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# Phosphorylation of Insulin-like Growth Factor I Receptor by Insulin Receptor Tyrosine Kinase in Intact Cultured Skeletal Muscle Cells<sup>†</sup>

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ABSTRACT: The interaction between insulin and insulin-like growth factor I (IGF I) receptors was examined by determining the ability of each receptor type to phosphorylate tyrosine residues on the other receptor in intact L6 skeletal muscle cells. This was made possible through a sequential immunoprecipitation method with two different antibodies that effectively separated the phosphorylated insulin and IGF I receptors. After incubation of intact L6 cells with various concentrations of insulin or IGF I in the presence of  $[^{32}P]$ orthophosphate, insulin receptors were precipitated with one of two human polyclonal anti-insulin receptor antibodies (B2 or B9). Phosphorylated IGF I receptors remained in solution and were subsequently precipitated by anti-phosphotyrosine antibodies. The identities of the insulin and IGF I receptor  $\beta$ -subunits in the two immunoprecipitates were confirmed by binding affinity, by phosphopeptide mapping after trypsin digestion, and by the distinct patterns of expression of the two receptors during differentiation. Stimulated phosphorylation of the  $\beta$ -subunit of the insulin receptor correlated with occupancy of the  $\beta$ -subunit of the insulin receptor by either insulin or IGF I as determined by affinity cross-linking. Similarly, stimulation of phosphorylation of the  $\beta$ -subunit of the IGF I receptor by IGF I correlated with IGF I receptor occupancy. In contrast, insulin stimulated phosphorylation of the  $\beta$ -subunit of the IGF I receptor at hormone concentrations that were associated with significant occupancy of the insulin receptor but negligible IGF I receptor occupancy. These findings indicate that the IGF I receptor can be a substrate for the hormone-activated insulin receptor tyrosine kinase activity in intact L6 skeletal muscle cells.

The insulin receptor contains an intrinsic protein kinase activity that phosphorylates tyrosine residues in the  $\beta$ -subunit of the receptor after hormone binding (Kasuga et al., 1982a,b; Roth & Cassell, 1983; Ullrich et al., 1985). Similarly, the structurally homologous receptor for insulin-like growth factor I (IGF I)<sup>1</sup> (Kasuga et al., 1981; Kull et al., 1983; Massague

& Czech, 1982; Rechler et al., 1980; Rechler & Nissley, 1985) becomes phosphorylated on tyrosine residues of its  $\beta$ -subunit in response to IGF I binding (Jacobs et al., 1983; Rubin et al., 1983; Zick et al., 1984). It has been hypothesized that tyrosine phosphorylation plays a role in mediating the biological actions of these two hormones, either via the phosphorylation of other proteins by the activated receptor kinases or as a result of the modified properties of the receptor that

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<sup>&</sup>lt;sup>1</sup> Abbreviations: IGF I, insulin-like growth factor I; IGF II, insulinlike growth factor II; HEPES, N-(2-hydroxyethyl)piperazine-N'-2ethanesulfonic acid; HPLC, high-pressure liquid chromatography; MSA, multiplication stimulating activity; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;  $\alpha$ -ptyr, anti-phosphotyrosine antibody; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

follow its autophosphorylation. Since insulin and IGF I have a similar spectrum of activities, it is also possible that the kinase of one type of receptor could phosphorylate the other type of receptor and thus initiate biological responses. Such a mechanism might help to explain the diversity of actions of these two hormones.

We have investigated the potential interactions between insulin and IGF I receptor phosphorylation in intact L6 cultured rat skeletal muscle cells (Yaffe, 1968). This cell line previously has been shown to have distinct receptors for insulin and IGF I, each of which mediates responses to low concentrations of its specific hormone (Beguinot et al., 1985, 1986). With anti-insulin receptor and anti-phosphotyrosine antibodies, we have been able to separate insulin and IGF I receptors from L6 cells and have characterized the phosphorylation of the two receptors. Using these methods, it was possible to study the relationship between occupancy and phosphorylation of both types of receptors and interactions between these receptors at the level of phosphorylation.

