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Insulin and Insulin-like Growth Factor I (IGF I) Stimulate Phosphorylation of a M_r 175,000 Cytoskeleton-associated Protein in Intact FRTL5 Cells*

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FRTL5 rat thyroid cells possess separate high affinity receptors for insulin and insulin-like growth factor I (IGF I) that undergo β-subunit phosphorylation upon interaction with the specific ligand. Phosphorylation is rapid and dose-dependent and occurs primarily on tyrosine residues. Within 2 min, both insulin and IGF I also give rise to a M_r 175,000 phosphoprotein (pp175) that can be immunoprecipitated by anti-phosphotyrosine antibody (α-Tyr(P)). Phosphorylation of pp175 occurs on serine and threonine as well as tyrosine residues. When FRTL5 cells are solubilized with 1% Triton X-100, α-Tyr(P) immunoprecipitates phosphorylated insulin and IGF I receptors but little pp175 from the Triton-soluble fraction. After treatment of the Triton-insoluble portion with 1% sodium dodecyl sulfate at 100 °C, pp175 can be identified by immunoprecipitation with α-Tyr(P). The fraction of FRTL5 cells that remains after extraction of an attached monolayer with 1% Triton for 5 min at 22 °C contains most of the cytoskeleton and also nuclei. Extraction of this ^{32}P-labeled cytoskeleton preparation with sodium dodecyl sulfate followed by α-Tyr(P) immunoprecipitation results in almost complete recovery of the pp175 content of the cells. When a nuclear fraction was prepared from FRTL5 cells by differential centrifugation, pp175 was not found in the nuclear pellet from labeled cells, but >80% of pp175 was recovered in the supernatant. We conclude that pp175 is a common substrate for insulin and IGF I receptor kinases which, in FRTL5 cells, is associated with the cytoskeleton. It is suggested that phosphorylation of proteins associated with cytoskeletal elements could be involved in insulin and IGF I action in cells.

Insulin and insulin-like growth factor I (IGF I)[1] are homologous hormones that generate similar biological responses in most cells (1, 2). Receptors for insulin and IGF I also exhibit a high degree of structural and functional homology (3–9). After hormone binding, insulin and IGF I receptors undergo autophosphorylation and enhancement of their tyrosine kinase activities (10–15). There is evidence that receptor autophosphorylation is important for insulin and IGF I action (16–19), but the relevance to hormonal action of other substrates for receptor kinase activity is unclear at this time.

In cell-free preparations, cytoskeletal proteins have been shown to serve as substrates for insulin and IGF I receptor kinases, raising the possibility that cytoskeletal components are important targets for these receptor kinases (20–22). In the case of microtubule-associated protein 2 (MAP-2), evidence has been provided that tyrosine phosphorylation by epidermal growth factor (EGF) and insulin receptor kinases alters its functional activity (23). Since these studies have been performed in broken cell systems, the question as to whether cytoskeletal proteins are also phosphorylated by insulin and IGF I receptor kinases in intact cells is still unanswered. In intact cells, insulin and IGFs cause rapid alterations of cytoskeletal structure (24–26). It has been suggested that these changes are related to tyrosine phosphorylation of some cytoskeletal proteins by insulin and IGF I receptor kinases (25), but none of these proteins has been identified as yet.

A few cellular proteins undergo tyrosine phosphorylation rapidly in response to insulin and IGF I (27–30). These include an insoluble species designated pp175 initially identified in L6 skeletal muscle cells (31). As it is the case for most other substrates described in intact cells, the subcellular localization, structure, and function of pp175 are not known.

In the present work we have investigated the subcellular localization of pp175 using the FRTL5 thyroid cells. These cells possess distinct receptors for insulin and IGF I and strongly depend on these factors for proliferation and other cellular functions (32–35). They also express higher levels of pp175 in comparison with other cell types,[2] thus representing an attractive model for this study. We report here that, in FRTL5 cells, the endogenous substrate for insulin and IGF I receptor kinases pp175 is associated with the cytoskeleton. This supports the concept that, also in intact cells, cytoskeletal components are important targets for insulin and IGF I action.

