-like growth factor I receptors in Chinese hamster ovary cells

M Amoui, B P Craddock and W Todd Miller

Department of Physiology and Biophysics, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794, USA
(Requests for offprints should be addressed to W T Miller, Department of Physiology and Biophysics, Basic Science Tower T-6, School of Medicine, SUNY at Stony Brook, Stony Brook, New York 11794-8661, USA; Email: miller@physiology.pnb.sunysb.edu)

Abstract

Insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) are closely related receptor tyrosine kinases. Despite their high degree of homology, recent evidence suggests that the two receptors have distinct biological roles. In several recent studies, the cytoplasmic tyrosine kinase domains of the two receptors have been shown to possess different signalling specificities. In this study, we examine the hypothesis that differential phosphorylation of insulin receptor substrate 1 (IRS-1) may contribute to these differences in signalling between the two receptors. Using Chinese hamster ovary (CHO) cells stably expressing human IR or IGF-IR and activated by their respective ligands, we show that there are differences between the two receptors with regard to the complement of SH2-containing proteins recruited to IRS-1. In particular, IGF-IR appears to couple IRS-1 preferentially

to Grb2 whereas, in contrast, IR appears to couple IRS-1 preferentially to the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase) and to Nck. The two receptors couple IRS-1 equally to the tyrosine phosphatase SHP2. We have also generated phosphospecific antibodies to three important tyrosine phosphorylation sites on IRS-1 (pY608, pY895 and pY1172). We used these antibodies to probe the phosphorylation status of these sites in intact CHO/IR and CHO/IGF-IR cells. In the case of pY608, these results also show evidence for differential phosphorylation of IRS-1 by the two receptors. Taken together, the results presented here support the notion that the cytoplasmic domains of IR and IGF-IR have differences in their intrinsic signalling potentials.

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Introduction

The polypeptide hormones insulin and insulin-like growth factor (IGF)-I are structurally homologous growth factors that regulate cell growth and metabolism. The two growth factors exert their biological effects by binding to distinct receptors on the surface of target cells. The receptors for insulin and for IGF-I (IR and IGF-IR respectively) are closely related (Yarden & Ullrich 1988, Fantl *et al.* 1993). They have the same oligomeric structure: in each case the receptor is made up of two extracellular α subunits containing the ligand-binding domain and two transmembrane β subunits possessing tyrosine kinase activity (Yarden & Ullrich 1988, Fantl *et al.* 1993). Activation of the receptors is believed to occur in a similar manner. Binding of ligand to the α subunits activates IR or IGF-IR, leading to autophosphorylation of tyrosine residues in the β subunits. Signalling via the IR and IGF-IR has been demonstrated to be dependent on their tyrosine kinase domains, which catalyze the phosphorylation of specific substrates (Chou *et al.* 1987, Ebina *et al.* 1987, Kato *et al.* 1993). The tyrosine kinase catalytic domains of IR and IGF-IR are very highly homologous

(~84% identity at the amino acid level) (Ullrich *et al.* 1986).

Recent evidence suggests that the receptors for insulin and IGF-I have some common biological roles, and others that are distinct (Blakesley *et al.* 1996, Lamothe *et al.* 1998, Urso *et al.* 1999). Evidence for distinct roles for the two receptors includes the study of IR- and IGF-IR-deficient mice, which have distinct phenotypes (Lamothe *et al.* 1998). Insulin is known to be a key regulator of physiological processes such as glucose transport and biosynthesis of glycogen and fat (Patti & Kahn 1998), but IGF-I has been shown to be more potent in stimulating cell growth by increasing DNA synthesis, and has a greater mitogenic potential than insulin (Lammers *et al.* 1989, Blakesley *et al.* 1996). Overexpression of IGF-IR is observed in many forms of human cancer, and interference with IGF-IR (by antisense strategies, antibodies or dominant negative mutants) in a variety of tumor cell lines reverses the transformed phenotype (Baserga *et al.* 1997).

