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# INSULIN-LIKE GROWTH FACTOR-I (IGF-I) STIMULATES TYROSINE KINASE ACTIVITY IN PURIFIED RECEPTORS FROM A RAT LIVER CELL LINE†

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SUMMARY: Solubilized, lectin-purified receptor preparations from BRL 3A2 rat liver cells are rich in Type I and Type II IGF receptors, but possess few insulin receptors. High concentrations of IGF-I or insulin stimulate phosphorylation of a Mr ≃ 98K membrane protein in these preparations. Phosphorylation of a synthetic polymer of tyrosine and glutamic acid was stimulated by IGF-I > IGF-II ≃ insulin. These relative potencies, together with the results of immunodepletion experiments using an autoantibody to the insulin receptor, suggest that the effects of each of these hormones is mediated by the Type I IGF receptor. Our results are consistent with the Type I IGF receptor having intrinsic tyrosine kinase activity capable of phosphorylating the receptor itself and other substrates.

The protein encoded by the transforming gene of Rous sarcoma virus ($pp^{60}src$) as well as the receptors for epidermal growth factor and for insulin possess tyrosine-specific kinase activity capable of autophosphorylation and phosphorylation of exogenous substrates (1-4). Recently, IGF-I†††-dependent phosphorylation of tyrosine residues in the Mr ≃ 90K β-subunit of the Type I IGF receptor (5) was reported (6,7). IGF-I is chemically homologous to insulin (8) and Type I IGF receptors have structural and immunologic similarities to insulin receptors (5,9-12). We now demonstrate that

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††† Abbreviations: IGF, insulin-like growth factor; MSA, multiplication-stimulating activity; Hepes, 4-(2-hydroxyethyl)-piperazineethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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lectin-purified solubilized membrane preparations from a rat liver cell line enriched in IGF receptors possess IGF-I-stimulated tyrosine kinase activity.

MATERIALS AND METHODS: IGF-I (I/4), IGF-II (9SEIV) and partially purified IGF (1932; 36 mU/mg) were kind gifts of R. E. Humbel (Zürich). Anti-insulin receptor IgG was purified from the serum of patient B-10 (12). Poly(glu,tyr) 4:1, a random copolymer of glutamic acid and tyrosine in a 4:1 ratio (mol. wt. 20-50,000) was purchased from Sigma (St. Louis, MO). Other hormones and reagents were as previously described (12,13).

Receptor Preparations. BRL 3A2 cells, a cloned line of rat liver cells, were grown to confluence in monolayer culture as previously described (14). Cells were detached mechanically in pH 7.6 buffer [50 mM Hepes, 2 mM PMSF, aprotinin (1 T.I.U./ml)] containing 0.25 M sucrose, homogenized at 4°C, and microsomal membranes isolated by differential centrifugation (15). The membranes were solubilized in pH 7.6 buffer containing 1% Triton X-100 (4°C, 30 min), centrifuged (200,000 x g, 60 min), and the supernate applied to a wheat germ agglutinin-agarose column (13). Rat liver membranes were solubilized and receptors partially purified as previously described (13).

Binding to solubilized receptors. $^{125}$I-labeled insulin, IGF-I or MSA (rat IGF-II) (12) were incubated (16 h, 4°C) with solubilized receptors and the indicated peptides in pH 7.6 buffer (50 mM Hepes, 10 mM $MgSO_4$, 1 mg/ml bovine serum albumin. Receptor-bound radioligand was precipitated with 12.5% polyethylene glycol (16) and quantitated in a gamma spectrometer. Non-specific binding (i.e., binding in the presence of 10 µg/ml of partially purified IGF or insulin) was subtracted from total binding to give specific binding.

Receptor phosphorylation. Partially purified, solubilized BRL 3A2 receptor preparations were incubated (60 min, 22°C) ± hormones in 50 mM Hepes (pH 7.6), 0.1% Triton X-100 and 5 mM $MnCl_2$ (70 µl). [$\gamma$-$^{32}$P]ATP (10 µl) was added (final concentration 7 µM; 28 Ci/mmol, New England Nuclear). After 20 min at 22°C, the reaction was stopped by adding: (i) 20 µl of 50 mM Hepes (pH 7.6), 50 mM sodium pyrophosphate, 250 mM NaF, 25 mM EDTA and 25 mM ATP, and (ii) 100 µl of 2X SDS gel sample buffer (9) ± 200 mM dithiothreitol (DTT), and boiling for 5 min (17). SDS-polyacrylamide gel electrophoresis and autoradiography were as described previously (9,12).

