Reconstitution of Insulin Signaling Pathways in Rat 3Y1 Cells Lacking Insulin Receptor and Insulin Receptor Substrate-1

EVIDENCE THAT ACTIVATION OF Akt IS INSUFFICIENT FOR INSULIN-STIMULATED GLYCOGEN SYNTHESIS OR GLUCOSE UPTAKE IN RAT 3Y1 CELLS

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Rat 3Y1 cells have endogenous insulin-like growth factor-1 receptors and insulin receptor substrate (IRS)-2, but lack both insulin receptor (IR) and IRS-1. To investigate the role of IR and IRS-1 in effects of insulin, we transfected IR and IRS-1 expression plasmids into cells and reconstituted the insulin signaling pathways. 3Y1 cells stably expressing the c-myc epitope-tagged glucose transporter type 4 (3Y1-GLUT4myc) exhibit no effects of insulin, at physiological concentrations. The 3Y1-GLUT4myc-IR cells expressing GLUT4myc and IR responded to phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P3) accumulation, Akt activation, the stimulation of DNA synthesis, and membrane ruffling but not to glycogen synthesis, glucose uptake, or GLUT4 translocation. The further expression of IRS-1 in 3Y1-GLUT4myc-IR cells led to stimulation of glycogen synthesis but not to glucose uptake or GLUT4 myc translocation in response to insulin, although NaF or phorbol 12-myristate 13-acetate did trigger GLUT4myc translocation in the cells. These results suggest that, in rat 3Y1 cells, (i) IRS-1 is essential for insulin-stimulated glycogen synthesis but not for DNA synthesis, PI-3,4,5-P3 accumulation, Akt phosphorylation, or membrane ruffling, and (ii) the accumulation of PI-3,4,5-P3 and activation of Akt are insufficient for glycogen synthesis, glucose uptake or for GLUT4 translocation.

Insulin elicits a remarkable array of biological effects in a wide range of cell types, including stimulation of glucose uptake and the enhanced synthesis of glycogen, lipid, protein, and DNA. Binding of insulin to cell surface receptors activates intrinsic tyrosine kinase, which leads to tyrosine phosphorylation of intracellular proteins such as IRS-1 (1), IRS-2 (2), IRS-3 (3), IRS-4 (4), Grb2-associated binder-1 (5), and Shc (6). IRS-1 has 20 potential tyrosine phosphorylation sites associated with Src homology 2 domain-containing proteins, including phosphatidylinositol (PI) 3-kinase (7), Grb2Ash (8), Nck (9), and SH-PTP2 (10).

To examine the physiological role of IRS-1 and its signaling molecules in insulin actions, we reconstituted insulin signaling pathways in the 3Y1 cells that lack both IR and IRS-1. One of the major physiological effects of insulin is stimulation of glucose transport, an effect mainly due to translocation of glucose transporter type 4 (GLUT4), from an intracellular pool to the cell surface. We developed a direct and quantitative method to assess GLUT4 translocation, using a c-myc epitope-tagged GLUT4 (GLUT4myc) (11), and have shown that the IRS-1 tyrosine phosphorylation and the subsequent PI 3-kinase activation are required for insulin-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes and Chinese hamster ovary (CHO) cells (12–14).

Here, in rat 3Y1 cells that lack both IR and IRS-1, we examined the effects of successive expressions of IR and IRS-1 on insulin-stimulated glucose uptake, GLUT4 translocation, glycogen synthesis, membrane ruffling, and DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO cells were grown in Ham’s F-12 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). 3Y1 cells, which were kindly provided by Dr. Shun Nakamura (the Institute of Medical Science, University of Tokyo) were grown in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo), supplemented with 10% fetal calf serum.

Stable Cell Lines—Clonal cell lines that stably express GLUT4myc, constructed by inserting a human c-myc epitope (14 amino acids) into the first ectodomain of GLUT4, are designated as follows: CHO-GLUT4myc, CHO cells expressing GLUT4myc; CHO-GLUT4myc-IR cells, CHO cells expressing GLUT4myc and insulin receptors (11). To obtain rat 3Y1 cells stably expressing GLUT4myc, SRE-GLUT4myc (11) and pSV2-neo were cotransfected into 3Y1 cells by calcium phosphate precipitation and selected with 400 μg/ml G418 (Sigma). The resultant 3Y1-GLUT4myc cells were subsequently cotransfected with SRE-IR and pSV2-bsr and selected with 10 μg/ml blasticidin S hydrochloride (Funakoshi, Tokyo). The resultant 3Y1-GLUT4myc-IR cells were further cotransfected with pCXN-IRS-1 (12) and pSV2-gpt and were selected with 25 μg/ml mycophenolic acid and 250 μg/ml xanthine to secure 3Y1-GLUT4myc-IR-IRS-1 cells. More than three independent

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‡ The abbreviations used are: IRS, insulin receptor substrate; PI, phosphatidylinositol; IR, insulin receptor; IGF-1-R, insulin-like growth factor-1 receptor; IGF, insulin-like growth factor; GLUT4, glucose transporter type 4; CHO, Chinese hamster ovary; PI-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline.
clones for each transfection were analyzed to avoid clonal deviations. Scatchard plot analyses were made as described (15).

