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Differential Signaling to Glycogen Synthesis by the Intracellular Domain of the Insulin *versus* the Insulin-like Growth Factor-1 Receptor

EVIDENCE FROM STUDIES OF TrkC-CHIMERAS*

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Insulin and insulin-like growth factor-1 (IGF-1) have similar cell-surface receptors yet subserve different physiological functions. To examine whether these differences relate to intrinsic signaling properties of the intracellular domains of their respective receptors, chimeric receptors were constructed using the extracellular domain of the neurotrophin-3 (NT-3) receptor, TrkC, and the intracellular domain of either the insulin receptor or the IGF-1 receptor. TrkC-IR (TIR) and TrkC-IGF-1R (TIGR) were stably expressed in 3T3-L1 cells. While TIR and TIGR cell lines expressing similar numbers of chimeric receptors showed a similar dose-response relationship in NT-3 stimulated DNA synthesis, NT-3 stimulated glycogen synthesis was greater in TIR than in TIGR cells (maximum TIGR response was 35% of maximum TIR response). Additionally, the concentration of NT-3 at which significant stimulation of glycogen synthesis was seen was 0.1 ng/ml in TIR and 1 ng/ml in TIGR cells. Basal levels of thymidine incorporation but not glycogen synthesis were consistently higher in TIR than in TIGR expressing cells. No detectable basal autophosphorylation of chimeric receptors was seen in any of the cell lines. However, exposure of cell lines to the phosphatase inhibitor bisperoxovanadate resulted in greater basal autophosphorylation of the TIR and endogenous murine IR than the TIGR and endogenous murine IGF-1R. Thus, in this system, the intracellular domain of the IR appears to couple more effectively to glycogen synthesis than that of the IGF-1R, whereas the intracellular domains of both receptors have a similar capacity to stimulate DNA synthesis.

Insulin and insulin-like growth factor-1 (IGF-1)[1] are homologous peptides that are essential for normal metabolism and growth regulation. In the intact organism these hormones have significantly different physiological roles (for review, see Refs. 1–3). Insulin primarily functions as a regulator of carbohydrate, fat, and protein metabolism. In contrast, IGF-1 is largely a mediator of cell growth and differentiation primarily stimulating DNA synthesis and mitogenic events (4–6). Despite these different functions, the cell surface receptors for insulin and IGF-1 (IR and IGF-1R, respectively) are highly homologous. They are both Type II tyrosine kinase receptors with the same disulfide-linked heterotetrameric structure consisting of two extracellular α-subunits containing the ligand-binding domain and two transmembrane β-subunits with the ligand-sensitive tyrosine kinase activity (7). The greatest homology (over 80%) is found in the tyrosine kinase domain while differences in the ligand-binding domain (overall homology 44–60%) account for ligand specificity. The greatest sequence divergence between the two receptors is found in their C termini (44% identity) (8).

In addition to the similarity of receptor structure the intracellular signaling events which result from ligand-induced receptor activation are remarkably similar (9). Both receptors phosphorylate insulin-receptor substrates IRS-1 (10–13), IRS-2 (14, 15), and Shc (16, 17) on tyrosine residues with the subsequent activation of a wide range of signaling molecules including those of the Ras-Raf-MAP kinase network (18–21) and phosphatidylinositol 3-kinase (22–24). It is widely assumed that the IR transduces a metabolic signal and the IGF-1 receptor a mitogenic one. However, it has been shown that both receptors are able to transduce both mitogenic and metabolic signals. IGF-1 has been shown to regulate glycogen metabolism in human hepatoma cells (25) and glucose transport in muscle (26) via the IGF-1R, while insulin can stimulate cell growth through its receptor in human fibroblasts (27) and Chinese hamster ovary (CHO) cells (28). Insulin has also been found to transduce a mitogenic signal through its own receptor when overexpressed in NIH 3T3 or CHO cells (29–31).

Both *in vitro* and *ex vivo* approaches have been taken to examine the issue of signaling specificity of the IR *versus* the IGF-1R. The former studies have examined the catalytic activity of semipurified IRs *versus* IGF-1Rs toward different peptide substrates and have suggested that consistent differences may exist (32, 33). *Ex vivo* approaches have either compared metabolic *versus* mitogenic effects in cell lines overexpressing the IR *versus* those overexpressing the IGF-1R (34–37) or have studied the signaling properties of chimeric receptors composed of parts of the IR and IGF-1R (38–40).

Issues such as cross-reactivity of the ligands for their non-

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[1] The abbreviations used are: IGF-1, insulin-like growth factor-1; IR, insulin receptor; CHO, Chinese hamster ovary; NT-3, neurotrophin-3; BSA, bovine serum albumin; PBS, phosphate-buffered saline; bpV-(phen), bisperoxovanadium phenanthroline; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein.

cognate receptor and the formation of hybrid receptors may have complicated interpretation of previous studies. To overcome some of the inherent limitations in previous systems, we have constructed chimeric receptors using the extracellular and most of the transmembrane domain of the neurotrophin-3 (NT-3) receptor TrkC (a monomeric transmembrane receptor tyrosine kinase) and the intracellular domain of either the IR or IGF-1R. As TrkC is known to be activated by ligand-induced dimerization, it was anticipated that the monomeric chimera would be inactive in the absence of ligand. Neither TrkC nor NT-3 are expressed in 3T3-L1 pre-adipocytes, nor is there any biological response to NT-3 in these cells. Thus these chimeras have allowed us to examine the relative abilities of the intracellular domains of the IR and IGF-1R to stimulate glycogen *versus* DNA synthesis in 3T3-L1 pre-adipocytes in response to comparable degrees of receptor activation by a single ligand.

EXPERIMENTAL PROCEDURES

Materials—Anti-IR and anti-IGF-1R antibodies were polyclonal antibodies raised to the C-terminal region of the IR and IGF-1R, respectively. Polyclonal anti-Trk C-terminal peptide antibody was as described (41). Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. (Bucks., United Kingdom). Unless otherwise stated, all biochemicals and cell culture materials were from Sigma (Poole, Dorset, UK) and general purpose laboratory reagents were of analytical grade and obtained from BDH (Poole, Dorset, UK). D-[^{14}C]Glucose, [*methyl*-^{3}H]thymidine, and Na^{125}I were from Amersham (Ayelsbury, Buckinghamshire, UK).

