The juxtamembrane but not the carboxyl-terminal domain of the insulin receptor mediates insulin's metabolic functions in primary adipocytes and cultured hepatoma cells

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ABSTRACT

Insulin-stimulated signaling pathways are activated upon interactions between the intracellular domains of the receptor and its downstream effectors. Insulin receptor substrate proteins (IRS-1, -2, -3 and -4) are the best-studied substrates for the insulin receptor kinase (IRK). We have previously shown that IRS-1 and IRS-2 interact with the juxtamembrane (JM) but not with the carboxyl-terminal (CT) region of the insulin receptor (IR) *in vitro*. However, the precise role of these IR regions in mediating insulin's bioeffects is still unresolved. In the present work we made use of vaccinia virus as a vector for quantitative expression of the JM and CT domains within the cytoplasm of physiologically insulin-responsive primary rat adipocytes and rat hepatoma Fao cells. We could demonstrate that overexpression of either the JM or the CT domains did not inhibit either insulin binding or insulin-stimulated receptor autophosphorylation. In contrast, metabolic effects such as insulin-induced glucose utilization in adipocytes,

and insulin-induced amino acid utilization in Fao hepatoma cells were inhibited (70–80%) in cells overexpressing the JM but not the CT domains of IR. The inhibitory effects of the overexpressed JM domain were accompanied by inhibition of insulin-stimulated IRS-1 phosphorylation, decreased IRS-1-associated PI3K activity, and decreased phosphorylation of the downstream effectors of PI3K, PKB and p70 S6K. Insulin-stimulated thymidine incorporation in Fao cells was also inhibited (40%) upon overexpression of the JM but not the CT region of IR. Our findings suggest that interactions between the JM region of IR and its downstream effectors are obligatory for insulin-stimulated metabolic functions in physiologically relevant insulin responsive cells. They also rule out the possibility that interaction of proteins, including PI3K, with the CT domain can provide an alternative pathway. *Journal of Molecular Endocrinology* (2000) **24**, 419–432

INTRODUCTION

The insulin receptor (IR) is a Tyr-specific protein kinase (IRK) that undergoes autophosphorylation following insulin binding. The activated receptor phosphorylates substrate proteins on multiple Tyr residues to further propagate insulin signal transduction and insulin action (Cheatham & Kahn 1995). The major targets of insulin receptor kinase (IRK) are the insulin receptor substrates (IRS-1, -2, -3 and -4) (Sun *et al.* 1991, 1995, Lavan *et al.* 1997a,b) and Shc (Pronk *et al.* 1993). IRS proteins

contain a conserved pleckstrin homology (PH) domain (Haslam *et al.* 1993, Mayer *et al.* 1993) at their amino terminus, followed by a P-Tyr binding (PTB) domain that functions as the IR binding region (Eck *et al.* 1996). The C-terminal region of IRS proteins contains multiple Tyr phosphorylation motifs that serve as docking sites for SH2 domain-containing signaling proteins like the p85 α regulatory subunit of PI3K (phosphatidylinositol 3-kinase), and other adaptors like Grb2, Nck, Crk, Fyn and SHP-2, which are involved in insulin signal transduction (Cheatham & Kahn 1995,

White 1997). Activation of PI3K leads to phosphorylation and activation of several Ser/Thr kinases, such as PDK (Alessi *et al.* 1997, Stokoe *et al.* 1997), protein kinase B (PKB; Alessi *et al.* 1996) and p70 S6K (Alessi *et al.* 1998) that mediate the metabolic and growth-promoting effects of insulin (Alessi *et al.* 1997), including glucose uptake and lipogenesis, protein synthesis and cell survival (Cheatham & Kahn 1995, White 1997). The relative roles of the different IRS proteins in mediating insulin action are still unclear, however studies of gene disruption revealed that IRS-2 compensates for the absence of IRS-1 in hepatocytes of IRS-1 null mice, while IRS-3 provides an alternative pathway for PI3K activation in adipocytes of these animals (Patti *et al.* 1995, Yamauchi *et al.* 1996, Smith *et al.* 1997). In contrast, IRS-2 null mice are severely glucose intolerant, suffer from impaired pancreatic β -cell functions, and develop classical type II diabetes (Withers *et al.* 1998).

Several structural regions have been defined within the intracellular part of the IR β subunit. These are the juxtamembrane (JM) region, the kinase region, and the carboxyl-terminal (CT) region (Ebina *et al.* 1985, Ullrich *et al.* 1985). The JM region (Arg⁹⁴⁰-Leu⁹⁸⁷) contains Tyr⁹⁶⁰ within a L-X₄-NPXYXSXSD motif. Mutation of Tyr⁹⁶⁰ to Phe or Ala decreases IR binding to IRS-1 (Gustafson *et al.* 1995) and IRS-2 (Chaika *et al.* 1999), impairs receptor signal transmission, and reduces the sensitivity and maximal responsiveness of cells transfected with the mutated IR, both to the metabolic and growth-promoting effects of insulin (Kaburagi *et al.* 1993, White *et al.* 1988, Chaika *et al.* 1999). This occurs even though autophosphorylation in other receptor regions is normal and the IRK is fully active *in vitro* (White *et al.* 1988, Kaburagi *et al.* 1993). The PTB domain of IRS proteins binds the NPEY motif within the JM region of IR (Eck *et al.* 1996), and these PTB-JM interactions are required for insulin-induced Tyr phosphorylation of IRS proteins (Gustafson *et al.* 1995, Sharma *et al.* 1997). Conversely, an isolated JM region of IR, but not its CT region, is sufficient to mediate quantitative interactions with IRS proteins *in vitro* (Paz *et al.* 1996, 1997), indicating that the JM domain contains all the structural information required for interaction with IRS proteins.