**Antibodies**—A monoclonal antibody (9E10) against human c-myc was obtained from the American Type Culture Collection. A monoclonal anti-IRS-1 antibody was obtained by immunizing mice with a glutathione-S-transferase fused C-terminal IRS-1 fragment. An anti-phosphotyrosine (anti-Tyr(P)) antibody was purchased from Transduction Laboratories. Phosphospecific Akt (Ser^473) antibody was purchased from New England Biolabs, Inc. Anti-Akt, anti-IR, anti-IRS-1, and anti-IRS-2 antibodies were prepared by immunizing rabbits with keyhole limpet hemocyanin-coupled peptides (COOH-terminal 20 amino acids of Akt1, 1.47 mM K_2HPO_4, 900 mM MgSO_4, 20 mM HEPES, pH 7.5, and 2 mg/ml bovine serum albumin) for 20 min at 37 °C, and lysed, and immunoprecipitated with anti-IR antibody. The immunoprecipitates were eluted around 22, 26, 47, 51, and 78 min, respectively.

**Immunoprecipitation**—Cell lysates were prepared with buffer containing 1% Nonidet P-40 after treatment with the indicated concentrations of insulin or insulin-like growth factor (IGF)-1 and precipitated with the indicated antibodies and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech), as described (16).

**Immunoblotting**—Cell lysates or the immunoprecipitated proteins described above were boiled for 3 min in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis; the separated proteins were transferred to nitrocellulose filter and then were probed with the indicated antibodies and detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and ECL systems (Amersham Pharmacia Biotech). Immunoblots were quantified by Image Master 1D (Amersham Pharmacia Biotech).

**[methyl-^3H]Thymidine Incorporation Assay**—Cells in 24-well plates were serum-starved for 24 h and incubated with the indicated ligands for 17 h at 37 °C. The cells were further incubated with 0.5 μCi of [methyl-^3H]thymidine (Amersham Pharmacia Biotech)/well for 1 h, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and precipitated with cold 10% trichloroacetic acid. The precipitates were washed twice with cold 10% trichloroacetic acid, solubilized with 0.2% NaOH, 0.1% SDS, and counted in a scintillation counter, as described (17).

**PI 3-Kinase Assay**—Cell lysates prepared as described above were immunoprecipitated with the indicated antibodies and protein A-Sepharose CL-4B. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis; the separated proteins were transferred to nitrocellulose filter and then were probed with the indicated antibodies and detected using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and ECL systems (Amersham Pharmacia Biotech). Immunoblots were quantified by Image Master 1D (Amersham Pharmacia Biotech).

**PI-3,4,5-P_3 Measurement in Vivo**—Cells in 35-mm glass dishes were labeled with 50 μCi of [32P]orthophosphate in phosphate-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) for 2 h at 37 °C, and stimulated with 100 nM insulin or 100 nM IGF-1 for 1 min at 37 °C. Phosphatidylinositol 3,4,5-trisphosphate was extracted twice with 930 μl of chloroform, methanol, 1% perchloric acid (50:25:18, v/v/v) and separated by the addition of 500 μl of chloroform and 500 μl of 1% perchloric acid. The lower organic phase was washed twice with chloroform-saturated 1% perchloric acid, 1 mM NaCl and dried. The lipids were deacetylated with mameylamine as described (18). The glycerol-PI-3,4,5-P_3 was separated using an anion exchange SAX HPLC column (Whatman Inc.) in a gradient of 0.04–0.85 M NH_4H_2PO_4; flow rate, 0.5 ml/min; a continuous gradient of B (50 mM NH_4H_2PO_4, pH 3.8, for 82 min (pump A, H_2O; pump B, 0.85 M NH_4H_2PO_4, pH 3.8, for 82 min, 13% at 20 min, 34% at 25 min, 42% at 55 min, 70% at 60 min, 100% at 80 min, and sustained 100% until 82 min), and counted in a scintillation counter. The deacylated forms of phosphatidylinositol 3-phosphate, phosphatidylinositol 4-phosphate, phosphatidylinositol 3,4-bisphosphate, phosphatidylinositol 4,5-bisphosphate, and PI-3,4,5-P_3 were eluted around 22, 26, 47, 51, and 78 min, respectively.

**Analysis of Akt Phosphorylation**—Cells in 24-well plates were incubated in 500 μl of Krebs-Ringer-Hepes buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl_2, 1.25 mM MgSO_4, 20 mM Hepes, pH 7.4, and 2 mg/ml bovine serum albumin) (11) for 30 min at 37 °C, stimulated with 100 nM insulin or 100 nM IGF-1 for 5 min at 37 °C, and lysed. The cell lysates were precipitated on 7% SDS-polyacrylamide gel electrophoresis gels and immunoblotted with an anti-Akt antibody or phosphospecific Akt (Ser^473) antibody.

**Analysis of Membrane Ruffling**—Cells in six-well plates were serum-starved for 12 h and stimulated with 100 nM insulin or 100 nM IGF-1 for 5 min at 37 °C. After fixation with 2% paraformaldehyde in PBS for 20 min at room temperature, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, stained with 25 milliunits/μl rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR) in PBS for 20 min, and visualized under a fluorescence microscope (Olympus) after an extensive wash with 0.05% Tween 20 in PBS.