Cell Culture—Parental 3T3-L1 pre-adipocytes (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum, 50 units/ml penicillin/streptomycin, and 2 mM glutamine. Stably transfected 3T3-L1 pre-adipocytes were maintained in the same medium containing 550 μg/ml G418 (Life Technologies, Inc.). CHO.IR cells are stably transfected CHO cells overexpressing the wild-type insulin receptor (42). These were maintained in F-12 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin/streptomycin, 2 mM glutamine, and 600 μg/ml G418. MG86/TrkC cells are stably transfected NIH 3T3 cells overexpressing the TrkC cDNA (43). These were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma C-5155), 50 units/ml penicillin/streptomycin, 2 mM glutamine, and 500 μg/ml G418. 3T3/IGF-1R are stably transfected NIH 3T3 cells overexpressing the IGF-1R (44). These were maintained in the same medium as wild-type 3T3-L1 pre-adipocytes.

Generation of Chimeric Receptors—Site-directed mutagenesis was used to introduce novel restriction sites in the transmembrane domain of the IR, IGF-1R, and TrkC. Mutants were generated using "Altered Sites II *in vitro* Mutagenesis System" (Promega). A *Dra*I site was introduced into the TrkC cDNA using a mutagenic oligonucleotide corresponding to nucleotides 2426–2451 (5′-CTTGTTGATCAT**TT**TA-AAGAGGACCA-3′; the *Dra*I site is underlined and the mutated nucleotides are boldfaced). A *Sna*BI site was introduced into the IR cDNA using a mutagenic oligonucleotide corresponding to nucleotides 2921–2954 (5′-TTGGAAGTATT**TACGTA**TTCCTGAGAAA-3′; the *Sna*BI site is underlined and the mutated oligonucleotides are boldfaced). A *Sna*BI site was introduced into the IGF-1R cDNA using a mutagenic oligonucleotide corresponding to nucleotides 2906–2926 (5′-TTCTATGGAA**T-**ACGTACAGCA-3′; the *Sna*BI site is underlined and the mutated nucleotide is boldfaced). The *Dra*I site does not alter any amino acids in the region of TrkC to be used in the chimeric receptor. The *Sna*BI sites produce a conservative L938V change in the IR and no change in the IGF-1R. TrkC was then cleaved with *Dra*I and the IR and IGF-1R with *Sna*BI followed by blunt-end ligation of the TrkC extracellular domain to the intracellular domain of either the IR or the IGF-1R producing the TIR and TIGR, respectively. The sequences of the transmembrane domains of the wild-type receptors are: IR, IIIGPLIFVFLFSVVIG-SIYLFL; IGF-IR, LIIALPVAVLLIVGGLVIMLYVHF; and TrkC, FGV-SIAVGLAAFACVLLVVLFIMI. The sequences of the transmembrane domains of the chimeric receptors are: TIR, FGVSIAVGLAAFACVLLV-VLFV**FL** and TIGR, FGVSIAVGLAAFACVLLVVLF**VHF**, where the boldfaced residues are derived from the IR and IGF-IR, respectively, and the underlined residue represents the conservative L938V change in the IR. The chimeric receptors were subcloned into pRc/CMV (Invitrogen, Leek, Norway) for expression in 3T3-L1 pre-adipocytes from the cytomegalovirus promoter. The entire transmembrane domain and 300 flanking nucleotides of both chimeras were sequenced in the final vector.

Generation of Stable Cell Lines—Semi-confluent 3T3-LI pre-adipocytes in 6-well dishes were transfected with 1.5 μg of either pRc/CMV/TIR or pRc/CMV/TIGR using 8 μl of the Lipofectamine reagent (Life Technologies, Inc., Paisley, Scotland) according to the manufacturer's protocol. Three days after transfection, selective media containing 550 μg/ml G418 was added. After 3 weeks of G418 selection, clonal lines were obtained by limiting dilution onto 96-well plates.

Labeling and Binding of [^{125}I]NT-3—G418-resistant clones expressing high levels of transfected receptor were selected by measurement of [^{125}I]NT-3 binding. NT-3 was labeled according to the following protocol from Regeneron Pharmaceuticals: 4 μg of NT-3 were mixed with 0.5 μg of lactoperoxidase, 400 μCi of Na^{125}I (2000 Ci/mmol), and H_2O_2 at a final concentration of 176 μM. All dilutions were done in 0.2 M sodium phosphate buffer, pH 6.0. After 12 min (vortexing vigorously after 6 min), the reaction was terminated by addition of 30 μl of CIP buffer (0.1 M sodium phosphate, 1 M NaCl, 0.1 M NaI, pH 7.5). The entire volume was then transferred to a BSA-coated Eppendorf tube and the reaction vial flushed with a further 40 μl of 1–2% BSA (radioimmunoassay grade). Half of the total volume was diluted to 100 μl and spun through a Sephadex G-25 spin column to remove unincorporated label. Labeled NT-3 was found to have a specific activity of 2000–3000 cpm/fmol. [^{125}I]NT-3 was stored at 4 °C and used within 2 weeks of preparation.

All binding studies were performed overnight at 4 °C with shaking, using confluent 24-well plates with a total volume of 300 μl/well. For selection of high-expressing clones, cells were incubated with 24 pM [^{125}I]NT-3, washed with ice-cold PBS and solubilized in 0.03% SDS. The radioactivity was measured in a NE1660 γ-counter. Nonspecific binding was determined by preincubating the cells for 45 min with a 1000-fold excess of cold NT-3. It was found to account for maximally 10–20% of total binding. For displacement experiments, cells were incubated with 12 pM [^{125}I]NT-3 and unlabeled NT-3 at concentrations between 0 and 600 pM.

Immunoprecipitation—Confluent 4-well plates were incubated in serum-free media overnight followed by addition of ligand (insulin, IGF-1, or NT-3) for 5 min. In experiments using bisperoxovanadium phenanthroline (bpV(phen)), the cells were exposed to bpV(phen) for 10 min followed by 5 min with or without ligand. Cells were then washed once with ice-cold PBS, solubilized in 0.5 ml of lysis buffer (50 mM Hepes, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 30 mM sodium fluoride, 30 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamidine, 1 μg/ml antipain, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and clarified by centrifugation. Supernatants were incubated for 4 h at 4 °C with 2 μl of either anti-IR or anti-IGF-1R antibody or 5 μl of anti-TrkC antibody plus 40 μl of a 5% slurry of protein A-agarose. After four washes with lysis buffer, pellets were resuspended in Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 3 min. For analysis of total cell lysates, aliquots of lysate supernatant were mixed with sample buffer containing dithiothreitol.