The differential signalling properties of IR and IGF-IR are believed to reflect, in part, different patterns of expression in different cell types (Blakesley *et al.* 1996). However, even when they are studied in the same cell

background, there are differences in the signalling pathways initiated by IR and IGF-IR. A chimeric receptor consisting of the ligand-binding domain of IR and the cytoplasmic domain of IGF-IR has a signalling specificity that resembles the IGF-IR (e.g. it is 10 times more active in stimulating DNA synthesis than the IR itself) (Lammers *et al.* 1989). In a recent study, chimeras were generated in which the extracellular portion of the neurotrophin receptor was fused to the intracellular portions of IR or IGF-IR. These chimeric molecules were stably expressed in 3T3-L1 adipocytes at levels comparable to those of endogenous receptors, and activated by treatment with neurotrophin. The chimeric receptors showed differences in signalling properties, with IR being more effective in stimulating physiologically relevant metabolic responses and IGF-IR more effective in promoting mitogenesis (Urso *et al.* 1999). In particular, IR is more effective than IGF-IR in promoting association of IRS-1 with phosphatidylinositol 3'-kinase (PI3-kinase), stimulation of glucose uptake, and translocation of glucose transporter (GLUT) 4. In contrast, IGF-IR is more effective in promoting Shc phosphorylation, association of Shc with Grb2, and activation of the mitogen-activated protein kinase cascade (Urso *et al.* 1999). In another series of experiments, IR was demonstrated to be more potent than IGF-IR in stimulating glycogen synthesis in murine hepatocytes (Park *et al.* 1999). Because the binding of insulin and IGF-I to their respective receptors can trigger distinct cellular responses, the signalling pathways emanating from the receptors are presumably different. At present, the molecular basis for these differences in signal transduction remain unclear, particularly in light of the structural homology of the receptors.

Several substrates are rapidly phosphorylated in response to IGF-I treatment (but not to insulin treatment), indicating that they may mediate effects specific for IGF-I (Jacobs *et al.* 1983). Proteins that are differentially phosphorylated by the insulin and IGF-I receptors include the β 2-adrenergic receptor (Karoo & Malbon 1996), 14-3-3 proteins (Furlanetto *et al.* 1997) and pp120, a plasma membrane glycoprotein expressed in hepatocytes (Najjar *et al.* 1997).

One of the major substrates for both IR and IGF-IR is the 185-kDa insulin receptor substrate (IRS)-1 protein (Sun *et al.* 1991, White & Yenush 1998). IRS-1 contains PH and PTB domains that are important for recruitment to the receptors (White & Yenush 1998). After phosphorylation, IRS-1 serves as an intermediate docking protein, providing binding sites for multiple downstream SH2 domain-containing proteins. For example, after IR and IGF-IR activation, the tyrosine-phosphorylated form of IRS-1 binds to the 85 kDa regulatory subunit of the PI3-kinase (White & Yenush 1998), and this interaction activates PI3-kinase. In addition to PI3-kinase, IRS-1 can also interact with the growth factor receptor bound-2 (Grb2) protein, the adaptor protein Nck, and the

SH2-containing tyrosine phosphatase SHP2, in each case by SH2 domains in the downstream proteins binding to specific phosphorylated tyrosine motifs within IRS-1 (White & Yenush 1998). In this paper, we examine whether IR and IGF-IR phosphorylate IRS-1 at different sites, leading to differential recruitment of downstream signalling partners.

Materials and Methods

Cell culture and antibodies

Chinese hamster ovary (CHO) cells stably overexpressing human IR or human IGF-IR were a kind gift from Dr Jonathan Whittaker (Hagedorn Research Institute, Denmark). The cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dialyzed fetal bovine serum, 300 µg/ml glutamine, 100 µg/ml non-essential amino acids, 50 µg/ml G418, 2 µM methotrexate, and antibiotics and antimycotics.

Anti-IRS-1 polyclonal antibody (anti-IRS-1), anti-Grb2 monoclonal antibody (anti-Grb2), anti-phosphoinositide 3-kinase monoclonal antibody (anti-PI3-kinase), anti-Nck monoclonal antibody (anti-Nck) and mouse anti-phosphotyrosine monoclonal antibody 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-SHP2 monoclonal antibody (anti-SHP2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell stimulation and lysis