EXPERIMENTAL PROCEDURES

Materials—Coon's modified Ham's F-12 medium was prepared from a lyophilized preparation purchased from KC Biological (Le-

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[1] The abbreviations used are: IGF I, insulin-like growth factor I; α-Tyr(P), anti-phosphotyrosine antibody; SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; MAP, microtubule-associated protein; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis.

[2] F. Beguinot, unpublished observations.

nexa, KS). Insulin was provided by Lilly, and Thr^{59} IGF I was purchased from AmGen (Thousand Oaks, CA). [^{32}P]Orthophosphate was from Du Pont-New England Nuclear, Pansorbin was from Calbiochem, wheat germ agglutinin-agarose was from Vector (Burlingame, CA), and albumin (fraction V from bovine serum) was from Armour. Polyclonal anti-phosphotyrosine antibody was a kind gift of Drs. C. R. Kahn and M. F. White. Polyclonal anti-tubulin antibodies and fluorescein-conjugated anti-rabbit immunoglobulin antibodies were purchased from Miles Co. (Kankakee, IL). All other chemicals were from Sigma.

Cell Culture—The FRTL5 cell line used in these studies is a subclone of the normal rat thyroid cells previously shown to retain the thyroid functional characteristics of iodide uptake and thyroglobulin synthesis over a long period of culture (34). These cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a mixture of three growth factors consisting of insulin (10 μg/ml), transferrin (5 μg/ml), and thyrotropin (10 mIU/ml). Cultures were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air and passaged every 2–3 weeks as described previously (34).

Identification of Phosphoproteins—For phosphorylation experiments, cells were plated in 100-mm tissue culture dishes (6×10^3 cells/cm^2) and grown as described above. The culture medium was aspirated, and the cells were rinsed three times with 150 mM NaCl and incubated in phosphate-free culture medium with no other supplement in a humidified atmosphere of 5% CO_2 and 95% air for 10 h at 37 °C. The cells then were incubated in phosphate-free medium containing 50 μCi/ml [^{32}P]orthophosphate for 8 h. Insulin or IGF I was subsequently added at the indicated concentrations and the incubation was continued for 5 more min, unless otherwise noted. Phosphorylation reactions were rapidly quenched as described previously (36), and cells were solubilized with 1 ml/dish of a solution containing 50 mM HEPES, pH 7.4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM phenylmethanesulfonyl fluoride, 0.2 mg/ml aprotinin (14 trypsin inhibitor units/mg), and either 1% Triton X-100 or 1% sodium dodecyl sulfate (SDS) as indicated (buffer A). The insoluble material was sedimented by centrifugation at 200,000 × *g* for 60 min at 4 °C and immunoprecipitated with anti-phosphotyrosine antibody as described by Pang *et al.* (37). The immunoprecipitated proteins were reduced with 5% (v/v) 2-mercaptoethanol and separated by SDS-PAGE on 7.5% resolving gels (38). The following proteins were used to estimate molecular weights: myosin, M_r = 200,000; β-galactosidase, M_r = 116,250; phosphorylase *b*, M_r = 94,000; bovine serum albumin, M_r = 66,000; and ovalbumin, M_r = 45,000. The ^{32}P-phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat films and an intensifying screen. The intensity of labeled bands 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.

Phosphoamino Acid Analysis—The ^{32}P-labeled protein bands in the dried gels were located by autoradiography and excised. Phosphorylated proteins were eluted from the gel pieces by incubating in 50 mM NH_4HCO_3 containing 0.1% SDS. The extracted material was then precipitated in 20% trichloroacetic acid at 4 °C using 50 μg of bovine serum albumin as carrier. Precipitated proteins were washed once with ethanol/ether (1:1 v/v) and hydrolyzed in 0.5 ml of 6 N HCl at 110 °C for 1 h. Hydrolysates were lyophilized and dissolved in 20 μl of H_2O containing 1 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine. The samples were spotted onto a cellulose-precoated plastic sheet and electrophoresed at 1000 V for 2 h in pyridine/acetic acid/water (1:10:189), pH 3.5. The location of the different phosphoamino acids was visualized by ninhydrin staining, and ^{32}P radioactivity associated with each phosphoamino acid spot was monitored by autoradiography.

