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Differences in Signaling Properties of the Cytoplasmic Domains of the Insulin Receptor and Insulin-like Growth Factor Receptor in 3T3-L1 Adipocytes*

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Birgitte Ursø, Diane L. Cope, Heidi E. Kalloo-Hosein, Amanda C. Hayward, Jon P. Whitehead, Stephen O'Rahilly, and Kenneth Siddle‡

From the University of Cambridge, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge CB2 2QR, United Kingdom

Insulin and insulin-like growth factors (IGFs) elicit distinct but overlapping biological effects *in vivo*. To investigate whether differences in intrinsic signaling capacity of receptors contribute to biological specificity, we constructed chimeric receptors containing the extracellular portion of the neurotrophin receptor TrkC fused to the intracellular portion of the insulin or IGF-I receptors. Chimeras were stably expressed in 3T3-L1 adipocytes at levels comparable to endogenous insulin receptors and were efficiently activated by neurotrophin-3. The wild-type insulin receptor chimera mediated approximately 2-fold greater phosphorylation of insulin receptor substrate 1 (IRS-1), association of IRS-1 with phosphoinositide 3-kinase, stimulation of glucose uptake, and GLUT4 translocation, compared with the IGF-I receptor chimera. In contrast, the IGF-I receptor chimera mediated more effective Shc phosphorylation, association of Shc with Grb2, and activation of mitogen-activated protein kinase compared with the insulin receptor chimera. The two receptors elicited similar activation of protein kinase B, p70S6 kinase, and glycogen synthesis. We conclude that the insulin receptor mediates some aspects of metabolic signaling in adipocytes more effectively than the IGF-I receptor, as a consequence of more efficient phosphorylation of IRS-1 and greater recruitment/activation of phosphoinositide 3-kinase.

Insulin and insulin-like growth factors (IGFs)[1] are structurally related polypeptides that exert diverse effects on mammalian tissues. The most prominent actions of insulin *in vivo* are concerned with the acute regulation of carbohydrate and lipid metabolism in liver, muscle, and fat, whereas IGFs act on skeletal and other tissues to promote growth, differentiation, and survival. The receptors for insulin and IGFs, which mediate these effects (IR and IGFR), are also structurally related and highly homologous, consisting of extracellular α-subunits responsible for ligand binding and transmembrane β-subunits possessing protein-tyrosine kinase activity, in a disulphide linked β-α-α-β configuration (1–3). Within the intracellular portion, the level of sequence identity between the receptors is greatest in the tyrosine kinase domain (84%) and somewhat less in the flanking juxtamembrane and carboxyl-terminal regions (61 and 44%, respectively). Not surprisingly, the signaling mechanisms of the insulin receptor (IR) and IGF-I receptor (IGFR) are broadly similar. Ligand binding activates tyrosine kinase activity, leading to phosphorylation of intracellular substrates, such as IRS and Shc proteins, and the recruitment and/or stimulation of signal transducing molecules, including phosphoinositide 3-kinase (PI 3-kinase) and Grb2·Sos (4, 5). These signal transducers in turn promote activation of protein-serine kinase cascades involving phosphoinositide-dependent kinase/PKB and MAPK/Erk kinase/MAPK, respectively, which modulate the activity of glucose transporters, enzymes, and transcription factors (6, 7).

Given the similarity in structure and signaling mechanism of the respective receptors, obvious questions arise concerning the basis of specificity in the actions of insulin and IGFs *in vivo*. In part, this specificity must reflect the different patterns of expression of receptors and responsive pathways in different cell types. Indeed, when examined in the same cell background, insulin and IGFs induce the same end point responses (8–10). However, such studies do not rule out the possibility of subtle differences in the coupling of the receptors to different responses, and there are various problems of interpretation in making detailed comparisons of IR and IGFR activity in a given cell type. First, neither receptor is completely specific for its ligand, and at high ligand concentrations, some cross-reaction with the heterologous receptor is inevitable. Second, it is difficult to make comparisons at equivalent occupancy of IR and IGFR because of differences in ligand affinity and levels of expression. Third, in cells that express both IR and IGFR a substantial fraction of receptors assemble as hybrid structures capable of binding both ligands (11). Finally, attempts to overcome these problems by overexpression of receptors in transfected cells might distort the signaling specificity, which could be seen at lower and more physiological levels of receptor expression.

In order to circumvent such problems, several laboratories have made use of chimeric receptors, in which the intracellular domains of the IR, IGFR, or insulin receptor-related receptor are coupled to a common extracellular ligand binding domain (12–15). It was reported that the IGFR was more efficient than the IR in mediating stimulation of DNA synthesis in NIH3T3 fibroblasts, with no difference in stimulation of glucose uptake (12). In contrast, in 3T3-L1 fibroblasts we found that the IR

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‡ To whom correspondence should be addressed. Tel.: 44-1223-336789; Fax: 44-1223-331157; E-mail: ks14@mole.bio.cam.ac.uk.

[1] The abbreviations used are: IGF, insulin-like growth factor; IR, insulin receptor; IGFR, type I insulin-like growth factor receptor; TIR, TrkC-insulin receptor chimera; TIGR, TrkC-IGF-I receptor chimera; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B/Akt; MAP, mitogen-activated protein; MAPK, MAP kinase; IRS, insulin receptor substrate; NT-3, neurotrophin-3; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

mediated a greater stimulation of glycogen synthesis compared with the IGFR, with no difference in the extent or efficiency of stimulation of DNA synthesis (14). Other work with truncated receptors, site-directed mutants, and chimeras has suggested that the efficiency of "metabolic" and "mitogenic" stimulations may depend on the carboxyl-terminal domains of the respective receptors (16–20), although the results have not been entirely consistent (21). A serious limitation of all these studies is that they have been carried out in cultured fibroblasts that display only a limited range of rather small responses to insulin and IGF-I compared with physiologically important target tissues. We have therefore examined the question of intrinsic signaling specificity by expressing IR and IGFR chimeras in differentiated 3T3-L1 adipocytes, which are highly insulin-responsive. We show here that glucose uptake is stimulated to a greater extent by IR than IGFR and that differences in the relative phosphorylation of distinct intracellular substrates by the IR and IGFR tyrosine kinases contribute to signaling specificity of the two receptors.

EXPERIMENTAL PROCEDURES

Materials—Antibodies were obtained from TCB (mouse monoclonal anti-phosphotyrosine, 4G10), Sigma (mouse monoclonal anti-phosphotyrosine PY20), Promega (rabbit anti-Active™ MAPK) or New England Biolabs (rabbit anti-phosphoAkt[Ser-473] and rabbit anti-pp70S6-kinase[Thr-389]). Other antibodies were produced in-house in rabbits using bacterially expressed GST fusion proteins as immunogens: anti-IR-carboxyl-terminal (carboxyl-terminal 98 amino acids of human IR), anti-IGFR-carboxyl-terminal (carboxyl-terminal 106 amino acids of human IGFR), anti-Shc (SH2-domain), anti-IRS-1 (carboxyl-terminal 236 amino acids of rat IRS-1), and anti-p85 (N-terminal SH2-domain). The anti-Grb2 antibody (mouse monoclonal) was a gift from Dr. J. Schlessinger (New York University Medical Center), rabbit anti-GLUT4 was a gift from Prof. G. Gould (University of Glasgow), rabbit anti-GLUT1 was a gift from Prof. S. Baldwin (University of Leeds), and goat anti-PKB and anti-p70S6 kinase antibodies were gifts from Dr. D. Alessi (University of Dundee). The TrkC cDNA and Fra-1 cDNA probes were gifts from Dr. J. M. Tavaré (University of Bristol). NT-3 was generously provided by Dr. E. Brewster (Regeneron Pharmaceuticals). Tissue culture media were purchased from Life Technologies, Inc., radiochemicals from Amersham Pharmacia Biotech, restriction enzymes from New England Biolabs, oligonucleotides from Genosys and other reagents from Sigma.

