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Differentiation-Dependent Phosphorylation of a 175,000 Molecular Weight Protein in Response to Insulin and Insulin-Like Growth Factor-I in L6 Skeletal Muscle Cells*

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ABSTRACT. Insulin and insulin-like growth factor-I (IGF-I) effects on protein phosphorylation were investigated in intact skeletal muscle cells at different stages of differentiation. In undifferentiated L6 myoblasts, stimulation by either insulin or IGF-I, but not IGF-II, led to a 3- to 5-fold increase in phosphorylation of insulin and IGF receptor β-subunits and the appearance of a 175,000 mol wt (Mr) phosphoprotein (pp175). These effects reached a maximum within 3 min, were maintained for 12 min, and then declined. Dose-response curves for pp175 phosphorylation in response to insulin (ED_{50} = 2 nM) and IGF-I (ED_{50} = 0.2 nM) were consistent with occupancy and stimulation of each receptor kinase by its specific hormone. The 175,000 Mr phosphoprotein was not precipitated by antireceptor antibodies, and the phosphoamino acid composition differed markedly from that of insulin and IGF-I receptors, with a 10-fold lower phosphotyrosine/phosphoserine ratio after insulin stimulation. In contrast to insulin and IGF-I receptors, pp175 was not extracted by the nonionic detergent Triton X-100, but required sodium dodecyl sulfate for solubilization. When experiments were carried out with L6 cells after differentiation into skeletal muscle myotubes, hormone-induced phosphorylation of pp175 was almost undetectable. We conclude that pp175 is a phosphoprotein distinct from insulin and IGF-I receptors that is involved in the early phosphorylation events that follow the activation of the insulin and IGF-I receptor kinases. Its disappearance after terminal differentiation of the L6 cells is consistent with a role in hormonal stimulation of cell proliferation. (*Endocrinology* **125:** 1599–1605, 1989)

THE INSULIN receptor is a tyrosine-specific protein kinase that undergoes activation and autophosphorylation after insulin binding (1–3). The insulin-like growth factor-I (IGF-I) receptor, which is highly homologous to the insulin receptor, also exhibits tyrosine kinase activity and hormone-induced autophosphorylation (4, 5). With the recognition that receptor phosphorylation is one of the earliest detectable events occurring after insulin and IGF-I binding, it has been postulated that the kinase activity of the receptors plays an important role in transducing the message of the hormone (6). This concept has been supported by transfection studies with mutant receptors, in which defective tyrosine kinase activity has been shown to correlate with a loss of insulin biological effects (7).

Several other receptor types contain an intrinsic hormone-activated tyrosine kinase activity, including the receptors for epidermal growth factor (8), platelet-derived growth factor (9), and macrophage colony-stimulating factor-1 (10). In addition, tyrosine kinase activity has been associated with oncogenes such as v-*erb*B (11), v-*fms* (10, 11), and *neu* (12). Since all of these receptors and oncogenes are thought to exert effects on cell growth, it appears likely that tyrosine phosphorylation may have an important role in growth regulation. This has prompted a search for tyrosine phosphoproteins that may be involved in the regulation of cell growth.

A number of phosphorylated proteins have been identified that may be substrates for receptor or viral protein tyrosine kinases. These include components of the cytoskeleton (13), certain glycolytic enzymes (14), and several as yet unidentified proteins (15–18). In addition, receptor tyrosine kinases may be able to phosphorylate other receptors or receptor-like molecules. We have previously presented evidence that the IGF-I receptor can

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serve as a substrate for the insulin (and IGF-I) receptor kinases in intact L6 skeletal muscle cells (19). Other investigators have shown that the *neu* proto-oncogene can be phosphorylated by the activated EGF receptor tyrosine kinase (20). Although hormone stimulation of these different tyrosine phosphoproteins has been demonstrated, a clear association between the appearance of specific phosphoproteins and cell growth state has not been apparent. In the present study, we have investigated an endogenous substrate common to both insulin and IGF-I receptor kinases in the differentiating L6 skeletal muscle cell line. This 175,000 mol wt (Mr) protein is readily identified in proliferating undifferentiated myoblasts, but essentially undetectable in myotubes that have undergone terminal differentiation and lost their capacity to replicate.