#### EXPERIMENTAL PROCEDURES

Materials. Insulin was provided by Lilly (Indianapolis, IN), and Thr<sup>59</sup>-IGF I was purchased from AmGen (Thousand Oaks, CA). Rat IGF II, alternatively designated multiplication stimulating activity (MSA), was purified from conditioned medium of BRL-3A cells by a modification of the procedure of Moses et al. (1980). [125I]Monoiodoinsulin (100 Ci/g) and [<sup>32</sup>P]orthophosphate were from New England Nuclear (Boston, MA). IGF I was labeled with <sup>125</sup>I by the chloramine-T method (Pilistine et al., 1984) to a specific activity of 420 Ci/g. Pansorbin was from Calbiochem-Behring (San Diego, CA), wheat germ agglutinin-agarose from Vector (Burlingame, CA), and albumin (fraction V from bovine serum) from Armour (Phoenix, AZ). Immunoglobulins were purified from sera of two patients (designated B2 and B9) with the type B syndrome of extreme insulin resistance (Kahn et al., 1976). Polyclonal anti-phosphotyrosine antibody was prepared in rabbits as previously described (Pang et al., 1985a).

Receptor Phosphorylation. The L6 rat skeletal muscle cells were cultured as previously described (Beguinot et al., 1986). For phosphorylation experiments, cells were plated in 100-mm tissue culture dishes ( $6 \times 10^3$  cells/cm<sup>2</sup>) and grown for the indicated periods of time. The culture medium was then aspirated; the plates were extensively washed with Eagle's minimum essential medium supplemented with 0.5% albumin and subsequently incubated for 15-18 h at 37 °C with the same medium. This medium was aspirated, and the dishes were rinsed 3 times with a solution containing 150 mM NaCl and 50 mM HEPES, pH 7.4. The cells then were incubated for 3.5 h with 6 mL of phosphate-free RPMI 1640 medium (Moore et al., 1967) containing 1 mCi/mL [<sup>32</sup>P]orthophosphate and 0.5% dialyzed albumin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Insulin, IGF I, or IGF II was subsequently added at the indicated concentrations, and the incubation was continued for 10 more min. The phosphorylation reaction was quenched by rapidly aspirating the medium and freezing the cell monolayer with liquid nitrogen (1.5 mL/dish).

The frozen cells were thawed and solubilized in 1 mL of a solution containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 4 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM phenylmethanesulfonyl fluoride, and 0.2 mg/mL aprotinin (14 trypsin inhibitor units/mg). The soluble material was sedimented by centrifugation at 225000g for 90 min, and the supernatant was applied to a 0.5-cm-diameter disposable column containing 0.3 mL of wheat germ agglu-

tinin-agarose. The column was washed with 100 mL of 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 4 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 2 mM phenylmethanesulfonyl fluoride. Bound glycoproteins were then eluted with 1 mL of this solution supplemented with 0.3 M *N*-acetylglucosamine. Insulin receptors were immunoprecipitated from this eluate with anti-insulin receptor antibodies (B2 or B9) as previously described (Kasuga et al., 1984). Immunoprecipitation of tyrosine-phosphorylated receptors was performed with anti-phosphotyrosine antibody as described by Pang et al. (1985b).

The immunoprecipitated proteins were reduced with 5% (v/v) 2-mercaptoethanol and separated by SDS-PAGE on 7.5% resolving gels (Laemmli, 1970). The following proteins were used to estimate molecular weight: myosin,  $M_r$  200000;  $\beta$ -galactosidase,  $M_r$  116250; phosphorylase b,  $M_r$  94000; bovine serum albumin,  $M_r$  66000; and ovalbumin,  $M_r$  45000. The [<sup>32</sup>P]phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The intensity of labeled bands on the autoradiographs was quantitated by densitometric scanning using an LKB 2202 laser densitometer. In some instances, this result was confirmed by quantitating the Cerenkov radiation from solubilized segments of the gels.

Analysis of Tryptic Phosphopeptides and Identification of Phosphoamino Acids. Tryptic phosphopeptides were obtained from receptor  $\beta$ -subunits contained in polyacrylamide gel fragments as previously described (White et al., 1984) and separated by high-performance liquid chromatography. Tryptic digests were applied to a 25-cm  $\mu$ Bondapak C<sub>18</sub> reverse-phase column (Waters, Milford, MA) and eluted at a flow rate of 1 mL/min with mobile phases composed of water with 0.05% trifluoroacetic acid and a nonlinear, concave-upward gradient of acetonitrile (from 0% to 40%) during 95 min. Fractions (1 mL) were collected, and the radioactivity in each fraction was measured as Cerenkov radiation with an efficiency of 40% using a Beckman LS 1801 liquid scintillation counter. Acetonitrile had no effect on the efficiency of Cerenkov counting. Trypsin digestion of the immunoprecipitated receptor  $\beta$ -subunits in polyacrylamide gel fragments generally released about 70% of the radioactivity. Approximately 80% of the radioactivity in the trypsin digests was routinely recovered from the reverse-phase column.