**Glycogen Synthesis Assay**—Cells in six-well plates were incubated in 2 ml of glycogen assay buffer (140 mM NaCl, 1.7 mM KCl, 900 μM CaCl_2, 1.47 mM KHPO_4, 900 mM MgSO_4, 25 mM Tris-HCl, pH 7.5, and 2 mg/ml bovine serum albumin) for 20 min at 37 °C and then with 100 nM insulin or 100 nM IGF-1 for 10 min at 37 °C. The assay was initiated by the addition of 100 μl of 40 mM d-glucose in glycogen assay buffer containing 3 μCi of 3H]glucose (Amersham Pharmacia Biotech) and terminated after 1 h at 37 °C by washing with ice-cold PBS. The glucose incorporated into glycogen was solubilized in 20% KOH, boiled for 20 min, and precipitated with ethanol, and the radioactivity was measured as described (19).

**Fig. 1. Expression of IR and IRS-1 in 3Y1 and CHO cells.** A, tyrosine phosphorylation of IR in 3Y1 and CHO cells. Cells were stimulated with 100 nM insulin for 5 min at 37 °C, lysed, and immunoprecipitated (IP) with anti-IR antibody. The immunoprecipitates were electrophoresed on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose filter, and immunoblotted with an anti-Tyr(P) antibody. B, detection of IRS-1 in 3Y1 and CHO cells. Cells were stimulated with 100 nM insulin for 5 min at 37 °C, and the lysates were subjected to immunoprecipitation with anti-IRS-1 polyclonal antibody, followed by immunoblotting with an anti-IRS-1 monoclonal antibody. C, tyrosine phosphorylation of IRS-1 or IRS-2 in 3Y1-GLUT4myc cells and CHO-GLUT4myc cells. Cells were stimulated with 100 nM insulin or 100 nM IGF-1 for 5 min at 37 °C, lysed, and immunoprecipitated with antibodies against IRS-1 or IRS-2. Tyrosine-phosphorylated IRS proteins were detected by immunoblotting with anti-Tyr(P) (Anti-PY) antibody.
Cells and CHO cells were estimated to be 2.8 × 10^4 IRs/cell, respectively. We next examined endogenous IR and IRS-1 in 3Y1-GLUT4myc 11 cells (Fig. 1C), which showed GLUT4 translocation in response to insulin or IGF-1 (Ref. 20 and Fig. 2A). These results indicate that 3Y1 cells carry IGF-1-R and IRS-2 but lack both endogenous IR and IRS-1.

To investigate the roles of IR and IRS-1, we reconstructed insulin signaling pathways by exogenously expressing IR and IRS-1 in the 3Y1-GLUT4myc cells.

**Stable and Exogenous Expressions of IR and IRS-1 in 3Y1 Cells**—Two typical clones of 3Y1 cells stably expressing GLUT4myc (3Y1-GLUT4myc 11 and 33) (Fig. 1, A and B, lanes 2 and 3), which showed GLUT4 translocation in response to phorbol 12-myristate 13-acetate (PMA) and NaF, were then analyzed (see Fig. 7B). The 3Y1-GLUT4myc clone 11 was transfected with an IR-expressing plasmid, and two clones stably expressing IR (3Y1-GLUT4myc-IR 134 and 140) were obtained (Fig. 1, A and B, lanes 4 and 5). The 3Y1-GLUT4myc-IR 134 clone was further transfected with an IRS-1-expressing plasmid, and two clones stably expressing IRS-1 (3Y1-GLUT4myc-IR-IRS-1 7 and 10) were obtained (Fig. 1B, lanes 6 and 7). The clones of CHO-GLUT4myc and CHO-GLUT4myc-IR have been described elsewhere (11).

**Insulin Stimulates DNA Synthesis in the Absence of IRS-1 in 3Y1 Cells Stably Expressing Exogenous IR**—Since 3Y1 cells have endogenous IGF-1-R and IRS-2, IGF-1-stimulated DNA synthesis was examined (Fig. 2B). Treatment of IGF-1 increases tyrosine phosphorylation of IRS-2 and DNA synthesis, in a dose-dependent manner, in the absence of IRS-1 in 3Y1-GLUT4myc 11 cells (Fig. 2, A and B). Insulin slightly stimulates DNA synthesis at a concentration of 100 nM. The insulin-induced DNA synthesis at 100 nM is likely to be mediated by IGF-1-R. Treatment of CHO cells with insulin or IGF-1 led to tyrosine phosphorylation of IRS-1 and IRS-2 (Fig. 1C, lanes 4–8). In contrast, 3Y1-GLUT4myc cells showed no tyrosine phosphorylation of IRS-1 in response to insulin or IGF-1 (Fig. 1C, lanes 1–3). However, a high concentration (100 nM) of insulin or IGF-1 stimulated tyrosine phosphorylation of IRS-2 (Fig. 1C, lanes 7–9). Low concentrations (1–10 nM) of IGF-1 also induced tyrosine phosphorylation of IRS-2, but low concentrations (1–10 nM) of insulin did not result in tyrosine phosphorylation of IRS-2 (Fig. 2A).