Materials and Methods

Materials

Purified porcine insulin was provided by Eli Lilly Co. (Indianapolis, IN), and [Thr^{59}]IGF-I was purchased from Amgen (Thousand Oaks, CA). Rat IGF-II, alternatively designated multiplication-stimulating activity, was purified from conditioned medium of BRL-3A cells by a modification of the procedure of Moses *et al.* (21). Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Rockville Centre, NY), and Pansorbin was from Calbiochem-Behring (San Diego, CA). Polyclonal antiphosphotyrosine antibody was prepared in rabbits as previously described (22). Albumin (fraction V, from bovine serum) was purchased from Armour (Phoenix, AZ), cell culture media were obtained from Gibco (Grand Island, NY), and all other chemicals were from Sigma (St. Louis, MO).

Cell culture and protein phosphorylation

The L6 rat skeletal muscle cells were cultured as previously described (23). Cells were plated in 100-mm tissue culture dishes (6000 cells/cm^2) and grown for 5 days (myoblasts) or 18 days (myotubes). Myotube cultures were treated with cytosine arabinoside to eliminate undifferentiated myoblasts (23). Cells were used for experiments no sooner than 4 days and 3 medium changes after treatment with cytosine arabinoside to assure removal of the antimetabolite. For phosphorylation experiments, the culture medium was aspirated, and the plates were extensively washed with Eagle's Minimum Essential Medium supplemented with 0.5% albumin and subsequently incubated for 15 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 were then incubated for 3.5 h with 6 ml phosphate-free RPMI-1640 medium containing 1 mCi/ml [^{32}P]orthophosphate and 0.5% dialyzed albumin at 37 C in a humidified atmosphere of 5% CO_2-95% air. Insulin, IGF-I, or IGF-II was subsequently added at the indicated concentration, and the incubation was continued for 10 more min. The phosphorylation reaction was quenched by rapidly aspirating the medium and freezing the cell monolayers with liquid nitrogen (1.5 ml/dish). The frozen cells were thawed and solubilized in 1 ml of a solution containing HEPES (50 mM; pH 7.4), sodium dodecyl sulfate (SDS; 1%), $Na_4P_2O_7$ (10 mM), NaF (100 mM), EDTA (4 mM), Na_3VO_4 (2 mM), phenylmethylsulfonylfluoride (2 mM), and aprotinin (0.2 mg/ml; 14 trypsin inhibitor U/mg). The preparation was then placed in a boiling water bath for 5 min, and the insoluble material was sedimented by centrifugation at 50,000 rpm in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 90 min. The supernatant was immunoprecipitated with antiphosphotyrosine antibody (α-pTYR), as described by Pang *et al.* (24).

The immunoprecipitated proteins were reduced with 5% (vol/vol) 2-mercaptoethanol and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% resolving gels (25). The following proteins were used to estimate Mr: myosin (Mr = 200,000), β-galactosidase (Mr = 116,250), phosphorylase-b (Mr = 94,000), BSA (Mr = 66,000), and ovalbumin (Mr = 45,000). The [^{32}P]phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen (Eastman Kodak, Rochester, NY). The intensity of labeled bands on the autoradiographs was quantitated by densitometric scanning using an LKB 2202 laser densitometer (LKB, Rockville, MD). In some instances, this result was confirmed by quantitating the Cerenkov radiation from solubilized segments of the gels.

Identification of phosphoamino acids

Fragments of polyacrylamide gels containing phosphoproteins of interest were excised and digested with trypsin as described previously (26). The resulting phosphopeptides were concentrated by lyophilization and subjected to acid hydrolysis. The phosphoamino acids were separated by electrophoresis and identified by autoradiography and comparison with phosphoamino acid standards (26, 27).