Western Blotting—Proteins were resolved by SDS-polyacrylamide gel electrophoresis (7.5% slab gels) and transferred by electroblotting onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was incubated in blocking buffer (PBS, 0.1% Tween 20, 3% BSA) for 1 h at room temperature. Blocking was followed by incubation at room temperature for 1 h with either anti-IR, anti-IGF-IR, or anti-phosphotyrosine antibody 4G10 in PBS, 0.1% Tween 20, 0.5% BSA. The membrane was then washed for 30 min: 3 × 5 min with PBS, 0.1% Tween and 3 × 5 min with water. Washing was followed by incubation with secondary antibody for 1 h at room temperature. This was followed by another 30-min wash as described above. For detection by the ECL system (Amersham, Ayelsbury, Bucks., UK), the secondary antibody was horseradish peroxidase-conjugated anti-mouse (or anti-rabbit) IgG (Dako Ltd., Denmark) diluted 1:10000 in PBS, 0.1% Tween 20, 0.5% BSA. For detection using ^{125}I, approximately 1 μCi of ^{125}I-labeled goat anti-mouse (or anti-rabbit) IgG (gift of Paul Bevan, Cambridge, UK) was used as secondary antibody. The washed filter was then exposed to x-ray film with an intensifier screen or exposed to a PhosphorImager plate. The results were viewed and quantified using a Fuji BAS2000 PhosphorImager.

Glycogen Synthesis Assay—Glycogen synthesis was assessed by measuring D-[^{14}C]glucose incorporation into glycogen. Confluent monolayers in 6-well dishes were serum-starved for 18 h followed by addition of either 0.001–10 ng/ml NT-3 or 100 nM insulin for 30 min and measurement of D-[^{14}C]glucose uptake as described (45). The unlabeled glucose concentration was 5 mM.

Thymidine Incorporation Assay—Confluent monolayers in 24-well

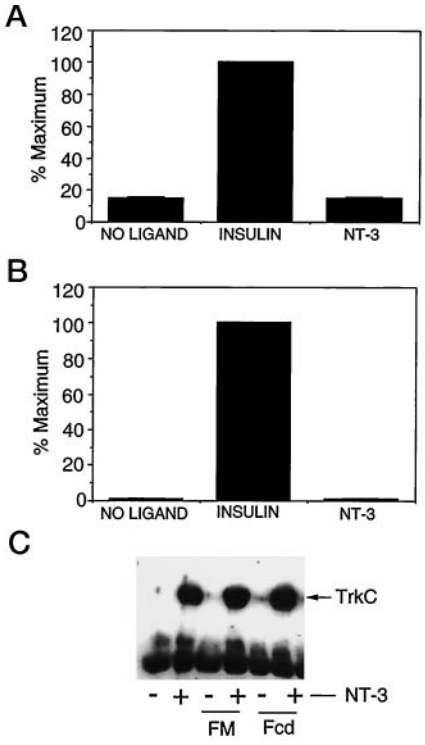

FIG. 1. *A*, stimulation of glycogen synthesis in 3T3-L1 pre-adipocytes. Cells were stimulated with 100 nM insulin or 50 ng/ml NT-3. *B*, stimulation of thymidine incorporation in 3T3-L1 pre-adipocytes. Cells were stimulated with serum or 50 ng/ml NT-3. All assays were carried out in triplicate. Results are the mean ± S.D. of three independent experiments and are expressed as fold over basal. *C*, phosphorylation of TrkC by NT-3. *Fcd*, fibroblast control media not exposed to cells; *FM*, fibroblast conditioned media. Cells were serum-starved for 2 h, treated with media ± NT-3 for 5 min, extracted, TrkC immunoprecipitated with anti-TrkC antibody, and blotted with anti-phosphotyrosine antibody 4G10.

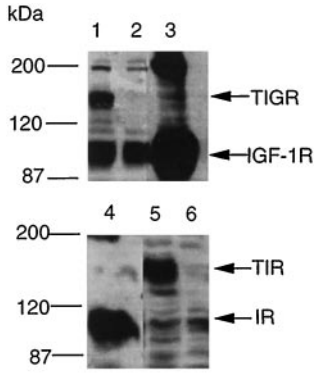

FIG. 2. **Expression of chimeric receptors in pools of stably transfected cells.** *1*, 3T3/TIGR pool; *2* and *6*, untransfected 3T3-L1; *3*, 3T3/IGF-1R; *4*, CHO.IR; *5*, 3T3-L1/TIR pool. Confluent 4-well plates were harvested and total cell lysates analyzed by SDS-PAGE followed by immunoblotting with anti-IR or anti-IGF-1R antibodies and detection by the ECL system.

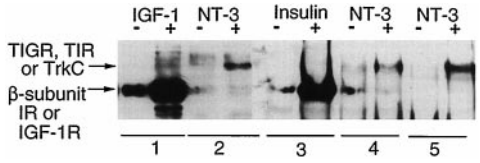

FIG. 3. **NT-3 stimulated tyrosine phosphorylation of chimeric receptors in pools of stably transfected cells.** *1*, 3T3/IGF-1R; *2*, 3T3-L1/TIGR pool; *3*, CHO.IR; *4*, 3T3-L1/TIGR pool; *5*, MG86/TrkC. Confluent 4-well dishes were serum-starved overnight, then treated with or without ligand (50 ng/ml NT-3, 100 nM insulin, or 10 nM IGF-1) for 5 min. Cells were harvested, receptor immunoprecipitated with anti-IR, anti-IGF-1R, or anti-TrkC antibody, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody 4G10 and detection by ECL.

dishes were serum-starved for 24 h in serum-free media. Cells were then washed in serum-free media followed by the addition of either 1 μM insulin or 0.001–1 μg/ml NT-3. After 22 h, thymidine incorporation was measured as described (46).