The CT region of IR (Leu¹²⁴⁵-Ser¹³⁴³) contains two autophosphorylation sites at Tyr¹³¹⁶ and Tyr¹³²² whose role in receptor signaling is still unresolved (Tavare & Siddle 1993). It has been postulated that autophosphorylation of this region generates docking sites for several adaptor proteins,

including the p85 α regulatory subunit of PI3K (Levy *et al.* 1994), whose direct binding to the carboxyl terminus region of IR could contribute to the effects of insulin on PI3K activity (Levy *et al.* 1994). Indeed, Chinese hamster ovary (CHO) cells expressing a receptor mutant whose C-terminal 43 amino acids (including these Tyr residues) were deleted (IR₄₃), display a compromised ability to stimulate glucose transport and glycogen synthesis (Maegawa *et al.* 1988), whereas mitogenic signaling and stimulation of the IRK activity remain intact (Maegawa *et al.* 1988). Likewise, cells expressing a chimeric IGF-I receptor whose CT domain is replaced by that of the IR, manifest IGF-I-stimulated glycogen synthesis and MAP kinase activity in correlation with the activity of the IR (Faria *et al.* 1994). The CT domain has been implicated in IRS-1-independent functions: it is an important element in mediating the effects of insulin on protein phosphatase-1 activity (Begum *et al.* 1993), and its phosphorylated Tyr¹³²² serves as the binding site for the SH2 domain of the adaptor protein Grb10 (Hansen *et al.* 1996).

Hence, while it is clear that the IR has the ability to induce diverse signals through distinct receptor regions, the exact role of the JM and CT regions of IR in mediating insulin action is not well defined. The difficulty stems from the fact that at least part of the relevant information was gathered from studies carried out in cells that are not physiological targets of insulin action. Primary adipocytes are a major target tissue for insulin action, however these fully differentiated cells are not readily amenable to genetic manipulations. Although introduction of foreign cDNAs into adipocytes by electroporation has been reported (Quon *et al.* 1994, Tanti *et al.* 1997), the relatively low yield ($\sim 10\%$) of the transfected cells required co-transfection with a reporter gene (e.g. HA-Glut4) whose translocation was monitored. Hence, no other effects of the transfected proteins could be studied. Previous studies have also reported on the successful infection of cultured 3T3-L1 adipocytes with vaccinia (Chen *et al.* 1997) or adeno-viruses (Sharma *et al.* 1997), however this system was not tested on primary adipocytes. To address this issue we set out to develop a system for gene transduction and protein overexpression in primary adipocytes using vaccinia virus. Individually expressed receptor domains were introduced into insulin responsive primary adipocytes and rat hepatoma Fao cells, utilizing recombinant vaccinia virus-mediated infection. Our findings suggest that interactions between the JM region of IR and its downstream effectors are obligatory

FIGURE 1. Schematic representation of recombinant vaccinia virus construction. The transfer vector, pSC65, contains vaccinia virus DNA sequences, TK_L and TK_R, that flank the genes coding for lacZ and for our gene of interest (g.o.i.) (the JM or CT regions of IR). BSC-1 cells were infected with vaccinia viruses prior to transfection with recombinant pSC65 plasmid. A double crossover event occurred between the TK_L and TK_R sequences and the homologous regions of the virus (L and R). This resulted in the generation of a recombinant virus into which lacZ and IR-JM or IR-CT genes were inserted. Recombinant viruses defined as Vcc-β-Gal, Vcc-CT and Vcc-JM were generated in such a manner.

for insulin-stimulated metabolic functions in physiologically relevant insulin responsive cells. Our results also rule out the possibility that interaction of proteins, including PI3K, with the CT domain can provide an alternative pathway.

MATERIALS AND METHODS

Materials

Recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). Wheat Germ Agglutinin-Agarose beads and protease inhibitor cocktail were from Sigma Chemicals Co. (St Louis, MO, USA). BrdU was from Calbiochem (La Jolla, CA, USA), and X-Gal was purchased from Promega (Madison, WI, USA). Lipofectamin and OptiMem were from GIBCO-BRL (Grand Island, NY, USA). Dinonylphthalate oil was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Collagenase was obtained from Worthington Biochemical Co. (Freehold, NJ, USA).

Antibodies

Monoclonal P-Tyr (PY-20) and polyclonal IR antibodies were obtained from Transduction Labs (Lexington, KY, USA). Polyclonal IRS-1 antibodies were prepared as described previously (Hadari *et al.* 1992). Monoclonal T7-tag antibodies were obtained from Novagene Inc. (Madison, WI, USA). Polyclonal phospho-specific PKB and p70 S6K antibodies were obtained from BioLab (Beverly, MA, USA). Polyclonal PKB antibodies were purchased from Sigma Chemicals Co. Polyclonal p85α-PI3K antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Generation of recombinant vaccinia virus