Detergent Treatment and Cytoskeleton Preparation—For the fluorescence microscopy studies, cells were grown on glass coverslips. The cells were washed five times with phosphate-buffered saline (PBS) and then incubated in PBS with 1% Triton X-100 for 5 min at 22 °C. After treatment with the detergent, the cells were washed five more times with PBS and then fixed in 3.7% formaldehyde dissolved in PBS containing 2% sucrose before being processed for immunofluorescence microscopy as described below. For the biochemical studies, cells were grown on plastic dishes, labeled with [^{32}P]orthophosphate, and stimulated with insulin as described above. After quenching phosphorylation reactions, the monolayers were incubated for 5 min at 22 °C with 1 ml/dish of buffer A containing 1% Triton X-100. Solubilized material was removed by suction and remaining cytoskeleton was scraped in 1 ml of buffer A containing 1% SDS, boiled at 100 °C for 5 min, and ultracentrifuged at 200,000 × *g* for 60 min at 4 °C. Solubilized proteins were immunoprecipitated with anti-phosphotyrosine antibody and analyzed as described above.

Fluorescence Microscopy—After being fixed as described above, cells were washed five times with PBS and incubated with either 5 mg/ml rhodamine-conjugated phalloidin (45 min at 22 °C) or 1:10 anti-tubulin antibody (20 min at 22 °C). For tubulin staining, a fluorescein-labeled second antibody was then added and the coverslips held for a further 20 min at 22 °C. Coverslips were finally mounted with Elvanol on microscope slides and viewed with a Zeiss microscope equipped with epifluorescent illumination using the appropriate filters for rhodamine or fluorescein fluorescence. Pictures were taken with a Planapo 63× oil immersion objective.

RESULTS

Receptor and pp175 Phosphorylation in FRTL5 Cells—^{32}P-Labeled cells were stimulated with IGF I or insulin and solubilized with 1% Triton X-100. Phosphotyrosine-containing proteins were then immunoprecipitated with anti-phosphotyrosine antibody (α-Tyr(P)) and analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In the absence of hormone, three bands were observed with M_r > 200,000, = 120,000 and = 70,000 (Fig. 1, *lane A*). The appearance of a doublet at M_r 120,000 was a frequent, although not constant, finding. We have no clues, at this time, to explain this apparent inconsistency and are investigating this problem further. Upon stimulation with IGF I (10^{-7} M) or insulin (10^{-6} M), a new band appeared with M_r = 95,000

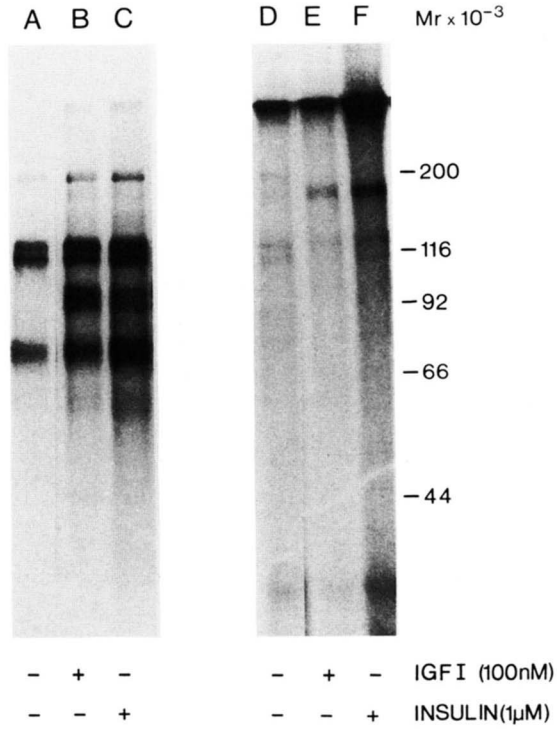

FIG. 1. **Identification of phosphoproteins precipitated by antiphosphotyrosine antibodies in intact FRTL5 cells.** FRTL5 cells were equilibrated with [^{32}P]orthophosphate, incubated for 2 min in the absence or the presence of 100 nM IGF I or 1 μM insulin, solubilized with Triton X-100 as described under "Experimental Procedures," and clarified by ultracentrifugation. Supernatants were recovered and Triton-insoluble pellets extracted with 1% SDS at 100 °C and centrifuged at 200,000 × *g* for 60 min. Triton-solubilized proteins (*lanes A–C*) and SDS-solubilized proteins (*lanes D–F*) were then immunoprecipitated with phosphotyrosine antibody, reduced with β-mercaptoethanol, and analyzed by SDS-PAGE. The autoradiograph shown was obtained by exposing the dried gel for 14 h at −70 °C. The difference in pp175 labeling in response to insulin and IGF I was not a consistent finding in all of the experiments.