Therefore, the insulin-stimulated phosphorylation of IRS-2 was considered to be mediated by IGF-1-R in 3Y1-GLUT4myc 11 cells (Fig. 1C, lane 8), because about 100-fold higher concentrations of insulin than IGF-1 stimulated IGF-1-R (Ref. 20 and Fig. 2A). These results indicate that 3Y1 cells carry IGF-1-R and IRS-2 but lack both endogenous IR and IRS-1.

**RESULTS**

**Lack of both IR and IRS-1 in 3Y1 Cells**—We found that rat 3Y1 cells have no detectable amounts of IR and IRS-1. The small amounts of tyrosine-phosphorylated IR were detected in precipitates of an anti-IR antibody from the insulin-stimulated CHO cells but not in those from 3Y1 cells (Fig. 1A, lanes 1 and 8). Scatchard plot analyses showed no detectable IR in 3Y1 cells and 2.8 × 10^3 IRs/cell in CHO cells. However, 3Y1 cells have endogenous IGF-1-R. The numbers of IGF-1-Rs of 3Y1 cells and 2.8 × 10^3 IRs/cell in CHO cells, respectively. We next examined endogenous IRS-1 and IRS-2 in 3Y1 cells. 3Y1 cells have no detectable IRS-1, while CHO cells have IRS-1 in immunoprecipitates obtained using an anti-IRS-1 antibody (Fig. 1B, lanes 1–5 and 8). We also investigated tyrosine phosphorylation of endogenous IRS-1 or IRS-2 in 3Y1 and CHO cells by immunoprecipitation with the specific antibodies (Fig. 1C). Treatment of CHO cells with insulin or IGF-1 led to tyrosine phosphorylation of IRS-1 and IRS-2 (Fig. 1C, lanes 4–10). In contrast, 3Y1-GLUT4myc cells showed no tyrosine phosphorylation of IRS-1 in response to insulin or IGF-1 (Fig. 1C, lanes 1–3). However, a high concentration (100 nM) of insulin or IGF-1 stimulated tyrosine phosphorylation of IRS-2 (Fig. 1C, lanes 7–9). Low concentrations (1–10 nM) of IGF-1 also induced tyrosine phosphorylation of IRS-2, but low concentrations (1–10 nM) of insulin did not result in tyrosine phosphorylation of IRS-2 (Fig. 2A).

Therefore, the insulin-stimulated phosphorylation of IRS-2 was considered to be mediated by IGF-1-R in 3Y1-GLUT4myc 11 cells (Fig. 1C, lane 8), because about 100-fold higher concentrations of insulin than IGF-1 stimulated IGF-1-R (Ref. 20 and Fig. 2A). These results indicate that 3Y1 cells carry IGF-1-R and IRS-2 but lack both endogenous IR and IRS-1.

**Effect of IR Expression of PI 3-Kinase Activity and the Accumulation of PI-3,4,5-P_3 Product**—Insulin- or IGF-1-stimulated
receptor kinases directly phosphorylate IRS-1 and IRS-2 at tyrosine residues. The binding of PI 3-kinase to the tyrosine-phosphorylated IRS-1 and IRS-2 induces PI 3-kinase activation and subsequent accumulation of the PI-3,4,5-P3 product. We examined the PI 3-kinase activities in immunoprecipitates with anti-IRS-1, anti-IRS-2, and anti-phosphotyrosine antibodies. In 3Y1-GLUT4myc and 3Y1-GLUT4myc-IR cells, activation of PI 3-kinase was detected in immunoprecipitates using anti-IRS-2 and anti-phosphotyrosine antibodies but not with anti-IRS-1 antibody (Fig. 3, A–C); these cells carry IRS-2 but not IRS-1 (Fig. 1C). Stable overexpression of exogenous IRS-1 in 3Y1-GLUT4myc-IR 134 cells (3Y1-GLUT4myc-IR-IRS-1 7) causes stimulation of PI 3-kinase activity by insulin and IGF-1 in immunoprecipitates with anti-IRS-1 antibody (Fig. 3A) and anti-phosphotyrosine antibody (Fig. 3C). On the other hand, IRS-1 overexpression reduced the PI 3-kinase activity in the immunoprecipitate in the case of anti-IRS-2 antibody (Fig. 3B). Thus, the affinity of PI 3-kinase for IRS-1 is apparently higher than that for IRS-2.

Next, we examined PI-3,4,5-P3 production in response to insulin or IGF-1 in vivo (Fig. 3D), because in vivo PI-3,4,5-P3 production does not always accompany PI 3-kinase activation in immunoprecipitates in vitro (21). In CHO-GLUT4myc cells, insulin treatment accumulated PI-3,4,5-P3, and this insulin-induced accumulation of PI-3,4,5-P3 was abolished by pretreatment with wortmannin. Although both insulin and IGF-1 treatments increased the activity of PI 3-kinase in 3Y1-GLUT4myc cells in immunoprecipitates obtained with anti-IRS-2 and anti-phosphotyrosine antibodies, only IGF-1 treatment increased significantly PI-3,4,5-P3 level in vivo (Fig. 3D). The expression of IR in the 3Y1-GLUT4myc 11 cells (3Y1-GLUT4myc-IR 134) led to accumulation of PI-3,4,5-P3 in response to insulin. Therefore, activation of PI 3-kinase via tyrosine-phosphorylated IRS-2 is not sufficient, and additional signaling via IR is required for insulin-induced accumulation of PI-3,4,5-P3.