Cells were starved for 16 h in serum-free F12 medium containing 0.5% BSA and then incubated for 5 min at 37 °C with or without 100 nM recombinant human insulin (IR-expressing cells) or 10 nM recombinant human IGF-I (IGF-IR-expressing cells). (We used a lower concentration of IGF-I to avoid possible cross-activation of IR, as suggested by Najjar *et al.* 1997.) Cells were then washed once with ice-cold PBS and lysed in 2 ml lysis buffer containing 25 mM Tris-HCl (pH 8.0), 140 mM NaCl, 2 mM EDTA, 1 mM NaVO₃, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin and 2 µg/mg leupeptin, and then incubated with occasional mixing for 30 min at 4 °C. The lysates were cleared by centrifugation at 12 000 g for 10 min. Protein concentrations of the postnuclear supernatants were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Levels of IR and IGF-IR overexpression were compared by fractionation of cell lysates on wheat germ agglutinin agarose, followed by SDS-PAGE with Coomassie staining, as described previously (Yoshimasa *et al.* 1990).

Glutathione S-transferase fusion proteins and in vitro binding assays

The glutathione S-transferase (GST) fusion protein containing the N-terminal SH2 domain (amino acids

333–430) of the p85 subunit of human PI3'-kinase was purchased from PharMingen (San Diego, CA, USA). A bacterial expression plasmid encoding full-length GST-Grb2 was a gift from Dr Dafna Bar-Sagi (SUNY at Stony Brook). Plasmids encoding GST fusions to the SH2 domains of Nck and SHP2 were gifts from Dr Bruce Mayer (University of Connecticut Health Center) and Dr Michael Hayman (SUNY Stony Brook) respectively.

GST fusion plasmids were used to transform *Escherichia coli* BL21 cells, and GST fusion proteins were purified and immobilized by affinity chromatography on glutathione-sepharose (Molecular Probes, Eugene, OR, USA). Postnuclear supernatants from insulin-stimulated, IGF-I-stimulated and unstimulated CHO cells were normalized for amounts of total protein (~8 mg) and incubated for 2 h at 4 °C with the immobilized GST fusion proteins. Unbound material was removed by five washes with lysis buffer, and bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE (10%) and transferred by electroblotting onto PVDF membrane. The membrane was incubated in blocking solution (Tris-buffered saline containing Tween 20 (TBST) plus 3% dry milk) for 2 h at room temperature, followed by incubation with anti-IRS-1 antibody (1 µg/ml in TBST, 3% dry milk) for 1 h at room temperature. The membrane was then washed five times with TBST and incubated for 2 h with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibody diluted 1:5000 in TBST plus 3% dry milk. This was followed with another set of washes as described above and the membrane-bound HRP was visualized with enhanced chemiluminescence (ECL) western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. For reprobing experiments, the membranes were stripped by incubation at 50 °C for 30 min in 62.5 mM Tris (pH 6.7), 100 mM β-mercaptoethanol, 2% SDS, washed in TBST, and reprobed with another antibody.

Co-immunoprecipitation experiments

Postnuclear supernatants from insulin-stimulated, IGF-I-stimulated, or unstimulated CHO cells were normalized for amounts of total protein (~8 mg). Lysates were incubated for 4 h at 4 °C with 5 µg anti-IRS-1 antibody, and the immunocomplexes were captured by addition of 30 µl 50% protein-A agarose slurry. After four washes with lysis buffer, pellets were resuspended in SDS-PAGE sample buffer and boiled for 3 min. Proteins were resolved by SDS-PAGE (10%) and transferred by electroblotting onto PVDF membrane. The membrane was incubated in blocking solution (TBST, 3% dry milk) for 1 h at room temperature followed by incubation with one of the following antibodies: anti-PI-3 kinase, anti-Nck, anti-Grb2, anti-SHP2 or anti-IRS-1 (1 µg/ml in TBST, 3%

dry milk) for 1 h at room temperature. Detection was with the ECL method, as described above.

Generation of phosphospecific antibodies

Three phosphopeptides containing relevant IRS-1 phosphorylation sites (pY608, pY895 and pY1172) were prepared by solid-phase synthesis using Fmoc chemistry. Phosphotyrosine was incorporated into the peptides using Na-Fmoc-O-phospho-L-tyrosine (Novabiochem, San Diego, CA, USA) (Ottinger *et al.* 1993). The sequences of the peptides were: pY608, CLHTDDGp YMPMSPG; pY895, CPKSPGEpYVNIEFG; pY1172, CLEKSLNpYIDLDLV. (Each peptide contained an N-terminal cysteine for coupling to carrier protein.) Peptides were purified by preparative reversed-phase HPLC. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to confirm the identity of the final products. The peptides were coupled to keyhole limpet hemocyanin using succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce, Rockford, IL, USA), and rabbit polyclonal antisera were produced by GeneMed Synthesis (South San Francisco, CA, USA).