Construction of Chimeras—Chimeras containing the extracellular and transmembrane portions of the neurotrophin receptor TrkC together with the intracellular portion of the human IR (TIR) or IGFR (TIGR) were constructed as described previously (14). The chimeras were expressed under the regulation of the elongation factor 1α promoter (a kind gift from Dr R. E. Lewis (Eppley Institute for Research in Cancer, University of Nebraska)) in the vector pcDNA3 (Invitrogen).

Tissue Culture—3T3-L1 fibroblasts (ATCC) were maintained at no higher than 70% confluency in DMEM containing 10% newborn calf serum, 4.5 g/liter glucose, 2 mM glutamine, and antibiotics (DMEM/newborn calf serum). For differentiation they were grown 2 days post-confluency in the same medium and then for 2 days in medium containing fetal calf serum instead of newborn calf serum (DMEM/fetal calf serum) supplemented with insulin (5 μg/ml), dexamethasone (0.1 μg/ml), and isobutylmethylxanthine (110 μg/ml), for 2 days in DMEM/fetal calf serum with insulin only and finally for 6 days in DMEM/fetal calf serum. Differentiated cells were used only when at least 90% of the cells showed adipocyte phenotype by accumulation of lipid droplets. 3T3-L1 fibroblasts were transfected using LipofectAMINE™ reagent (Life Technologies, Inc.) and selected in medium containing 600 μg/ml G418 (Life Technologies, Inc.) to produce stable expressing clones (obtained by subculturing at limiting dilution on microtitre plates).

NT-3 Binding—The expression levels of the chimeras in 3T3-L1 cells were analyzed by radioligand binding assay. NT-3 was radioiodinated as described (14). The cells were incubated with ^{125}I-NT-3 (30,000 dpm) and increasing concentrations of unlabeled NT-3 in binding buffer (100 mM Hepes, 120 mM NaCl, 1 mM EDTA, 15 mM $NaC_2H_4O_2$) overnight at 4 °C, washed twice in cold phosphate-buffered saline, solubilized in 0.1 M NaOH and counted in a NE1600 gamma counter. IC_{50} values were obtained from calculations with GraphPad PRISM, version 2.01.

Western Blotting—Serum-starved adipocytes were stimulated for 5 min and solubilized in lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM $Na_2P_2O_7$, 0.1 mM 4-(2-aminoethyl)benzene sulfonyl fluoride, HCl, 2.5 mM benzamidine, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin A), and the lysate was clarified by centrifugation at 13,500 × *g* for 15 min at 4 °C. Lysates were subjected to immunoprecipitation using anti-IRS-1 (1:200) or anti-Shc (1:200) antibodies plus protein A-agarose (2 mg/sample), and immunoprecipitates were washed once in lysis buffer and twice in phosphate-buffered saline on ice. Crude cell extracts or specific immunoprecipitates were resolved by SDS-PAGE before electroblotting to polyvinylidene difluoride membranes (Millipore), and specific proteins were detected by incubation with appropriate antibodies in TBST (150 mM NaCl, 50 mM Tris, 0.1% Tween 20) after blocking in 1% bovine serum albumin, followed by ^{125}I-labeled secondary antibodies (approximately 0.2 μCi per blot), and quantified on a Fujix BAS2000 phosphorimager.

Glucose Uptake—Assays were performed as described previously (22). In short, differentiated adipocytes in six-well plates were serum-starved for 2 h in DMEM containing 25 mM glucose and 2 mM glutamine and incubated in 1 ml of KRH (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM Hepes, pH 7.4) with hormone at 37 °C for 30 min and 2-deoxy-D-[2,6-^{3}H]glucose (0.33 μCi/ml; final specific activity, 6.7 μCi/μmol) for an additional 5 min. Uptake was stopped by three rapid washes on ice with KRH, the cells were solubilized in 1 ml of 0.1 M NaOH and neutralized (50 μl of concentrated HCl), and radioactivity was determined by liquid scintillation counting.

Plasma Membrane Lawn Assays of GLUT4 Translocation—3T3-L1 adipocytes, grown on collagen-coated glass coverslips, were serum-starved for 2 h. Cells were either left untreated or stimulated for 15 min with 100 nM insulin or 4 nM NT-3. Coverslips were rapidly washed in ice-cold buffer for the preparation of plasma membrane lawns exactly as described (23). After fixation in paraformaldehyde, plasma membrane lawns were incubated with either anti-GLUT4 or anti-GLUT1 antibody (1:100 dilution) for 2 h at room temperature. After washing, the coverslips were incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG, washed, and mounted on glass slides. Coverslips were viewed using a × 60 objective lens on a Nikon Optiphot-2/Bio-Rad MRC-1000 microscope operated in laser scanning confocal mode. Samples were illuminated at 488 nm, and images were collected at 510 nm. Duplicate coverslips were prepared at each experimental condition, and five random images of plasma membrane lawn were collected from each. Five fields from each image were quantified using Bio-Rad MRC-1000 confocal microscope operating software (CoMOS, version 6.05.8), on an AST premmia SE P/60 personal computer.

Glycogen Synthesis—Assays were performed as described previously (24). In short, fully differentiated adipocytes in six-well plates were serum-starved for 3 h in DMEM with 5.5 mM glucose and 2 mM glutamine, stimulated with hormone for 30 min, and incubated with D-[U-^{14}C]glucose (1 μCi/ml; final specific activity, 0.18 μCi/μmol) for 30 min. Incubations were stopped by three rapid washes on ice with phosphate-buffered saline, the cells were solubilized in 1 ml of 0.1 M NaOH, carrier glycogen was added (2 mg), and the samples were boiled for 30 min. Glycogen was precipitated with 70% ethanol overnight at −20 °C; the samples were centrifuged at 1720 × *g*, washed once with 70% ethanol, and resuspended in water; and radioactivity was determined by scintillation counting.

Glycogen Synthase Activity Assay—Assays were performed as described (25). In short, differentiated adipocytes in six-well plates were starved for 3 h in serum-free DMEM without glucose supplemented with 20 mM Hepes and 1% bovine serum albumin, incubated with hormone for 30 min, washed in 100 mM NaF-10 mM EDTA, scraped off, and sonicated. The enzyme activity was determined in a Tris (50 mM), NaF (25 mM), EDTA (20 mM) buffer with glycogen (10 mg/ml) and uridine-5′-diphospho-D-[U-^{14}C]glucose (5 μCi/ml; final specific activity, 1.3 μCi/mg) and in the absence or presence of glucose 6-phosphate (2 mg/ml) for 20 min at 30 °C. The samples were spotted on filter paper and washed three times in 70% ethanol and dried, and radioactivity was determined by liquid scintillation counting. Activity was calculated as a percentage of the maximum: that is, 100 × (activity without glucose 6-phosphate)/(activity with glucose 6-phosphate).

Phosphoinositide 3-Kinase Assay—Cells were stimulated for 5 min, lysed, and immunoprecipitated with either anti-IRS-1 (1:200) or anti-phosphotyrosine PY20 (1:50) antibody. The immunoprecipitates were washed and assayed as previously described (26). In short, the immunoprecipitates were incubated with phosphatidylinositol and [^{32}P]ATP for 15 min at 37 °C, and the reaction was stopped with chloroform: methanol (1:2). The lipids were extracted twice by acidic chloroform extraction, and the lower phase was collected and dried in a speed-vacuum drier. The lipid film was resuspended and spotted on TLC

plates (kieselgel 60F254 from Merck) and run in chloroform:methanol:ammonia:water (300:210:45:75 (v/v)). The resolved radiolabeled phosphatidylinositol 3-phosphate was quantified on a Fujix BAS2000 phosphorimager.