**FIG. 3.** Effects of exogenously expressed IR and IRS-1 on PI 3-kinase activity and in vivo PI-3,4,5-P3 production in 3Y1 and CHO cells. PI 3-kinase activity was determined in immunoprecipitates (IP) using an anti-IRS-1 antibody (A), anti-IRS-2 antibody (B), or anti-Tyr(P) (Anti-PY) antibody (C) and after treatment of the indicated cells with 100 nM insulin or 100 nM IGF-1 for 10 min at 37 °C. D, cells labeled with [32P]orthophosphate for 2 h at 37 °C were exposed to 100 nM insulin or 100 nM IGF-1 for 1 min at 37 °C. The [32P]-labeled phospholipids were deacylated and analyzed by HPLC as described under "Experimental Procedures." Values represent means ± S.E. of three determinations.

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PI-3,4,5-P₃ in 3Y1 cells (see “Discussion”). Successive IRS-1 expression enhanced the insulin- and IGF-1-dependent PI-3,4,5-P₃ accumulation, consistent with activation levels of PI 3-kinase.

**Effect of IR Expression on Insulin-stimulated Akt Kinase Activity**—Insulin activates Akt (protein kinase B) presumably via PI 3-kinase (22). Phosphorylations on both serine 473 and threonine 308 of Akt are required for Akt activation, although bindings of phosphatidylinositol 3,4-bisphosphate or PI-3,4,5-P₃ produced by PI 3-kinase to Akt or to Akt upstream kinase do affect Akt kinase activation (23, 24). Treatments with insulin and IGF-1 phosphorylated and activated Akt in CHO-GLUT4myc cells (Fig. 4, A and B, lanes 10–12). In 3Y1-GLUT4myc cells, IGF-1 but not insulin induced Akt activation (Fig. 4, A and B, lanes 1–3). Expression of IR in 3Y1-GLUT4myc cells (3Y1-GLUT4myc-IR 134) restored Akt phosphorylation during insulin stimulation, concomitant with restoration of the electrophoretic mobility of the Akt. Phosphorylated Akt are indicated as p-Akt.

**Effect of IRS-1 Expression on Insulin-stimulated Membrane Ruffling**—In various species of cells, stimuli including insulin induce rapid rearrangement of actin filaments beneath the plasma membrane, an event termed “membrane ruffling.” PI 3-kinase has been shown to play an important role in the membrane ruffling (25–27). As described previously (25), 5-min treatment with insulin or IGF-1 in CHO cells after serum starvation for 12 h induced membrane ruffling around peripheral regions (Fig. 5, K and L). Both IGF-1 and insulin stimulate PI 3-kinase activity in 3Y1-GLUT4myc cells after precipitations by anti-IRS-2 and anti-phosphotyrosine antibodies (Fig. 3, B and C). While IGF-1 strongly induced membrane ruffling, insulin did not induce membrane ruffling in 3Y1-GLUT4myc cells (Fig. 5, B and C). IGF-1, but not insulin, stimulates the accumulation of PI-3,4,5-P₃ product (Fig. 3D) and Akt activity (Fig. 4). Expression of IR restored the membrane ruffling induced by insulin (Fig. 5E), concomitant with restoration of the insulin-stimulated accumulation of PI-3,4,5-P₃ product and Akt activation (Fig. 3D and Fig. 4). Therefore, only activation of PI 3-kinase is insufficient for insulin-stimulated membrane ruffling, and accumulation of PI-3,4,5-P₃ and Akt activation seems to be required for such events. Further expression of IRS-1 in 3Y1-GLUT4myc-IR cells led to responses to low concentrations of insulin.

**Effect of IRS-1 Expression on Insulin-stimulated Glycogen Synthesis**—In CHO-GLUT4myc cells, both insulin and IGF-1 stimulated glycogen synthesis severalfold, whereas in 3Y1-GLUT4myc and 3Y1-GLUT4myc-IR cells, IGF-1 but not insulin stimulated glycogen synthesis (Fig. 6). These findings indicate that IGF-1 stimulates glycogen synthesis via IGF-1-R and IRS-2 but that insulin did not stimulate glycogen synthesis via IR and IRS-2 in 3Y1 cells. Insulin-stimulated glycogen synthesis is mediated by PI 3-kinase and Akt kinase. Akt kinase phosphorylates and inhibits glycogen synthetase kinase 3, which plays an important role in regulating glycogen synthesis (28). PI 3-kinase activation accompanying PI-3,4,5-P₃ accumulation, and Akt phosphorylation via IR and IRS-2 were insufficient for insulin-induced glycogen synthesis in 3Y1-GLUT4myc-IR cells. Insulin-stimulated glycogen synthesis was observed after expression of IRS-1 (3Y1-GLUT4myc-IR-IRS-1 7). On the other hand, IGF-1 treatment induced glycogen synthesis even in 3Y1-GLUT4myc cells devoid of IR and IRS-1. Therefore, IGF-1-induced glycogen synthesis may be transmitted via IGF-1-R/IGF-2-R and heterotrimeric GTP-binding proteins, regardless of IR and IRS-1 (see “Discussion”).