(Fig. 1, *lanes B* and *C*, respectively). As it was the case for the M_r 120,000 band, also the M_r 95,000 band appeared frequently, but not constantly, as a doublet. This M_r 95,000 band was also immunoprecipitated with polyclonal anti-insulin receptor antibodies and migrated as a $M_r > 300,000$ complex when analyzed under nonreducing conditions (data not shown). These properties have been described for both insulin and IGF I receptor β-subunits in other cells (10, 11, 13, 14). In a previous report (31), we have shown that Triton-insoluble residues of the L6 myoblasts contain a M_r 175,000 phosphoprotein (pp175) believed to be the endogenous substrate for insulin and IGF I receptor kinases in these cells. We investigated therefore whether the same protein is also present in FRTL5 cells. For this purpose, the insoluble residue from Triton-extracted cells was further extracted with 1% SDS. Solubilized proteins were immunoprecipitated with α-Tyr(P) and analyzed by PAGE and autoradiography. In unstimulated cells, no phosphoproteins were found other than those detected in the Triton extract (Fig. 1, *lane D*). Upon stimulation with IGF I or insulin, a M_r 175,000 band was observed (Fig. 1, *lanes E* and *F*). A similar band was barely visible in the Triton extract. This phosphoprotein in FRTL5 cells exhibited the same molecular weight, hormone sensitivity, and detergent solubility as the previously described pp175 of L6 cells.

Characterization of Receptor and pp175 Phosphorylation—In intact FRTL5 cells, both insulin and IGF I increased phosphorylation of their respective receptor β-subunits in a dose-dependent fashion (Fig. 2, *top panel*). The effect of insulin was half-maximal at a concentration of 8×10^{-10} M and reached a plateau at 10^{-8} M. IGF I exhibited a similar dose-response curve indicating that the M_r 95,000 band contains both insulin and IGF I receptor β-subunits. In the case of pp175 phosphorylation, insulin and IGF I showed similar potencies with half-maximal effects in the nanomolar range (Fig. 2, *bottom panel*). The similarity between insulin and IGF I dose-response curves suggests that phosphorylation of pp175 results from the occupancy of the two receptor types by insulin and IGF I, respectively.

In cells that were solubilized with SDS, the anti-phosphotyrosine antibody immunoprecipitated both receptors and pp175. These proteins were almost undetectable in the basal state, but the amount immunoprecipitated increased to a maximum level within 2 min after insulin stimulation (Fig. 3, *lanes A* and *B*). After 10–60 min with insulin the level of phosphorylation of receptor β-subunits was essentially unchanged (Fig. 3, *lanes C* and *D*). In contrast, phosphorylation of pp175, measured by scanning densitometry and Cerenkov counting, decreased by 40% after 10 min and by 80% after 60 min. Nearly identical results were obtained when the cells were treated with IGF I. Thus, dephosphorylation of insulin or IGF I-induced pp175 occurred more rapidly than dephosphorylation of the respective receptors. Alternatively, insulin and IGF I action may have led to the segregation of pp175 into an SDS-insoluble protein pool unavailable for immunoprecipitation. Since we could not detect any pp175 in the SDS-insoluble material (data not shown), we favored the possibility that, after phosphorylation, pp175 undergoes rapid dephosphorylation. Based on the rate of loss of radioactivity from maximally phosphorylated pp175, in three experiments the half-life of phosphorylated pp175 was determined to be approximately 25 min.