Affinity Labeling of Receptors. Covalent labeling of insulin and IGF I receptors was performed as previously described (Beguinot et al., 1985). Briefly, cells were incubated for 4 h at 15 °C with  $(1-4 \times 10^{-10} \text{ M}^{125}\text{I} \text{ insulin or } ^{125}\text{I} \text{ IGF I and}$ the indicated concentrations of unlabeled peptides in HEPES buffer (120 mM NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 10 mM NaHCO<sub>3</sub>, 1.3 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, and 1% bovine serum albumin, pH 7.8). Unbound hormone was subsequently removed by aspiration and washing with the same buffer, and bound radioligand was chemically crosslinked by addition of 100  $\mu$ M disuccinimidyl suberate in albumin-free HEPES buffer for 30 min. Cell proteins then were solubilized in SDS buffer (0.5 M Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, and 5%  $\beta$ -mercaptoethanol, pH 6.8) and analyzed on 7.5% polyacrylamide gels. The intensity of the labeled bands on autoradiographs was quantitated by densitometric scanning.

### RESULTS

Insulin Receptor Phosphorylation in L6 Cells. After equilibration for 3.5 h with  $[^{32}P]$  orthophosphate, intact L6 myotubes were incubated for 10 min with  $10^{-7}$  M insulin and solubilized with a mixture designed to inhibit both phospha-



FIGURE 1: Identification of two distinct  $M_r$  95000 phosphoproteins precipitated by anti-insulin receptor and anti-phosphotyrosine antibodies in L6 cells. L6 myotubes were equilibrated with  $\int_{1}^{32}P$ ]orthophosphate for 3.5 h, incubated for 10 min with 0 or  $10^{-7}$  M insulin as indicated, and solubilized as described under Experimental Procedures. After partial purification by wheat germ-agarose chromatography, the cell extracts were incubated with anti-insulin receptor antibody (B9) for 8 h at 4 °C. Immunocomplexes were then precipitated (lanes 1 and 2), and supernatant from the insulin-stimulated sample was incubated again with B9 (lane 3) or anti-phosphotyrosine antibody ( $\alpha$ -ptyr, lane 4). In each case, equal volumes of sample were incubated with antibody. Proteins from the immunoprecipitates were dissolved in Laemmli buffer and analyzed by gel electrophoresis under reducing conditions (Laemmli, 1970).

tases and kinases. A glycoprotein fraction was then prepared by wheat germ agglutinin-agarose affinity chromatography, and phosphoproteins were immunoprecipitated from this fraction by anti-insulin receptor antibodies (B2 or B9), separated by polyacrylamide gel electrophoresis under reducing conditions, and identified by autoradiography. In the absence of insulin, two bands of  $M_r$  95000 and 88000 were immunoprecipitated (Figure 1, lane 1). After stimulation with insulin, <sup>32</sup>P incorporation into the  $M_r$  95000 band increased about 6-fold, and labeling of the M, 88 000 species increased approximately 3-fold (Figure 1, lane 2). Although the insulin receptor  $\beta$ -subunit usually appears as a single protein of molecular weight approximately 90 000, precipitation of the two bands by the B2 and B9 antibodies suggested that both represent the insulin receptor. A similar doublet pattern has also been observed by Tamura et al. (1983) in studies of insulin receptors in adipocytes and by this laboratory in hepatocytes (unpublished results). Under nonreducing conditions, both species migrated as a complex of greater than  $M_r$  300000 (data not shown).

The action of insulin on phosphorylation of the  $M_r$  95000 and 88 000 bands was rapid and sustained, with maximal effects achieved within 1 min and maintained for at least 15 min. The effect of insulin on phosphorylation of the  $M_r$  95000 species was half-maximal at a concentration of 10-9 M and reached a plateau at 10<sup>-8</sup> M (Figure 2A). IGF I also stimulated phosphorylation of this protein, but much higher concentrations were required. Similar dose-response relationships were observed for the  $M_r$  88 000 species (Figure 2B). The potency of insulin in stimulating phosphorylation of these bands was almost identical with its potency in stimulating the uptake of glucose and amino acids by the L6 cells (Beguinot et al., 1986). In contrast, IGF I was at least 50-fold less potent in stimulating phosphorylation of the immunoprecipitated bands than in stimulating metabolite uptake (Beguinot et al., 1985). IGF II at 10<sup>-7</sup> M was 100-fold less effective than IGF I in stimulating <sup>32</sup>P incorporation into the two bands. On the basis