Phosphospecific antibodies were purified by successive rounds of affinity chromatography using immobilized phosphopeptides and unphosphorylated peptides. The unphosphorylated peptides were produced by treatment of the phosphorylated peptides with yersinia tyrosine phosphatase and were repurified by HPLC. To prepare columns for affinity purification, the peptides were reduced by addition of β-mercaptoethanol and repurified by HPLC. Peptides were then coupled to iodoacetyl agarose support (Pierce). Non-specific binding sites on columns were blocked by addition of free cysteine (8 mg/ml) and phosphospecific antibodies were purified according to the manufacturer's instructions.

ELISA

The purified phosphospecific antibodies were analyzed for specificity against unphosphorylated and various phosphorylated peptides using ELISA. Purified peptides (Y608, Y895 and Y1172) and phosphopeptides (pY608, pY895 and pY1172) were bound to 96-well Reacti-bind maleimide-activated plates (Pierce) overnight at room temperature on a shaker. Wells were washed three times with 400 µl/well PBS and then excess maleimide groups were blocked overnight at 4 °C with 150 µl free cysteine (10 µg/ml in PBS). Purified antibodies (100 µl in 1% BSA-TBST) were added to the wells and incubated for 2 h at room temperature on a shaker, followed by four washes with TBST. HRP-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech) (100 µl of a 1:5000 dilution in 1% BSA-TBST) was added to each well and incubated for 2 h at room temperature with shaking,

Figure 1 Insulin and IGF-I induce tyrosine phosphorylation of IRS-1. CHO cells expressing IR and IGF-IR were stimulated as described in Materials and Methods. Top panel: IRS-1 was immunoprecipitated (IP) from postnuclear supernatants with anti-IRS-1 antibody, subjected to SDS-PAGE (10%), and analyzed by immunoblotting using anti-phosphotyrosine mAb 4G10. Bottom panel: the membrane was stripped and reprobed with IRS-1 antibody.

followed by four washes with TBST. For detection, 100 µl 1-Step Turbo TMB (Pierce) were added to each well and incubated for 10 min at room temperature. The color reaction was halted by the addition of 100 µl of 100 mM H₂SO₄. After thorough mixing, absorbance was read at 450/630 nm on a Biotek EL311 plate reader.

Results

To compare the specificities of insulin and IGF-I receptors, we adopted a model cell system developed by Dr Jonathan Whittaker and his collaborators, who have achieved high-level expression of IR and IGF-IR in CHO cells to a level of >10⁷ molecules/cell (Yoshimasa *et al.* 1990). (The endogenous levels of receptors in these cells are approximately 30 000 IR per cell and 200 000 IGF-IR per cell (Myers *et al.* 1993), so background activities of wild-type receptors make a relatively small contribution to overall phosphorylation.) Using the procedures reported previously (Yoshimasa *et al.* 1990), we confirmed that, in our hands, the levels of overexpression for IR and IGF-IR were similar (data not shown). Thus we have been able to compare phosphorylation of endogenous IRS-1 by IR and IGF-IR in a similar cell background.

In our initial experiments, we measured overall tyrosine phosphorylation of IRS-1 in the CHO/IR and CHO/IGF-IR cells. Quiescent CHO/IR and CHO/IGF-IR cells (16 h serum-deprived) were incubated with or without 100 nM insulin or 10 nM IGF-I respectively for 5 min at 37 °C and cell lysates were prepared. Equal amounts of total protein were immunoprecipitated with

Figure 2 Association of IRS-1 from insulin-stimulated or IGF-I-stimulated cells with SH2-containing fusion proteins. Immobilized GST-fusion proteins were incubated with lysates from unstimulated and stimulated CHO/IR cells (left two lanes) and CHO/IGF-IR cells (right two lanes). The proteins bound to the beads were fractionated by SDS-PAGE (10%). After transfer to PVDF membrane, IRS-1 was detected by anti-IRS-1 western blotting. Immobilized GST served as the negative control.

anti-IRS-1 and the immunocomplexes were analyzed by SDS-PAGE, followed by western blotting with anti-Tyr(P) antibody (4G10). As shown in Fig. 1, both insulin and IGF-I induce a significant increase in tyrosine phosphorylation of IRS-1 as compared with unstimulated control cells. The concentrations of IRS-1 protein in the stimulated and unstimulated CHO/IR and CHO/IGF-IR cells were similar, as judged by reprobing the membrane with anti-IRS-1 antibody (Fig. 1).