Results

Identification of phosphoproteins stimulated by insulin and IGF-I

Intact L6 myoblasts were equilibrium-labeled with [^{32}P]orthophosphate, then incubated with 10^{-7} M insulin, IGF-I, or IGF-II for 10 min and extracted with 1% SDS as described in *Materials and Methods*. Immunoprecipitates with antiphosphotyrosine antibody (α-pTYR) revealed several bands ranging in Mr from 15,000–220,000 on SDS-PAGE and autoradiography (Fig. 1, lane A). Upon stimulation with insulin, two new major bands with apparent Mr of 175,000 and 95,000 were apparent (lane B). Increased phosphorylation of minor bands of Mr greater than 200,000 and less than 45,000 and several other bands was also observed, but these were not consistent findings in all of the experiments. A similar pattern was observed after IGF-I (Fig. 1, lane D), but not IGF-II (lane C), stimulation of the cells. The appar-

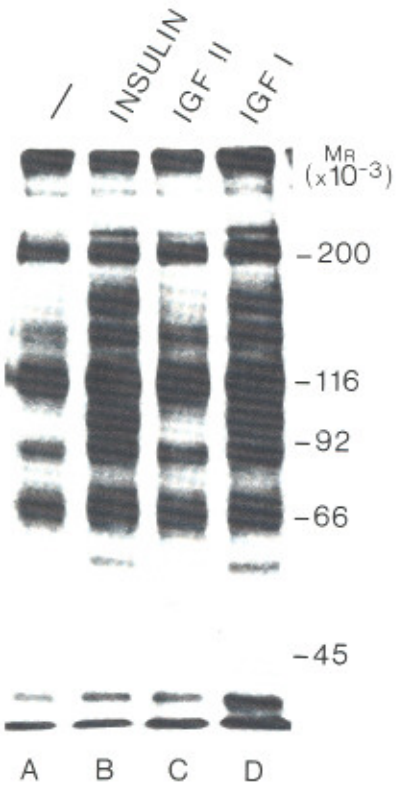

FIG. 1. Identification of insulin- and IGF I-stimulated phosphoproteins in L6 cells. L6 myoblasts were labeled with [^{32}P]orthophosphate for 3.5 h and incubated for 10 min with either no addition (lane A) or 10^{-7} M insulin, IGF-I, or IGF-II (lanes B, D, and C). Cells were then solubilized with 1% SDS as described in *Materials and Methods*. After centrifugation at 150,000 × *g* for 90 min, the cell extracts were immunoprecipitated with antiphosphotyrosine antibody. Proteins from the immunocomplexes were dissolved in Laemmli buffer and analyzed on 7.5% polyacrylamide gels under reducing conditions. The autoradiogram shown in the figure was obtained by exposing the dried gel for 24 h with an enhancing screen.

ent Mr and hormone sensitivity of the 95,000 Mr species is appropriate for insulin and IGF-I receptor β-subunits in these cells (28). The 175,000 Mr species (pp175) has not been previously described in L6 cells.

The phosphorylation of pp175 was further explored in the experiment described in Fig. 2. Before the addition of insulin, pp175 was not detectable in cell extracts by α-pTYR immunoprecipitation. This is consistent with the absence of phosphotyrosine residues in pp175 in the basal state. After incubation of the cells with insulin or IGF-I, the amount of pp175 precipitated by α-pTYR increased rapidly (Fig. 2). A maximal effect was achieved within approximately 3 min and was maintained for 12 more min, declining thereafter.

Insulin and IGF-I both increased pp175 phosphorylation, with half-maximal effects between 2×10^{-9} and 2×10^{-10} M and maximal stimulation at approximately 10^{-8} M (Fig. 3). These concentrations are similar to those required for insulin and IGF-I stimulation of biological

