Expression and function of insulin/insulin-like growth factor I hybrid receptors during differentiation of 3T3-L1 preadipocytes

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During the assembly of cell surface receptors, insulin proreceptors are sometimes joined to insulin-like growth factor (IGF) receptor precursors to form covalently linked hybrid receptors. To address the biological consequences of hybrid receptor formation, we studied 3T3-L1 cells known to undergo a 50–70-fold increase in insulin binding while maintaining nearly constant levels of IGF-I binding during differentiation from preadipocytes into adipocytes. The presence of insulin/IGF receptor hybrids in 3T3-L1 adipocytes was demonstrated by the immunoprecipitation of phosphorylated receptors and a novel enzyme-linked immunoassay. Hybrid receptor levels were very low in the early stages of differentiation and increased rapidly between days 4 and 6, reaching a level about 100-fold higher in the mature adipocyte. Coincident with the hybrid assembly, the formation of archetypal (α_2,β_2) IGF receptors decreased. In fully differentiated adipocytes, virtually all of the IGF receptors were in hybrid form. Stimulation by IGF-I of receptors isolated from mature adipocytes caused autophosphorylation of IGF receptor β subunits in hybrid complexes, whereas autophosphorylated IGF holoreceptors were not demonstrable. Insulin and IGF-I were equipotent in stimulating glucose uptake in the differentiated adipocytes, leading to the conclusion that hybrid insulin/IGF receptors can transduce a transmembrane signal when activated by IGF-I. We conclude that hybrid formation constitutes a novel post-translational mechanism whereby increased synthesis of insulin receptors limits the cell surface expression of the homologous IGF receptor. Furthermore, biological actions in 3T3-L1 adipocytes, previously attributed to archetypal IGF receptors, are in fact mediated through hybrid receptors.

INTRODUCTION

During the synthesis of insulin receptors (INSRs) and type I insulin-like growth factor (IGF) receptors (IGFRs), two identical proreceptors are joined by class I disulphide bonds. The proreceptors are subsequently cleaved into α and β subunits to form mature tetrameric (α_2,β_2) holoreceptors. Several laboratories have demonstrated that the fidelity of the assembly is not absolute, such that an INSR proreceptor is sometimes linked to an IGFR proreceptor, forming a hybrid INSR/IGFR molecule [1–13]. Hybrid receptors have been demonstrated in multiple tissues in rabbits [4] and humans, including heart, kidney, liver, fat, spleen, placenta and muscle [5]. Recent clinical studies have demonstrated that humans with type II diabetes mellitus [6] or with primary hyperinsulinaemia [7] have increased hybrid receptor expression in skeletal muscle. In cells that have a disproportionate production of one type of proreceptor, formation of the homologous holoreceptor might be limited by the availability of identical proreceptors (i.e. the less abundant proreceptors will be incorporated into hybrids rather than holoreceptors). The latter situation commonly occurs in transfection experiments in which large numbers of the expressed receptors are synthesized on a background of relatively few wild-type receptors [8]. We reasoned that a similar phenomenon might occur in cells undergoing a natural change in the ratio of INSR to IGFR receptors, such as the developing adipocyte.

Because hybrid receptors are necessarily found in the presence of classical INSRs and IGFRs, the signalling properties of these molecules have been difficult to discern [4,9]. From studies *in vitro* we know that INSR/IGFR hybrids bind IGF-I avidly but have a rather low affinity for insulin [10,11]. IGF-I is more potent than insulin in stimulating autophosphorylation of the hybrid receptors [10], but the biological significance of this phosphorylation has not been established. Although INSR/IGFR hybrids undergo ligand-induced internalization and degradation in a manner comparable to that of IGFR holoreceptors [3], hormonal responses such as hexose uptake, amino acid uptake and thymidine incorporation have not yet been demonstrated. Thus the role of INSR/IGFR hybrid formation *in vivo* remains to be established.

In the present study we addressed questions about the formation and function of INSR/IGFR hybrids, with the 3T3-L1 cell line as a model. The 3T3-L1 preadipocytes can be induced to undergo a reproducible process of differentiation into adipocytes, during which the expression of INSR increases from 3000–20000 receptors per cell to 170000–250000 receptors per cell in the mature cells [12–15]. In contrast, IGFRs were reported to be expressed at a constant level (13000 sites per cell) in both preadipocytes and adipocytes [14]. Thus 3T3-L1 cells undergo a natural process in which INSRs are overexpressed by a factor of 20–50-fold relative to IGFRs. We predicted that the mature adipocytes would express a large number of INSR/IGFR hybrids because of the relative abundance of INSRs. We further hypothesized that the above changes in INSR expression would alter IGFR assembly such that, in mature adipocytes, most of the IGFRs would be contained in hybrids, whereas most of the INSRs would be assembled as holoreceptors. Because cultured adipocytes are known to be responsive to both insulin and IGF-I with respect to glucose uptake [16], amino acid uptake [17] and lipoprotein lipase activity [18], we believed that the 3T3-L1

Abbreviations used: IGF-I, insulin-like growth factor I; IGFR, type I insulin-like growth factor receptor; INSR, insulin receptor; WGA, wheatgerm agglutinin.