DNA corresponding to the JM or the CT region of IR (a.a. 943–984 and 1245–1331 respectively), tagged by immunogenic sequence derived from T7 bacteriophage, was subcloned into a plasmid transfer vector, pSC65 (Chakrabarti *et al.* 1997, Moss & Earl 1991), using 5' Bgl-II site and 3' Kpn-I site (see Fig. 1). Within pSC65, the genes of

interest (g.o.i.) are flanked by DNA from nonessential region of the vaccinia virus genome, that enables homologous recombination between the genes of interest and the vaccinia virus gene. BSC-1 (ATCC#CCL26) cells (10^6 cells/30 mm dish) were infected at a multiplicity of infection (m.o.i.) of 0.05 pfu (plaque-forming units) per cell with wild-type vaccinia virus. After a 2 h incubation at 37 °C the medium was discarded and the cell monolayers were transfected with 5 µg pSC65, pSC65-JM or pSC65-CT, using Lipofectin. Forty-eight hours post infection the cells were harvested by discarding the medium (save for 1 ml) and scraping the cells off the well using a rubber stick. Cells were ruptured by three cycles of freezing and thawing and were sonicated for 30 s using a bath sonicator. HuTK⁻ 143B cells (ATCC#CRL8303, 10^6 cells/30 mm dish) were incubated with 10^{-1} , 10^{-2} and 10^{-3} dilutions of BSC-1 cell extracts in 1 ml minimum essential medium (MEM) containing 2% fetal calf serum (FCS), in a CO₂ incubator at 37 °C. After 2 h incubation the medium was discarded, and 2 ml 'plaque-medium' (1% LMP agarose in MEM medium containing 5% FCS), containing 25 µg/ml BrdU were added, and incubation in a CO₂ incubator at 37 °C was continued. Forty-eight hours later, 2 ml 'plaque-medium' containing 25 µg/ml BrdU and 0.4% X-Gal were added, and incubation was continued for 6–12 h in a CO₂ incubator at 37 °C, until blue plaques appeared. The blue plaques were picked with pipette into 0.5 ml MEM medium containing 10% FCS, and the cells were ruptured by three cycles of freezing and thawing followed by a 30 s sonication in a bath sonicator. The extracts served for another cycle of infection of HuTK⁻ 143B cells as described above. Following three selection cycles, plaques were picked up, frozen and thawed three times and sonicated for 30 s. Hela S3 (ATCC#CCL2.2) cells (10^8 cells/150 mm dish), were incubated for 3 days in a CO₂ incubator at 37 °C with 2 ml MEM medium containing 10% FCS and 10^{-2} dilution of HuTK⁻ 143B cells extract. Seventy-two hours post infection the cells were harvested by discarding the medium, adding 2 ml fresh MEM medium containing 2% FCS, and scraping the cells off the plate using a rubber stick. Cells were frozen and thawed three times and were sonicated for 30 s using a bath sonicator. These extracts served as the recombinant vaccinia virus stock ($4.5\text{--}8.5 \times 10^9$ pfu/ml), and were subsequently used for infection.

Generation of primary adipocytes culture

Male 6–7 week-old Wistar rats were decapitated. The distal halves of the epididymal fat pads were

pooled and isolated fat cells were prepared by incubation of the tissue with collagenase at 37 °C for 1 h, as described (Cushman & Salans 1978, Lawrence & Lerner 1978), save for the fact that 1% insulin-free BSA was added to the digestion buffer. The cells were gently filtered through mesh, and were washed several times with Krebs Ringer buffer (KRB) (100 mM NaCl, 4 mM KH₂PO₄, 2 mM MgSO₄, 10 mM NaHCO₃, 1 mM CaCl₂, 30 mM Hepes, pH 7.4) to get rid of blood cells, undigested tissue, and excess of collagenase.

Infection of primary adipocytes

Primary adipocytes were prepared by collagenase digestion (Cushman & Salans 1978). The cytocrite was adjusted to 50% in KRB buffer, and fractions of 0.2 ml (10^6 cells) were incubated with 10^7 m.o.i. recombinant vaccinia virus at 37 °C for 1 h. At the end of incubation, cells were washed once with KRB buffer and once with Dulbecco's modified Eagle's medium (DMEM) medium, and were further incubated for 5–24 h in DMEM medium containing 1% insulin-free BSA in a CO₂ incubator at 37 °C.

Infection of Fao cells

Fao cells were grown in RPMI medium supplemented with 10% fetal calf serum. Confluent monolayers, grown in 60 mm dishes (10^7 cells) were incubated with 10^8 m.o.i. recombinant vaccinia virus at 37 °C for 1 h. At the end of incubation cells were washed twice with RPMI medium, and were further incubated for 5–24 h in serum-free RPMI medium in a CO₂ incubator at 37 °C.

Assay of β-galactosidase activity

Infected cells were grown in 35 mm dishes. Cells were washed with PBS, incubated with 1 ml fixation solution (2% formamide, 0.2% glutaraldehyde) for 10 min at 4 °C, and were washed twice with PBS. Following fixation, 1 ml staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 1 mg/ml X-Gal in PBS) was added to each well, for an incubation of 10–60 min at 37 °C. When blue color was observed, cells were washed with distilled water to stop the reaction.

Insulin-induced amino acid uptake and utilization in Fao cells

Confluent Fao cells in 35 mm dishes were incubated in serum-free RPMI medium for 16 h. Cells were then treated with 100 nM insulin for 3 h at 37 °C,

following an additional incubation with 1 ml of 5 $\mu\text{Ci/ml}$ [^3H]leucine in RPMI medium for 30 min at 37 °C. Cells were washed three times with PBS and solubilized with 1 ml lysis buffer (5% SDS, 20 mM Na_2CO_3 and 2 mM EDTA). Cellular radioactivity was measured in a scintillation counter.