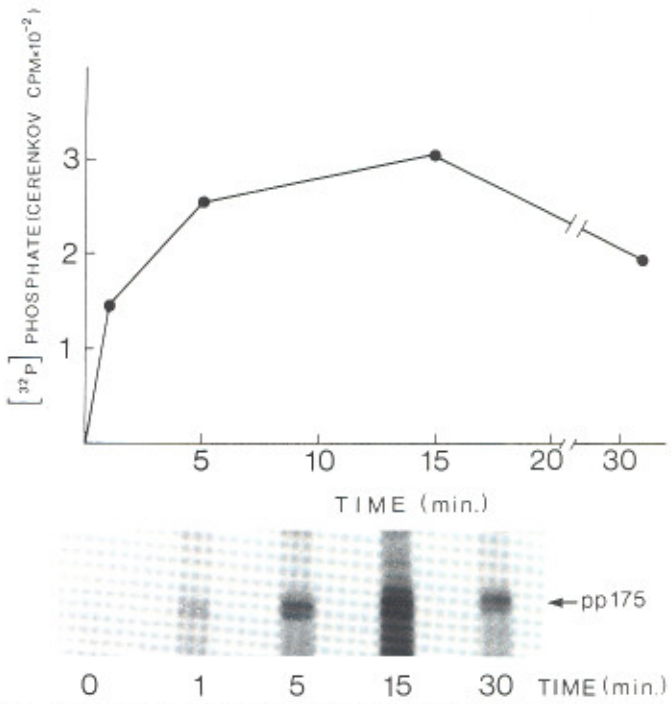

FIG. 2. Time course of insulin-stimulated phosphorylation of pp175 in L6 cells. L6 myoblasts were equilibrium labeled with [^{32}P]orthophosphate and incubated with 10^{-7} M insulin for 0, 1, 5, 15, or 30 min as indicated. Cells were then solubilized, and ^{32}P-labeled pp175 was analyzed by PAGE as described in Fig. 1. The autoradiogram shown in the figure was obtained by exposing the gel for 24 h with an enhancing screen. ^{32}P incorporated into pp175 was quantitated by Cerenkov counting of the corresponding gel pieces excised from the gel.

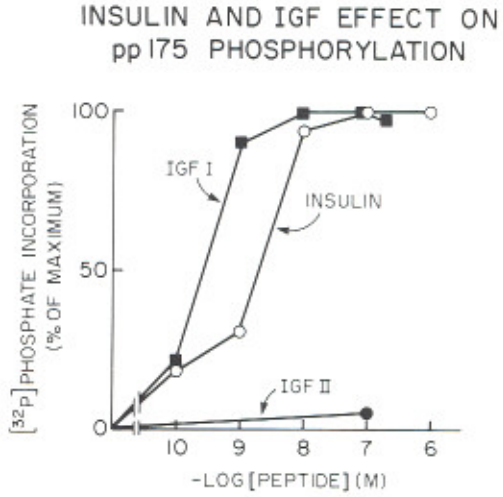

FIG. 3. Dose-response curves for insulin and IGF effects on pp175 phosphorylation in L6 cells. Myoblasts were equilibrium labeled with [^{32}P]orthophosphate and then incubated for 10 min with increasing concentrations of insulin, IGF-I, or IGF-II as indicated. The cells were subsequently solubilized, and cell proteins analyzed by PAGE as described in Fig. 1. ^{32}P incorporated into pp175 was quantitated by excision of the bands from the gel and Cerenkov counting. *Data points* are from one representative experiment.

responses such as glucose and amino acid uptake in L6 cells, which are thought to be mediated by distinct high affinity receptors (23, 28). In contrast, IGF-II did not elicit a significant effect at concentrations as high as 10^{-7} M.

Characterization of pp175

To further characterize the 175,000 Mr phosphoprotein, we analyzed the partitioning of this protein in Triton X-100 and SDS extracts of the cells. For this purpose, L6 myoblasts were labeled with [^{32}P]orthophosphate for 3.5 h and then incubated in the absence or the presence of 10^{-7} M insulin for 10 additional min. Cells were solubilized with 1% Triton X-100, and the insoluble fraction was sedimented by centrifugation at 150,000 × *g* for 90 min. The supernatant was immunoprecipitated with α-pTYR and analyzed by gel electrophoresis and autoradiography.

In the absence of insulin, one major phosphoprotein of 120,000 Mr was immunoprecipitated (Fig. 4, lane A). The precise identity of this species is unknown. Upon stimulation with 10^{-7} M insulin, ^{32}P incorporation into the 120,00 Mr band did not change significantly, while a 95,000 Mr band appeared (Fig. 4, lane B). This band is thought to represent both insulin and IGF-I receptor β-subunits, both of which bind insulin at high concentrations (19). No phosphoproteins were detected in the 175,000 Mr range in the Triton X-100 extract of L6 cells. In the absence of insulin stimulation, extraction of the Triton-insoluble fraction of the cells with 1% SDS did not yield additional phosphoproteins (Fig. 4, lane C). However, in insulin-stimulated cells, sequential extraction with SDS revealed the 175,000 Mr phosphoprotein and further recovery of the 95,000 Mr band.