PKB Kinase Activity—The assay was performed as described (27). Cells were serum-starved overnight, stimulated with hormone for 5 min, and extracted in PKB lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM pyrophosphate, 0.27 M sucrose, 1 μM microcystin, 0.1% β-mercaptoethanol, protease inhibitors). The extracts (250 μg) were immunoprecipitated with PKB-β-specific antibody followed by PKB-α-specific antibody or PKB-γ-specific antibody (all at 5 μg/sample) and protein G-Sepharose (2 mg), and the precipitates were washed twice in lysis buffer with 0.5 M NaCl and once in Buffer A (50 mM Tris, 0.1 mM EGTA, 0.1% β-mercaptoethanol). The kinase activity was measured against cross-tide substrate in assay buffer (50 mM Tris, 0.1 mM EGTA, 2.5 μM PKI, 0.1 mM ATP, 10 mM $MgCl_2$, 300 μM cross-tide) with [^{32}P]ATP (0.3 μCi/sample) for 30 min at 30 °C. Incubations were stopped by spotting on p81 Whatman filter paper and washing in phosphoric acid (85 mM); the papers were washed three additional times, dipped in acetone, and dried; and radioactivity was determined by scintillation counting.

Phosphorylation of PKB, p70S6-Kinase, and MAP Kinase—The serine phosphorylation of PKB/Akt (α- and β-isoforms) after 5 min of stimulation was determined by Western blotting (as described above) with antibody specific for the phosphorylated (Ser-473) form of the enzyme (rabbit anti-phosphoAkt[Ser473] from Upstate Biotechnology, Inc.). The phosphorylation of MAPK was similarly determined by blotting with an antibody specific for the doubly phosphorylated (Thr/Tyr) form of the enzyme (rabbit anti-Active™ MAPK from Promega). The phosphorylation of p70S6-kinase after 15 min of stimulation was determined by immunoprecipitation of clarified lysates with goat-anti-p70S6-kinase (1 μg/sample) and protein G-agarose (20 μg/sample), followed by SDS-PAGE and Western blotting with an antibody specific for the phosphorylated form of the enzyme (rabbit anti-pp70S6-kinase[Thr-389] from New England Biolabs). Blotting data were quantified on a Fujix BAS2000 phosphorimager.

Induction of Fra-1 mRNA—Fully differentiated 3T3-L1 adipocytes were serum-starved overnight and stimulated for 4 h with hormone, and RNA was extracted with TriReagent (Sigma). Northern blots were performed as described (28), transferred to Hybond™ N membrane (Amersham Pharmacia Biotech), and cross-linked by UV irradiation. Probe for Fra-1 (generously provided by Dr. J. M. Tavaré, University of Bristol) was labeled according to the protocol with [^{32}P]CTP using Rediprime labeling system (Amersham Pharmacia Biotech) and purified on Nick™ columns (Amersham Pharmacia Biotech). The membranes were incubated in QuickHyb (Stratagene) for 1 h at 68 °C with the boiled probe and washed twice in 2× SSC (1× SSC: 0.15 M NaCl, 15 mM sodium citrate) with 0.1% SDS at room temperature and twice in 0.5× SSC with 0.1% SDS at 65 °C. The later samples were analyzed by dot blots after verification of the probe specificity on Northern blots. The results were quantified on a Fujix BAS2000 phosphorimager.

RESULTS

Expression of TIR and TIGR Constructs—Chimeric constructs containing the intracellular portions of the IR or IGFR together with the extracellular portion of the TrkC receptor (TIR and TIGR, respectively) were expressed in 3T3-L1 cells under the control of the elongation factor 1α promoter, which was found to drive similar levels of expression in both fibroblasts and adipocytes. Clonal lines of transfected fibroblasts were selected with G418 and screened for expression of chimeras by Western blotting cell extracts with anti-IR and anti-IGFR antibodies as appropriate, no suitable anti-TrkC antibody being available. Following differentiation into adipocytes, clones were rescreened by Western blotting of extracts with anti-phosphotyrosine antibodies after stimulation with NT-3 or insulin. Two clones were selected for further study that expressed similar levels of TIR and TIGR, as judged by relative intensity of NT-3-stimulated autophosphorylation in adipocytes (Fig. 1 and Table I). The levels of expression were reproducible on repeated differentiation of adipocytes from fibroblast stocks. The levels of expression of chimeras were approximately 2-fold (Table I) higher than those of endogenous insulin receptors, as judged by Western blotting with anti-IR and/or anti-phosphotyrosine antibodies after stimulation with NT-3 or insulin. By the same criteria, the levels of endogenous insulin receptors were identical in both clones. There was no evidence of cross-phosphorylation of chimeras following stimulation with insulin or of insulin receptors following stimulation with NT-3. Radioligand binding assays were carried out on the selected clones to verify that the chimeras bound NT-3 with an affinity comparable to the wild-type TrkC (Fig. 1*C*). There was no detectable binding of NT-3 to untransfected adipocytes.

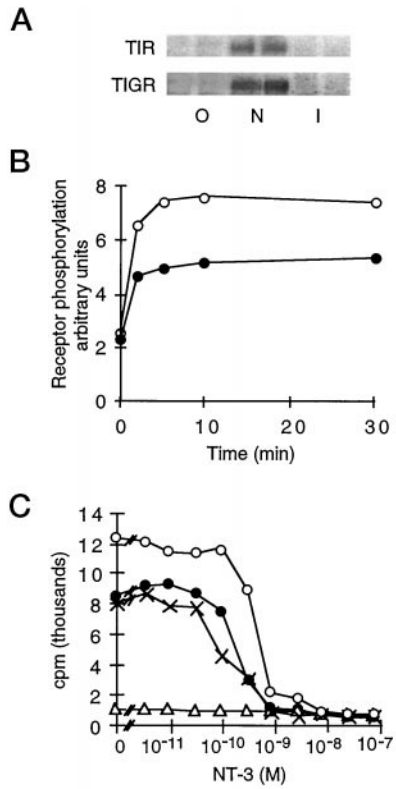

FIG. 1. **Expression of chimeras in 3T3-L1 adipocytes.** *A,* anti-phosphotyrosine Western blotting. Serum-starved cells were stimulated for 5 min with 4 nM NT-3 (*N*) or 10 nM insulin (*I*) or left unstimulated (*O*) and lysed, and 100 μg of protein from each lysate was subjected to SDS-PAGE in duplicate and immunoblotted with anti-phosphotyrosine antibody (4G10). *B,* time course of receptor autophosphorylation. Anti-phosphotyrosine blots obtained as in *A* were quantified by phosphorimaging. *C*, NT-3 binding. Cells grown in 24-well plates were incubated in duplicate with ^{125}I-NT-3 and the indicated concentrations of unlabeled NT-3 at 4 °C overnight and washed, and bound radioactivity was counted. *Filled circles,* TIR; *open circles,* TIGR; *triangles,* untransfected 3T3 L1 adipocytes; *crosses,* TrkC-transfected CHO cells (MG86). Estimated IC_{50} values are as follows: TIR, 0.14 nM; TIGR, 0.10 nM; TrkC, 0.14 nM.

TABLE I

Receptor levels in 3T3-L1 adipocytes of chimeric expressing clones

Lysates from clonal cell lines that had been stimulated with 10 nM insulin or 4 nM NT-3, containing equivalent amounts of protein, were subjected to SDS-PAGE and Western blotting.

Cell line	Phospho-IR levels[a]	Phospho-chimera levels[b]	IR levels[c]
TIR	0.7	1	0.8
TIGR	0.7	1.7	

[a] Insulin receptor phosphorylation in response to insulin relative to TIR phosphorylation in response to NT-3, assessed by Western blotting with anti-phosphotyrosine antibody.