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system would constitute an ideal model in which to assess the biological consequences of hybrid receptor formation.

EXPERIMENTAL

Antibody preparation

The anti-INSR antibody was previously developed in our laboratory by immunizing rabbits with the synthetic peptide Cys-Asn-Gly-Arg-Val-Leu-Thr-Leu-Pro-Arg-Ser-Asn-Pro-Ser, corresponding to the C-terminal 13 amino acid residues of the mouse INSR β subunit [19]. The antibody is effective in the immunoprecipitation and immunoblotting of both mouse and human INSRs. It does not cross-react with human type I IGF receptors because it does not immunoprecipitate or immunoblot IGFRs derived from I-24 cells, a stably transfected NIH 3T3 fibroblast line that overexpresses the human IGFR [20,21]. For use in the immunoassay, the immunoglobulin fraction was purified from anti-INSR serum with a Protein A–Sepharose column by a standard technique [22]. Biotinylation was performed with an enhanced chemiluminescence protein biotinylation module (Amersham Life Science) as described by the manufacturer. For development of the anti-IGFR antibody, rabbits were immunized with the synthetic peptide Cys-Ser-Leu-Pro-Leu-Pro-Asp-Arg-His-Ser-Gly-His-Lys, corresponding to residues 1313–1324 of the human IGFR [23]. This sequence was chosen because there is no homologous portion in the INSR, and because it is fully conserved in the 3T3-L1 (i.e. mouse) IGFR (S. J. Casella, unpublished work). The antibody was affinity-purified on a peptide–Sepharose column before use. The antibody immunoprecipitates the phosphorylated human IGFR β subunit derived from I-24 cells.

Cell lines

All tissue culture supplies were purchased from Gibco-BRL. Cells were cultured at 37 °C under air/CO_2 (19:1). Monolayers of 3T3-L1 cells were cultured and differentiated as described previously [24]. In brief, 3T3-L1 fibroblasts were grown to confluence; 2 days later the conversion to adipocytes was induced by the addition of methylisobutylxanthine, dexamethasone and insulin to Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum. The culture medium was supplemented with insulin for a further 2 days, followed by 4–6 days in Dulbecco's modified Eagle's medium supplemented only with 10 % (v/v) fetal bovine serum. Clone 7, I-24 cells, a gift from Dr. A. Ullrich (Max-Planck Institut für Biochemie, München, Germany), were propagated in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, 2 mM L-glutamine and antibiotics. This is a stably transfected NIH-3T3 fibroblast cell line that overexpresses the human IGFR cDNA [20]. Human lymphoblastoid IM-9 cells were grown in continuous culture in RPMI-1640 medium with L-glutamine supplemented with 10 % (v/v) fetal bovine serum and antibiotics. At 48–72 h the cells were divided 1:10, and fresh medium was added to each flask; the rest of the cells were harvested for the preparation of membranes.

Solubilization of cell membranes

3T3-L1 cells were harvested at 2 days after confluence (undifferentiated), and at days 3, 4, 5, 6, 7, 8 and 10 after induction of differentiation. I-24 cells were harvested at confluence. One dish from each batch was treated with trypsin and cells were counted (Coulter Counter; Coulter Electronics). Cell membranes were solubilized by a protocol modified after Flores-Riveros et al. [19]. Cell monolayers were washed twice with PBS, pH 7.4, followed by a quick wash with buffer B [10 mM Hepes/10 mM EDTA (pH 7.4)]. Cell layers were then incubated for 10 min at 4 °C with 1 ml per 10 cm dish of buffer B containing 1 μl each of protease inhibitor cocktails PIC-1 [1 mg/ml leupeptin/2 mg/ml antipain/10 mg/ml benzamidine/10000 i.u./ml aprotinin] and PIC-2 [1 mg/ml chemostatin/1 mg/ml pepstatin in DMSO] [25], then scraped off the plates, collected in 1.5 ml Microfuge tubes and centrifuged at 19000 ***g*** for 45 min. The pellet was solubilized in a buffer containing 50 mM Hepes (pH 7.5), 1 mM EDTA, 1 % (v/v) Triton X-100 and protease inhibitors, each at 1 μl/ml, incubated for 45 min at 4 °C with rotation, and centrifuged for 45 min at 19000 ***g***. The supernatant was aspirated and saved at −20 °C until used. IM-9 cell membranes were prepared as described by Misra and Hintz [26]. Approx. 10^9 cells were washed three times in PBS, suspended in 40 % (v/v) sucrose/50 mM Hepes/4 mM iodoacetic acid (pH 7.4) and homogenized by 20 passes with a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 750 ***g*** for 10 min; the supernatant was centrifuged at 20000 ***g*** for 30 min. The pellet was washed three times with 100 mM Hepes buffer containing 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 15 mM sodium acetate and 10 mM dextrose, pH 7.5. The membranes were then solubilized in 1 % (v/v) Triton X-100 buffer as described above.