The phosphoamino acid composition of the insulin/IGF I receptor β-subunits and pp175 was compared after acid hydrolysis and separation of the phosphoamino acids by high voltage electrophoresis. Both proteins exhibited detectable amounts of phosphotyrosine (Fig. 4). Both phosphoserine and

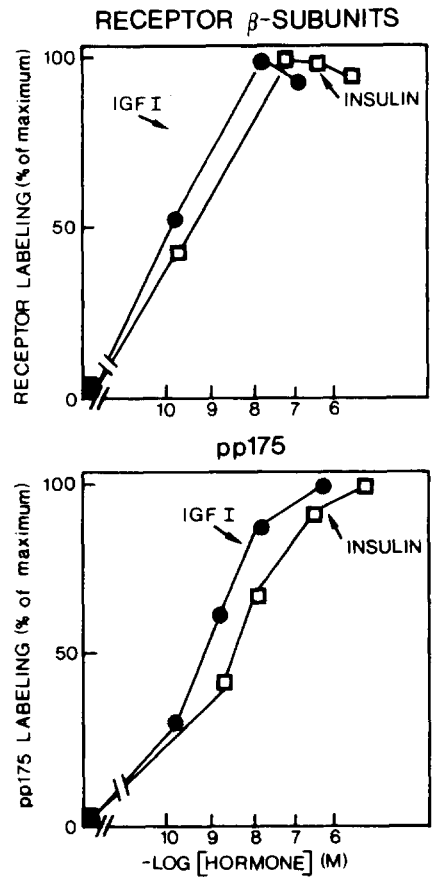

FIG. 2. **Dose-response relationship between insulin (□) and IGF I (●) concentrations and phosphorylation of receptor β-subunits (*top*) and pp175 (*bottom*).** FRTL5 cells, labeled with [^{32}P]orthophosphate, were incubated for 2 min at 37 °C with the indicated concentrations of insulin or IGF I. Cells were solubilized with 1% SDS at 100 °C, clarified by ultracentrifugation, and precipitated with phosphotyrosine antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, receptor subunits localized by autoradiography, and the corresponding fragments of the gel were removed for determination of radioactivity by Cerenkov counting. A representative experiment is shown.

small amounts of phosphothreonine, was detected in the receptor β-subunits. We could not detect any labeled phosphoamino acid in either pp175 or the receptor β-subunits in the absence of insulin or IGF I stimulation of FRTL5 cells (data not shown).

Subcellular Localization of pp175—Extraction of FRTL5 cells with 1% Triton leaves an insoluble residue that contains pp175. In other cultured cells and tissues, the Triton-insoluble residue has been shown to contain most of the nuclei and cytoskeletal elements (39, 40). We investigated therefore whether an association could be established between these subcellular structures and pp175. Upon incubating an intact monolayer of FRTL5 cells with Triton X-100 for 5 min at room temperature, 80–90% of the proteins were extracted. The Triton-insoluble residue was further studied by fluorescence microscopy using fluorescein-conjugated phalloidin. This revealed strongly fluorescent actin fibers and the outlines of nuclei with relatively little fluorescence in the nuclei (Fig. 5, *left*). Using anti-tubulin antibodies and fluorescein-conjugated anti-IgG, ghosts also stained positive for tubulin (data not shown). Thus, the Triton-insoluble fraction of FRTL5 cells, as it is the case for other cells (39), contains nuclei and cytoskeletal elements. Control experiments were

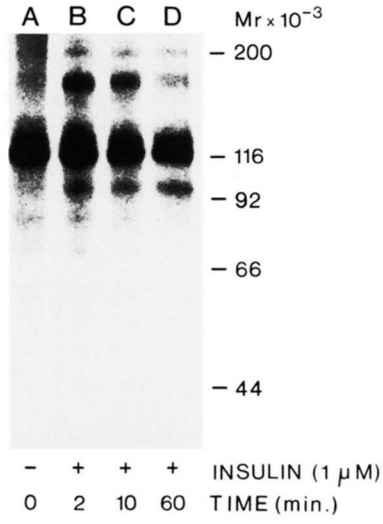

FIG. 3. **Time course of insulin effect on receptor and pp175 phosphorylation.** FRTL5 cells labeled with [^{32}P]orthophosphate were incubated with 10^{-6} M insulin at 37 °C for the indicated time. Cells were then solubilized with 1% SDS, clarified by ultracentrifugation, and precipitated with phosphotyrosine antibody. Immunoprecipitated phosphoproteins were separated by SDS-PAGE, receptors and pp175 bands were localized by autoradiography, and the corresponding fragments of the gel removed for determination of radioactivity by Cerenkov counting. A representative experiment is shown.