[b] Chimera phosphorylation in response to NT-3 relative to TIR phosphorylation levels, assessed by Western blotting with anti-phosphotyrosine antibody.

[c] Insulin receptor levels relative to TIR levels, assessed by Western blotting with insulin receptor-specific antibody. Data shown are mean values from three independent determinations in all cases.

Metabolic Responses in 3T3-L1 Adipocytes—Previous work on 3T3-L1 fibroblasts had shown that TIR mediated a greater stimulation of glycogen synthesis than TIGR, although the magnitude of response to NT-3 or insulin in these cells was relatively small (14). The present studies were conducted with an independently derived set of clones, in which expression of chimeras was under the control of a different promoter from that used previously. We tested metabolic responses following stimulation of TrkC chimeras in 3T3-L1 adipocytes, which express the GLUT4 glucose transporter and are much more insulin-responsive than fibroblasts.

Glucose uptake was stimulated similarly (typically 6–8-fold) by 10 nM-insulin in both clones of transfected cells. However, when cells were treated with NT-3, stimulation of glucose uptake was consistently 2-fold greater via TIR than via TIGR, at all concentrations of NT-3 (Fig. 2*A*). There was no detectable response to NT-3 in untransfected cells.

To confirm that the stimulation of glucose uptake by the two chimeras reflected primarily the translocation of GLUT4 glucose transporters to the plasma membrane, we performed plasma membrane lawn assays. Plasma membrane lawns from disrupted cells were probed with anti-GLUT4 or GLUT1 antibody followed by immunofluorescence detection (Fig. 3). In all cell lines (TIR, TIGR, and untransfected), insulin induced 4–5-fold increases in GLUT4 immunostaining, but only 1.5–2-fold increases in GLUT1 immunostaining. In agreement with the results on glucose uptake, the TIR chimera mediated a significantly greater increase in GLUT4 at the plasma membrane than the TIGR chimera (Fig. 3*B*). There was no effect of NT-3 on GLUT4 distribution in untransfected cells lacking chimera. Similar trends were apparent for the translocation of GLUT1 in TIR and TIGR cells, although the small magnitude of stimulations made it difficult to draw clear conclusions in this case.

Incorporation of glucose into glycogen was stimulated approximately 15-fold by insulin in both clones. Maximum stimulation of glycogen synthesis and glycogen synthase by 4 nM NT-3 was similar in TIR and TIGR cells (Fig. 2, *B* and *C*), but TIR cells responded significantly better than TIGR at submaximal NT-3 concentrations (Fig. 2*B*). Again, stimulation of glycogen synthesis by NT-3 was undetectable in untransfected cells. The fact that the difference in maximum stimulation of glucose uptake by NT-3 in TIR and TIGR cells was not reflected in differential maximal stimulation of glucose incorporation into glycogen suggests that glycogen synthase activity, rather than glucose transport, was rate-limiting for glycogen synthesis under these conditions. Consistent with this hypothesis, glycogen synthesis was near maximally stimulated at concentrations of NT-3 that produced little stimulation of glucose uptake. Moreover, glycogen synthesis was similarly more sensitive than glucose uptake to stimulation by insulin (data not shown).

Phosphorylation of Intracellular Substrates—The initial step in signal transduction from the IR and IGFR is the tyrosine phosphorylation of intracellular substrates, such as IRS and Shc proteins, which then recruit other signaling intermediates, such as PI 3-kinase and Grb2·Sos, respectively (4, 29). Substantial evidence implicates the PI 3-kinase pathway as an essential component of signaling to glucose transport and glycogen synthesis (30), whereas Grb2·Sos is required for activation of the MAP kinase pathway (7). To examine whether the differences in metabolic responses mediated by TIR and TIGR in 3T3-L1 adipocytes were a consequence of differential activation of known signaling pathways, we used sequential immunoprecipitation and Western blotting to determine the tyrosine phosphorylation of IRS-1 and Shc and the association of these proteins with the p85 regulatory subunit of PI 3-kinase and Grb2, respectively (Fig. 4). Data were normalized by expression as a percentage of signals obtained following stimulation with 10 nM insulin, which produced similar responses in both clonal lines.

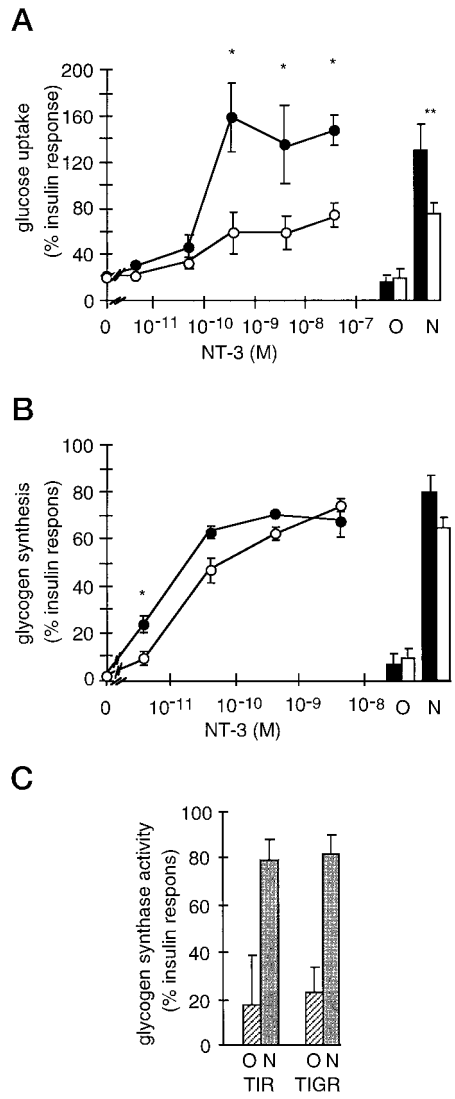

FIG. 2. **Stimulation of glucose uptake and glycogen synthesis.** *A,* 2-deoxy-glucose uptake. Cells grown in six-well plates were serum-starved for 2 h and stimulated for 30 min with the indicated concentrations of NT-3 (*O,* unstimulated; *N,* 4 nM NT-3) before measurement of 2-deoxyglucose uptake over 5 min as described under "Experimental Procedures." Data are means ± SD from three (dose-response) or five (inserted bars) independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin (mean insulin responses, 3635 cpm/well for TIR and 4265 cpm/well for TIGR). *Filled circles* and *filled columns,* TIR; *open circles* and *open columns,* TIGR (*$p < 0.05$, TIR *versus* TIGR). *B,* glycogen synthesis. Cells grown in six-well plates were serum-starved for 3 h and stimulated for 30 min with the indicated concentrations of NT-3 (*O,* unstimulated; *N,* 4 nM NT-3) before measurement of [^{14}C]glucose incorporation into glycogen over 90 min as described under "Experimental Procedures." Data are means ± SD from three (*data points*) or five (*columns*) independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin (mean insulin responses, 18,450 cpm/well for TIR and 18,900 cpm/well for TIGR). Symbols as in *A. C,* glycogen synthase activity. Cells grown in six-well plates were serum-starved for 3 h and stimulated for 30 min without (*hatched columns*) or with (*gray columns*) NT-3 (4 nM) before measurement of glycogen synthase fractional activity (with or without glucose 6-phosphate) as described under "Experimental Procedures." Data are means ± SD from six (TIR) or three (TIGR) independent experiments performed in duplicate, normalized to activity with 10 nM-insulin (mean insulin responses, 11.7% in TIR and 9.85% in TIGR). Differences between values for TIR and corresponding values for TIGR were not significant ($p > 0.1$).