The phosphoamino acid composition of receptor β-subunits and pp175 was determined after acid hydrolysis and separation of the amino acids by high voltage electrophoresis. After insulin stimulation, both proteins contained substantial amounts of phosphoserine. The pp175 band contained smaller amounts of phosphotyrosine and phosphothreonine. Compared with pp175, more phosphotyrosine, but no phosphothreonine, was detected in receptor β-subunits.

Developmental changes in pp175 phosphorylation

At all stages of differentiation, L6 cells express many more receptors for IGF-I than for insulin (28). In previous reports we have demonstrated that the effect of insulin on insulin receptor autophosphorylation in L6 cells increases during differentiation, while IGF-I receptor phosphorylation decreases, in keeping with an increase in insulin receptor number and a decrease in IGF-I receptor number (23, 28). We, therefore, investigated

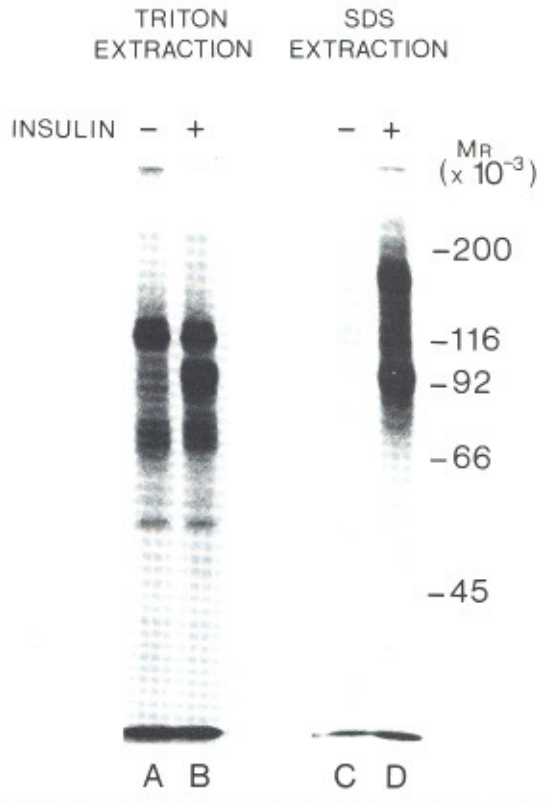

FIG. 4. Differential solubility of pp175 in Triton X-100 and SDS extracts of the L6 cells. L6 myoblasts were equilibrium labeled with [^{32}P]orthophosphate and incubated for 10 more min with or without 10^{-7} M insulin as indicated. Cells were then solubilized with 1% Triton X-100 as described in *Materials and Methods* and centrifuged at 150,000 × *g* for 90 min, and the supernatants were immunoprecipitated with antiphosphotyrosine antibody (lanes A and B). Pellets were then resuspended in 1% SDS at 100 C for 10 min and centrifuged at 150,000 × *g* for 90 min, and supernatants were immunoprecipitated with antiphosphotyrosine antibody (lanes C and D). Immunoprecipitated proteins were analyzed by PAGE as described in Fig. 1. The autoradiogram shown was obtained by exposing the dried gel for 24 h with an enhancing screen.

whether pp175 phosphorylation also changes during development. For this purpose, we added insulin at a high concentration (10^{-7} M) to assure its binding to both insulin and IGF-I receptors and compared hormone effects in undifferentiated L6 myoblasts and differentiated myotubes. At the myoblast stage of development, which was used in all of the experiments described above, insulin increased ^{32}P incorporation into the 175,000 and 95,000 Mr species by at least 10-fold (Fig. 5, lanes A and B). In differentiated myotubes, the 95,000 Mr band was also phosphorylated in response to insulin, although the magnitude of this effect was about 5-fold less than that in myoblasts (Fig. 5, lanes C, D). The decrease in the 95,000 Mr band is consistent with the decline in the number of IGF-I receptors during L6 cell differentiation and the predominance of this receptor species relative to the insulin receptor in the 95,000 Mr band (28). At the