Autophosphorylation of partly purified INSRs and IGFRs

Partly purified INSRs and IGFRs were prepared by solubilizing 3T3-L1 mature (day 9) adipocyte membranes in Triton X-100 and subjecting the solubilized membranes to wheatgerm agglutinin (WGA) affinity chromatography as described previously [19,27]. Approx. 5×10^6 cell equivalents of WGA eluate were incubated with 30 nM of insulin or IGF-I in 50 mM Hepes buffer, pH 7.4, containing 0.1 % Triton X-100 and 1 mM EDTA for 1 h at room temperature. The kinase reaction was initiated by the addition of manganese acetate (final concentration 2.5 mM), $MgCl_2$ (final concentration 2.5 mM) and 20 μM ATP supplemented with 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; New England Nuclear). The phosphorylation reaction proceeded for 30 min and was then quenched by the addition of 0.5 M EDTA to a final concentration of 50 mM. For experiments involving free $\alpha\beta$ dimers, phosphorylated receptors were treated with 1 mM dithiothreitol at pH 8.5 for 30 min at 37 °C and quenched with 3 mM *N*-ethylmaleimide before immunoprecipitation.

Immunoprecipitation of phosphorylated receptors

Aliquots (20 μl) of phosphorylated receptors were diluted to 50 μl with immunoprecipitation buffer [10 mM Tris (pH 7.4)/2 mM EDTA/0.15 M NaCl/0.1 % (v/v) Triton X-100] containing 1 μl of anti-INSR antiserum and 50 μl of Protein A–Sepharose 4B. After incubation overnight at 4 °C, the supernatant was recovered and the pellet was washed three times with cold immunoprecipitation buffer. The supernatant or the pelleted beads were boiled in SDS sample buffer containing 4 % (v/v) 2-mercaptoethanol. Samples were subjected to electrophoresis for approx. 1800 V h on 7.5 % (w/v) SDS gels with a 3 % (w/v) stack. Autoradiograms were exposed at −70 °C with intensifying screens for 1–3 days.

Development of a double-antibody ELISA for detection of hybrids

The assay was performed in 96-well polystyrene microtitre plates (Immulon-2; Dynatech). Unless specified otherwise, the standard volume added to each well was 50 μl; the wells were washed by filling them three times with PBS, pH 7.4, containing 0.05 %

(v/v) Tween 20 (Sigma). Working dilutions of reagents were determined by chequerboard titrations. Affinity-purified anti-IGFR antibody was diluted 1:1000 in PBS, pH 7.4, and the wells were coated at 37 °C. After 90 min, 200 μl of blocking solution [1 % (w/v) BSA in PBS] was added and the plates were stored for 1–7 days at 4 °C. The solubilized cell preparation was diluted in blocking solution to a final volume of 50 μl, then incubated in the washed wells for 60 min at 37 °C. Unbound material was removed by washing; the plates were then incubated with biotinylated anti-INSR antibody, diluted 1:1000 in blocking solution, for 60 min at 37 °C. The plates were then washed and incubated with horseradish peroxidase-conjugated streptavidin (Amersham-Life Science), diluted 1:1500 in PBS/0.05 % Tween, for 45 min at room temperature. After a final wash, peroxidase activity was detected by adding 100 μl of the substrate *o*-phenylenediamine dihydrochloride (Sigma). After 1 h in the dark at room temperature, the reaction was stopped with 100 μl 1 M HCl and the plates were read spectrophotometrically at 490 nm with an automated plate reader (V_{max} kinetic plate reader; Molecular Devices). The software package STATLIA® (Brendon Scientific) was used for establishing titration curves, calculations and statistical analysis. A mean and S.D. for receptor equivalents were calculated for each sample.