RESULTS

Design of Chimeric Receptors—To select an appropriate extracellular chimeric partner for these studies a number of ligands were examined to ensure they did not activate downstream signaling in 3T3-L1 pre-adipocytes. Both NGF and HGF were found to produce biological responses in 3T3-L1 pre-adipocytes. In contrast, NT-3 did not stimulate glycogen synthesis or thymidine incorporation in 3T3-L1 pre-adipocytes (Fig. 1, *A* and *B*), while these processes responded to insulin and serum, respectively. Conditioned medium was harvested from 3T3-L1 pre-adipocytes and assayed for the presence of ligand that could activate TrkC in MG86/TrkC cells. There was no increase in TrkC phosphorylation in cells exposed to the supernatants suggesting these cells do not express NT-3 (Fig. 1*C*). Therefore, TrkC was chosen as the receptor to be used in the construction of the chimeric receptors.

Expression and Autophosphorylation of Chimeric Receptors—Chimeric receptors consisting of the entire extracellular domain and 21 amino acids of the transmembrane domain of TrkC and the intracellular domain of the insulin or IGF-1 receptor (TIR and TIGR, respectively) were cloned into the mammalian expression vector pRc/CMV which also contains an SV40 promoter-driven G418 resistance gene. The resulting TIR and TIGR expression vectors were transfected into 3T3-L1 pre-adipocytes followed by growth in G418 to generate a pool of stably transfected cells. To determine whether the chimeric receptors were correctly synthesized and expressed, total cell lysates from these pools were analyzed by 7.5% SDS-PAGE, followed by anti-IR or anti-IGF-1R blotting of proteins transferred to polyvinylidene difluoride membrane (Fig. 2). Both chimeric receptors were expressed and migrated on SDS-PAGE with an apparent molecular mass of 145–150 kDa. Neither receptor was expressed in parental 3T3-L1 cells while all cell lines expressed the IR and IGF-1R.

We examined the functionality of the chimeric receptors in the pools of stably transfected 3T3-L1 stimulated with NT-3. In the absence of suitable antibody to immunoprecipatate chimeric receptors via TrkC extracellular domain, receptors were immunoprecipitated with antibodies to the intracellular domain of the IR or IGF-1R then immunoblotted with an anti-phosphotyrosine antibody (Fig. 3). The anti-IR and anti-IGF-1R antibodies both recognized the respective endogenous murine receptors but they could be clearly distinguished from the chimeric receptor because of major size differences. Both the TIR and TIGR showed NT-3-dependent autophosphorylation of the chimeric receptor but not of the endogenous insulin or IGF-1 receptor. NT-3 also stimulated autophosphorylation of TrkC in MG86/TrkC cells while insulin and IGF-1 stimulated autophosphorylation of their cognate receptor in CHO.IR and 3T3/IGF-1R cells, respectively.

Ligand Binding—Limiting dilution of the 3T3-L1 pool was used to generate several independent G418-resistant clonal cell lines expressing the TIR or TIGR. [^{125}I]NT-3 binding was used to select clones expressing high levels of chimeric receptor and

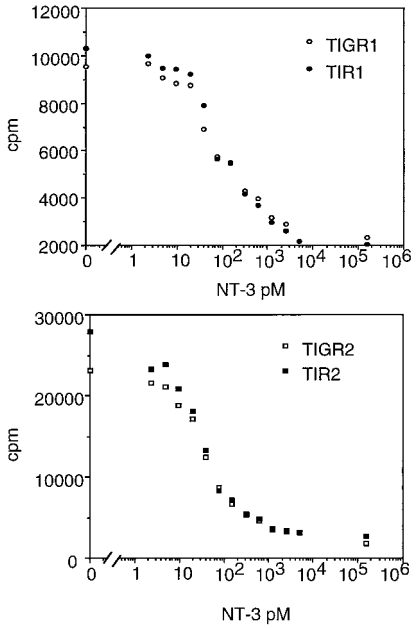

FIG. 4. **Displacement of bound [^{125}I]NT-3 by cold NT-3 in clones expressing either the TIGR or TIR.** Cells were incubated overnight at 4 °C with 12 pM [^{125}I]NT-3 and unlabeled NT-3 at concentrations between 0 and 600 pM, washed with ice-cold PBS, and solubilized in 0.03% SDS. The radioactivity was measured in a NE1660 γ-counter.

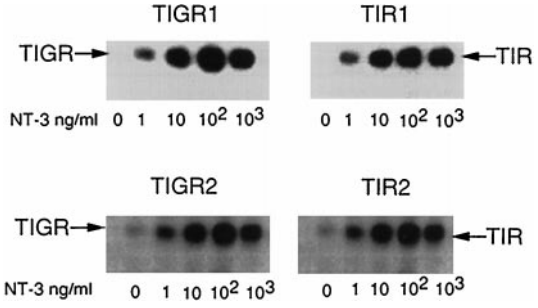

FIG. 5. **Dose-response of tyrosine phosphorylation in paired 3T3-L1/TIR and 3T3-L1/TIGR cell lines.** Confluent 4-well dishes were serum-starved overnight then exposed to 0–1000 ng/ml NT-3 for 5 min. Cells were harvested, receptor immunoprecipitated with anti-IR or anti-IGF-1R antibody, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody 4G10. Tyrosine-phosphorylated receptor was detected using 1 μCi of ^{125}I-labeled goat anti-mouse IgG as secondary antibody.

to identify pairs of clones expressing similar levels of TIR and TIGR. Each pair was analyzed in triplicate on at least two different occasions with MG86/TrkC cells and parental 3T3-L1 pre-adipocytes as positive and negative controls, respectively. Two pairs of clones were selected for use in further studies: TIGR1/TIR1 and TIGR2/TIR2 where both clones of each pair bound similar amounts of [^{125}I]NT-3 (data not shown). The displacement of [^{125}I]NT-3 tracer by unlabeled NT-3 was comparable in the pairs of TIR *versus* TIGR clones (Fig. 4).