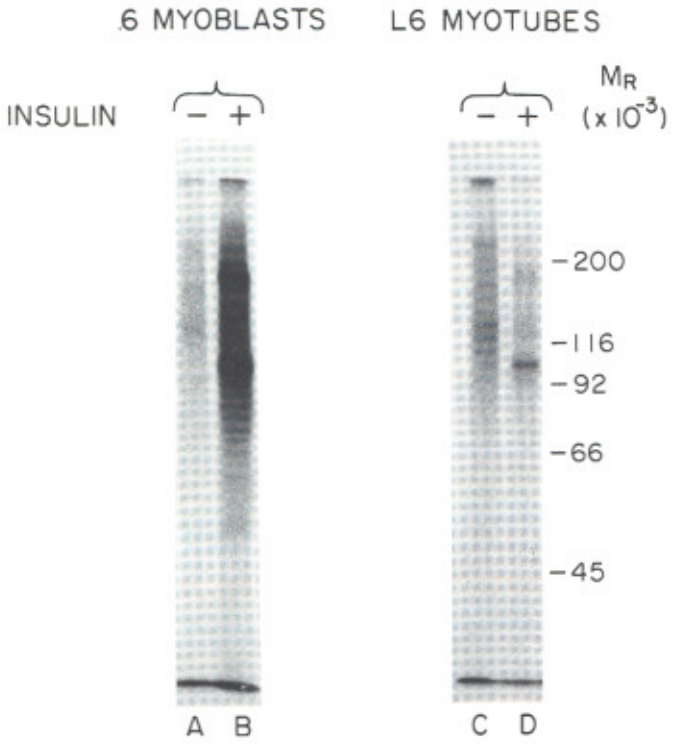

FIG. 5. Changes in pp175 phosphorylation during L6 cell differentiation. Undifferentiated L6 myoblasts and differentiated myotubes were equilibrium labeled with [^{32}P]orthophosphate and incubated for 10 min in the presence or absence of 10^{-7} M insulin, as indicated. After Triton X-100 solubilization, cells were centrifuged at 150,000 × *g* for 90 min. Pellets were resuspended in 1% SDS and centrifuged as described in Fig. 4. Supernatants were immunoprecipitated with antiphosphotyrosine antibody, and precipitated proteins were analyzed by PAGE. The autoradiogram shown in the figure was obtained by exposing the dried gel for 36 h with an enhancing screen.

same time, the 175,000 Mr phosphoprotein became undetectable in myotubes (Fig. 5, lanes C and D), suggesting that either the expression of pp175 decreased or some other step involved in its phosphorylation was altered during L6 cell differentiation.

Discussion

The relationships between the postreceptor pathways of insulin, IGF-I, and other growth-stimulating hormones are largely unknown. Since the insulin and IGF-I receptors contain intrinsic hormone-activated tyrosine kinase activity (1–5), we have investigated the existence of cellular substrates common to the two kinases in the differentiating L6 skeletal muscle cell line. Using antiphosphotyrosine antibody, we observed that insulin and IGF-I, but not IGF-II, rapidly stimulate phosphorylation of a 175,000 Mr protein (pp175). Other than receptor β-subunits, this was the only species that was consistently stimulated by the two hormones. Both insulin and IGF-I were effective at nanomolar concentrations, suggesting that pp175 phosphorylation occurred in response to binding and activation of each receptor kinase by its specific hormone. Although IGF-II at the concentration used in this study (10^{-7} M) has been shown to partially displace bound [^{125}I]insulin and [^{125}I]IGF-I from L6 cells (23, 28), affinity labeling studies indicate that this results from the displacement of insulin and IGF-I from IGF-II receptors (28). The absence of pp175 phosphorylation in L6 cells treated with 10^{-7} M IGF-II is consistent with its failure to bind to insulin or IGF-I receptors at this concentration.