Radioligand binding

Iodinated IGF-I (specific radioactivity 2000 Ci/mmol) was prepared as described previously [28]. Monolayers of 3T3-L1 cells were incubated in KRP buffer [128 mM NaCl/4.7 mM KCl/1.25 mM $CaCl_2$/1.25 mM $MgSO_4$/5 mM $NaPO_4$ (pH 7.4)] supplemented with 0.5 % BSA, 50 pM ^{125}I-IGF-I and various concentrations of unlabelled hormone. After incubation overnight at 4 °C, the cells were washed twice and lysed in NaOH/0.1 % SDS, then the bound radioligand was measured in a γ-spectrophotometer. Dissociation constants and receptor numbers were estimated with the curve-fitting program LIGAND [29].

Insulin- and IGF-I-stimulated hexose uptake

Preadipocytes and adipocytes were assayed for 2-deoxyglucose uptake as previously described [24,27]. In brief, cell monolayers were washed and incubated in serum-free medium for 2 h before the experiment was initiated. Monolayers were washed twice with KRP buffer [128 mM NaCl/4.7 mM KCl/1.25 mM $CaCl_2$/1.25 mM $MgSO_4$/5 mM $NaPO_4$ (pH 7.4)] and equilibrated in KRP buffer at 37 °C for 10 min. Cell monolayers were incubated with various concentrations of insulin or IGF-I, followed by a 10 min incubation with the radiolabelled sugar (final concentration 200 μM 2-[^{14}C]deoxyglucose; 0.1 μCi/ml). Cell monolayers were then washed three times with PBS and solubilized in NaOH/SDS; radioactivity was determined by liquid scintillation.

RESULTS

Immunoprecipitation of autophosphorylated INSRs and IGFRs in 3T3-L1 cells

WGA-purified receptors, derived from mature (day 9) 3T3-L1 adipocytes, exhibited autophosphorylation when incubated with [γ-^{32}P]ATP. Most of the incorporated radioactivity appeared in a band at 97 kDa corresponding to the INSR β subunit (Figure 1, lane 1), and the intensity increased in a concentration-dependent manner in response to insulin (Figure 1, lanes 1–5). IGF-1 produced only a modest increase in labelling of the 97 kDa band as well as the appearance of a slightly larger phosphoprotein (estimated as 105 kDa) that increased in intensity

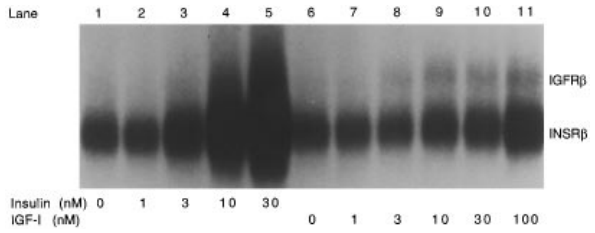

Figure 1 Ligand-induced autophosphorylation of insulin and IGF-I receptors isolated from 3T3-L1 adipocytes

Affinity-purified, solubilized receptors were incubated with graded amounts of ligand, then autophosphorylated in the presence of [γ-^{32}P]ATP. The samples were fractionated by SDS/PAGE [7.5% (w/v) gel] for 1800 V h to resolve the IGFR and INSR β subunits, then analysed by autoradiography.

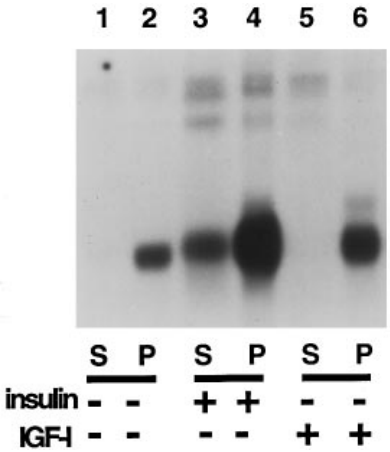

Figure 2 Immunoprecipitation of phosphorylated receptors

WGA-purified receptors, isolated from fully differentiated adipocytes, were autophosphorylated with [γ-^{32}P]ATP in the presence of 30 nM insulin (lanes 3 and 4) or 30 nM IGF-I (lanes 5 and 6) and immunoprecipitated with anti-INSR antibody. Supernatants (S) and pellets (P) were run on adjacent lanes of an SDS/7.5% PAGE gel and receptor subunits were detected by autoradiography.

at higher ligand concentrations (Figure 1, lanes 6–11). Immunoblotting with an anti-phosphotyrosine antibody confirmed that the proteins were phosphorylated on tyrosine residues, as expected (results not shown). On the basis of immunoprecipitation experiments presented below, and immunoblots (results not shown), we concluded that the 97 kDa band represented the INSR β subunit and that the band with the smaller electrophoretic mobility (i.e. 105 kDa) was the IGFR β subunit. Previous studies [1,9] have shown that the mobility of the IGFR β subunit is smaller than that of the INSR β subunit.