Phosphorylation of poly(glu,tyr) 4:1. Solubilized receptors, hormones, poly(glu,tyr) 4:1 (2.2 mg/ml) and $Mg(CH_3COO)_2$ (10 mM) were incubated in pH 7.6 buffer (30 min, 22°C). [$\gamma$-$^{32}$P]ATP was added (8-18 min), the reaction terminated in trichloroacetic acid/Na pyrophosphate and $^{32}$P incorporated into the artificial substrate determined (15,18).

Effect of immunodepletion with anti-insulin receptor IgG (B-10) on tyrosine kinase activity. Receptor preparations (300 µl) from rat liver membranes (250 µg/ml) or BRL 3A2 cell membranes (55 µg/ml) were incubated (16 h, 4°C) with 30 µl of control IgG (0.9 mg/ml) or IgG B-10 (0.5 and 2.5 mg/ml). Protein A (150 µl, 20% Pansorbin w/v in 50 mM Hepes, pH 7.6) was added (4°C, 1 h). Receptor-antibody-protein A complexes were removed by centrifugation (8,000 x g, 5 min, 4°C). Residual hormone-sensitive tyrosine kinase activity in the supernatant medium was determined on aliquots (50 µl) after incubation with hormones, poly(glu,tyr) 4:1 (2.2 mg/ml) and $Mg(CH_3COO)_2$ (10 mM) as described above.

RESULTS: Solubilized and lectin-purified BRL 3A2 membranes possess Type I and Type II IGF receptors but not insulin receptors. Microsomal membrane preparations from BRL 3A2 cells were solubilized with Triton X-100 and purified

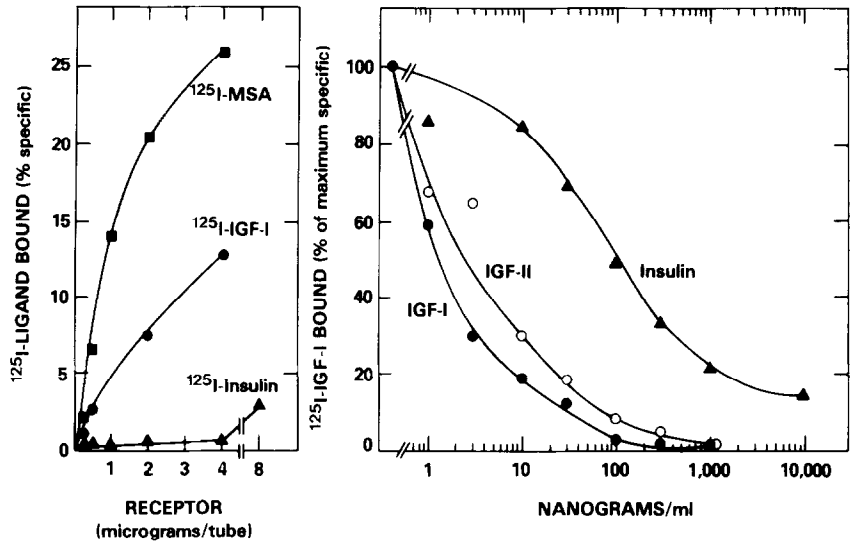


Figure 1: Binding to solubilized BRL 3A2 cell membranes. (Left): Different concentrations of solubilized membranes were incubated with $^{125}$I-IGF-I (250 pg, ●), $^{125}$I-MSA (200 pg, ■) or $^{125}$I-insulin (40 pg, ▲). Specific binding (Bound/Total radioactivity) is plotted against receptor protein added (μg/tube). (Right): $^{125}$I-IGF-I (250 pg) was incubated with solubilized BRL 3A2 membranes (1.6 μg) and different concentrations of unlabeled IGF-I (●), IGF-II (○), and insulin (▲). The percentage of maximal specific binding of $^{125}$I-IGF-I (18.3% of total) is plotted against ng/ml of peptide.

by lectin chromatography. These preparations bound $^{125}$I-IGF-I and $^{125}$I-MSA specifically at low protein concentrations (0.25-0.5 μg), whereas binding of $^{125}$I-insulin was low even at high concentrations of protein (4-8 μg) (Fig. 1, left). $^{125}$I-IGF-I and $^{125}$I-MSA bound predominantly to Type I and Type II IGF receptors (5), respectively. $^{125}$I-IGF-I binding was inhibited by IGF-I > IGF-II >> insulin (Fig. 1, right). $^{125}$I-MSA binding was inhibited by unlabeled MSA and IGF, but only minimally inhibited by insulin (results not shown). The presence of both IGF receptor subtypes and the paucity of insulin receptors in solubilized membranes are consistent with previous observations in intact BRL 3A2 cells (9,14).