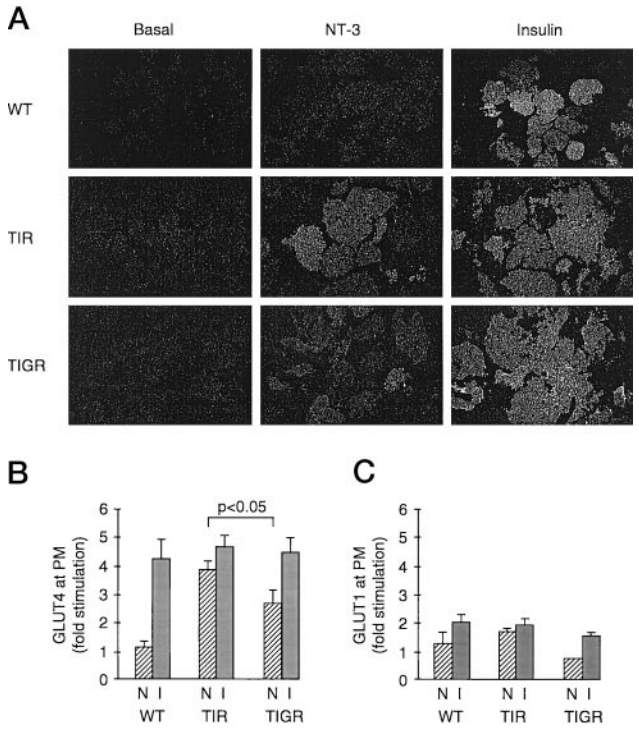

FIG. 3. **Plasma membrane lawn assay of GLUT4 and GLUT1 translocation.** Serum-starved 3T3-L1 adipocytes were either left untreated (*O*) or stimulated with 100 nM insulin or 4 nM NT-3 (*N*) for 15 min before preparation of plasma membrane lawns and assay of glucose transporter translocation. Data from each experiment, utilizing 50 fields for each condition, were quantified as described under "Experimental Procedures." *A,* representative images from a typical experiment. *B,* GLUT4 at plasma membrane (*PM*), calculated as mean ± S.E. fold increase over basal for three (wild-type untransfected 3T3-L1 adipocytes (*WT*)), five (TIR), and four (TIGR) independent experiments. *C,* GLUT1 at plasma membrane, calculated as mean ± S.E. fold increase over basal for three independent experiments. Statistically significant differences between responses to NT-3 in TIR and TIGR cells are indicated.

The level of IRS-1 tyrosine phosphorylation induced by NT-3 after 5 min was approximately 2-fold higher in TIR cells compared with TIGR, at all concentrations (Fig. 4*A*). It was confirmed by stripping and reprobing blots with anti-IRS antibody that equal amounts of IRS-1 were immunoprecipitated from TIR and TIGR cells. The level of p85 in the same IRS-1 immunoprecipitates was higher in TIR cells than TIGR, although the basal was also higher (Fig. 4*B*). The differences in stimulation of IRS-1 phosphorylation and p85 association were maintained throughout the 30-min period used to measure glucose uptake (Fig. 5). Thus, the relative NT-3-induced IRS-1 phosphorylation and p85 association mediated by TIR and TIGR closely paralleled stimulation of glucose uptake.

In contrast, NT-3-induced tyrosine phosphorylation of the 52-kDa isoform of Shc, and its association with Grb2, were each approximately 2.5-fold higher in TIGR cells compared with TIR (Fig. 4, *C* and *D*). Weak stimulation of phosphorylation of the 66-kDa isoform of Shc was seen in TIGR but not TIR cells (Fig. 4*C*).

Activation of Phosphoinositide 3-Kinase—We next examined whether differences in p85 association with IRS-1 were paralleled by differences in PI 3-kinase activity in anti-IRS-1, anti-phosphotyrosine, and anti-p85 immunoprecipitates. There was no stimulation of PI 3-kinase activity by NT-3 in anti-IRS-1 or anti-phosphotyrosine immunoprecipitates in untransfected cells. Activity of PI 3-kinase in anti-IRS-1 immunoprecipitates was approximately 70% greater in TIR cells than in TIGR cells following NT-3 stimulation (Fig. 6*A*). As with the other differential responses, the maximum stimulation by NT-3 was somewhat greater than that with 10 nM insulin in TIR cells, but appreciably less in TIGR cells. No significant difference was observed in the PI 3-kinase activities in anti-phosphotyrosine precipitates from NT-3-stimulated TIR and TIGR cells (Fig. 6*B*). The maximum activity in response to NT-3 was very similar to the response to 10 nM insulin in both cell lines and approximately 30% of the maximum PI 3-kinase activity observed in anti-IRS-1 immunoprecipitates. As previously observed (26), PI 3-kinase activity in anti-p85 precipitates was not significantly stimulated by insulin, nor was it stimulated by NT-3, but the activities measured were the same in TIR and TIGR cells (data not shown). Only approximately 20% of the p85 precipitable PI 3-kinase activity was recovered in IRS-1 precipitates from insulin-stimulated TIGR cells.

Activation of Akt/PKB—Recent evidence has indicated that signaling downstream from PI 3-kinase involves translocation and/or activation of one or more phosphoinositide-dependent kinases, which in turn phosphorylate and activate Akt/PKB, a serine/threonine-specific protein kinase that exists in three isoforms α, β, and γ (6). The three isoforms were present at similar levels in 3T3-L1 adipocytes, as reflected in the basal or insulin-stimulated kinase activity measured in specific immunoprecipitates (Fig. 7). In both TIR and TIGR-expressing cells, NT-3 (4 nM) induced levels of kinase activity of all three isoforms that were approximately the same as those induced by insulin (10 nM) (all 2–3 fold over basal), with no significant difference between the two chimeras. We also assessed the phosphorylation of Ser-473 of PKB (α- and β-isoforms) by Western blotting with phosphopeptide-specific antibody, as an index of stimulation (6). Again, NT-3 and insulin induced similar levels of phosphorylation (6-fold over basal), with no significant difference between TIR or TIGR cells at either 5 or 30 min of stimulation (Fig. 8*A*).

Phosphorylation of p70S6 Kinase—Activation of p70S6 kinase is, like the activation of PKB, dependent on PI 3-kinase (30). Stimulation of glucose transport by insulin is clearly independent of p70S6 kinase activity, but the enzyme may play a role in the activation of glycogen synthase in some cell types including 3T3-L1 adipocytes (31). Activation of p70S6 kinase by growth factors is associated with phosphorylation at multiple sites, of which Thr-412 in the mouse enzyme (corresponding to Thr-389 in the human enzyme) correlates most closely with activity (32). We used a phospho-specific antibody to determine phosphorylation of p70S6 kinase in TIR or TIGR cells after 15 min of stimulation with NT-3 or insulin (Fig. 8*B*). The two chimeras were equally effective in mediating p70S6 kinase phosphorylation, at both submaximal and maximal concentrations of NT-3, and the stimulation at the higher concentration of NT-3 was very similar to that induced by insulin.