FIGURE 2: Dose-response relationships between insulin (•) and IGF I (•) concentrations and insulin receptor phosphorylation in intact L6 cells. L6 myotubes labeled with [ $^{32}P$ ]orthophosphate were incubated for 10 min at 37 °C with the indicated concentrations of insulin or IGF I. Cells were solubilized, and a glycoprotein fraction was purified by wheat germ-agarose chromatography and incubated with anti-receptor antibody B9. Immunocomplexes were precipitated by gel electrophoresis under reducing conditions. The  $M_r$  95000 (A) and 88 000 (B)  $\beta$ -subunits of the insulin receptor were localized by autoradiography and the corresponding fragments of the gel removed for determination of radioactivity by Cerenkov counting. A representative experiment is shown.

of these dose-response data and the fact that anti-insulin receptor antibodies B2 and B9 have a higher affinity for insulin receptors than IGF I receptors in L6 cells (data not shown), it was tentatively concluded that the labeled bands immunoprecipitated by the anti-insulin receptor antibodies represented insulin receptors and not IGF I receptors.

IGF I Receptor Phosphorylation. We previously have shown that L6 cells possess both insulin and IGF I receptors (Beguinot et al., 1985, 1986). Since anti-insulin receptor antibodies do not effectively immunoprecipitate the IGF I receptors, we investigated whether phosphorylated receptors for IGF I could be identified by anti-phosphotyrosine antibodies ( $\alpha$ -ptyr) after depletion of insulin receptors by immunoprecipitation. For this purpose, <sup>32</sup>P-labeled insulin receptors were first immunoprecipitated from glycoprotein extracts with the B9 antibody, and the remaining supernatant then was treated with  $\alpha$ -ptyr (Figure 1). As described above, antiinsulin receptor antibody immunoprecipitated two phosphoproteins ( $M_r$  88 000 and 95 000) (Figure 1, lane 1). In the presence of 10<sup>-7</sup> M insulin, phosphorylation of these proteins was increased (Figure 1, lane 2). Repeat immunoprecipitation of the supernatant with the B9 antibody yielded no further phosphoproteins, indicating the completeness of the first immunoprecipitation reaction (Figure 1, lane 3). Subsequent treatment of the supernatant with  $\alpha$ -ptyr, however, yielded an additional  $M_r$  95000 phosphoprotein (Figure 1, lane 4). These findings indicated that the phosphoproteins precipitated by B9 are distinct from the phosphoprotein precipitated by  $\alpha$ -ptyr after B9 immunodepletion.

Figure 3 shows dose-response curves for IGF I and insulin stimulation of <sup>32</sup>P incorporation into the phosphoprotein precipitated by  $\alpha$ -ptyr after insulin receptor immunodepletion. Stimulation of <sup>32</sup>P incorporation by IGF I was half-maximal at  $5 \times 10^{-10}$  M and reached a plateau at  $5 \times 10^{-9}$  M. Insulin was about 15% as potent as IGF I in stimulation of this phosphorylation (Figure 3), and IGF II was 50-fold less potent (data not shown). Thus, in contrast to the phosphoproteins precipitated by the B9 anti-insulin receptor antibody, the species precipitated by  $\alpha$ -ptyr was more potently phosphorylated in cells treated with IGF I than with insulin. When gels were run under nonreducing conditions, both the band precipitated by B9 antibody and that sequentially precipitated by  $\alpha$ -ptyr migrated at approximately  $M_r$  320000, with no material present at M, 95000. On the basis of these experiments, it was tentatively concluded that the proteins precip-



FIGURE 3: Dose-response relationships between insulin (**I**) and IGF I (**•**) concentrations and IGF I receptor phosphorylation in intact L6 cells. L6 myotubes labeled with [<sup>32</sup>P]orthophosphate were incubated for 10 min at 37 °C with the indicated concentrations of insulin or IGF I. Cells were solubilized as described under Experimental Procedures, and glycoproteins were partially purified by wheat germ-agarose chromatography. The extracts were depleted of insulin receptors with B9 anti-insulin receptor antibody, and the supernatants were then further immunoprecipitated with anti-phosphotyrosine antibody. Proteins from the sequential immunoprecipitation were dissolved in Laemmli sample buffer and analyzed by gel electrophoresis under reducing conditions (Laemmli, 1970). The  $\beta$ -subunit of the IGF I receptor was localized by autoradiography and quantitated by Cerenkov counting. A representative experiment is shown.

itated by the anti-insulin receptor antibody and visualized on gels under reducing conditions represented phosphorylated insulin receptor  $\beta$ -subunits whereas the protein remaining after depletion of insulin receptors and precipitated by antiphosphotyrosine antibody was the IGF I receptor  $\beta$ -subunit. This conclusion was further supported by phosphopeptide mapping and by comparative studies on the phosphoproteins during L6 cell differentiation as described below.