To establish the identity of the phosphoproteins, the phosphorylated WGA-purified receptors were immunoprecipitated with the anti-(insulin receptor) antibody and both the supernatant and pellet were analysed by SDS/PAGE. As expected, the supernatant was depleted and most of the labelled INSR β subunits appeared in the pellet (Figure 2, compare lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6). Notably, the IGFR β subunits, phosphorylated in the presence of IGF-I, co-precipitated with the INSR β subunit (Figure 2, lanes 5 and 6), implying that the two β subunits were associated. To demonstrate that the co-precipitation of IGFR β subunits by the anti-INSR

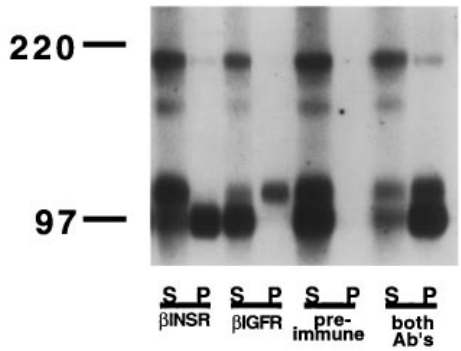

Figure 3 Antibody specificity: immunoprecipitation of $\alpha\beta$ dimers

Adipocyte receptors were phosphorylated in the presence of [γ-^{32}P]ATP and 30 nM IGF-I. Class I disulphide bonds were reduced under mild conditions and quenched with *N*-ethylmaleimide to create free INSR and IGFR $\alpha\beta$ dimers. The mixture was immunoprecipitated with anti-INSR (βINSR) or anti-IGFR (βIGFR) as indicated, and analysed as described previously. The numbers at the left are molecular masses in kDa.

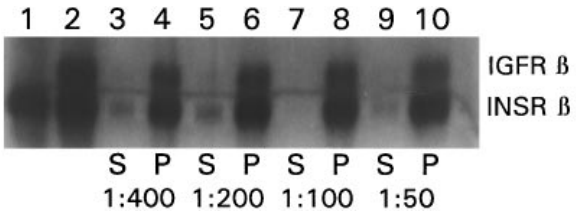

Figure 4 Quantitative immunoprecipitation of phosphorylated IGFR β subunits by anti-INSR antibody

Partly purified receptors, isolated from day 9 adipocytes, were stimulated with 100 nM IGF-I in the presence of [γ-^{32}P]ATP and immunoprecipitated with various concentrations of anti-INSR antibody. Laser densitometry of the autoradiogram revealed that more than 95% of the phosphorylated IGFR appeared in the pellet at all antibody concentrations tested.

antibody was not due to antibody cross-reactivity, purified receptors were treated with dithiothreitol to reduce class I disulphide bonds, after which the immunoprecipitation experiment was repeated. Under these conditions, the IGFR β subunits remained in the supernatant (Figure 3, lane 1), proving that the anti-(insulin receptor) antibody did not react directly with IGFR. Similarly, the anti-IGFR antibody readily immunoprecipitated the upper band, thereby confirming its identity as the IGFR β subunit, but did not react with the INSR β subunit (Figure 3, lane 4). Thus the co-precipitation of INSR and IGFR β subunits could not be explained by a lack of antibody specificity. Moreover, this experiment demonstrates that the association of the INSR and IGFR β subunits is dependent on the most easily reduced (i.e. class 1) disulphide bonds. To exclude the remote possibility that the association was due to an unidentified linker protein, insulin-stimulated INSRs were mixed with autophosphorylated human IGFRs (purified from I-24 cells) and the pooled mixture was immunoprecipitated. Under these conditions no co-precipitation was observed with either anti-INSR or anti-IGFR antibodies (results not shown). The appearance of a phosphoprotein doublet on IGF-I stimulation, in the context of the co-precipitation of IGFR β subunit with an INSR-specific antibody, verifies that insulin/IGF-I hybrids exist in 3T3-L1 adipocytes and that they undergo autophosphorylation.