Phosphorylation of MAP Kinase—Tyrosine phosphorylation of Shc and its association with the Grb2·Sos complex is a major pathway leading via Ras, Raf, and MAPK/Erk kinase to the phosphorylation and activation of MAP kinase (7). This pathway has been implicated in signaling to transcriptional and mitogenic events, but it appears not to be important in regulating acute metabolic responses (7). We investigated the activity of MAP kinase by immunoblotting cell extracts with an antibody specific for the doubly (Thr/Tyr) phosphorylated and hence activated form of the enzyme (33). In 3T3-L1 cells, the p42 isoform of MAP kinase was more abundant than p44 isoform, as judged by immunoblotting with a pan-specific anti-MAP kinase antibody, or with anti-phospho-MAP kinase in stimulated cells (data not shown). Phosphorylation of $p42^{MAPK}$ in response to a maximally effective concentration of NT-3 (4 nM) was consistently and significantly greater in TIGR cells

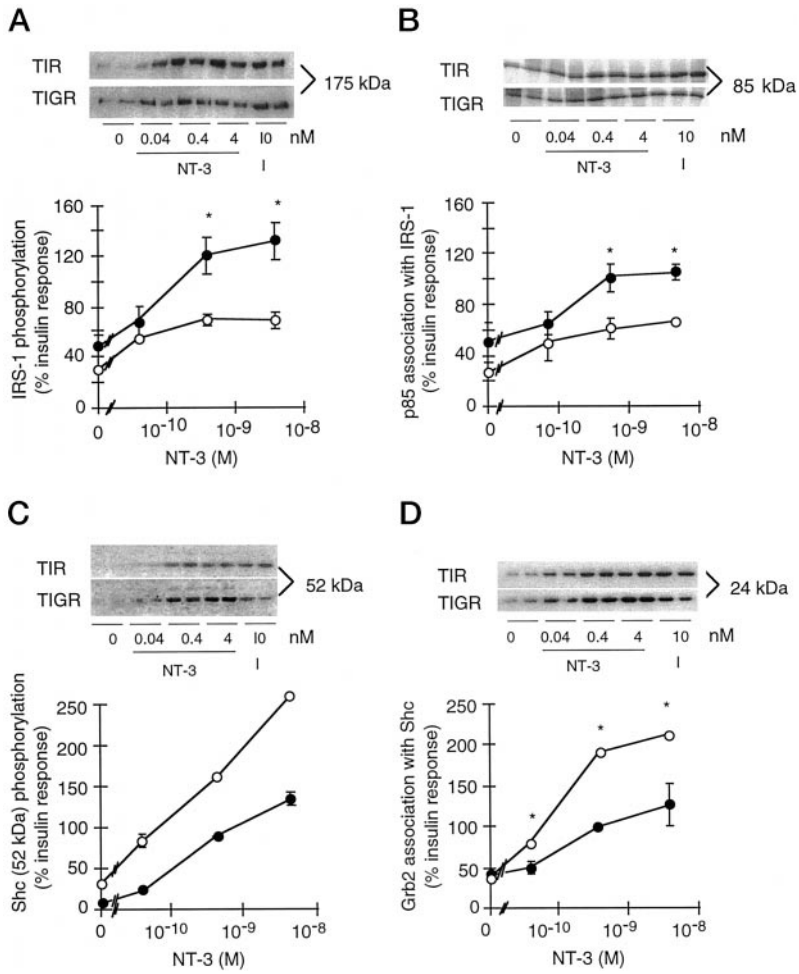

FIG. 4. **Phosphorylation and SH2-association of IRS-1 and Shc.** *A,* IRS-1 tyrosine phosphorylation. Serum-starved cells were stimulated for 5 min with NT-3 or insulin as indicated and lysed, and 500 μg of protein was immunoprecipitated with anti-IRS-1 antibody and subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. A representative gel is shown with each data set, and numerical data shown are means ± SD obtained by quantification of gels from three independent experiments performed in duplicate, normalized to stimulation with 10 nM insulin (mean insulin responses, 110 arbitrary phosphorimager units for TIR and 145 units for TIGR). *Filled circles,* TIR; *open circles,* TIGR; $^*p < 0.05$, TIR *versus* TIGR. *B,* IRS-1 association with p85 regulatory subunit of PI 3-kinase. Lysates from stimulated cells were prepared, immunoprecipitated and electrophoresed as in *A* before immunoblotting with anti-p85 antibody. Data presentation is as in *A* (mean insulin responses, 320 arbitrary phosphorimager units in TIR and 400 units in TIGR). *C,* Shc tyrosine phosphorylation. Lysates from stimulated cells were prepared as in *A*, immunoprecipitated with anti-Shc antibody, and electrophoresed and immunoblotted with anti-phosphotyrosine antibody. Data presentation is as in *A* (mean insulin responses, 174 arbitrary phosphorimager units in TIR and 144 units in TIGR). *D,* Shc association with Grb2. Lysates from stimulated cells were prepared, immunoprecipitated, and electrophoresed as in *C* before immunoblotting with anti-Grb2 antibody. Data presentation is as in *A* (mean insulin responses, 515 arbitrary phosphorimager units in TIR and 450 units in TIGR).

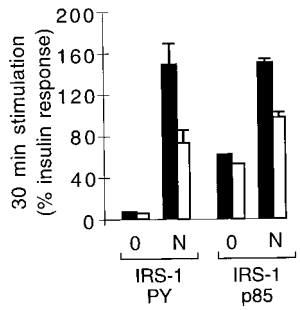

FIG. 5. **Persistence of IRS-1 phosphorylation and p85 association.** IRS-1 tyrosine phosphorylation and association with p85 were determined after 30 min of incubation without (*0*) or with (*N*) 4 nM NT-3, as described in legend to Fig. 4. Data were calculated relative to the effect of 10 nM insulin for three independent experiments, as in Fig. 4. *Filled columns*, TIR; *open columns*, TIGR.

than in TIR cells (Fig. 8*C*). The response to NT-3 in TIGR cells was also greater than the response to insulin. Similar differences were evident with $p44^{MAPK}$ (data not shown).

Induction of Fra-1 mRNA—Fos-related antigen 1 (Fra-1) is a nontransforming Fos analogue involved in G_0 to G_1 transition and asynchronous cell growth. Fra-1 mRNA is induced by insulin or serum (34). Because measurement of mitogenesis/DNA synthesis is not meaningful in terminally differentiated 3T3-L1 cells, which do not divide, we used induction of Fra-1 mRNA as an alternative end point readout of the MAPK pathway. We found no difference between TIR or TIGR cells, both of which showed 4–5-fold induction of Fra-1 mRNA after 4 h of incubation with 4 nM NT-3, similar to the response to 10 nM insulin (data not shown).

DISCUSSION

Although insulin and IGFs exert distinct physiological effects *in vivo*, the contribution of differences in receptor function, as opposed to receptor distribution, to this specificity remains unclear. Functional differences might arise from the kinetics of ligand association and dissociation, affecting the lifetime and/or intracellular itinerary of the activated receptors (35, 36). Alternatively, the receptor intracellular domains

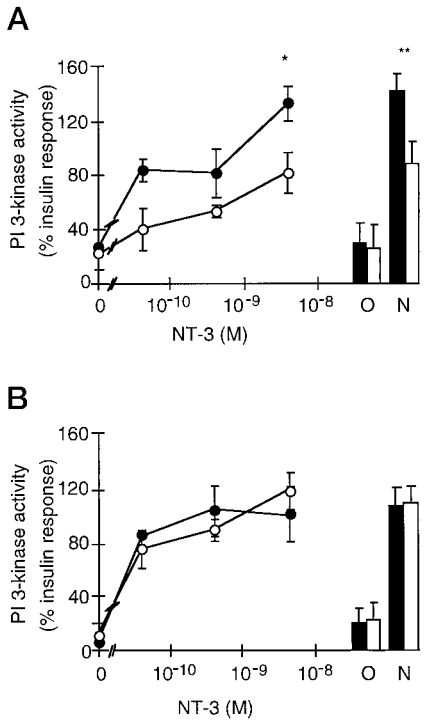

FIG. 6. **Activation of PI 3-kinase.** *A,* PI 3-kinase activity in anti-IRS-1 immunoprecipitates. Serum-starved cells were stimulated for 5 min with NT-3 as indicated (*O,* unstimulated; *N,* 4 nM NT-3) and lysed, and 100 μg of protein was immunoprecipitated with anti-IRS-1 antibody before measurement of PI 3-kinase activity as described under "Experimental Procedures." Data are means ± range/SD from two (*data points*) or four (*columns*) independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin (mean insulin responses, 1130 arbitrary phosphorimager units in TIR and 1280 units in TIGR). *Filled circles* and *filled columns,* TIR; *open circles* and *open columns,* TIGR; (*$p < 0.05$, TIR *versus* TIGR). *B,* PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. Cells were treated and extracted as in *A* except that immunoprecipitation was carried out with anti-phosphotyrosine antibody prior to determination of PI 3-kinase activity. Data presentation and symbols are as in *A* (mean insulin responses, 450 arbitrary phosphorimager units in TIR and 390 units in TIGR).