IGF-I and insulin stimulate phosphorylation of a Mr ≃ 98K membrane protein that exists as part of a Mr > 300K complex. Partially purified BRL 3A2 receptors were incubated with IGF-I or insulin, or without added polypeptide (60 min, 22°C). [γ-$^{32}$P]ATP was added (20 min), and aliquots of the incubation mixture examined by SDS-gel electrophoresis and autoradiography

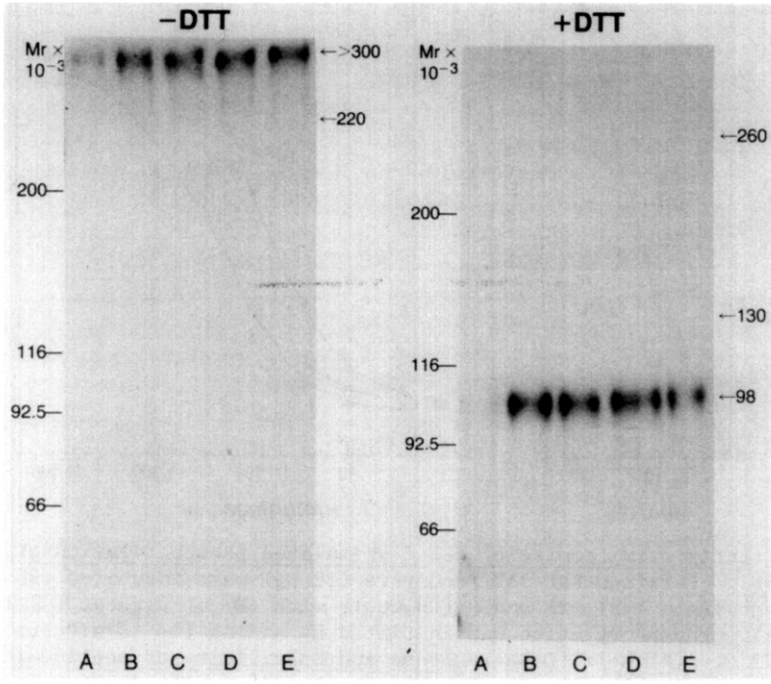


Figure 2: IGF-I and insulin-stimulated phosphorylation of proteins in partially purified BRL 3A2 cell membranes. Solubilized, purified BRL 3A2 cell membranes (0.3 µg/lane) were incubated (60 min, 22°C, 5 mM $MnCl_2$) without peptide (A) or with IGF-I [(100 ng/ml (B), 1000 ng/ml (C)] or insulin [(100 ng/ml (D), 1000 ng/ml (E)] as described in Materials and Methods. Electrophoresis was performed in the absence (-DTT, left) or in the presence (+DTT, right) of 100 mM dithiothreitol.

$^{125}$I-IGF-I or $^{125}$I-MSA were crosslinked to BRL 3A2 cells with disuccinimidyl suberate (9). The electrophoretic mobilities of crosslinked IGF-I/Type I-receptor complexes (Mr ≃ 130K, +DTT; Mr > 300K, -DTT) and MSA/Type II-receptor complexes (Mr ≃ 260K, +DTT; Mr ≃ 220K, -DTT) are shown by arrows.

(Fig. 2). High concentrations (100 and 1000 ng/ml) of both hormones significantly stimulated phosphorylation of a single major protein of Mr ≃ 98K (+DTT) that was part of a Mr > 300K complex (-DTT). We propose that the Mr ≃ 98K phosphoprotein represents the β-subunit of the Type I-IGF receptor. Phosphorylation of the α-subunit of the Type I-IGF receptor (Mr ≃ 130K, +DTT), or of the Type II-IGF receptor (Mr ≃ 220K, -DTT; Mr ≃ 260K, +DTT) was not observed.