Characterization of Phosphorylated Insulin and IGF I Receptors. (A) Phosphopeptide Mapping of the M, 95000 Phosphoproteins. The Mr 95000 phosphoproteins immunoprecipitated by B9 anti-insulin receptor antibody or by sequential immunoprecipitation with  $\alpha$ -ptyr anti-phosphotyrosine antibody were excised from polyacrylamide gels and digested with trypsin. This yielded a mixture of phosphopeptides which was separated by reverse-phase HPLC. In the absence of insulin stimulation, a single major [<sup>32</sup>P]phosphopeptide was obtained from the  $M_r$  95000 B9-precipitated protein (Figure 4A). After incubation with insulin for 10 min, at least four additional phosphopeptides appeared (Figure 4B). The peptide map of this species was almost identical with that of the insulin receptor  $\beta$ -subunit purified from the rat Fao hepatoma cell line (White et al., 1985). The 95000-dalton band precipitated by  $\alpha$ -ptyr after insulin receptor immunodepletion yielded a different pattern of phosphopeptides (Figure 4C). This included a new prominent peak in the early fractions (peak 1) and the absence of peak 4 of the insulin receptor. Phosphopeptides 2 and 3 showed similar elution in the two protein species, but an additional peak appeared between them. Thus, by HPLC analysis, the  $M_r$  95000 protein precipitated from L6 cells by anti-insulin receptor antibodies had a phosphopeptide map identical with that of the insulin receptor  $\beta$ subunit of Fao cells. After insulin receptor immunodepletion,  $\alpha$ -ptyr antibody precipitated a different M, 95000 protein, consistent with our identification of this species as the IGF I receptor  $\beta$ -subunit.

(B) Changes in Insulin and IGF I Receptors during Differentiation. We previously have shown that insulin receptors increase during L6 cell differentiation whereas IGF I receptors decrease (Beguinot et al., 1985, 1986). We therefore investigated whether hormone-stimulated phosphorylation of the two receptors identified with anti-insulin receptor and  $\alpha$ -ptyr



FIGURE 4: Characterization of tryptic phosphopeptides from the  $M_r$ 95000  $\beta$ -subunits of insulin and IGF I receptors: (A)  $M_r$  95000 B9-precipitated species in the absence of insulin; (B)  $M_r$  95000 B9-precipitated species after insulin treatment; (C)  $M_r$  95000 insulin-stimulated species precipitated by  $\alpha$ -ptyr after B9 immunodepletion. L6 myotubes labeled for 3.5 h with [<sup>32</sup>P]orthophosphate were incubated without insulin or with 10<sup>-7</sup> M insulin as indicated for 10 min. Insulin and IGF I receptors were identified by immunoprecipitation with antibodies B9 and  $\alpha$ -ptyr, respectively, as shown in Figure 1. Proteins in the immunoprecipitates were dissolved in Laemmli buffer and separated by gel electrophoresis under reducing conditions (Laemmli, 1970). The  $M_r$  95000  $\beta$ -subunits immunoprecipitated by antibodies B9 (A, B) and  $\alpha$ -ptyr (C) were identified by autoradiography, excised from the gels, and digested with trypsin as described under Experimental Procedures. The resulting phosphopeptides were separated by reverse-phase HPLC. The solid line represents the Cerenkov radiation measured in each fraction, and the dashed line represents the acetonitrile gradient.

antibodies exhibited similar changes. For this purpose, undifferentiated myoblasts and differentiated myotubes were equilibrated with [32P]orthophosphate for 3.5 h and then stimulated with 10<sup>-7</sup> M insulin for 10 min. At this concentration, insulin binds to both insulin and IGF I receptors. Labeled glycoproteins were obtained with wheat germ agglutinin and immunoprecipitated either with the B9 antibody or with anti-phosphotyrosine antibody after B9 immunodepletion. As shown in Figure 5, after differentiation to myotubes, L6 cells showed a 4-6-fold increase in phosphorylation of the  $M_r$  95 000 and 88 000 subunits of the insulin receptor in comparison with undifferentiated myoblasts (lane 2 vs lane 1). Opposite changes were observed for the presumed IGF I receptor  $\beta$ -subunit (lanes 3 and 4). In this case, phosphorylation decreased 4-fold during differentiation from myoblasts to myotubes. Thus, during L6 cell differentiation, changes in hormone-stimulated phosphorylation of the two species identified as insulin and IGF I receptors closely paralleled the known changes in receptor number.