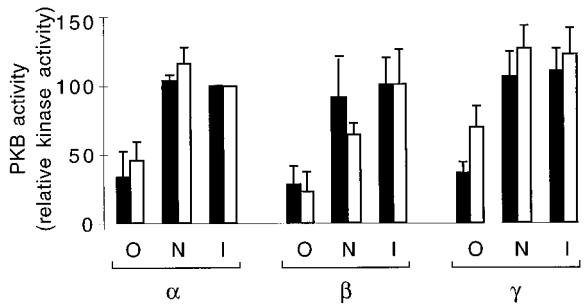

FIG. 7. **Activation of protein kinase B.** Serum-starved cells were stimulated for 5 min with 4 nM NT-3 (*N*) or 10 nM insulin (*I*) or left unstimulated (*O*) and lysed, and 200 μg of protein from each lysate was immunoprecipitated with antibodies specific for the α-, β-, or γ-isoform of PKB before assaying PKB activity as described under "Experimental Procedures." Data are means ± SD from three independent experiments performed in duplicate, normalized to stimulation of the α-isoform with 10 nM-insulin (α, 494 cpm/mg protein in TIR and 398 in TIGR). *Filled columns,* TIR; *open columns,* TIGR.

might possess different signaling capacities, reflecting their differences in primary sequence (nonidentity, 16% in the tyrosine kinase domain, 39% in the juxtamembrane domain, and 56% in the carboxyl-terminal domain). To focus on the activity of intracellular domains without interference from endogenous receptors and to allow examination of a range of physiologically

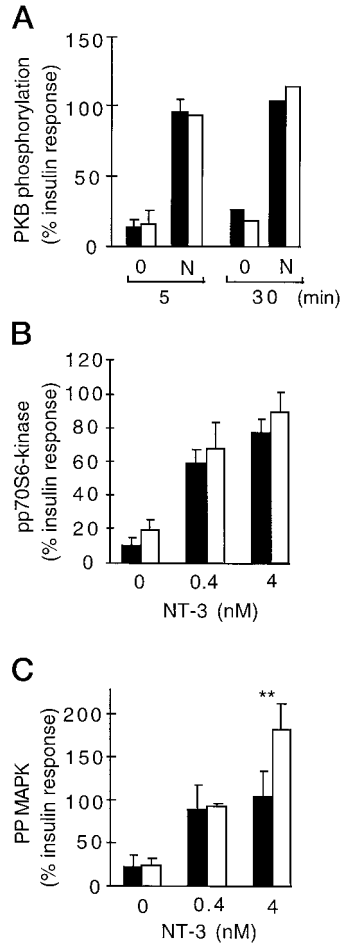

FIG. 8. **Phosphorylation of PKB, p70S6 kinase, and MAP kinase.** *A,* phosphorylation of PKB on Ser-473. Serum-starved cells were stimulated for 5 or 30 min as in Fig. 7, and lysates were subjected to SDS-PAGE and immunoblotted with antibody specific for the phospho-S473 form of PKB (isoforms α and β). Data are means ± SD from three independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin. *B,* phosphorylation of p70S6-kinase on Thr-389. Serum-starved cells were stimulated for 15 min as indicated, and lysates were immunoprecipitated with anti-p70S6-kinase antibody, subjected to SDS-PAGE, and immunoblotted with antibody specific for the phospho-Thr 389 form of the p70S6 kinase. Data are means ± SD from four independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin. *C,* phosphorylation of 42-kDa MAPK. Serum-starved cells were stimulated for 5 min with NT-3 as indicated, and lysates were subjected to SDS-PAGE and immunoblotted with antibody specific for the doubly (Thr/Tyr) phosphorylated form of MAPK. Data are mean ± range from two independent experiments performed in duplicate, normalized to stimulation with 10 nM-insulin (**$p < 0.005$ TIGR *versus* TIR). In all panels, *filled columns* represent TIR, and *open columns* represent TIGR.

relevant metabolic responses, we expressed TrkC-IR (TIR) or TrkC-IGFR (TIGR) chimeras in 3T3-L1 adipocytes. Signaling capacities of the IR and IGFR have previously been compared only in transfected fibroblasts, in which reaction of ligands with endogenous as well as transfected receptors complicates interpretation of data, and the spectrum and magnitude of metabolic responses are rather small; the results of such studies have been inconsistent. For instance, it was reported that the IGFR was more effective than the IR in stimulating DNA synthesis and MAP kinase activity (12, 37), although in other work, no such difference was observed (14, 38). The IR appeared to be more effective than IGFR in phosphorylating IRS-1 and activating PI 3-kinase in one study (38) but not in another (39).

We found that stimulation of glucose uptake and GLUT4

translocation in 3T3-L1 adipocytes was more effectively mediated by TIR than TIGR (Figs. 2 and 3). TIR also mediated greater stimulation of IRS-1 phosphorylation and IRS-1-associated PI 3-kinase activity at both early and late time points (Figs. 4–6). These differences were consistently observed when paired clones of 3T3-L1 fibroblasts, selected for similar levels of expression of TIR and TIGR, were differentiated on multiple occasions. The differences were not a consequence of unequal or excessive expression of the respective chimeras, as if anything, the TIGR was expressed at a slightly higher level than TIR in the differentiated adipocytes, and the level of expression of both chimeras was of the same order as that of endogenous insulin receptors. We also rule out the possibility that the different responses of TIR and TIGR cells to NT-3 reflect chance clonal variation, as insulin acting via its own receptor elicited very similar responses in both sets of cells. Moreover, phosphorylation of Shc showed the opposite relationship to phosphorylation of IRS-1 in the same cells, being more effectively induced via the TIGR than via TIR (Fig. 4).

The more effective stimulation of glucose uptake via the IR than the IGFR chimera was manifest as a difference in maximum response rather than half-maximally effective concentration of NT-3 (EC_{50} approximately 0.2 nM), although the dose-response relationships were not defined with sufficient precision to rule out a difference in sensitivity. It is generally believed that glucose uptake reflects the number of GLUT4 transporters at the plasma membrane (29), and we showed that the translocation of GLUT4 was indeed greater in response to stimulation of TIR than TIGR (Fig. 3). This result implies that the IR signals to a larger intracellular pool of transporters than the IGFR, which might reflect differential efficiency of mobilization from a single pool of transporters, or differential recruitment from more than one pool. In relation to the latter possibility, it has been shown that GLUT4 vesicles cycle through several distinct intracellular compartments in adipocytes (40), and in skeletal muscle, discrete pools of GLUT4 vesicles are responsive to stimulation by insulin and contraction (41).

There is ample evidence that PI 3-kinase activity is both necessary and sufficient for stimulation of glucose transport (29, 30). Differences in glucose uptake and GLUT4 translocation mediated by TIR and TIGR were paralleled by greater IRS-1-associated PI 3-kinase activity, and the stimulations of glucose uptake and IRS-1 phosphorylation showed similar NT-3 concentration dependence (EC_{50} approximately 0.2 nM) (Figs. 2–4). In contrast, TIR and TIGR induced similar PI 3-kinase activity measured in anti-phosphotyrosine precipitates, and this activity was increased at very low concentrations of NT-3 (<0.04 nM). Our finding that relative stimulations of glucose transport correlate better with IRS-associated than with anti-phosphotyrosine-precipitable PI 3-kinase activity are at variance with studies suggesting that IRS-1-associated PI 3-kinase was not essential for glucose transport stimulation (42, 43).