Although the co-precipitation of some IGFRs with anti-INSR antibody (indicative of hybrid formation) was expected, we predicted that a portion of the IGFR β subunits representing IGFR holoreceptors would remain in the supernatant because they are not recognized by the INSR antibody. To our surprise, the anti-INSR antibody quantitatively immunoprecipitated almost all of the phosphorylated IGFR β subunits. To verify this finding, WGA-purified receptors isolated from day 9 adipocytes were stimulated with IGF-I and immunoprecipitated with graded concentrations of anti-INSR (Figure 4). Laser densitometry revealed that more than 97% of the radiolabelled IGFR β subunit was immunoprecipitated even at the lowest antibody concentration tested (1:400 dilution). Thus stimulation of the mature adipocytes by IGF-I results in phosphorylation of both the INSR and IGFR β subunits of the hybrid receptors but there were few, if any, stimulated archetypal IGFRs demonstrable after immunodepletion of the hybrids.

Development of immunoassay for hybrid receptors

To quantify hybrid receptor formation at different stages of adipocyte development, we developed a novel sandwich immunoassay. Triton X-100 extracts of cultured cells were first incubated in microtitre plates coated with anti-IGFR antibody to bind both hybrid and archetypal IGFRs. The plates were washed, to remove INSRs, and incubated with biotinylated anti-INSR antibody to detect INSR β subunits incorporated in hybrid receptors. Bound antibody was quantified colorimetrically with horseradish peroxidase-conjugated streptavidin. In a typical assay, the incubation of 250000 cell equivalents of adipocyte extract resulted in an attenuance of 1.8–2.0 after a 60 min incubation. The sensitivity was estimated by serially diluting solubilized cell preparations from mature adipocytes. Using a threshold of twice the non-specific binding, the assay could reproducibly detect 350 adipocyte cell equivalents per well (approx. 5×10^6 receptors). Specificity was evaluated by competition with the peptide against which the anti-IGFR antibody was produced. The peptide inhibited 80% of hybrid receptor binding, whereas an irrelevant peptide had minimal effect. The ELISA does not seem to detect IGF-I holoreceptors because it gave a negligible signal with I-24 cells, a cell line overexpressing the human IGFR cDNA. Similarly the assay does not seem to detect insulin holoreceptors because it gave a very low signal with an INSR-rich preparation of IM-9 cells. To correlate the changes in attenuance with receptor number, a standard curve was constructed by using day 10 adipocyte membranes and fitted to a four-parameter logistic curve with STATLIA®. Because it was not practicable to purify a sufficient quantity of hybrid receptor for direct quantification, it was not possible to derive absolute receptor numbers directly from the immunoassay. The receptor number was therefore approximated by assuming that all IGF-I-binding sites in the fully differentiated cells (see below) represented hybrids. The intra-assay coefficient of variation was 4–10%. Interassay variation was 16–25% for high-concentration receptor preparations (more than 2.6×10^9 receptors per well) and 40–50% for lower receptor concentrations (up to 1.5×10^9 receptors per well).

Changes in hybrid receptor expression during adipocyte differentiation

Differentiating 3T3-L1 cells were harvested on days 0, 3, 4, 5, 6, 7, 8 and 10 after induction of differentiation and INSR/IGFR hybrid receptor levels were determined with solubilized, partly purified membranes with the double-antibody ELISA. Hybrid

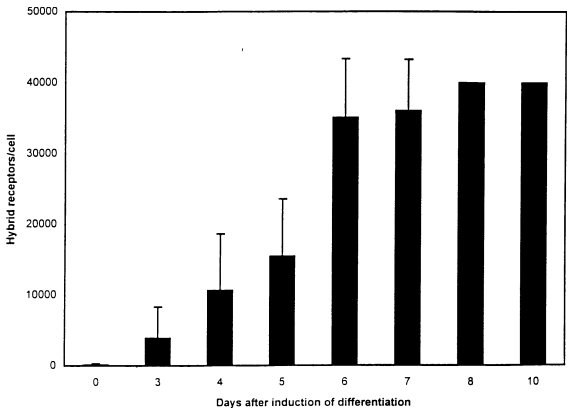

Figure 5 Changes in insulin/IGF-I hybrid receptor during 3T3-L1 adipocyte differentiation

Solubilized 3T3-L1 membranes (250000 cell equivalents), harvested during differentiation, were assayed for hybrid receptors by immunoassay. Raw attenuance readings were converted to relative receptor number as described in the Materials and methods section. Assuming that all IGF-I-binding sites in the fully differentiated cells represent hybrid receptors, 100% corresponds to approx. 37000 receptors per cell.

Table 1 Binding of IGF-I to 3T3-L1 cells during differentiation

Radiolabelled IGF-I was bound to 3T3-L1 monolayers at different stages of differentiation. Dissociation constants and total binding site estimates were based on competitive displacement with unlabelled ligand.