Response of Paired Clones to NT-3—We then examined the dose-response of NT-3-induced chimera autophosphorylation in the paired clones. Serum-starved cells were incubated for 5 min with 0.1–1000 ng/ml NT-3 followed by immunoprecipitation with anti-IR or anti-IGF-1R antibody and immunoblotting with anti-phosphotyrosine antibody 4G10 (Fig. 5). Paired clones showed a similar dose-response to NT-3 reaching a maximum at 100 ng/ml. Chimera autophosphorylation was reduced at the higher NT-3 concentration of 1000 ng/ml, a finding consistent with the known properties of transmembrane receptors which

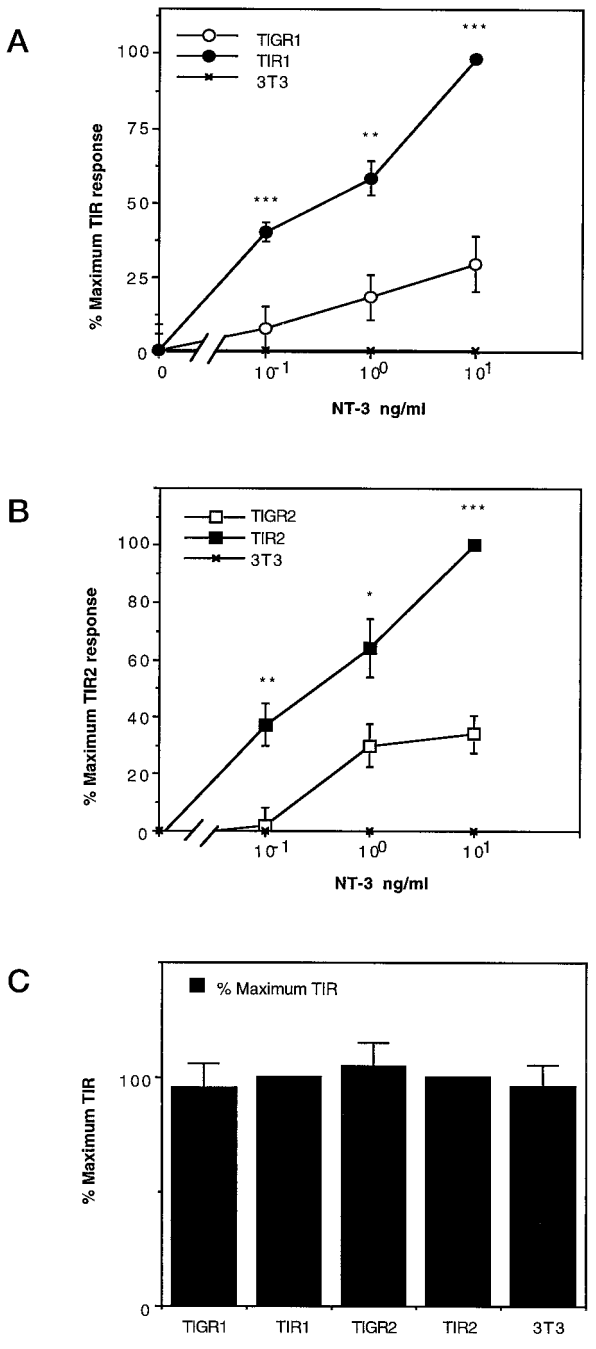

FIG. 6. **Stimulation of glycogen synthesis.** Confluent 6-well dishes were serum-starved overnight followed by addition of 1 pg/ml to 10 ng/ml NT-3 (or 100 nM insulin) for 30 min and D-[^{14}C]glucose for 1.5 h. *A*, stimulation of glycogen synthesis in TIGR1 and TIR1. ○, TIGR1; ●, TIR1; ×, parental 3T3-L1 pre-adipocytes (3T3). *B*, stimulation of glycogen synthesis in TIGR2 and TIR2. □, TIGR2 cells; ■, TIR2 cells; and ×, parental 3T3-L1 pre-adipocytes (3T3). *C*, all cell lines showed a similar response to 100 nM insulin. 3T3-L1 is expressed as % maximum TIR2. Results are presented as % maximum TIR response calculated as: (dpm-basal)/(maximum dpm of TIR clone-basal) × 100. Basal levels of D-[^{14}C]glucose incorporation were comparable for TIGR clones, TIR clones, and 3T3-L1 pre-adipocytes at approximately 3.3 pmol of [^{14}C]glucose incorporated per 10^6 cells/h). Typically, maximum NT-3 stimulation for TIR clones was 7.4 pmol of [^{14}C]glucose incorporated per 10^6 cells/h while maximum insulin stimulation for TIR clones was typically 9 pmol of [^{14}C]glucose incorporated per 10^6 cells/h. Results are mean ± S.E. of three independent experiments performed in duplicate. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.005$ using the nonparametric Mann-Whitney test.

are dimerized by ligand (47, 48).

Biological Activity and Specificity of Chimeric Receptors—The paired clones TIGR1/TIR1 and TIGR2/TIR2 were then used to compare the effect of NT-3 on glycogen synthesis in cells (Fig. 6). Serum-starved cells were exposed to various concentrations of NT-3 for 30 min followed by a 1.5-h pulse of D-[U-^{14}C]glucose. Results are shown separately for each of the matched pairs and represent the mean results from three independent experiments. Parental 3T3-L1 cells did not respond to NT-3 but glycogen synthesis was stimulated by NT-3 in both pairs of chimeric expressing cell lines. The dose-response curves show that, in both pairs, NT-3 stimulated glycogen synthesis was greater in TIR than in TIGR expressing clones. The maximal response seen in both TIR1 and TIR2 was almost 3 times that of TIGR1 and TIGR2, respectively. Additionally, the concentration at which significant stimulation of glycogen synthesis was seen was 10-fold higher in TIGR1 and TIGR2 than in TIR1 and TIR2 (1 *versus* 0.1 ng/ml). All clones showed a similar response to 100 nM insulin suggesting that the differences seen are specific to NT-3.

The effect of NT-3 on thymidine incorporation in TIGR1/TIR1 and TIGR2/TIR2 was also examined. Serum-starved cells were exposed to various concentrations of NT-3 for 22 h followed by a 1.5-h pulse of [*methyl*-^{3}H]thymidine. Parental 3T3-L1 cells did not respond to NT-3 but [^{3}H]thymidine incorporation was stimulated by NT-3 in both pairs of chimeric expressing cell lines (Fig. 7). In contrast to the studies of glycogen synthesis, the dose-response characteristics of DNA synthesis were comparable in both pairs of TIR *versus* TIGR expressing clones. All clones showed a similar response to 1 μM insulin.