The serine-specific protein kinase PKB/Akt has been identified as a downstream effector of PI 3-kinase, which is activated by phosphoinositide-dependent kinases secondarily to recruitment of both PKB and phosphoinositide-dependent kinase to membranes via association with the PI 3-kinase product phosphatidylinositol 3,4,5 trisphosphate (6). Studies of the role of PKB in stimulation of glucose transport have produced conflicting data. On the one hand, glucose uptake was increased in 3T3-L1 cells expressing constitutively active or inducible PKB (44–47). However, expression of a dominant negative PKB failed to block insulin-stimulated glucose uptake (48, 49). We found no difference in levels of PKB activity in isoform-specific immunoprecipitates or in phosphorylation of serine 473 at early and late time points, in TIR and TIGR cells stimulated with NT-3 (Figs. 7 and 8), despite the substantial difference in IRS-1-associated p85 and PI 3-kinase activity at the same NT-3 concentration. It may be that a small activation of PI 3-kinase is sufficient for maximum activation of PKB or that the IRS-1-associated pool of PI 3-kinase does not have as much influence on PKB activity as the anti-phosphotyrosine-precipitable pool. Regardless of the relationship between PKB and PI 3-kinase activities, our data suggest that PKB activity alone is not the sole determinant of stimulated glucose uptake, as identical PKB activities were observed in TIR and TIGR cells exhibiting significantly different stimulations of glucose uptake.

In contrast to the stimulation of glucose transport, incorporation of glucose into glycogen was stimulated to the same maximum by NT-3 in TIR and TIGR cells, although TIR cells were somewhat more responsive at low NT-3 concentrations (EC_{50} values in the range of 0.01–0.03 nM) (Fig. 2). The disparity in NT-3 concentration dependence and fold stimulation of glycogen synthesis compared with glucose uptake suggests that the stimulation of synthesis reflected activation of glycogen synthase rather than increased glucose uptake. The similar effectiveness of IR and IGFR in stimulating glycogen synthesis in 3T3-L1 adipocytes contrasts with the substantial difference observed previously in 3T3-L1 fibroblasts (14). However, it has been reported that the activation of glycogen synthase involves predominantly inhibition of glycogen synthase kinase-3 in 3T3-L1 fibroblasts but stimulation of PP1 in adipocytes (50), and it may be that this difference in mechanism underlies the difference in responsiveness to IR and IGFR in the two cell types. Like the stimulation of glucose uptake, activation of glycogen synthase requires PI 3-kinase (30). In terms of NT-3 concentration dependence and relative effectiveness of IR and IGFR, stimulation of glycogen synthesis correlates better with anti-phosphotyrosine-precipitable than IRS-1-associated PI 3-kinase activity, raising the possibility that separate pools of PI 3-kinase are involved in stimulation of glucose uptake and glycogen synthase. Certainly, the signaling pathways to GLUT4 translocation and glycogen synthase activation appear to diverge downstream of PI 3-kinase. PKB has been proposed as the principal route to inactivation of glycogen synthase kinase-3, which in turn is thought to be the major mechanism of activation of glycogen synthase in many tissues (51). However, in 3T3-L1 adipocytes, levels of glycogen synthase kinase-3 are low, and glycogen synthesis is not activated by constitutively active PKB (44, 46). There is evidence that p70S6 kinase contributes to activation of glycogen synthase in 3T3-L1 adipocytes (31). Consistent with the similar stimulations of glycogen synthesis mediated by TIR and TIGR, we found no difference between the chimeras with respect to the activation of p70S6 kinase.

It seems clear, therefore, that the contribution of different signaling pathways to end point responses may vary not only with respect to different responses in a given cell but also with respect to a given response in different cell types. Against this background, it is difficult to predict relative efficiency of IR and IGFR in mediating metabolic effects in other tissues based on the present data for glucose transport in 3T3-L1 adipocytes. Interestingly, in hepatocytes, as in 3T3-L1 fibroblasts, glycogen synthesis was more effectively stimulated via IR than IGFR, although both receptors mediated similar activation of PKB (52). The insulin-specific stimulation of glycogen synthesis in hepatocytes appeared to involve a rapamycin-sensitive pathway.

It is not meaningful to study classical mitogenic responses (proliferation/DNA synthesis) in a terminally differentiated and nondividing cell line such as the 3T3-L1 adipocyte. How-

ever, we examined various components of the MAP kinase pathway that have been implicated in transcriptional and mitogenic responses in other cells (7). TIGR cells were more responsive than TIR with regard to stimulation of Shc phosphorylation, association of Shc with Grb2, and activation of MAP kinase (Figs. 4 and 8). No difference was observed in the induction of Fra1 mRNA in TIR and TIGR cells stimulated by a near-maximal concentration of NT-3, although it is possible that relatively modest activation of MAP kinase is sufficient for maximal Fra1 induction and that a differential effect of TIGR and TIR on Fra1 mRNA might have been observed at lower NT-3 concentrations. Because TIGR was expressed at a slightly higher level than TIR in the cell lines studied, it cannot be definitely concluded that the IGFR is more efficient than the IR in phosphorylating Shc. However, the relative Shc responses do provide excellent controls for the IRS-1 phosphorylation measured in the same cells, and the ratio of IRS/Shc phosphorylation was approximately 4-fold higher in TIR cells than in TIGR cells across the full range of NT-3 concentrations tested.

Various mechanisms might contribute to relative specificity in substrate phosphorylation by the IR and IGFR, including differences in the intrinsic specificity of the tyrosine kinases themselves (53, 54), differences in the association of substrates with receptors via PTB or other domains (55–58), the modulation of such interactions by receptor-specific adaptor proteins, such as Grb10 (59, 60), or differences in endocytosis and subsequent trafficking of receptors (61, 62). No major differences between the two receptor kinases were apparent in the phosphorylation of recombinant IRS-1 *in vitro* (39), although this result does not exclude possible differences in activity of receptors toward IRS-1 in intact cells, where receptors and IRS-1 may be subject to regulatory phosphorylation on serine residues or modulation by other proteins. The carboxyl-terminal domains of the IR and IGFR have been implicated in signaling specificity (18–20), although the influence of this region must be relatively subtle, as carboxyl-terminally truncated IR and IGFR signal normally in at least some cell types (63, 64). Surprisingly, the insulin receptor-related receptor, which is more similar to IGFR than to IR in its carboxyl-terminal domain, signaled with similar efficiency to the IR in fibroblasts and in 3T3-L1 cells (13). Further studies on chimeric constructs involving exchange or mutagenesis of carboxyl-terminal or other subdomains may help to define the residues responsible for IR and IGFR signaling specificity.

In conclusion, our data suggest that the IR and IGFR activate a common pool of PI 3-kinase that is coupled to activation of PKB and p70S6 kinase and to translocation of some GLUT4 glucose transporters. We propose that the IR, but not the IGFR, activates an additional pool of PI 3-kinase, which may be IRS-1-associated, and thereby mobilizes additional GLUT4 transporters, resulting in greater stimulation of glucose uptake. The biochemical basis of these putative discrete pools of PI 3-kinase and their specificity in coupling to downstream responses remains to be determined.

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Birgitte Ursø, Diane L. Cope, Heidi E. Kalloo-Hosein, Amanda C. Hayward, Jon P. Whitehead, Stephen O'Rahilly and Kenneth Siddle

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