In contrast to insulin and IGF-I receptors, pp175 was not extracted by the nonionic detergent Triton X-100, but was solubilized by SDS. After insulin stimulation, the phosphoamino acid composition of pp175 exhibited marked differences compared with the receptors, with a 10-fold lower phosphotyrosine/phosphoserine ratio. Antireceptor antibodies also failed to precipitate the 175,000 Mr phosphoprotein (data not shown). Based on these observations, we conclude that pp175 does not represent a precursor of insulin and IGF-I receptors, but is either a common substrate for the insulin and IGF-I receptor kinases or a substrate of another kinase activated by the receptors. The exact nature of pp175 is presently unknown. It does not appear to be the epidermal growth factor receptor or the platelet-derived growth factor receptor, since both of these receptors exhibit Mr that differ slightly from that of pp175 in L6 cells and can be effectively extracted with Triton X-100 (data not shown). We cannot at present exclude the possibility that pp175 is a receptor for some other growth factor, such as the colony-stimulating factor-1 receptor (10), or the product of a proto-oncogene, such as *neu* (12), although these proteins also should be solubilized by Triton.

In Triton X-100 and in detergent-free extracts of Fao hepatoma cells, White *et al.* (15, 29) identified a phosphotyrosine-containing protein of 185,000 Mr (pp185) which appeared early after insulin binding. Phosphorylation of pp185 by insulin was shown to occur mostly on tyrosine residues and with a dose-response curve similar to that for insulin receptor autophosphorylation. Based on these findings, the researchers suggested that pp185 represents an endogenous substrate for the insulin receptor kinase in the Fao cells. Subsequently, Izumi *et al.* (16) reported that IGF-I also stimulated phosphorylation of a 185,000 Mr protein in NRK and MDCK cells. These cells are rich in IGF-I receptors but have no insulin receptors. The researchers suggested that the 185,000 Mr species in these and the Fao cells is the same protein, which might serve as a common substrate for insulin and IGF-I receptor kinases. Shemer *et al.* (30) have identified a similar 185,000 Mr phosphoprotein in N18 mouse neuroblastoma cells. The relationship between the 175,000 Mr phosphoprotein observed in the L6 cells and these 185,000 Mr phosphoproteins is presently unclear.

After insulin stimulation, pp175 exhibited a 10-fold lower phosphotyrosine/phosphoserine ratio than pp185. In contrast to pp185, the pp175 protein required SDS for extraction and was not solubilized by Triton X-100. Based on these distinguishing features, it is probable that pp175 is distinct from pp185.

In rat liver cells, Okamoto *et al.*[1] have observed a 175,000 Mr phosphoprotein which also required SDS for extraction. We have identified a similar and possibly identical protein in another cell line, the FRTL-5 thyroid cells.[2] As in the L6 cells, this protein is phosphorylated within minutes after insulin or IGF-I stimulation and contains phosphotyrosine, phosphoserine, and phosphothreonine. These observations suggest that pp175 may not be unique to L6 cells and may be present in other cell types as well. The failure to identify pp175 in previous studies with other cells could be attributable to differences in detergents used to solubilize proteins after phosphorylation.

A remarkable feature of pp175 in L6 cells is its almost complete disappearance at the time of cell differentiation. This decrease in pp175 occurs at the same time that the cells lose their capacity to undergo continued cell division (31), thus suggesting a possible relationship between pp175 and cell replication. Phosphorylation of the 95,000 Mr receptor band also decreased after differentiation, consistent with the known decrease in IGF-I receptors. Since high affinity insulin-binding sites and insulin-stimulated glucose uptake are known to increase in L6 cells after differentiation (23), and low concentrations of insulin stimulate pp175 phosphorylation in myoblasts, the failure of insulin to stimulate pp175 in myotubes cannot be explained by a loss of insulin receptors with differentiation. At present, it is not known whether the marked decrease in the amount of phosphorylated pp175 in differentiated cells is related to the decrease in IGF-I receptors, altered expression of other cellular tyrosine kinases, or decreased expression of the pp175 protein itself. With the recognition that the appearance of phosphorylated pp175 is dependent on the differentiation state of L6 cells, comparative studies with myoblasts and myotubes may make it possible to identify the pp175 protein and its function. This information should provide important insight into the relationship between insulin and IGF-I receptor kinases and the mechanism of insulin and IGF action.

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