Receptor Autophosphorylation in the Presence of BpV-(phen)—While the NT-3 dose-response of thymidine incorporation was very similar in TIR and TIGR expressing clones, a consistent increase in basal levels of thymidine incorporation (but not glycogen synthesis) was seen in the TIR *versus* TIGR expressing clones. The basal levels of thymidine incorporation in TIGR clones were around 4000 dpm (approximately 2% of maximal NT-3 response), whereas in TIR clones the basal levels of thymidine incorporation were around 20,000 dpm (approximately 10% of maximal NT-3 response).

These differences in basal levels of thymidine incorporation were not associated with parallel differences in cell proliferation as the doubling times of all cell lines was similar (data not shown). The differences on basal levels of thymidine incorporation could not be explained by a greater degree of basal receptor autophosphorylation of the TIR as none could be detected in any of the clonal cell lines studied. However, it was possible that a small amount of such basal activity might have been undetectable by immunoblotting.

To amplify basal autophosphorylation, cells were exposed to the phosphatase inhibitor bpV(phen) prior to addition of ligand (Fig. 8). In the absence of ligand and bpV(phen) (*lanes 1* and *6*) there is no detectable basal autophosphorylation of either the chimeric or endogenous receptors. When NT-3 is added, there is stimulation of autophosphorylation of the TIGR (*lane 2*) and the TIR (*lane 7*). In the presence of bpV(phen) and absence of NT-3, the extent of basal chimeric receptor autophosphorylation of the TIR was greater than that of the TIGR (comparing TIR in *lanes 8* and *10* with TIGR in *lanes 3* and *5*).

This raised the possibility that the structural alteration of the TIR chimera might have resulted in a degree of "artificial" constitutive activation which did not occur to a similar extent with the TIGR chimera. However, in the presence of bpV(phen) alone, the endogenous murine IR showed a comparable hypersensitivity to bpV(phen) compared with the endogenous murine

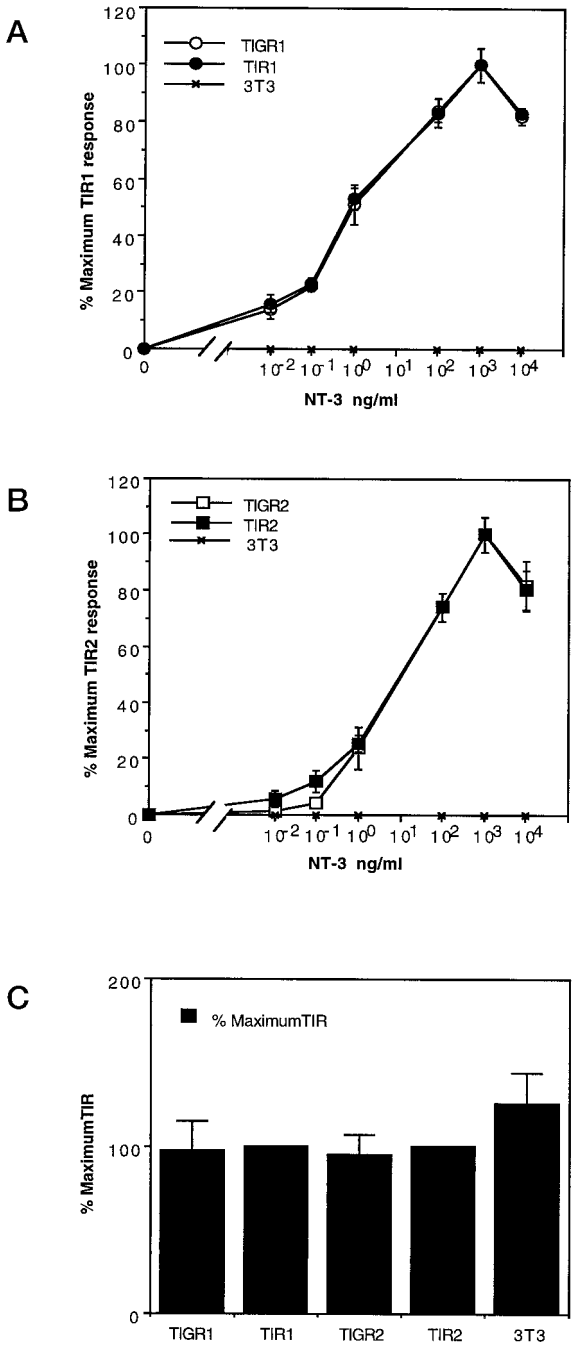

FIG. 7. **Stimulation of thymidine incorporation.** Confluent 24-well plates were serum-starved for 24 h. Cells were then washed in serum-free media followed by the addition of either 1 μM insulin or 0.001–1 μg/ml NT-3. After 22 h, 1 μCi of [*methyl*-^{3}H]thymidine was added to each well for 1.5 h. *A*, stimulation of thymidine incorporation in TIGR1 and TIR1. ○, TIGR1; ●, TIR1; ×, parental 3T3-L1 pre-adipocytes (3T3). *B*, stimulation of thymidine incorporation in TIGR2 and TIR2. □, TIGR2 cells; ■, TIR2 cells; and ×, parental 3T3-L1 pre-adipocytes (3T3). Basal levels of thymidine incorporation were approximately 3000–4000 dpm for 3T3-L1 pre-adipocytes, 4000 dpm for TIGR clones (approximately 2% of maximal TIGR response), and 20,000 dpm for TIR clones (approximately 10% of maximal TIR response). *C*, all clones showed a similar response to 1 μM insulin. 3T3-L1 is expressed as % maximum TIR2. Results are presented as % maximum TIR incorporation calculated as: (dpm-basal)/(maximum dpm of TIR-basal) × 100. Results are mean ± S.E. of four independent experiments performed in duplicate.