Relationship between Occupancy and Phosphorylation of Insulin and Type I IGF Receptors. Using the B9 and  $\alpha$ -ptyr antibodies to separate phosphorylated insulin and type I IGF receptors, it was possible to investigate the relationship between phosphorylation and occupancy of the two receptor types by the two hormones. For this purpose, insulin and IGF I receptors were affinity labeled by cross-linking <sup>125</sup>I insulin or



FIGURE 5: Changes in insulin and IGF I receptor phosphorylation during L6 cell differentiation. Undifferentiated L6 myoblasts and differentiated myotubes were equilibrium labeled with [<sup>32</sup>P]orthophosphate, incubated for 10 min with 10<sup>-7</sup> M insulin, and solubilized as described under Experimental Procedures. After partial purification by wheat germ-agarose chromatography, the cell extracts were immunoprecipitated by B9 (lanes 1 and 2) or sequentially by B9 and  $\alpha$ -ptyr antibodies (lanes 3 and 4) as shown in Figure 1. Proteins from the immunocomplexes were dissolved in Laemmli buffer and analyzed on polyacrylamide gels under reducing conditions (Laemmli, 1970). The autoradiogram shown in the figure was obtained by exposing lanes 1 and 2 for 24 h and lanes 3 and 4 for 7 h.

<sup>125</sup>I IGF I with disuccinimidyl suberate in the presence of increasing concentrations of unlabeled insulin or IGF I. Competition of each ligand for labeling of the two receptor types was quantitated by densitometric scanning of the resulting autoradiographs, and occupancy was expressed as the percentage of maximal inhibition of receptor labeling (Beguinot et al., 1985). In parallel experiments, the phosphorylation of insulin and IGF I receptors was quantified under similar conditions. According to this approach, the dose-response curve for IGF I effects on phosphorylation of the type I IGF receptor was superimposable with the IGF I receptor occupancy curve (Figure 6A). In contrast, the affinity of IGF I for the insulin receptor as measured by inhibition of labeling was about 50-fold less. Thus, the IGF I effect on phosphorylation of the IGF I receptor correlated with the occupancy of its own receptor and not with the ability of IGF I to occupy the insulin receptor. Together with the greater potency of IGF I versus insulin in stimulating the phosphorylation of the IGF I receptor, it can be concluded that type I IGF receptor phosphorylation occurs as a result of IGF I receptor occupancy and that there are no spare IGF I receptors in L6 cells. This observation is consistent with previous findings on insulin and IGF I receptor stimulated biological responses in the L6 cells, which demonstrated the absence of spare receptors for IGF I (Beguinot et al., 1985, 1986).

The stimulatory effect of insulin on type I IGF receptor phosphorylation was examined next. Since studies on IGF I action revealed an absence of spare IGF I receptors, one would predict a close correlation between occupancy of the type I receptor and effects of any ligand that are mediated by the receptor. In contrast, there was a poor correlation between type I receptor occupancy and stimulation of its phosphorylation by insulin; i.e., 50% of the maximal insulin effect on type I IGF receptor phosphorylation occurred when IGF I receptor occupancy by insulin was undetectable (Figure 6B). Under the same conditions, insulin receptor occupancy was approximately 8%, a value similar to the occupancy associated



FIGURE 6: Relationship between insulin and IGF I stimulatory effects on IGF I receptor phosphorylation and their inhibitory effects on <sup>125</sup>I affinity labeling of insulin and IGF I receptors in L6 myotubes. The symbols are as follows: IGF I receptor phosphorylation ( $\bullet$ ); IGF I receptor occupancy (O); insulin receptor occupancy ( $\Box$ ). Insulin and IGF I receptors were labeled by <sup>125</sup>I insulin or <sup>125</sup>I IGF I in the presence of the indicated concentrations of unlabeled IGF I (A) or insulin (B). Inhibition of receptor labeling was quantitated by densitometric scanning of the autoradiographs and expressed as percentage inhibition of IGF I receptor phosphorylation were derived from those shown in Figure 3.

with half-maximal insulin effects on glucose and amino acid uptake in the L6 cells (Beguinot et al., 1986). Thus, the insulin effect on phosphorylation of the type I IGF receptor appears to be mediated via the insulin receptor. The possibility that insulin acts via the IGF I receptor with 50-fold greater efficiency than IGF I itself is an unlikely alternative.

As was the case for insulin stimulation of type I IGF receptor phosphorylation, 50% of the maximal phosphorylation of insulin receptors in response to insulin occurred with less than 10% insulin receptor occupancy and with undetectable IGF I receptor occupancy (Figure 7B). These data indicate that the insulin receptor catalyzes its own phosphorylation in response to insulin. Phosphorylation of the insulin receptor by IGF I also more closely reflected occupancy of the insulin receptor than the type I IGF receptor (Figure 7A). Almost 60% of type I IGF receptors were occupied by insulin in the absence of any effect, whereas the same degree of insulin receptor occupancy by IGF I elicited almost maximal phosphorylation.