Phosphorylation of poly(glu,tyr) 4:1 is stimulated by IGF-I, IGF-II and insulin. The preceding results did not distinguish whether the β-subunit of the Type I IGF receptor was merely a substrate for a protein kinase or whether, like the insulin receptor (4,19), the receptor itself might possess

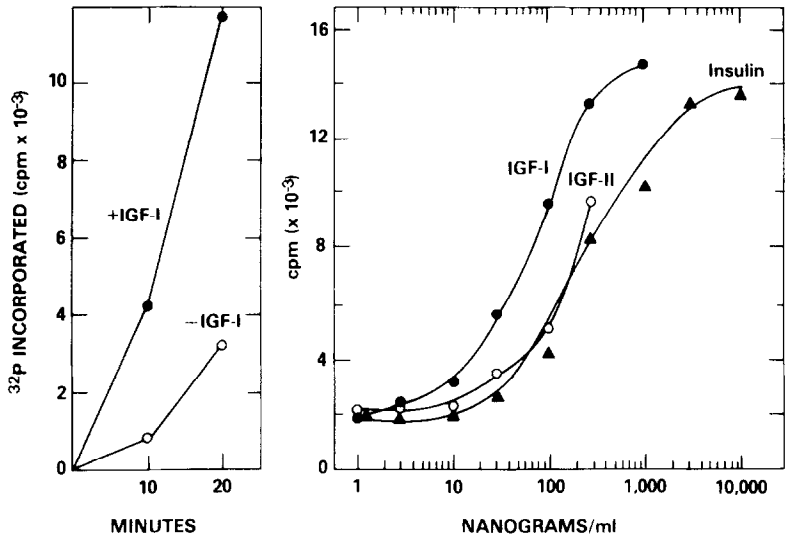


Figure 3: (Left): Time course of IGF-I stimulated phosphorylation of poly (glu,tyr) 4:1. Purified BRL 3A2 receptors (12 µg) were incubated (30 min, 22°C) with poly(glu,tyr) 4:1 and $Mg(CH_3COO)_2$ with (●) or without (○) IGF-I (0.54 µg/ml). Phosphorylation was initiated by adding [$\gamma$-$^{32}$P]ATP for 8 or 16 min at 22°C. (Right): Dose response and specificity of peptide-induced phosphorylation of poly(glu,tyr) 4:1. Solubilized, purified BRL 3A2 receptors (4.5 µg) were incubated (30 min, 22°C) with IGF-I (●), IGF-II (○) or insulin (▲) at the indicated concentrations in the presence of poly(glu,tyr) 4:1 (2.2 mg/ml) and $Mg(CH_3COO)_2$ (10 mM). Reaction with [$\gamma$-$^{32}$P]ATP was for 12 min.

intrinsic tyrosine kinase activity. IGF-I-stimulated tyrosine kinase was demonstrated using [$\gamma$-$^{32}$P]ATP and a synthetic tyrosine-containing polymer. IGF-I stimulated $^{32}$P incorporation into poly(glu,tyr) 4:1 by partially purified BRL 3A2 receptors by 4-10-fold (Fig. 3, left). Phosphorylation also was stimulated by IGF-II and insulin, (Fig. 3, right). Maximally effective concentrations of IGF-I and insulin gave 6-7 fold stimulation. IGF-I was most potent (half-maximal stimulation at ≃ 40 ng/ml); IGF-II was ≃ 5-fold less potent. The relative potencies of IGF-I and IGF-II were similar to their ability to bind to solubilized Type I IGF receptors (Fig. 1). Insulin was similar to IGF-II in its ability to stimulate phosphorylation. The high potency of IGF-I and IGF-II virtually excludes the possibility that these hormones stimulate artificial substrate phosphorylation via the insulin receptor. The greater potency of IGF-I than IGF-II is consistent with both peptides stimulating phosphorylation via the Type I-IGF receptor.