**Reconstitution of Insulin Signaling Pathway of Glucose Uptake or GLUT4 Translocation**—GLUT4 is exclusively expressed in skeletal muscle and fat cells and is rapidly translocated to the cell surface from an intracellular pool, in response to insulin. The translocation of GLUT4 is thought to be a major mechanism of insulin-stimulated glucose uptake in these cells. We earlier developed a highly sensitive, quantitative method to detect immunologically the translocation of GLUT4 in intact cells, using c-myc epitope-tagged GLUT4 (11). Not only muscle and fat cells but also CHO and NIH-3T3 fibroblast cells that exogenously express GLUT4myc showed insulin-dependent GLUT4myc translocation and glucose uptake, albeit the extent differing among cells (11, 29). Fibroblasts may have a basic mechanism to translocate exogenously expressed GLUT4myc, and we attempted to reconstitute the insulin signaling pathway of GLUT4myc translocation in 3Y1 cells (see “Discussion”). In CHO-GLUT4myc cells, insulin or IGF-1 stimulated glucose uptake 1.6–1.7-fold (Fig. 7A), and PMA and NaF besides these hormones triggered GLUT4myc translocation approximately 3-fold. This discrepancy is due to the low level expression of exogenous GLUT4myc compared with endogenous GLUT1 in CHO cells. Therefore, insulin-stimulated glucose uptake is mainly regulated by endogenous GLUT1, not by exogenous expressed GLUT4myc, in the CHO-GLUT4myc cells. As we described previously (11), exogenously expressed GLUT4myc is
functional, and we got several CHO clones that have different expression levels of GLUT4myc. Exogenously overexpressed GLUT4myc, which cannot remain in the intracellular pool of CHO cells, existed directly on the cell surface, disturbed to examine the -fold increase of insulin-stimulated GLUT4myc translocation because of the high background. Treatments with PMA and NaF triggered GLUT4myc translocation in three kinds of 3Y1 clones (Fig. 7B). Thus, 3Y1 cells may have a basic machinery to recruit intracellular vesicles containing GLUT4myc to the cell surface. In 3Y1-GLUT4myc-IR-IRS-1 cells, neither insulin nor IGF-1 stimulated glucose uptake (Fig. 7A) or GLUT4myc translocation (Fig. 7B), although each treatment did activate PI 3-kinase and Akt kinase and accumulated PI 3,4,5-P_3 (Figs. 3 and 4). We examined the expressions of exogenous GLUT4myc in 3Y1 and CHO clones by Western blot, and the expressions of GLUT4myc, which are observed as broad bands between 50 and 65 kDa, were almost equal in these cells (Fig. 7C). These results indicate that activation of PI 3-kinase and Akt kinase was insufficient for insulin-induced glucose uptake or GLUT4myc translocation in 3Y1 cells and that 3Y1 cells lack the downstream molecule(s) from Akt kinase. However, Kohn et al. (30) reported that the expression of constitutively active Akt induced GLUT4 translocation in 3T3-L1 adipocytes.

DISCUSSION

Insulin-induced DNA Synthesis in 3Y1 Cells in the Absence of IRS-1—To access the function of insulin receptor and IRS-1, we reconstituted insulin signaling pathways in the 3Y1 cells that lack both IR and IRS-1. PI 3-kinase activity is involved in numerous insulin actions, including GLUT4 translocation (12), glycogen synthesis (31, 32), DNA synthesis (17, 39), and protein synthesis (34). Insulin and IGF-1 stimulated mitogenic response in 3Y1 cells. It has been reported that IRS-2 could not be substituted for IRS-1 for IGF-1 stimulation of [3H]thymidine incorporation into DNA in primary embryonic fibroblasts derived from IRS-1 knockout mice (35) and also reported that IRS-1 is essential for insulin-stimulated mitogenesis in the 32D

![Fig. 5. Effects of exogenously expressed IR and IRS-1 on membrane ruffling in 3Y1 and CHO cells. The cells were serum-starved for 12 h and then incubated in the absence or presence of 100 nM insulin or 100 nM IGF-1 for 5 min. Actin filaments were stained with rhodamine-phalloidin after fixation with 2% paraformaldehyde as described under “Experimental Procedures.”](image-url)

![Fig. 6. Effects of exogenously expressed IR and IRS-1 on glycogen synthesis in 3Y1 and CHO cells. The amount of [3H]glucose incorporated into glycogen was measured during a 1-h treatment with 100 nM insulin or 100 nM IGF-1. Values represent means ± S.E. of at least three determinations.](image-url)
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myeloid progenitor cell line (36). However, IGF-1 and insulin stimulate DNA synthesis in the absence of IRS-1 in 3Y1-GLUT4myc cells and 3Y1-GLUT4myc-IR cells, respectively (Fig. 2, B and C). The discrepancy of the results between these cells and 3Y1 cells is attributed to the fact that 1) alternative pathway(s) for the growth factor-stimulated DNA synthesis may exist in established cultured 3Y1 cells that have no IRS-1, 2) insulin- or IGF-1-induced tyrosine phosphorylation of IRS-2 is sufficient to stimulate DNA synthesis in 3Y1 cells, and 3) an Shc-Grb2-Sos complex is more important for insulin- or IGF-1-induced DNA synthesis than is the IRS-1-Grb2-Sos complex in 3Y1 cells.