Next, we examined whether IR and IGF-IR differentially phosphorylate IRS-1 in CHO cells. We addressed this first by measuring the interaction of a panel of SH2-containing proteins with IRS-1. We immobilized bacterially-expressed GST fusion proteins containing the SH2 domains from four known downstream substrates of IRS-1: Grb2, the p85 subunit of PI3-kinase, SHP2 and Nck. We used these immobilized SH2 domains to probe the binding of phosphorylated IRS-1 from lysates of CHO/IR and CHO/IGF-IR cells before and after stimulation with ligand. Bound proteins were analyzed by

Figure 3 Co-immunoprecipitation (IP) of IRS-1 and SH2-containing proteins. (A) Top: NP40 lysates from unstimulated and stimulated CHO/IR cells (left two lanes) and CHO/IGF-IR cells (right two lanes) were incubated with protein A beads and anti-IRS-1 antibody. Immunocomplexes were fractionated by SDS-PAGE (10%), transferred to membrane, and analyzed by western blot using antibody to the p85 subunit of PI3-kinase. Bottom: The same membrane was stripped and reprobed using anti-IRS-1 antibody. The same experiment was performed using anti-Grb2 antibody (B), anti-SHP2 antibody (C) and anti-Nck antibody (D).

SDS-PAGE, followed by western analysis using anti-IRS-1 antibody. These data show that the GST fusion proteins containing the SH2 domain of PI3-kinase and Nck preferentially bind IRS-1 phosphorylated by IR, whereas the GST fusion protein containing Grb2 preferentially binds to IRS-1 phosphorylated by IGF-IR (Fig. 2). (These preferences did not appear to be absolute; for example, in the experiment with the p85 subunit, upon longer exposure of the blot a band was visible for IRS-1 in stimulated CHO/IGF-IR cells. However, this signal was ~10% of the signal for CHO/IR cells.) The GST fusion protein containing SH2 domains of SHP2 shows no preference in binding IRS-1 phosphorylated by IR over that phosphorylated by IGF-IR. As shown above for Fig. 1, the cell lysates used in these experiments contained approximately equal amounts of IRS-1. Binding to the immobilized SH2 domains was specific, as pull-down assays with GST alone failed to detect binding to IRS-1 from CHO/IR or CHO/IGF-IR cells (Fig. 2).

In the next set of experiments, we studied the binding of tyrosine-phosphorylated IRS-1 from CHO/IR and CHO/IGF-IR cells to SH2 containing downstream signaling proteins by co-immunoprecipitation. Quiescent

CHO/IR or CHO/IGF-IR cells were incubated with or without insulin or IGF-I. After cell lysis, equal amounts of protein were immunoprecipitated with anti-IRS-1 antibody and the immunocomplexes were fractionated by SDS-PAGE. We analyzed the bound proteins by western blotting with antibodies against Grb2, the p85 subunit of PI3-kinase, SHP2 and Nck (Fig. 3). The data obtained from these experiments are in agreement with GST pulldown experiments shown in Fig. 2, and can be summarized as follows: IGF-IR appears to couple IRS-1 preferentially to Grb2, whereas IR appears to couple IRS-1 preferentially to the p85 subunit of PI3-kinase and to Nck. The two receptors couple IRS-1 equally to SHP2. The data presented in Figs 2 and 3 suggest that IRS-1 is phosphorylated differentially in CHO/IR and CHO/IGF-IR cells when receptors are stimulated with the appropriate ligand. In both cases, the preferences observed in these experiments were not absolute (e.g., some Grb2 did co-immunoprecipitate with IRS-1 from CHO/IR cells, as shown in Fig. 3).