The fact that insulin was more potent in stimulating phosphorylation than in binding to the Type I-IGF receptor suggested the possibility that part of its effects might be mediated by the low level of insulin receptor. To investigate this possibility, anti-insulin receptor immunoglobulin B-10 was used to selectively deplete insulin receptors (and insulin receptor-associated tyrosine kinase) by immunoprecipitation without affecting Type I-IGF receptors (N. Sasaki, unpublished results). When BRL 3A2 receptors were incubated with IgG B-10 and immune complexes removed with protein A, insulin-stimulated and IGF-I-stimulated kinase activity in the supernate was not decreased (Fig. 4, bottom). By contrast, when purified rat liver membrane preparations that contain insulin receptors and insulin-stimulated kinase but lack IGF receptors and IGF-I-stimulated kinase were immunoprecipitated with IgG B-10, insulin-stimulated phosphorylation by the supernate was depleted. We conclude that although insulin stimulates substrate phosphorylation via

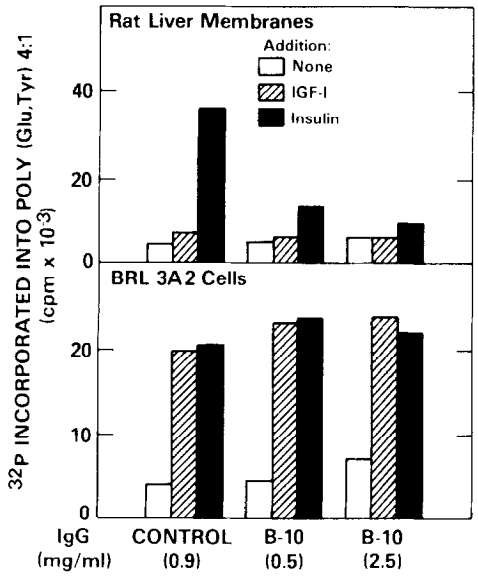


Figure 4: Effect of immunodepletion with control and B-10 IgG on insulin- and IGF-I-stimulated tyrosine kinase. Receptor preparations from rat liver membranes (top) or BRL 3A2 cell membranes (bottom) were incubated with control or B-10 IgG. Receptor-antibody complexes were removed by precipitation with protein A. Supernate (50 µl) was incubated (30 min, 22°C) without added peptide ( ☐ ) or with IGF-I (100 ng/ml, ▨ ) or insulin [(600 ng/ml (top) or 3000 ng/ml (bottom), ■ )] in the presence of poly(glu,tyr) 4:1 and $Mg(CH_3COO)_2$. [$\gamma$-$^{32}$P]ATP was added for 18 min.

the insulin receptor in rat liver membranes, in BRL 3A2 cells insulin, like IGF-I and IGF-II, appears to act via the Type I-IGF receptor.

## DISCUSSION

Partially purified receptor preparations from BRL 3A2 cells exhibit IGF-I and insulin-stimulated tyrosine kinase activity toward a synthetic substrate. The relative potencies of the IGFs and insulin (i.e., IGF-I > IGF-II ≃ insulin) and the results of immunodepletion experiments using an autoantibody to the insulin receptor strongly suggest that the effects of each of these hormones are mediated by the Type I-IGF receptor.

Indirect evidence suggests that the IGF-I-stimulated tyrosine kinase is associated with the Type I-IGF receptor. IGF-I and insulin at high concentrations stimulate phosphorylation of a protein of Mr ≃ 98K under reducing conditions and Mr > 300K without disulfide reduction. Although this size is compatible with either the β-subunit of the insulin receptor or the Type I IGF receptor, the paucity of insulin receptors in the preparation favors assignment to the IGF receptors. IGF-I-dependent phosphorylation of β-subunits of Type I IGF receptors in human lymphocytes and placenta recently has been reported (6,7). Our results with the artificial substrate suggest that β-subunit phosphorylation results from stimulation of receptor tyrosine kinase activity, rather than reduced activity of a hypothetical IGF-I-stimulated phosphatase or modification of the Type I receptor as a substrate for kinase.

Higher concentrations of IGF-I and IGF-II were required to stimulate synthetic substrate phosphorylation than to inhibit $^{125}$I-IGF-I binding. The basis for this difference is unknown, but may reflect different experimental conditions. Similar results were obtained for 3T3-L1 cell insulin receptors (20). On the other hand, increasing the time of preincubation with peptide increased the sensitivity of phosphorylation by insulin in rat liver membranes (13).

Although our preparations contain abundant Type II-IGF receptors, we have no evidence that under our assay conditions the Type II receptor is phosphorylated (i.e., substrate) or that it possesses kinase activity toward poly(glu,

tyr) 4:1. The relative potencies of IGF-I and IGF-II are most compatible with mediation by a Type I-IGF receptor. Thus, once again, the Type I-IGF receptor seems more homologous to insulin receptors than to Type II-IGF receptors (5). Preliminary results suggest that Type I-IGF receptor and insulin receptor kinases have similar cation and substrate specificities.

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