Differentiation time (days)	Dissociation constant (nM)	Binding sites per cell
1	0.9	46000
6	2.3	36000
10	3.6	37000

receptor levels were very low in the preadipocytes (day 0) as well as in the early stages of differentiation (day 3) and increased rapidly between days 4 and 6 after the induction of differentiation (Figure 5). After day 6, hybrid receptor levels gradually rose to a maximum at day 10. During this period the number of INSRs increased from approx. 10000 per cell (day 0) to approx. 250000 per cell (day 9) [12,14]. In contrast, radioligand studies of IGF-I binding at days 0, 6 and 9 showed that there was little change in the number of binding sites (Table 1). These results are consistent with the report of Smith et al. [14] in that there was no significant change in total binding between undifferentiated and differentiated 3T3-L1 cells. We did, however, find a higher absolute number of binding sites (37000 sites per cell compared with 13000 sites per cell). In addition we found that the affinity of IGF-I binding was higher in the undifferentiated cells. Although the lower affinity of IGF-I for binding to mature adipocytes might reflect a lower affinity for the hybrid receptor, the cell binding results must be interpreted with caution because of the known interference of cell-surface IGF-binding proteins [30] that are expressed in 3T3-L1 cells [31].

Insulin- and IGF-I-stimulated hexose uptake

To determine whether hybrid receptors were capable of modulating responses in the intact adipocyte, insulin- and IGF-I-stimulated hexose uptake was determined (Figure 6). As expected, the adipocytes were exquisitely sensitive to insulin, with an ED_{50}

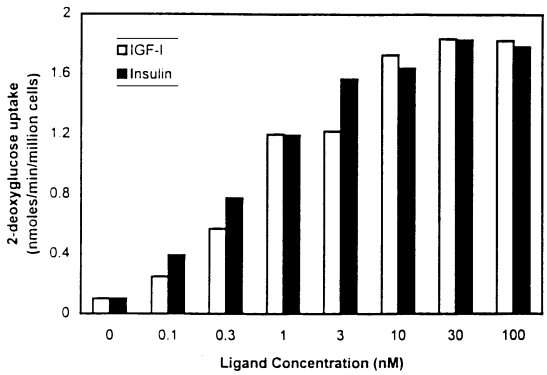

Figure 6 Insulin- and IGF-I-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes

Mature adipocytes (day 9) were incubated with 2-[^{14}C]deoxyglucose in the presence of graded concentrations of insulin or IGF-I for 10 min. Cells were washed and the radioactivity incorporated was measured as an indicator of hexose uptake.

of 0.3–1 nM and a maximal stimulated 2-deoxyglucose uptake of 1.7 nmol/min per 10^6 cells. Surprisingly, IGF-I was equally effective in inducing glucose uptake, despite the paucity of classic IGF receptors. The half-maximal response at 0.3–1 nM is indicative of the relatively high affinity of IGF-I for the hybrids and is in agreement with the IC_{50} of 0.15 nM observed in ligand-binding studies of isolated INSR/IGFR hybrids [4]. It seems that excitation of the relatively limited number of hybrids is sufficient to induce the maximal glucose uptake in adipocytes, since saturation of the more abundant insulin receptors did not result in a higher V_{max}. We therefore conclude that hybrid receptors can transmit the signal for hexose uptake when stimulated by IGF-I.

DISCUSSION

By using classic co-precipitation techniques and a novel sandwich immunoassay, we have demonstrated that 3T3-L1 adipocytes express an increasing number of INSR/IGFR hybrids during the process of differentiation. As a consequence of hybrid formation, IGF holoreceptor expression is limited and IGF-stimulated glucose uptake, previously attributed to the activation of classic type I IGFRs, occurs through activation of the hybrid INSR/IGFR molecule.

Because hybrids have been well described in multiple tissues and the increased expression of INSRs in adipocytes is well known, it is not surprising that IGFR/INSR hybrids are demonstrable in 3T3-L1 cells. Because 3T3-L1 adipocytes are known to be responsive to IGF-I [14,16,32] and have relatively abundant IGF-binding sites [14], we expected to find a mixture of classic IGF holoreceptors with hybrids. By comparing the amount of radiolabelled IGF-I that was immunoprecipitated by a specific anti-(insulin receptor) antibody with that immunoprecipitated with a specific anti-IGFR antibody, Bailyes et al. [5] reported that $68 \pm 7\%$ of the IGF-I binding to human fat represented interaction with hybrids. If one presumes that each IGFR holoreceptor binds two radioligands, then up to 81% of the receptors were in hybrid formation. Our study, however, demonstrates that IGFR holoreceptor formation is more limited in the cultured 3T3-L1 adipocyte. Remarkably, the anti-INSR antibody co-precipitated virtually all of the receptors that exhibited IGF-I-stimulated autophosphorylation. Because the antibody is highly specific and does not immunoprecipitate