The experiments described in Figs 2 and 3 used SH2 binding to give an indirect readout of IRS-1 phosphorylation. In order to determine directly the location of the

Figure 4 ELISA experiments on phosphospecific antibodies. The antibodies were analyzed for specificity against unphosphorylated and phosphorylated peptides, as described under Materials and Methods. Purified peptides (Y608, Y895 and Y1172) and phosphopeptides (pY608, pY895 and pY1172) were bound to maleimide-activated plates. After washing and blocking excess maleimide groups, we added the purified antibodies to the wells. After additional wash steps, HRP-conjugated donkey anti-rabbit antibody was added to each well. Detection was with 1-Step Turbo TMB, and the color reaction was halted by the addition of H_2SO_4 . Absorbance readings were taken at 450/630 nm. Peptides Y608 and pY608 are shown by open bars, Y895 and pY895 by gray bars, and Y1172 and pY1172 by black bars. The antibody-peptide combinations not shown in this figure (e.g. anti-pY608 antibody with unphosphorylated Y895 or Y1172 peptides) all gave background levels of absorbance.

sites that are differentially phosphorylated in CHO/IR and CHO/IGF-IR cells, we developed phosphospecific antibodies against three relevant IRS-1 phosphorylation sites: pY608, pY895 and pY1172. These sites were selected because, once phosphorylated, they become binding sites for the SH2 domains of the p85 subunit of PI3-kinase, Grb2 and SHP2 respectively. As described in the Materials and Methods section, the antibodies were raised against phosphotyrosine-containing peptides. To assess their specificity, the purified antibodies were tested against cognate phosphorylated peptides, cognate non-phosphorylated peptides and non-related phosphopeptides, using an ELISA. As shown in Fig. 4, the anti-pY608, anti-pY895 and anti-pY1172 antibodies were highly specific toward their cognate phosphopeptides. In particular, the antibodies did not react well with the unphosphorylated versions of the peptides (Fig. 4).

To assess the phosphorylation status of the IRS-1 sites in intact cells, we immunoprecipitated IRS-1 from unstimulated or stimulated CHO/IR and CHO/IGF-IR cells. Proteins in the immunoprecipitates were fractionated by SDS-PAGE and transferred to PVDF membrane as

described above. The state of IRS-1 phosphorylation in each case was determined by immunoblotting with phosphospecific antibodies (Figs 5–7). As shown in Fig. 5A, Y608 of IRS-1 is phosphorylated more heavily in stimulated CHO/IGF-IR cells than in stimulated CHO/IR cells. Unstimulated cells do not show reactivity with anti-pY608. The membrane was stripped and re-probed with anti-IRS-1 to confirm that approximately equal amounts of IRS-1 were present in the cell lysates (Fig. 5B). This experiment also showed a slight shift in IRS-1 mobility in lysates from stimulated CHO/IR and CHO/IGF-IR cells that was comparable in the two cell lines. The membrane was also re-probed with anti-phosphotyrosine antibody to measure phosphorylation of IRS-1 on all sites (Fig. 5C). The results of this last experiment indicated that the overall levels of IRS-1 phosphorylation were comparable in the stimulated CHO/IR and CHO/IGF-IR cells.

Similar experiments were carried out with the anti-pY895 antibody (Fig. 6) and with the anti-pY1172 antibody (Fig. 7). Both the Y895 and the Y1172 sites were phosphorylated upon stimulation of the cells with the

Figure 5 Y608 of IRS-1 is differentially phosphorylated in insulin- and IGF-I-stimulated CHO cells. IRS-1 was isolated from lysates of unstimulated and stimulated CHO/IR and CHO/IGF-IR cells by immunoprecipitation (IP). (A) Immunocomplexes were fractionated by SDS-PAGE (7.5%) and transferred to membrane. The phosphorylation status of IRS-1 Y608 was detected with pY608-specific antibody. The same membranes were stripped and reprobed by anti-IRS-1 antibody (B) and anti-pTyr mAb 4G10 (C).