IGF-1-induced mitogen-activated protein kinase or DNA synthesis is mediated by a G_i protein-coupled pertussis toxin-sensitive pathway (37, 38). IGF-2-R directly interacts with G_i (39) in response to IGF-2 and stimulates DNA synthesis (40). IGF-1 cross-reacts with IGF-2 receptor (41). We found that the G_i protein-coupled receptor exogenously expressed in CHO GLUT4myc cells stimulated DNA synthesis, PI-3,4,5-P_3 accumulation, Akt phosphorylation, glycogen synthesis, and GLUT4myc translocation. These responses were abolished by pertussis toxin treatment. From these results, it is likely that IGF-1-induced biological effects in 3Y1 cell line are partly mediated by the IGF-2-receptor.

Insulin Signaling Pathways of the Accumulation of PI-3,4,5-P_3—Insulin- or IGF-1-induced PI 3-kinase activation is closely related to Akt phosphorylation and to membrane ruffling (22, 25–27). In this report, we showed that insulin increases PI 3-kinase activities almost equally in the precipitates of anti-IRS2 antibody in 3Y1-GLUT4myc and 3Y1-GLUT4myc-IR cells (Fig. 3B). However, the increase in PI-3,4,5-P_3 in 3Y1-GLUT4myc-IR cells is much larger than the increase in 3Y1-GLUT4myc cells in response to insulin (Fig. 3D). This discrepancy may be explained by the activation of Ras signaling pathway via IR in 3Y1-GLUT4myc-IR cells. Insulin-activated tyrosine kinase of IR directly phosphorylates Shc and consequently activates Ras via the Grb2-Sos complex. The activated Ras directly activates PI 3-kinase, which cannot be detected in the precipitates of anti-IRS2 or anti-phosphotyrosine antibodies (27, 42, 43). Activations of PI 3-kinase via both IRS-2 and Ras signaling pathways may trigger insulin-induced PI-3,4,5-P_3 accumulation in 3Y1-GLUT4myc-IR cells but not in 3Y1-GLUT4myc cells after treatment with insulin. Expression of constitutively active PI 3-kinase resulted in increased PI-3,4,5-P_3 concentrations in vivo (44) and induced Akt phosphorylation and membrane ruffling (27, 44). In response to insulin or IGF-1, Akt is phosphorylated and activated by Akt upstream kinase but only in the presence of PI-3,4,5-P_3 (23). Taken together, these observations indicate that PI-3,4,5-P_3 accumulation plays an important role in Akt phosphorylation and membrane ruffling. Our results are in accord with these reports.

Insulin-induced Glycogen Synthesis, Glucose Uptake, and GLUT4 Translocation—PI 3-kinase also plays an important role in the insulin- or IGF-1-dependent glycogen synthesis (31). We showed that activation of PI 3-kinase and Akt are insufficient for insulin-induced glycogen synthesis and that other signaling molecule(s) mediated by IRS-1 are required. 3Y1 cells contain endogenous IRS-2, which is not effective for insulin-induced glycogen synthesis. Insulin- or IGF-1-stimulated glycogen synthesis is approximately 5–7-fold in CHO-GLUT4myc cells, and the effects in 3Y1-GLUT4myc-IR-IRS-1 cells are small (approximately 2-fold) compared with the CHO clones (1.6–1.7-fold). This may be one of the reasons why the effects of insulin or IGF-1 on glycogen synthesis are very small in 3Y1 clones.

However, we cannot deny the possibility that 3Y1 clones do not contain the insulin regulatory machinery for the physiological control of the glucose metabolic process (46).

Several lines of evidence support the requirement of PI 3-kinase in the insulin-dependent glucose uptake or GLUT4 translocation (12–14, 47). However, it was unclear whether a sole PI 3-kinase activation would recruit intracellular GLUT4 to the cell surface and stimulate glucose uptake; there are controver-
sial reports on similar experiments. Platelet-derived growth factor (PDGF)-stimulated PI 3-kinase somewhat enhanced glucose uptake, but GLUT4 translocation in 3T3-L1 adipocytes did not occur, indicating that sole activation of PI 3-kinase does not translocate GLUT4 (48, 49). However, this may not be the case, because our team and another group of investigators reported that PDGF activated PI 3-kinase activity and triggered GLUT4 translocation (50, 51).