IGFR directly, one must conclude that there are few classic IGF holoreceptors in fully differentiated 3T3-L1 adipocytes. When INSRs and IGFRs were co-transfected in fibroblasts, the extent of hybrid formation was proportional to the molar ratio of the insulin and IGF-I receptor expression plasmid [33], suggesting that the assembly mechanism does not discriminate between the two proreceptors. If one assumes that proreceptors segregate freely and that INSR synthesis is 10-fold that of IGFR, random assembly would result in 81% INSRs, 18% hybrids and 1% IGFRs. Thus elevated INSR production limits the expression of IGFR at the cell surface through post-translation mechanisms. Assembly of INSRs occurs in the endoplasmic reticulum before cleavage of the proreceptors [34], and we have previously shown that β subunit exchange does not occur among the fully formed INSRs [35]. Therefore it is highly unlikely that the process of hybrid formation is reversible once the receptors have been transported to the cell surface [4]. Thus our results also imply that the cell-surface IGFRs must turn over relatively rapidly during differentiation, because previously synthesized holoreceptors would not participate in hybrid formation. This is consistent with the relatively short (9 h) half-life of insulin receptors in 3T3-L1 adipocytes [35].

To provide a practical means of quantifying hybrid production, we developed a highly specific ELISA technique that allowed us to quantify hybrid formation over the 9-day course of adipocyte differentiation. Previous studies have usually quantified INSR/IGFR hybrids by measuring the percentage of IGF-I binding reactive with an insulin receptor-specific antibody [2,3,5,6,8,36,37]. The latter approach is limited by competition with other IGF-I-binding moieties, including classic IGF receptors, IGF-binding proteins and type II IGF/mannose 6-phosphate receptors. Our assay measures hybrid receptor protein immunologically, overcoming the problems inherent in interpreting ligand binding data. The assay is specific and sensitive, and lends itself easily to an analysis of multiple samples because it is performed in microtitre plates. Our results show that the hybrid receptor level increases during 3T3-L1 adipocyte differentiation, closely following the pattern of increase in insulin receptors; this supports the hypothesis that hybrid receptor formation is determined by the relative number of INSRs and IGFRs present in the cell.

Having demonstrated that IGFR receptors were limited in 3T3-L1 adipocytes, we re-examined the biological response to IGF-I in these cells. In keeping with earlier reports [16,32], hexose uptake in these cells was very responsive to stimulation by IGF-I, with half-maximal stimulation occurring between 0.3 and 1 nM. In fact IGF-I was as potent as insulin in stimulating hexose uptake in the fully differentiated cells. This response cannot be explained by interaction with the INSR, because IGF-I binds INSRs weakly; neither is it plausible that a small number of classic IGFR receptors (undetected by our assay) could produce such a vigorous response with a V_{max} similar to that of insulin. The logical conclusion is therefore that hybrid receptors can induce hexose uptake when stimulated by IGF-I. Although we have demonstrated that the IGF half of the hybrid is phosphorylated after binding of IGF-I, it is not clear that this *cis*-phosphorylation is the primary mode of action. Elegant studies of kinase-deficient INSR mutants suggest that *trans*-phosphorylation of the β subunit might be necessary for activation of the intracellular cascade [33]. Thus the increased glucose uptake produced by IGF-I might be a consequence of *trans*-activation of the INSR β subunit in the hybrid receptor. The recent reports of intracellular substrates that are exclusively activated by the respective β subunits [38] will allow us to test this hypothesis directly.

The theory that IGFR and INSR evolved from a common ancestral gene has been strengthened by the recent discovery of daf-2, in *Caenorhabditis elegans*, which shares a high degree of homology with both receptors [39]. It is therefore plausible that both the INSR and IGFR share a common assembly machinery in the endoplasmic reticulum and that there has been no selective advantage to confer specificity. It is, however, interesting to speculate that the hybrid formation is advantageous in the developing adipocyte. If one accepts the hypothesis that hybrid signalling is exclusively through the INSR β subunit, a possible selective advantage emerges. Through the synthesis of hybrids, the adipocyte might limit the propagative signals of the IGFR that are no longer needed in the growth-arrested, fully differentiated cell, while preserving the ability of the cell to respond metabolically to both hormonal signals.

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