Figure 6 Similar phosphorylation of IRS-1 Y895 in insulin- and IGF-I-stimulated CHO cells. IRS-1 was isolated from lysates of unstimulated and stimulated CHO/IR cells (left two lanes) and CHO/IGF-IR cells (right two lanes) by immunoprecipitation (IP). (A) Immunocomplexes were fractionated by SDS-PAGE (7.5%) and transferred to membrane. The phosphorylation status of IRS-1 Y895 was detected with pY895-specific antibody. The same membranes were stripped and reprobed by anti-IRS-1 antibody (B) and anti-pTyr mAb 4G10 (C).

appropriate ligand. In contrast to results for Y608, these experiments indicated that there was no significant difference in the level of phosphorylation of Y895 or Y1172 in CHO/IR and CHO/IGF-IR cells (Figs 6 and 7). Each blot was stripped and reblotted with anti-IRS-1 to demonstrate that equal amounts of IRS-1 were present in the immunoprecipitate. Reprobing with anti-phosphotyrosine antibody confirmed that the overall tyrosine phosphorylation of IRS-1 was comparable in the two cell types. Taken together, the results presented in Figs 5–7 indicate that, although all three sites examined (Y608, Y895 and Y1172) were phosphorylated by IR and IGF-IR in response to activation, only in the case of Y608 was a significant difference observed between CHO/IR and CHO/IGF-IR cells.

Discussion

In the present study, we investigated the binding between IRS-1 and four SH2-containing proteins in CHO cells overexpressing IR or IGF-IR. The results presented in Figs 2 and 3 indicate that there are differences between IR and IGF-IR with regard to the population of SH2-containing proteins recruited to IRS-1 (Table 1). Although this system is artificial in the sense that the receptors are overexpressed in a heterologous cell type,

there are several reasons to believe that the observed differences mirror real differences in signalling potential between the two receptors. The two receptors were expressed at comparable levels in the two cell types. The concentrations of IRS-1 protein were also similar in the CHO/IR and CHO/IGF-IR cells (Fig. 1), and the overall phosphorylation of IRS-1 occurred at similar levels in the two cell types (Fig. 1). Furthermore, we observed cases in which IR was more effective at promoting an association with IRS-1 (p85 and Nck) and, conversely, others in which IGF-IR is more effective (Grb2), arguing against one receptor having an overall greater level of activity toward IRS-1 (Figs 2 and 3). In the case of SHP2, the two receptors appeared to be approximately equal in promoting binding to IRS-1 in this experimental system.

The differences observed in these experiments agree with results obtained in other cells (Urso *et al.* 1999) and with the results we obtained previously using synthetic IRS-1 peptides and the IR and IGF-IR catalytic domains (Xu *et al.* 1995). For example, in the *in vitro* studies IGF-IR preferred a peptide modelled on the Y895 sequence of IRS-1. In the present work (Figs 2 and 3), IGF-IR appears to be more effective at phosphorylating IRS-1 on a site (or group of sites) that couples to Grb2, which is known to bind at Y895 (White & Yenush 1998). Similarly, IR exhibited preferential binding to a peptide

Figure 7 Similar phosphorylation of IRS-1 Y1172 in insulin- and IGF-I-stimulated CHO cells. IRS-1 was isolated from lysates of unstimulated and stimulated CHO/IR and CHO/IGF-IR cells by immunoprecipitation (IP). (A) Immunocomplexes were fractionated by SDS-PAGE (7.5%) and transferred to membrane. The phosphorylation status of IRS-1 Y1172 was detected with pY1172-specific antibody. The same membranes were stripped and reprobed by anti-IRS-1 antibody (B) and anti-pTyr mAb 4G10 (C).

modelled on the Y987 sequence of IRS-1. Our pulldown and co-immunoprecipitation data (Figs 2 and 3) showed that IRS-1 phosphorylated by IR had a greater affinity for binding to the p85 subunit of PI3-kinase than IRS-1 phosphorylated by IGF-IR. The p85 subunit has previously been shown to bind to Y987 of IRS-1, in addition to five other YMXM motifs (Backer *et al.* 1992). These

Table 1 Summary of comparisons between IR and IGF-IR. In each case, + represents a positive signal in the assay, – represents no significant signal, and +/- represents a weak signal. Pulldown data and co-immunoprecipitation (co-IP) data are from Figs 2 and 3 respectively; +, binding with IRS-1 in stimulated CHO cells. Results using phosphospecific antibodies are from Figs 5–7: +, reactivity with that antibody in lysates from stimulated CHO cells