The left shift in the dose-response curves for insulin receptor autophosphorylation and insulin receptor phosphorylation of the IGF I receptor in comparison with the insulin binding curve differs from previous observations in cultured Fao hepatoma cells, in which there appears to be a closer correlation between dose-response curves for hormone binding and receptor autophosphorylation (Haring et al., 1984). At present, it is not clear whether these differences reflect unique characteristics of the different cell types or changes in experimental methodology. It should be noted that phosphorylation was studied after 10 min of incubation with hormone at 37 °C, whereas equilibrium binding was measured after 5 h at 15 °C.



FIGURE 7: Relationship between insulin and IGF I stimulatory effects on insulin receptor phosphorylation and their inhibitory effects on <sup>125</sup>I affinity labeling of insulin and IGF I receptors in L6 myotubes. The symbols are defined as follows: insulin receptor phosphorylation ( $\bullet$ ); insulin receptor occupancy ( $\Box$ ); IGF I receptor occupancy (O). Insulin and IGF I receptors were labeled by <sup>125</sup>I insulin and <sup>125</sup>I IGF I, in the presence of the indicated concentrations of the unlabeled IGF I (A) or insulin (B). Inhibition of receptor labeling was quantitated by densitometric scanning of the autoradiographs and expressed as the percentage inhibition of maximal labeling. The curves for insulin and IGF I stimulation of insulin receptor phosphorylation were derived from those shown in Figure 2A.

### DISCUSSION

Cultured rat skeletal muscle cells of the L6 line have distinct high-affinity receptors for insulin and IGF I (Beguinot et al., 1985, 1986). In contrast to most cell types, in which insulin effects on cell proliferation are mediated by an IGF receptor (King et al., 1980), insulin and IGF I both stimulate growth responses in the L6 cells through their own high-affinity receptors (Beguinot & Smith, 1985). This could result from direct effects of the two receptors on pathways that regulate cell growth or, alternatively, could result from an interaction between the two receptors. Since tyrosine phosphorylation is thought to play a central role in the action of these hormones (Kasuga et al., 1982), we have investigated receptor interactions by studying the abilities of the insulin and IGF I receptors to catalyze autophosphorylation and to phosphorylate each other in intact L6 cells.

In order to approach this problem, it was necessary to separate the phosphorylated receptors for insulin and IGF I. This was accomplished by first immunoprecipitating insulin receptors with anti-insulin receptor antibodies and then precipitating phosphorylated IGF I receptors with antiphosphotyrosine antibodies. We found that human polyclonal anti-insulin receptor antibodies (either B2 or B9) precipitated two phosphoproteins of  $M_r$  95 000 and 88 000. These were identified as phosphorylated  $\beta$ -subunits of the insulin receptor on the basis of (1) the greater potency of insulin compared to IGF I in stimulating their phosphorylation, (2) the generation of phosphopeptides on trypsin digestion that were very similar to those of the rat Fao cell insulin receptor, and (3) an increase in these receptor species during differentiation that parallels the known increase in insulin receptors. After immunodepletion of insulin receptors with the B2 or B9 antibodies, an additional 95 000-dalton phosphoprotein was precipitable with anti-phosphotyrosine antibodies. This was identified as the IGF I receptor  $\beta$ -subunit on the basis of (1) its lack of precipitation by anti-insulin receptor antibodies, (2) its phosphorylation by lower concentrations of IGF I than insulin, (3) its tryptic phosphopeptide map that was similar to but clearly distinct from the insulin receptor peptide map, and (4) its decreased expression during differentiation that paralleled the known decrease in IGF I receptors. As expected from their subunit structures, phosphorylated insulin and IGF I receptors both migrated at slightly above  $M_r$  300 000 on nonreducing gels.

The separation of insulin and IGF I receptors by sequential immunoprecipitation with B2 or B9 anti-insulin receptor antibodies followed by anti-phosphotyrosine antibody is possible because of the high degree of specificity of B2 and B9 antibodies for the insulin receptor in L6 cells. A similar specificity has been observed with B9 antibodies in human placenta (Kasuga et al., 1983) and with B2 antiserum in BRL-3A2 cells, in which immunoprecipitation of the IGF I receptor requires higher concentrations of antibody than are necessary for insulin receptor immunoprecipitation. The B2 antiserum inhibited both insulin and IGF I binding in placenta and IM-9 lymphoblasts, however, indicating that the high specificity of B2 and B9 antibodies observed in the L6 cells does not apply to all cell types. It is also possible that these antibodies would precipitate IGF I receptors in L6 cells if used at higher concentrations.