The discrepancy between these two different results can relate to the following. Activation of PI 3-kinase and GLUT4 translocation triggered by PDGF in 3T3-L1 adipocytes are transient (52). Therefore, traditional methods to detect the GLUT4 translocation, (i.e. Western blot analysis using anti-GLUT4 antibody after fractionating by sucrose density gradient) cannot be used to detect PDGF-triggered GLUT4 translocation. On the other hand, expression of constitutively active PI 3-kinase in 3T3-L1 adipocytes enhanced glucose uptake and increased the amount of GLUT4 on the cell surface, indicating that sole activation of PI 3-kinase is sufficient for insulin-stimulated translocation of GLUT4 (26, 53, 54). The introduction of a phosphopeptide that activates PI 3-kinase as strongly as insulin via binding to the p85 subunit into 3T3-L1 adipocytes increased glucose uptake only 20%, and insulin and GLUT4 translocation occurred to some extent (55). These results suggest that one or more other insulin-triggered signaling pathways, besides PI 3-kinase, participate in the stimulation of glucose transport and GLUT4 translocation. These discrepancies seem to stem from the use of different assay systems, especially when evaluating GLUT4 translocation. It has been reported that PDGF receptors are expressed in 3T3-L1 fibroblasts, and full effects of PDGF on glucose transport are evident, but in contrast to GLUT4 and insulin receptor mRNAs, levels of mRNA of PDGF receptors gradually decrease after differentiation of 3T3-L1 cells (56). Therefore, it seems to be important when the PDGF-stimulated GLUT4 translocation and glucose uptake are examined before losing PDGF receptors during differentiation.

To address the question of whether sole activation of IR or IRS-1-dependent activation of PI 3-kinase stimulates glucose uptake or translocates GLUT4 and to identify the molecule(s) responsible for the glucose uptake or the translocation, we attempted to reconstitute the insulin signaling pathway from IR to glucose transporters. As mentioned by Kandror and Pilch (57), we may speculate that GLUT4, being expressed in heterologous cells, would be targeted to the small compartment maximally related to genuine GLUT4-containing vesicles from insulin-sensitive tissues. Rat 3Y1 cells seem to have a basic machinery to translocate GLUT4, by stimulation of PMA or NaF, but do not have IR or IRS-1 (Figs. 1 and 7). However, the activation of PI 3-kinase did not stimulate glucose uptake or recruit intracellular GLUT4myc to the cell surface, although PI 3-kinase activated with IRS-1/IRS-2 caused Akt activation, glycosgen and DNA synthesis, and membrane ruffling, which are tentatively downstream events of PI 3-kinase (Figs. 2–6). These results suggest that 3Y1 cells lack downstream molecule(s) of PI 3-kinase or Akt kinase, which may be responsible for insulin-triggered glucose uptake or GLUT4myc translocation. However, we cannot deny the possibility that the translocation of GLUT4myc in 3Y1 cells by stimulation of PMA or NaF might reflect movement through an endosomal recycling pathway but not through the insulin-sensitive GLUT4-containing vesicles present in muscle and adipose cells. In addition to PI-3,4,5-P3-dependent protein kinase-1 and Akt, molecules such as protein kinase Ca (58), protein kinase Ce (59), protein kinase Cε (60, 61), and general receptors for phosphoinositides-1 (62) have been identified as the target of PI 3-kinase or Akt kinase, which may be responsible for insulin-triggered glucose uptake or GLUT4myc translocation. In summary, the association between tyrosine-phosphorylated IRS proteins and PI 3-kinase is not sufficient for production of PI-3,4,5-P3. Insulin-induced PI-3,4,5-P3 production leads to Akt activation and membrane ruffling but does not stimulate glycogen synthesis or glucose uptake or translocate GLUT4 in 3Y1 cells (Table I). We hope to identify the molecule(s) responsible for insulin-dependent glucose uptake or GLUT4 translocation; reconstituted 3Y1 cells are useful to search for candidate molecules related to insulin-stimulated glucose uptake or GLUT4 translocation and to examine the roles of these molecules in stimulation of glucose uptake or GLUT4 translocation.

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**Table I** Summary of insulin- and IGF-1-dependent effects in 3Y1 cells via IR and IRS-1

| Effects* | Ligands* | 3Y1-GLUT4myc (IGF-1-R, IRS-2) | 3Y1-GLUT4myc-IR, IRS-2 | 3Y1-GLUT4myc-IR-IRS-1 (IGF-1-R, IRS-1, IRS-2) | CHO-GLUT4myc (IGF-1-R/IR, IRS-1/IRS-2) |
|---|---|---|---|---|---|
| DNA synthesis | Insulin | + | + | + | + |
| | IGF-1 | + | + | + | + |
| PI-3,4,5-P3, accumulation, Akt phosphorylation, membrane ruffling | Insulin | + | + | + | + |
| Glycogen synthesis | Insulin | + | + | + | + |
| | IGF-1 | + | + | + | + |
| Glucose uptake | Insulin | + | + | + | + |
| | IGF-1 | + | + | + | + |
| GLUT4myc translocation | Insulin | + | + | + | + |
| | IGF-1 | + | + | + | + |
| | NaF or PMA | + | + | + | + |

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*100 nM insulin, 100 nM IGF-1, 10 mM NaF, or 1 μM PMA-dependent effects were observed (+) or not (−) with regard to DNA synthesis, PI-3,4,5-P3, accumulation, Akt phosphorylation, membrane ruffling, glycogen synthesis, and GLUT4myc translocation.

*This effect seems to be mediated by IGF-1-R.
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