	IR	IGF-IR
p85 pulldown	+	–
p85 co-IP	+	+/-
Grb2 pulldown	–	+
Grb2 co-IP	+/-	+
SHP2 pulldown	+	+
SHP2 co-IP	+	+
Nck pulldown	+	–
Nck co-IP	+	–
anti-pY608	–	+
anti-pY895	+	+
anti-pY1172	+	+

results suggest that the intrinsic specificities of IR and IGF-IR are important in selective phosphorylation of IRS-1. However, the differences we observed in Figs 2 and 3 are clearly not absolute: for example, we observed some degree of coupling between IRS-1 and the p85 subunit of PI3-kinase in CHO/IGF-IR cells (Fig. 3).

We have also developed phosphospecific antibodies to three important phosphorylation sites on IRS-1 (pY608, pY895 and pY1172). These antibodies allowed us to examine, for the first time, the phosphorylation status of these sites in intact cells. Western blotting with the phosphospecific antibodies showed that the antibodies specifically recognized phosphorylated IRS-1; no reactivity was seen with IRS-1 isolated from unstimulated cells (Figs 5A, 6A, 7A). In stimulated CHO/IR and CHO/IGF-IR cells, varying amounts of reactivity were observed for the phosphospecific antibodies. The results with anti-pY608 (Fig. 5) provide additional evidence for differential phosphorylation of IRS-1 in cells overexpressing IR or IGF-IR. Y608 of IRS-1 was phosphorylated more efficiently in cells expressing IGF-IR than in cells expressing IR (Fig. 5A). This finding was somewhat unexpected, because Y608 has been reported to bind the p85 subunit of PI3-kinase (Backer *et al.* 1992) and we observed more efficient binding of the p85 subunit in CHO/IR cells (Figs 2 and 3). This discrepancy could be due to the fact that five other YMXM motifs on IRS-1 are alternate binding sites for the p85 subunit; the results presented in Figs 2 and 3 may reflect differential phosphorylation of one or more of these sites.

Western blotting of IRS-1 with anti-pY895 and anti-pY1172 showed comparable phosphorylation of these sites in CHO/IR and CHO/IGF-IR cells (Figs 6A and 7A). In the case of pY1172, this site was previously shown to be a binding site for SHP2 phosphatase (Kuhne *et al.* 1993, White & Yenush 1998). In our pulldown and co-immunoprecipitation experiments, we also observed no significant differences between CHO/IR and CHO/IGF-IR cells with regard to the association of SHP2 with IRS-1 (Figs 2 and 3). In the case of Grb2, we observed stronger binding to IRS-1 in CHO/IGF-IR cells than in CHO/IR cells. There are a number of possible explanations for this observation in light of the fact that Y895 appears to be phosphorylated approximately equally in the two cell types. In insulin-stimulated cells, an alternative signalling pathway may be engaged that causes the sequestration of Grb2 away from Y895 of IRS-1. An IR-specific signalling pathway may promote the formation of a complex between IRS-1 and the non-receptor tyrosine kinase, Fyn; Fyn has also been shown to bind at Y895 (Sun *et al.* 1996). Alternatively, another uncharacterized site on IRS-1 (besides Y895) might serve as a Grb2 docking site in these cells.

In summary, we have provided evidence for specific signalling by IR and IGF-IR through IRS-1 in CHO cells. The results are consistent with earlier work in other

cell types (Lammers *et al.* 1989, Park *et al.* 1999, Urso *et al.* 1999) and with our previous *in vitro* experiments on the isolated catalytic domains of IR and IGF-IR (Xu *et al.* 1995). Our results do not identify the regions of the receptors that are important in conferring differences in intrinsic signaling capacity. Previous work suggested that the C-terminal domains of the receptors are involved in selective signalling (Tartare *et al.* 1994, Blakesley *et al.* 1996, Najjar *et al.* 1997). The receptors may also couple to different potential substrates through specific interactions with phosphotyrosine-binding domains (Sawka-Verhelle *et al.* 1996, Xu *et al.* 1999). Future studies will use the experimental system described here to examine the importance of the juxtamembrane and C-terminal domains of the receptors, and residues within the catalytic domains that may be involved in specific substrate recognition.

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