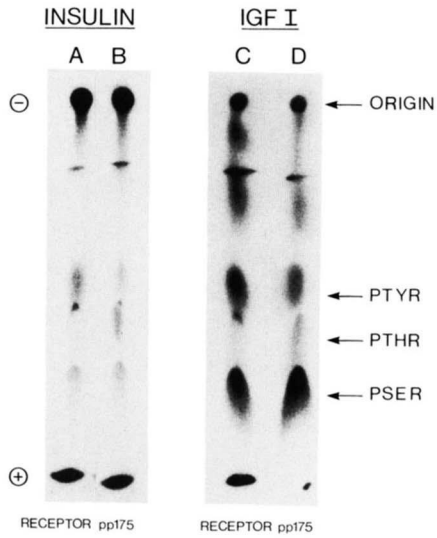

FIG. 4. **Phosphoamino acid analysis of insulin and IGF I receptor β-subunits and pp175.** Receptor β-subunits and pp175 were purified from ^{32}P-labeled FRTL5 cells by immunoprecipitation and polyacrylamide gel electrophoresis. The bands were identified by autoradiography, excised from the gels, and digested with trypsin. The tryptic peptides were hydrolyzed with 6 N HCl and resulting free amino acids separated by high voltage electrophoresis as described under "Experimental Procedures." The radioactive amino acids were identified by the migration of known standards. In this figure, insulin-stimulated and IGF I-stimulated phosphoproteins were derived from separate experiments.

performed to verify pp175 association with these cytoskeleton preparations.

^{32}P-Labeled proteins were obtained by extracting labeled cells with Triton X-100 and then solubilizing the Triton-resistant material with SDS. The cytoskeletal protein-enriched fraction was immunoprecipitated with α-Tyr(P) and analyzed by SDS-PAGE and autoradiography. In unstimulated cells, the M_r 175,000 band could not be detected, whereas a protein migrating as pp175 appeared in response to insulin

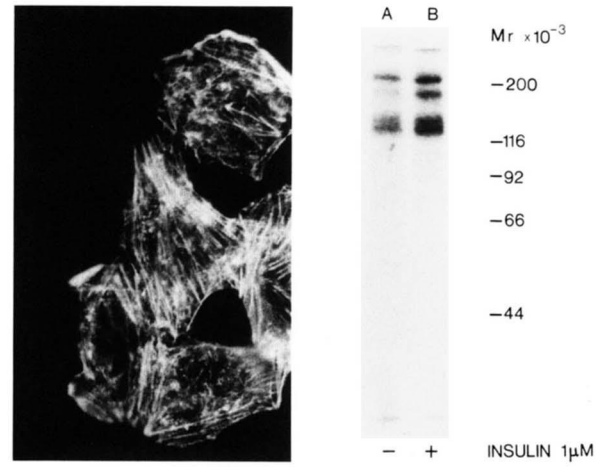

FIG. 5. **Association of pp175 with the FRTL5 cytoskeleton.** *A*, FRTL5 cells were extracted with Triton X-100, fixed in 3.7% formaldehyde, and stained with rhodamine-conjugated phalloidin as described under "Experimental Procedures." After mounting, cytoskeletons were studied by fluorescence microscopy and pictures taken with a 63× oil immersion objective. *B*, cells were labeled with [^{32}P] orthophosphate and stimulated with 10^{-6} M insulin for 2 min. After Triton extraction, cytoskeletons were solubilized with 1% SDS and immunoprecipitated with phosphotyrosine antibody. Precipitated proteins were analyzed by SDS-PAGE and autoradiography.