Glucose uptake and lipogenesis in primary adipocytes

Fractions (200 μl) of primary adipocytes (with a cytotrite of 50%), incubated in DMEM medium containing 1% insulin-free BSA, were stimulated with 100 nM insulin for 30 min at 37 °C. Cells were then incubated at 37 °C with 200 μl 0.5 $\mu\text{Ci/ml}$ [^{14}C]glucose in the same buffer. Glucose uptake (and generation of glucose metabolites) as well as lipogenesis were measured for a period of 30 min and 2 h respectively. Cells were separated from free [^{14}C]glucose using dinonylphtalate oil and cellular radioactivity was measured using regular or hydrophobic extraction scintillation as described previously (Karnieli *et al.* 1981, Cherqui *et al.* 1986).

Western blot analysis

Infected cells were washed two times with PBS, and harvested in buffer A (25 mM Tris-HCl, 2 mM sodium orthovanadate, 0.5 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β -glycerophosphate, 25 mM NaCl, 1% Tx-100 and protease inhibitor cocktail 1:1000, pH 7.4). For primary adipocytes, four freeze-thaw cycles were added. Cells were centrifuged at 12 000 g for 20 min at 4 °C, and the supernatant was collected. Samples of 100 μg were resolved by SDS-PAGE under reducing conditions, and were transferred into nitrocellulose membrane for Western blotting with the indicated antibodies.

Thymidine incorporation

Fao cells (at 50% confluence) in 35 mm dishes were incubated for 30 h in serum-free RPMI medium, prior to infection with vaccinia virus. Six hours post infection cells were treated with 100 nM insulin for 14 h at 37 °C. At the end of incubation the medium was replaced with 1 ml serum-free RPMI medium, containing 0.5 $\mu\text{Ci/ml}$ [^3H]thymidine, and incubation was continued for 2 h at 37 °C. Cells were washed three times with PBS and were incubated for 30 min at 4 °C in 0.5 ml ice-cold 7.5% trichloroacetic acid. The cells were washed twice with 98% ice-cold ethanol, dissolved in 0.5 ml 0.1 M NaOH, and counted using a scintillation cocktail.

Concentration of IR on wheat germ agglutinin column

Primary adipocytes or Fao cells were solubilized at 4 °C in buffer A. Cells were centrifuged at 12 000 g for 15 min at 4 °C, and the supernatants were collected. Aliquots containing 0.5–1.0 mg protein were incubated for 1 h at 4 °C with wheat germ agglutinin agarose beads. The complexes were washed three times with buffer B, and once with buffer B deprived of detergents. Immobilized IR was released from the beads by boiling in Laemmli's 'sample buffer' (Laemmli 1970), resolved by means of 7.5% SDS-PAGE, and Western-blotted with IR or P-Tyr antibodies.

Immunoprecipitation

Primary adipocytes were solubilized at 4 °C in buffer A. Cells were centrifuged at 12 000 g for 15 min at 4 °C, and the supernatants were collected. Aliquots containing 0.5–1.0 mg protein were incubated for 2 h at 4 °C with IRS-1 antibodies coupled to 60 μl protein A-Sepharose beads (50%). Immunocomplexes were washed three times with buffer A, and once with buffer A deprived of detergents. Immunocomplexes were resolved by means of 7.5% SDS-PAGE and Western-blotted using IRS-1, P-Tyr or p85 α -PI3K antibodies.

RESULTS

Infection with a recombinant vaccinia virus is an efficient means to quantitatively introduce proteins and peptides into primary adipocytes and rat hepatoma Fao cells

Recombinant vaccinia viruses Vcc- β -Gal, Vcc-CT and Vcc-JM (i.e. recombinant vaccinia viruses containing β -Gal gene, IR-CT region gene and IR-JM region gene respectively) were isolated as outlined in Fig. 1 and described in the Materials and Methods section. In addition to the JM or the CT polypeptides, these viruses also expressed a variant of the bacterial β -galactosidase gene, under the control of the early/late vaccinia virus promoter, so that it is continuously expressed (Chakrabarti *et al.* 1997, Moss & Earl 1991). Primary adipocytes and rat hepatoma Fao cells were infected with the recombinant viruses. The efficiency of infection was assessed by *in vivo* X-Gal staining of cells infected with Vcc- β -Gal at a m.o.i. of 10 pfu/cell. As shown in Fig. 2B, more than 95% of the infected Fao cells produced β -Gal activity, as judged by the number of blue cells, while non-infected cells exhibited no blue color in the presence of the β -galactosidase

FIGURE 2. *In vivo* β -Gal staining of infected cells. Rat hepatoma Fao cells (A and B), or primary adipocytes (C and D) were infected with a recombinant vaccinia virus coding for the β -Gal gene (Vcc- β -Gal). Twenty-four hours post infection, an *in situ* β -Gal staining was performed as described in the Materials and Methods section. The infection efficiency was evaluated by the number of cells stained blue in the presence of the β -galactosidase chromogenic substrate, X-Gal.

chromogenic substrate (Fig. 2A). Similar results were obtained with primary rat adipocytes (Fig. 2C and D); about 80% of the Vcc- β -Gal-infected cells were stained blue when treated with X-Gal. The infection did not alter the morphological features of either Fao cells or primary adipocytes. These results indicate that infection with a vaccinia virus is a promising technique to produce high expression levels of proteins and polypeptides in hepatoma cells and primary adipocytes.