The relationship between the two phosphoproteins precipitated by the anti-insulin receptor antibodies ( $M_r$  95 000 and 88 000) has yet to be clearly defined. The peptide maps were generally similar, except that an additional phosphopeptide was present in the smaller species (data not shown). The presence of this additional tryptic phosphopeptide suggests that the  $M_r$  88 000 phosphoprotein is not simply a proteolytic product of the  $M_r$  95 000 band but that both may be alternative forms of the insulin receptor  $\beta$ -subunit. The  $M_r$  88 000 band showed more marked basal phosphorylation than the  $M_r$  95 000 band but was not recognized by anti-phosphotyrosine antibody. Since this indicated that the  $M_r$  88 000 species contains little or no phosphotyrosine, we focused on the  $M_r$  95 000 phosphoprotein in this study on receptor tyrosine phosphorylation.

Stimulation of insulin receptor phosphorylation in intact L6 cells by insulin or IGF I correlated with occupancy by each hormone of the insulin receptor. Thus, increasing IGF I stimulation of insulin receptor phosphorylation occurred over a range of concentrations at which IGF I binds to a progressively greater degree to the insulin receptor, but the IGF I receptor is fully occupied. Similarly, stimulation of IGF I receptor phosphorylation by IGF I correlated with occupancy of the IGF I receptor. Insulin stimulation of IGF I receptor phosphorylation, on the other hand, appeared to occur through a different mechanism. Although insulin can bind to the IGF I receptor at high concentrations, insulin stimulation of IGF I receptor phosphorylation occurred at concentrations that correlated with insulin receptor occupancy and the known presence of spare insulin receptors. Thus, 50% of the maximal insulin effect on IGF I receptor phosphorylation occurred in the absence of detectable IGF I receptor occupancy but with the same level of insulin receptor occupancy associated with half-maximal stimulation by insulin of insulin receptor phosphorylation and of glucose and amino acid uptake in the L6 cells (Beguinot et al., 1986). These data indicate that the IGF I receptor in intact cells is a substrate for the insulin

receptor kinase or for another intermediate kinase which is activated by the insulin receptor.

In a previous study with IM-9 cells, Jacobs et al. (1983) showed that insulin and somatomedin C (IGF I) each stimulated phosphorylation of receptors for the other hormone. Because high hormone concentrations were required, it was considered most probable that this resulted from the binding of each hormone to the receptor for the other. However, the authors suggested the possibility that one receptor type could function as a substrate for the tyrosine kinase of the other. Recent studies on the neu oncogene, which is a receptor-like membrane protein, have suggested that it may be a substrate in a similar manner for the activated epidermal growth factor receptor kinase (Stern et al., 1986). In the L6 cells, we have presented experimental evidence indicating that the hormone-activated insulin receptor can phosphorylate tyrosine residues on the IGF I receptor in intact cells. Future work will be required to establish whether this is a unique characteristic of L6 cells or whether a similar interaction occurs in other cell types.

The observation that insulin receptors phosphorylate IGF I receptors in L6 cells raises the possibility that some of the biological actions of insulin in the L6 cells may be mediated by the IGF I receptor as a result of its phosphorylation by the insulin receptor kinase. Receptors for other growth factors also have been shown to contain intrinsic tyrosine kinase activity and to undergo tyrosine phosphorylation after hormone binding. These include receptors for epidermal growth factor (Ushiro & Cohen, 1980), platelet-derived growth factor (Ek et al., 1982), and colony-stimulating factor 1 (Scherr et al., 1985). In addition, a number of retroviral oncogene products and their cellular homologues are tyrosine-specific protein kinases and substrates for tyrosine phosphorylation (Heldin & Westermark, 1984). Although the final pathways have not yet been defined, it has been postulated that tyrosine kinase activity and tyrosine phosphorylation may be involved in both normal and abnormal cell proliferation (Heldin & Westermark, 1984). This could result from the action of  $\varepsilon$  number of different tyrosine kinases on a single critical substrate. Alternatively, as suggested by our observations in the L6 cells, tyrosine kinases such as the insulin receptor could potentially regulate cell growth indirectly by phosphorylating other growth factor receptors or cellular oncogene homologues.

**Registry No.** IGF I, 67763-96-6; insu'in, 9004-10-8; insulin receptor protein kinase, 88201-45-0; tyrosine, 00-18-4.

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