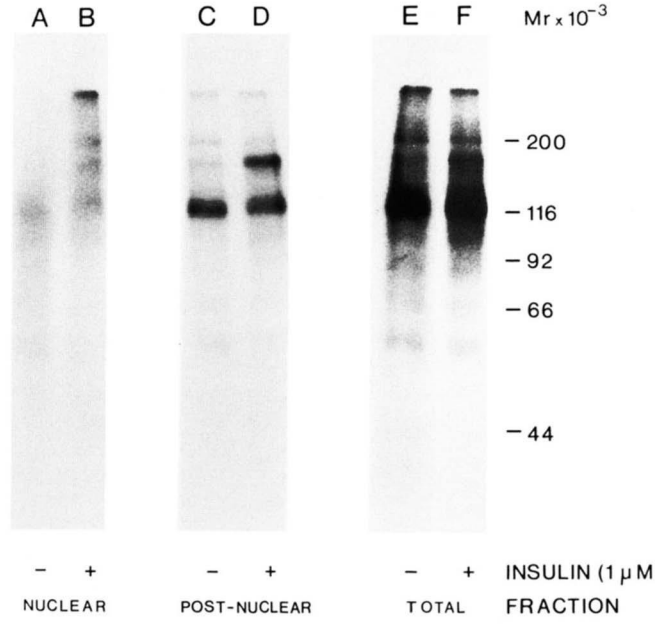

FIG. 6. **Subcellular localization of pp175.** FRTL5 cells were labeled with [^{32}P]orthophosphate and incubated for 2 min in the absence or the presence of 10^{-6} M insulin. The cells were then broken by stirring in buffer A containing 1% Triton X-100 and centrifuged for 20 min at 800 × *g*. The crude nuclear pellet was recovered and the supernatant centrifuged for 60 min at 200,000 × *g*. The nuclear and post-nuclear pellets were extracted with 1% SDS and precipitated with phosphotyrosine antibody. In a parallel experiment, labeled cells were solubilized with 1% SDS (total cell extracts) and precipitated with phosphotyrosine antibody. Proteins immunoprecipitated from the nuclear (*lanes A* and *B*), post-nuclear (*lanes C* and *D*), and total cell extracts (*lanes E* and *F*) were separated by SDS-PAGE and analyzed by autoradiography. A representative experiment is shown.

(Fig. 5, *right, lanes A* and *B*). Since the Triton-insoluble fraction contains both nuclear and cytoskeletal components, we further investigated the potential association of pp175 with nuclei. For this purpose, ^{32}P-labeled cells were stimulated with insulin, and crude nuclear and supernatant fractions

were obtained by differential centrifugation. Both fractions were then extracted with SDS, immunoprecipitated with α-Tyr(P), and analyzed by SDS-PAGE. In extracts of the nuclear fraction almost no pp175 was precipitated by α-Tyr(P) either before or after insulin stimulation (Fig. 6, *lanes A* and *B*). In contrast, pp175 was precipitated in the post-nuclear supernatant fraction from insulin-stimulated cells (Fig. 6, *lanes C* and *D*). This indicated that pp175 was associated with cytoskeletal elements that did not sediment with the nuclei. The amount of pp175 in this supernatant, measured by Cerenkov counting upon subtraction of background radioactivity, was >80% of the total pp175 content of the cell (Fig. 6, *lanes E* and *F*).

DISCUSSION

Several substrates for insulin and IGF I receptor kinases have been recognized in broken as well as in intact cell systems (20–22, 27–30, 41–43). However, the relevance of these proteins to insulin and IGF action is still unclear. To approach this problem we have studied the subcellular localization of the endogenous substrate for insulin and IGF I receptor kinases designated pp175. This protein was first described in myoblasts of the L6 skeletal muscle cell line (31). Based on our findings in these cells, we have suggested that pp175 phosphorylation in response to insulin and IGF I may have a role in stimulation of cell proliferation.[3] In the present work, we have studied pp175 in the FRTL5 thyroid cell line since we found that, in these cells, the level of phosphorylation of pp175 is constantly and significantly higher than L6 cells.[2] In addition FRTL5 cells possess distinct receptors for insulin and IGF I (32, 33) and are dependent on the presence of one or the other of these two hormones in the culture medium for proliferation (34, 44) and a number of differentiated functions (35).