The JM and CT regions of IR are quantitatively expressed in hepatoma cells and primary adipocytes

Rat adipocytes and Fao cells were infected at m.o.i. of 10 pfu/cell with Vcc- β -Gal, Vcc-CT or Vcc-JM. As shown in Fig. 3, high expression levels of both the CT and the JM regions of IR were observed in both cell types, 6 and 20 h following infection. Hence, a 6-h infection period was sufficient to obtain maximal expression levels of the peptides of interest. Using the T7-tag antibodies we found that Fao cells expressed similar levels of the JM and CT polypeptides, while primary adipocytes expressed higher levels of the CT domain. These observations

FIGURE 3. Overexpression of IR-derived JM and CT domains in Fao cells and primary adipocytes. Rat hepatoma Fao cells (A) or primary adipocytes (B) were infected with a recombinant vaccinia virus coding for T7-tagged fusion peptides corresponding to 41 amino acids (a.a. 943–984) of the JM region of IR (Vcc-JM), or 86 amino acids (a.a. 1245–1331) of the C-terminal region of IR (Vcc-CT). Cellular extracts were prepared, and samples (100 μ g) were resolved by means of 12% SDS-PAGE, and immunoblotted with T7-tag antibody. n.i., non infected.

FIGURE 4. Insulin-induced amino acid uptake and utilization in infected Fao cells. Rat hepatoma Fao cells were infected with the indicated recombinant vaccinia virus as described in the Materials and Methods section. Following 6 h infection, cells were incubated at 37 °C with or without 100 nM insulin for 30 min, then amino acid uptake and utilization were measured using [^3H]leucine, as described in Materials and Methods. Results are means $\pm$ s.d. of a representative experiment repeated twice in duplicates.

were confirmed following Coomassie Blue staining of cell extracts that revealed the presence of protein bands corresponding to the JM and CT polypeptides.

Overexpression of the JM polypeptide inhibits insulin-stimulated uptake and utilization of amino acid by Fao cells

An important biological effect of insulin is the regulation of nutrient content by increased hepatic amino acid uptake (Prentki *et al.* 1981). To determine the effects of the overexpressed IR domains on insulin-induced utilization of amino acids, Fao hepatoma cells were infected with Vcc-β-Gal, Vcc-CT or Vcc-JM and the cellular content of [^3H]leucine (and its metabolites) was measured 6 h post infection. In non-infected cells insulin stimulated Leu uptake twofold, which reflects the combined effects of insulin on Leu transport, Leu incorporation into proteins and Leu metabolism (Fig. 4). Leucine uptake was not affected by infection and overexpression of β-Gal or the CT region of IR. In contrast, overexpression of the JM region of IR presumably acted as a negative dominant inhibitor, and markedly inhibited ($\sim 70\%$) the stimulatory effects of insulin on amino acid uptake. These results suggest that the JM region of IR plays an important role in mediating insulin-induced utilization of amino acids by hepatoma cells.

FIGURE 5. Insulin-induced glucose uptake and lipogenesis in infected primary adipocytes. Primary adipocytes were infected with the indicated recombinant vaccinia virus as described in the Materials and Methods section. Following 6 h infection, cells were incubated at 37 °C with or without 100 nM insulin for 30 min, then glucose uptake and utilization (A) and lipogenesis (B) were assayed using [^{14}C]glucose, as described in Materials and Methods. Results are means $\pm$ s.d. of a representative experiments repeated three times in duplicates.

Overexpression of the JM, but not the CT domain of IR, inhibits insulin-stimulated glucose utilization in primary rat adipocytes

The effects of overexpressing the JM or CT domains of IR on insulin-stimulated metabolic functions in primary adipocytes were next evaluated. Consistent with previous studies carried out with 3T3-L1 adipocytes (Chen *et al.* 1997), vaccinia virus infection *per se* did not interfere with insulin-stimulated glucose uptake. As shown in Fig. 5A, insulin stimulated a three- to fourfold increase in the cellular content of [^{14}C]glucose (and its metabolites) in non-infected adipocytes or in adipocytes infected with Vcc-β-Gal or Vcc-CT. In

contrast, overexpression of the JM domain abolished the stimulatory effects of insulin on glucose uptake and utilization, without affecting the basal activity. To further characterize the effects of the overexpressed JM domain, insulin-stimulated incorporation of [^{14}C]glucose into lipids was measured. As demonstrated in Fig. 5B, insulin stimulated threefold the rate of lipogenesis in non-infected, Vcc- β -Gal infected or Vcc-CT infected cells, while overexpression of the isolated JM domain inhibited insulin-stimulated lipogenesis. These findings suggest that the overexpressed JM domain acts in a dominant negative fashion, and support the notion that this region of IR plays a crucial role in mediating insulin's metabolic functions.

Overexpression of the isolated JM domain does not interfere with insulin binding or receptor autophosphorylation

To determine whether the inhibitory effects of the isolated JM domain on insulin-induced metabolic functions were not secondary to inhibition of insulin binding, the ability of cell surface receptors to bind insulin was measured in control and infected cells. Non-infected as well as Vcc- β -Gal infected Fao cells or primary adipocytes bound [^{125}I]insulin to a similar extent as Vcc-CT and Vcc-JM infected cells (not shown). Likewise, insulin-induced autophosphorylation of the receptor β -subunits in both cell types was unaltered following overexpression of the isolated JM or CT domains (Fig. 6). These results indicate that the presence of free intracellular domains of IR has no effect on the assembly of IR heterotetramer, nor does it interfere with insulin binding to the receptor or with the intrinsic function of the receptor as a protein tyrosine kinase.