IGF-1R (IGF-IR in *lane 3*). This indicates that these findings are more likely to relate to intrinsic differences in the properties of the IR *versus* IGF-1R intracellular domains rather than

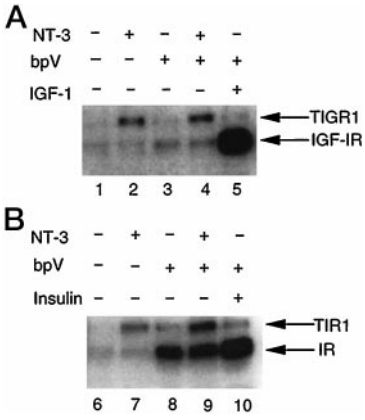

FIG. 8. **Potentiation of autophosphorylation by bisperoxovanadium phenanthroline (bpV(phen)) in the TIGR1/TIR1 pair.** Confluent 4-well plates were incubated in serum-free media overnight followed by incubation with or without 0.1 mM bpV(phen) for 10 min then with or without ligand (100 ng/ml NT-3, 10 nM insulin, or 10 nM IGF-1) for a further 5 min. Cells were harvested, receptor immunoprecipitated with anti-IR or anti-IGF-1R antibody, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody 4G10. Tyrosine-phosphorylated receptor was detected using 1 μCi of ^{125}I-labeled goat anti-mouse IgG as secondary antibody. *A*, the chimeric TIGR receptor and the endogenous IGF-IR. *1*, basal autophosphorylation in the absence of ligands; *2*, stimulation of TIGR autophosphorylation by NT-3; *3*, TIGR and IGF-IR in the presence of bpV(phen) and absence of ligands showing potentiation of basal autophosphorylation; *4*, stimulation of TIGR autophosphorylation by NT-3 in the presence of bpV-(phen); and *5*, stimulation of IGF-IR autophosphorylation by IGF-I in the presence of bpV(phen). *B*, the chimeric TIR receptor and the endogenous IR. *Lane 6*, basal autophosphorylation in the absence of ligands; *7*, stimulation of TIR autophosphorylation by NT-3; *8*, TIR and IR in the presence of bpV(phen) and absence of ligands showing potentiation of basal autophosphorylation; *9*, stimulation of TIR autophosphorylation by NT-3 in the presence of bpV(phen), and *lane 10*, stimulation of IR autophosphorylation by insulin in the presence of bpV(phen). Similar results were obtained with the TIGR2/TIR2 pair. All experiments were performed on two separate occasions.

merely representing accidental properties of artificial chimeric receptors. NT-3 is still able to stimulate autophosphorylation of the TIGR and TIR in the presence of bpV(phen) (*TIGR lane 4, TIR lane 9*) while IGF-I and insulin also stimulate autophosphorylation of their cognate receptor in the presence of bpV-(phen) (*IGF-IR lane 5, IR lane 10*).

DISCUSSION

In the present study, we have found that the intracellular domain of the IR is more active at stimulating glycogen synthesis than that of the IGF-1R while both have a similar capacity to stimulate DNA synthesis. In the absence of ligand, however, experiments with a phosphatase inhibitor suggest that the IR has a higher level of basal kinase activity than the IGF-1R. The fact that comparable results were obtained with two independent pairs of clones provides reassurance that the differences seen are likely to be biologically meaningful.

In the intact organism, these homologous peptide hormones have very different functions. Insulin acts largely as a regulator of metabolic processes while IGF-1 is predominantly involved in the control of cell growth and differentiation. At least four possible explanations for this specificity are apparent. 1) *In vivo* differences in the properties of insulin and IGF-1 might result from the different tissue distribution of the IR and IGF-1R. The fact that there are considerably more IRs than IGF-1Rs on adult adipocytes and hepatocytes could explain the greater metabolic effects of insulin on these tissues. However, there are tissues, such as skeletal muscle (the major site of whole body glucose disposal) which express both IRs and IGF-1Rs to high levels. 2) Insulin secretion from pancreatic β-cells increases rapidly in response to nutrients and acts immediately on target tissues. In contrast, IGF-1 forms stable complexes with specific binding proteins. Since insulin has a short plasma half-life, the insulin receptor is exposed to rapidly changing ligand concentrations *in vivo* whereas the effects of IGF-1 on its receptor is likely to be more "tonic." 3) The time course of activation of the receptor kinase may differ because of differences in ligand-binding kinetics (43, 44). DeMeyts *et al.* (43) have demonstrated that a higher dissociation rate of ligands from the IR compared with the IGF-1R would result in tighter binding of IGF-1 to its receptor resulting in receptor activation for a longer time compared with insulin and its receptor. 4) There could be differences in the intrinsic signaling properties of the receptor kinases. There have been several approaches used to investigate intrinsic signaling differences. These have included *in vitro* studies of purified or partially-purified receptors and studies of receptors overexpressed in cell lines.

Using highly purified preparations of IGF-1R and IR, Sahal *et al.* (31) demonstrated intrinsic differences in substrate specificity between the two kinases toward polymeric substrates. Xu *et al.* (32) showed that the IR catalytic domain preferentially phosphorylates peptides based on Tyr-987 and Tyr-727 of IRS-1 while the IGF-1R preferentially phosphorylated a synthetic peptide containing Tyr-895 of IRS-1, the Grb2-binding site.

Several studies comparing cells overexpressing IRs or IGF-1Rs have been reported. Lammers *et al.* (35) have demonstrated that, while they stimulate glucose transport with similar efficiency, when overexpressed in NIH 3T3 fibroblasts the K_m for IGF-1 stimulated DNA synthesis in IGF-1R overexpressing cells was an order of magnitude lower than that seen with insulin in IR overexpressing cells. In studies of NIH 3T3 fibroblasts, Tartare *et al.* (36) reported that the maximum stimulation of glycogen synthesis by insulin in IR overexpressing cells was 2-fold greater than that of IGF-1 in IGF-1R overexpressing cells. Additionally, in these studies, insulin was reported to result in a 5-fold stimulation of $p44^{mapk}$ in IR overexpressing cells, whereas IGF-1 only stimulated $p44^{mapk}$ 2.5-fold in IGF-1R overexpressing cells. Other studies have examined the relative abilities of both receptors to act as tumor promoter agents. Thus, Kaleko *et al.* (33) demonstrated that overexpression of IGF-1R in NIH 3T3 cells as well as human and rat fibroblasts resulted in IGF-1-induced transformation and rapid tumor formation in nude mice. In contrast, overexpression of the IR in 3T3 cells while resulting in loss of contact inhibition and focal growth, did not promote tumor formation in nude mice (34).