We have shown that insulin and IGF I receptors in FRTL5 cells undergo rapid autophosphorylation in response to nanomolar concentrations of the respective ligands. Phosphorylation of a single M_r 175,000 protein, in addition to the receptors, follows insulin and IGF I binding to the cells. This protein is believed to be the same protein designated pp175 in L6 cells because it exhibits similar molecular size and insulin-induced phosphorylation kinetics (31). As shown previously in the L6 myoblasts (31), tyrosine phosphorylation of pp175 in FRTL5 cells occurs in response to nanomolar concentrations of both insulin and IGF I suggesting that it serves as a common substrate for both the insulin and the IGF I receptor kinases. The finding of the same substrate in thyroid and in muscle cells suggest that pp175 may be a ubiquitous protein that occurs in tissues of different embryonic origin. In support of this conclusion, using dissociated hepatocytes, Okamoto *et al.*[4] also have observed a M_r 175,000 protein undergoing rapid tyrosine phosphorylation in response to insulin. Substrates for insulin and/or IGF I receptor kinases exhibiting molecular size close but not identical to pp175 (pp185 and pp150) have been observed in different cell types (17, 27). The relationship between these phosphoproteins and the M_r 175,000 phosphoprotein is presently unclear. In all cases, phosphorylation occurs within seconds upon insulin or IGF I binding although the rate of dephosphorylation of pp175 appears to be greater than that of pp185 (45). A unique feature of pp175 is its association with the Triton-insoluble cytoskeleton, whereas pp185 and pp150 are aqueous-soluble proteins or readily solubilized by Triton X-100 (17, 27, 45). Based on these features, it appears that pp175 is distinct from pp185 and pp150. Alternatively, it is possible that the interaction of these phosphoproteins with the cytoskeleton varies in different cell types and, as a consequence, their solubility characteristics may vary in different cells. The cellular cytoskeleton includes several proteins that differ in structure and function (46) and interact with different cellular organelles (46, 47). Although the present study does not identify a specific cytoskeletal component with which pp175 is associated, electron microscopy studies failed to show structures other than cytoskeletal fibers and nuclei in the Triton-insoluble fraction where pp175 segregates (data not shown). Since pp175 was not detected in a purified nuclear fraction, we conclude that pp175 is either a cytoskeletal protein or that it becomes associated with the cytoskeleton upon phosphorylation. Availability of specific antibodies and purification of the protein will help to elucidate this last point.

Using cell-free systems, several investigators have shown that purified insulin and EGF receptor kinases phosphorylate cytoskeletal proteins (20–22). These include tubulin, microtubule-associated proteins (MAPS), and proteins associated with the microfilaments. Nishida *et al.* (23) also showed that tyrosine phosphorylation by the EGF receptor kinase induces functional alterations in MAP-2. These include inhibition of MAP-2 ability to promote tubulin depolymerization and cross-linking of actin filaments. Since insulin and EGF receptors phosphorylate MAP-2 on the same site(s) and to a similar extent (22), it has been proposed that tyrosine phosphorylation by the insulin receptor kinase induces similar functional alterations in MAP-2 (23). Thus, *in vitro*, insulin receptor-mediated tyrosine phosphorylation may be able to modify the function of a unique cytoskeletal protein. This is the only known protein, other than the receptor kinase itself, in which functional activity has been shown to be directly modulated by the receptor kinase. However, it remains to be demonstrated whether the effects on MAP-2 also occur "*in vivo*."

In intact KB cells, Kadowaki *et al.* (25) reported that occupancy of the insulin, type I IGF, and EGF receptors causes rapid membrane ruffling and cytoskeletal reorganization. Because these effects require the intrinsic tyrosine kinase activity of the receptors (26), the authors suggested that they are consequent to tyrosine phosphorylation of cytoskeletal proteins. Our finding that, in intact FRTL5 cells, insulin and IGF I receptors phosphorylate a cytoskeletal or a cytoskeleton-associated protein provides direct evidence that receptor-induced phosphorylation of these proteins also occurs *in vivo*, supporting the view that the cytoskeleton is an important target for insulin and IGF I action.

Insulin mediates translocation of glucose transporters (48) and the insulin-like growth factor II receptors (49) from intracellular storage sites to the plasma membrane. It has been reported that cytoskeletal elements may play a role in these processes (50, 51). Protein phosphorylation is an important mechanism regulating function and organization of cytoskeletal fibers (24, 47). Thus, the phosphorylation of cytoskeletal or cytoskeleton-associated proteins by insulin and IGF I receptor kinases that we have described might provide a molecular link between insulin/IGF I action and cytoskeleton function.

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