Overexpression of the isolated JM domain inhibits insulin-stimulated Tyr phosphorylation of IRS-1, and activation of PI3K, PKB and p70 S6 kinase

Tyrosine phosphorylation of IRS proteins is required for insulin-mediated glucose transport and amino acid uptake (Cheatham & Kahn 1995). We have previously shown that IRS-1 and IRS-2 selectively interact with immobilized peptides comprising the JM region of IR (Paz *et al.* 1996, 1997), an interaction that presumably involves the PTB domain of the IRS proteins (Eck *et al.* 1996). Hence, the overexpressed IR-derived JM domains could competitively inhibit the interactions between the IR and the PTB domains of the endogenous

FIGURE 6. Effect of overexpression of the JM or CT domains on IR autophosphorylation. Rat hepatoma Fao cells or primary adipocytes were infected with the indicated recombinant vaccinia virus. Following 6 h infection cells were incubated with 100 nM insulin at 37 °C for the indicated periods of time. Cellular extracts were prepared and samples (500 μg Fao extracts; 1 mg adipocytes extracts) were incubated with immobilized WGA, as described in Materials and Methods. Complexes were eluted with Laemmli's 'sample buffer', resolved by means of 7.5% SDS-PAGE and immunoblotted with P-Tyr and IR antibodies. Results are of a representative experiment, reproduced twice.

IRS proteins and, as a result, inhibit their Tyr phosphorylation following activation of IRK. To address this possibility, extracts, derived from insulin-stimulated Fao cells and primary adipocytes, overexpressing the CT or the JM domains of IR, were immunoprecipitated with IRS-1-specific antibodies followed by Western immunoblotting with P-Tyr antibodies. As shown in Fig. 7A, the 180 kDa band, representing the IRS proteins, underwent Tyr phosphorylation in response to insulin in non-infected Fao cells, and in cells infected with Vcc- β -Gal or Vcc-CT. In contrast, insulin-stimulated Tyr phosphorylation of IRS proteins in Vcc-JM-infected Fao cells was inhibited 70–80%. Similar results were obtained with primary adipocytes (Fig. 7B). These findings support the notion that Tyr phosphorylation of IRS proteins depends upon interactions between IRS proteins and the JM domain of IR, which is required for mediating insulin-induced metabolic effects.

Tyrosine-phosphorylated IRS-1 can associate with, and stimulate the activity of, PI3K (Hadari *et al.* 1992). This results in phosphorylation and activation of downstream Ser/Thr kinases such as PDK-1, PKB and p70 S6K (Stokoe *et al.* 1997, Alessi *et al.* 1996, 1997, 1998). To determine which of the downstream effectors of IRS-1 are affected when its Tyr phosphorylation is inhibited we assayed for the association of p85 α , the regulatory subunit of PI3K, with immunoprecipitated IRS-1 in infected Fao cells. As demonstrated in Fig. 8,

FIGURE 7. Effect of overexpression of the JM or CT domains on insulin-stimulated IRS-1 phosphorylation. Rat hepatoma Fao cells (A) or primary adipocytes (B) were infected with the indicated recombinant vaccinia virus as described in Materials and Methods. Following 6 h infection, cells were incubated with 100 nM insulin at 37 °C for the indicated periods of time. Cellular extracts of Fao cells (A) or primary adipocytes (B) were prepared and 500 µg (A) or 1 mg (B) samples were subjected to immunoprecipitation (IP) with IRS-1 antibody. Immunocomplexes were resolved by means of 7.5% SDS-PAGE and immunoblotted with P-Tyr and IRS-1 antibodies. Quantitation of the intensity of the bands corresponding to P-Tyr-IRS-1 is presented as bar graphs. Results are means $\pm$ s.d. of two independent experiments.

insulin stimulation resulted in the association of p85 α with Tyr-phosphorylated IRS-1 in non-infected cells or cells infected with Vcc- β -Gal or Vcc-CT. In contrast, overexpression of the JM region largely inhibited the insulin-induced association of IRS-1 with p85 α . The selective inhibitory effects of the overexpressed JM domain were accompanied by a decrease in insulin-dependent phosphorylation of both PKB (Fig. 9A) and p70 S6K (Fig. 9B). These results are consistent with the notion that the interactions of IRS-1 with the JM but not the CT domain of the IR are required for proper Tyr phosphorylation of IRS-1, its interactions with downstream effectors, and the induction of metabolic functions such as utilization of glucose and amino acids in physiologically insulin-responsive cells.

Overexpression of the JM domain inhibits insulin-stimulated DNA synthesis

The effects of the overexpressed receptor domains on insulin-stimulated DNA synthesis were next evaluated. As shown in Fig. 10, insulin stimulated three- to fourfold [^{3} H]thymidine incorporation into DNA in non-infected Fao cells or in cells infected

FIGURE 8. Effect of overexpression of the JM or CT domains on IRS-1-PI3K complex formation. Rat hepatoma Fao cells were infected with the indicated recombinant vaccinia virus. Following 6 h infection, cells were incubated with 100 nM insulin for 1 min at 37 °C, and cellular extracts were prepared. Samples (500 µg) were subjected to immunoprecipitation (IP) with IRS-1 antibody. Immunocomplexes were resolved by means of 7.5% SDS-PAGE and immunoblotted with p85 α -PI3K antibodies. Quantitation of the intensity of the bands corresponding to p85-PI3K is presented as bar graphs. Results are means $\pm$ s.d. of two independent experiments.