A complementary approach has involved the study of chimeric receptors comprised of portions of the IR and IGF-R. Lammers *et al.* (35) reported that a receptor consisting of the ligand-binding domain of the IR and the cytoplasmic domain of the IGF-1R is 10 times more active in stimulating DNA synthesis than the IR itself in response to insulin. This is consistent with their demonstration of a greater mitogenic signaling capacity of the intact IGF-1R compared with the IR. Tartare *et al.* (36) studied a chimeric IGF-1R in which the C-terminal 112 amino acids was replaced by the equivalent sequences of the IR. This chimera behaved more like the intact IR than IGF-1R in studies of glycogen synthesis and MAP kinase activation. When studied *in vitro* together with partially-purified IRs and IGF-1Rs (45), this chimera displayed an array of effects similar to the IR rather than the IGF-1R.

Faria *et al.* (37) showed that substitution of the IR C terminus with that of the IGF-1R resulted in an 80% decrease in

c-*fos* mRNA induction and a significant reduction in thymidine incorporation in response to insulin. In contrast, substitution of the IGF-1R C-terminal with that of the IR did not affect ligand-stimulated thymidine incorporation which was similar in NIH 3T3 cells overexpressing either the IR, IGF-1R, or the chimeric receptor (30). These studies suggest that the C-terminal region, poorly conserved between the two receptors, may be involved in defining the specificity of downstream events.

The use of the TrkC-based chimeric receptor approach in our studies has allowed the avoidance of several factors which may have confounded previous approaches to the question. Thus, 1) NT-3 has no effects on untransfected 3T3-L1 cells. 2) Hybrid formation between endogenous IR and IGF-1R receptors appears unlikely, particularly as no transphosphorylation of the endogenous receptors was seen upon NT-3 treatment of the chimera-expressing cells. 3) The use of the same extracellular domain for both IR and IGF-1R-containing chimeras removes any confounding effects of differences in ligand binding. The similarities between the two chimeric receptors in terms of displacement of labeled NT-3 by cold NT-3 and the similarities in the dose-response characteristics of NT-3 stimulated chimera autophosphorylation provides further reassurance in this regard. Thus, it appears likely that the clear differences in the coupling of the two chimeric receptors to ligand-induced glycogen synthesis is most likely to represent intrinsic differences in the signaling properties of the intracellular domains of the IR *versus* the IGF-1R.

The finding of a modest but consistent increase in the basal amount of radiolabeled thymidine incorporation in cells expressing the TIR *versus* TIGR chimera could not be explained by detectable differences in the basal extent of chimera autophosphorylation. However, when exposed to bpV(phen), TIR autophosphorylation was greatly enhanced whereas only a small effect on TIGR autophosphorylation was seen. This did not appear to reflect artificial properties of the chimeras as the same differential response to bpV(phen) was seen with the endogenous murine IR and IGF-1R. It is therefore possible that the intracellular domain of the insulin receptor exhibits a greater degree of ligand independent autophosphorylation than the equivalent domain of the IGF-1R. Alternatively, bpV-(phen) may have some specificity for particular phosphatases which preferentially act upon the IR. These findings are in accordance with findings of marked enhancement of glucose disposal when various animal models of NIDDM are treated with vanadate or its derivatives (peroxovanadate).

Glycogen synthase is activated by net dephosphorylation either by activation of protein phosphatase-1 or inhibition of glycogen synthase kinase-3. Hubbard and Cohen (46) initially described a model in which the inactivation of protein phosphatase-1 was mediated by the MAP kinase pathway. However, several recent studies question this model (reviewed in Ref. 47). Using the phosphatidylinositol 3-kinase inhibitor wortmannin and the $p70^{S6K}$ inhibitor rapamycin, Shepherd *et al.* (48) reported that phosphatidylinositol 3-kinase and $p70^{S6K}$ are involved in stimulation of glycogen synthesis in 3T3-L1 adipocytes. Both $p70^{S6K}$ and protein kinase B have been shown to phosphorylate and inactivate glycogen synthase kinase-3 *in vitro* (49, 50). However, $p70^{S6K}$ does not inactivate glycogen synthase kinase-3 in all cells. Studies in L6 myocytes have led to a new model in which the activation of phosphatidylinositol 3-kinase leads to activation of PKB which phosphorylates and inactivates glycogen synthase kinase-3 (50). Differences in post-receptor signaling through these pathways could underly the differences demonstrated in our system.

In summary, these studies provide strong support for the existence of intrinsic differences in the signaling properties of the intracellular domains of the insulin *versus* the IGF-1 receptors. The greater efficiency of coupling of the insulin receptor to glycogen synthesis was readily demonstratable in the system studied despite the fact that 3T3-L1 pre-adipocytes are relatively poorly insulin responsive compared with mature adipocytes. Studies of TrkC-based chimeras in differentiated 3T3-L1 adipocytes should allow further testing of the relative metabolic signaling capacities of the insulin *versus* the IGF-1 receptor intracellular domains including the examination of specialized functions such as glucose transporter translocation and the inhibition of lipolysis. Further studies will also be required to precisely delineate the intracellular molecules linking the receptor to glycogen synthesis which are preferentially activated by the IR *versus* IGF-1R.

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Additions and Corrections

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Differential signaling to glycogen synthesis by the intracellular domain of the insulin *versus* the insulin-like growth factor-1 receptor. Evidence from studies of TrkC-chimeras.

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Several reference citations are incorrect.

Page 24325, right-hand column, line 12 from the bottom: (29–31) should be (**28–30**); line 6 from the bottom, (32, 33) should be (**31, 32**); line 4 from the bottom, (34–37) should be (**33–37**); and line 2 from the bottom, (38–40) should be (**35–37**).

Page 24326, left-hand column, paragraph 2, line 4: (41) should be (**38**); in paragraph 3, line 8, (42) should be (**39**); line 11, (43) should be (**40**); and in line 15, (44) should be (**35**). In the right-hand column, line 3 from the bottom, (45) should be (**39**).

Page 24327, left-hand column, line 4: (46) should be (**39**).

Page 24329, left-hand column, line 1: (47, 48) should be (**41, 42**).

Differential Signaling to Glycogen Synthesis by the Intracellular Domain of the Insulin *versus* the Insulin-like Growth Factor-1 Receptor: EVIDENCE FROM STUDIES OF TrkC-CHIMERAS

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