FIGURE 9. Effect of overexpression of the JM or CT domains on PKB and p70 S6K phosphorylation. Rat hepatoma Fao cells were infected with the indicated recombinant vaccinia virus. Following 6 h infection, cells were incubated with 100 nM insulin for 1 min at 37 °C, and cellular extracts were prepared. (A) Samples (100 µg) were resolved by means of 7.5% SDS-PAGE and immunoblotted with PKB and phospho-specific PKB antibodies. Results are means $\pm$ s.d. of three independent experiments; (B) same as in (A) except that p70 S6K and phospho-specific p70 S6K antibodies were used. Quantitation of the intensity of the bands corresponding to phospho-specific PKB and phospho-specific p70 S6K is presented as bar graphs. Results are means $\pm$ s.d. of two independent experiments.

with Vcc-β-Gal or Vcc-CT. In contrast, overexpression of the JM domain partially abolished (~40%) the stimulatory effects of insulin on

FIGURE 10. Insulin-induced thymidine incorporation in infected Fao cells. Rat hepatoma Fao cells were infected with the indicated recombinant vaccinia virus. Following 6 h infection, cells were incubated at 37 °C with or without 100 nM insulin for 14 h. Thymidine incorporation was measured using [^3H]thymidine, as described in the Materials and Methods section. Results are means $\pm$ s.d. of a representative experiment carried out four times in duplicates.

[^3H]thymidine incorporation into DNA. Hence, insulin-induced growth-promoting functions were also inhibited by the overexpressed JM polypeptide. These results implicate the JM domain as an essential element required for mediating both metabolic and growth-promoting effects of insulin.

DISCUSSION

The introduction of truncated/mutated forms of the IR into a number of cell lines enabled distinct roles to be assigned to different receptor regions in modulating the biological effects of insulin (Maegawa *et al.* 1988, Thies *et al.* 1989, Tavare & Siddle 1993, Gustafson *et al.* 1995, Chaika *et al.* 1999). Still, all cell lines studied, including differentiated 3T3-L1 adipocytes, were at best high-fidelity models but they fall short of being physiological insulin-responsive tissues. Studies with transgenic mice, harboring altered receptors could provide the desired answers, but the current repertoire of these models is rather limited.

Primary adipocytes are a major target tissue for insulin action, however these fully differentiated cells are not readily amenable to genetic manipulations. To overcome this problem we set out to develop an alternative method that enables a rapid and quantitative expression of proteins of interest in primary adipose cells. This system makes use of a vaccinia virus that serves as an efficient vector for expressing genes of interest within the cytoplasm of

eukaryotic cells (Moss & Earl 1991). This approach provides several advantages: first, infection with vaccinia virus enables quantitative, high expression level of peptides/proteins in the majority (>80%) of the infected cells 6 h post-infection. This enables us to study the effects of the introduced protein on any desired cellular function using the entire cell population, while eliminating the need to co-infect with a reporter gene that monitors a selective cellular response. Secondly, infection with a vaccinia virus *per se* or with an irrelevant protein such as β -galactosidase does not impair insulin responsiveness of the transfected cells; it does not alter insulin binding, nor does it affect the ability of insulin to activate the IRK and induce receptor autophosphorylation. Similarly, infection and expression of an irrelevant protein did not have any detrimental effects on insulin action and comparable levels of insulin-stimulated glucose utilization and lipogenesis (in adipocytes) or amino acids utilization (in Fao cells) were observed in non-infected cells, or in cells infected with β -galactosidase.

Previous studies furthered the notion that the IR mediates its interaction with downstream effector molecules through different domains within its cytoplasmic tail (Maegawa *et al.* 1988, White *et al.* 1988, Tavaré & Siddle 1993, Gustafson *et al.* 1995, Hansen *et al.* 1996, O'Neill *et al.* 1996, Chaika *et al.* 1999). Here we assessed the contribution of these domains to the metabolic effects of insulin in physiological insulin-responsive primary rat adipocytes, as well as in Fao hepatoma cells. We show that infection with a recombinant virus results in quantitative expression of the JM and CT domains in amounts exceeding by far the concentration of the endogenous receptor. We have previously shown (Paz *et al.* 1996) that expression of recombinant JM or CT domains in bacteria yields functional polypeptides that effectively interact with downstream effectors of the IR, such as IRS and Shc proteins. Therefore, we can assume that the JM and CT domains, overexpressed *in vivo* in primary adipocytes and hepatoma cells, act as dominant negative inhibitors of the corresponding domains of the endogenous receptors. Interestingly, fundamental differences were found when the effects of the overexpressed polypeptides were compared. When fat cells were infected with recombinant virus expressing the JM region of IR, insulin-stimulated glucose uptake and utilization is almost completely blunted, without any significant effect on the basal activity. In contrast, infection with the CT domain of IR had no significant effect. These findings suggest that glucose uptake and utilization in primary adipocytes is presumably mediated by proteins that selectively interact with the JM domain of IR.

Overexpression of the JM and CT domains could interfere with any of the elements constituting the signaling pathways leading to insulin-stimulated glucose transport and lipogenesis. Overexpression of the JM or CT domains did not affect insulin binding nor did it affect the insulin-stimulated receptor kinase activity, assessed by autophosphorylation of the IR *in vivo*. These findings suggest that the intramolecular interactions of the endogenous IR domains, a prerequisite for the receptor kinase activation (Hubbard 1997), are strong enough and therefore insensitive to the presence of the overexpressed JM and CT domains. In contrast, overexpression of the JM, but not the CT domains, eliminated the ability of the IRK to interact with, and phosphorylate the IRS proteins.

IRS proteins are key mediators of insulin-stimulated glucose transport, mainly due to binding and activation of PI3K (Cheatham & Kahn 1995). These proteins interact with the JM domain of IR through their PTB domains (Eck *et al.* 1996). Indeed, we have previously shown that an individually expressed JM region of IR contains all the structural information that enables it to interact with IRS-1 and IRS-2 (Paz *et al.* 1996, 1997). Our present findings place the interactions between the JM region of IR and the IRS proteins as a central event required to propagate insulin metabolic functions. These results are somewhat at variance with previous observations demonstrating that overexpression of the PTB/SAIN domains of IRS proteins in 3T3-L1 adipocytes inhibited insulin's ability to enhance the phosphorylation of IRS proteins, without interference with insulin-stimulated glucose transport in these cells (Sharma *et al.* 1997). Since glucose transport assayed in 3T3-L1 adipocytes was still inhibited by wortmannin, a PI3K inhibitor, it led to the conclusion that insulin may stimulate glucose transport by an alternative pathway, independent of the engagement of IRS proteins with the IR (Sharma *et al.* 1997). This alternative pathway could involve direct binding of PI3K to the CT domain of the IR. While we cannot rule out the possibility that proteins, different from IRS, interact with the JM domain to further propagate glucose transport through activation of PI3K, our findings rule out the possibility that interaction of proteins, including PI3K, with the CT domain can provide an alternative pathway. Our results suggest that glucose transport and utilization in primary rat adipocytes depends solely upon interactions between downstream effectors and the JM domain of the IR, making the latter the key region involved in relaying insulin's metabolic function in physiological insulin responsive cells.

The overexpressed JM domain presumably acted as a dominant negative inhibitor of IR-IRS-1 complex formation, thus interfering with the ability of IRS proteins to undergo insulin-induced Tyr phosphorylation. Since Tyr-phosphorylated IRS proteins serve as docking sites for several SH2-domain containing proteins such as p85, the regulatory subunit of PI3K (Backer *et al.* 1992, Hadari *et al.* 1992), overexpression of the JM domain also inhibited activation of PI3K and its downstream effectors PKB (Alessi *et al.* 1996) and p70 S6K (Ming *et al.* 1994, Alessi *et al.* 1998), supporting the established notion that these enzymes are downstream targets of IRS/PI3K in rat adipocytes (Egawa *et al.* 1999).

In hepatocytes, a major insulin-induced biological effect is the regulation of nutrient uptake by increased amino acid uptake, leading to enhanced protein synthesis. Insulin-induced amino acid uptake requires several hours (Reid & Reid 1987), however, the initiation of the insulin signal involves early changes in Ser/Thr phosphorylation of multiple intracellular proteins. Enhanced Ser/Thr phosphorylation of the ribosomal protein S6 (Lane *et al.* 1992) through activation of p70 S6 kinase (Mukhopadhyay *et al.* 1992, Thomas 1992) is one of the early biological responses to insulin that induces protein synthesis (Krieg *et al.* 1989). As shown here, interference with the ability of the JM domain to interact with downstream effectors decreased the ability of insulin to induce amino acid uptake and utilization. Hence, similar to lipogenesis, JM-effectors interactions are indispensable for insulin-induced metabolic effects in Fao cells. Insulin-induced DNA synthesis in Fao cells was less sensitive to the inhibitory effects of the overexpressed JM domain. These results could simply reflect the differences in assay conditions for measurements of amino acid utilization vs DNA synthesis. Alternatively, the growth-promoting activities of insulin could be mediated by proteins that better interact with Tyr-phosphorylated endogenous JM receptor domain, making them somewhat less sensitive to competition by the overexpressed non-phosphorylated JM region.

Several reasons could account for the insensitivity of the cells to the overexpressed CT domain. While we have previously shown (Paz *et al.* 1996) that the isolated CT region, expressed in bacteria as a His₆-fusion peptide, maintains its structural characteristics and is capable of interacting with downstream effectors such as Shc, we cannot rule out the possibility that this domain when overexpressed in mammalian cells does not assume its native conformation or localization and therefore fails to act in a negative dominant fashion. Alternatively,

the interactions of the isolated CT domain with its putative downstream effectors *in vivo* might be of low enough affinity such that it fails to act as a true negative dominant inhibitor. In that respect the overexpressed CT domain resembles the overexpressed CSF-1/IR chimera mutant, lacking 12 amino acids from its JM region, including the NPEY motif, which does not induce CSF-1 mediated glucose transport when overexpressed in 3T3-L1 adipocytes, but fails to act as a negative dominant inhibitor of insulin-stimulated glucose transport, mediated by the endogenous IRs in these cells (Boehm *et al.* 1998). Finally, the inhibitory effects exerted by the JM, but not the CT, domain could not be attributed to selective Tyr phosphorylation of the former, since neither domain undergoes significant Tyr phosphorylation in response to insulin in infected cells (K Paz & Y Zick, unpublished observations).

In summary, our data demonstrate the use of vaccinia virus-mediated gene transfer to introduce and functionally express various protein domains in physiologically insulin-responsive cells. Our findings suggest that interactions between the JM region of IR and its downstream effectors are obligatory for insulin-stimulated metabolic functions in physiologically relevant insulin-responsive cells. They also rule out the possibility that interaction of proteins, including PI3K, with the CT domain can provide an alternative pathway. In contrast, insulin-induced growth-promoting effects depend only partially upon interactions between the JM domain of IR and its downstream effectors. Further studies are therefore required to elucidate the molecular components that interact with the different IR domains and mediate insulin signal transduction in hepatoma cells and terminally differentiated primary adipocytes. The vaccinia virus-mediated protein expression system sets the stage for such studies.

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