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Thyrotropin and Insulin-like Growth Factor I Regulation of Tyrosine Phosphorylation in FRTL-5 Cells

INTERACTION BETWEEN cAMP-DEPENDENT AND GROWTH FACTOR-DEPENDENT SIGNAL TRANSDUCTION*

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Pretreatment of rat FRTL-5 thyroid cells with thyrotropin (TSH) markedly potentiated the mitogenic response to insulin-like growth factor I (IGF-I) (Tramontano, D., Moses, A. C., Veneziani, B. M., and Ingbar, S. H. (1988) *Endocrinology* 122, 127–132; Takahashi, S.-I., Conti, M., and Van Wyk, J. J. (1990) *Endocrinology* 126, 736–745). The present study was undertaken to determine whether this synergism between TSH and IGF-I in FRTL-5 cells was correlated with changes in tyrosine phosphorylation of intracellular proteins. Tyrosine phosphorylation in intact cells was determined by gel electrophoresis and immunoblotting using monospecific anti-phosphotyrosine antibodies. Cells were preincubated for up to 24 h with TSH, dibutyryl cAMP, forskolin, or cholera toxin and then incubated for an additional 1 min in the absence or presence of IGF-I. As reported by others, IGF-I rapidly increased tyrosine phosphorylation of a 175-kDa protein as well as a less intense band of 90–100 kDa. Pretreatment for 6–12 h with either TSH or other agents that elevate intracellular cAMP potentiated the IGF-I-dependent tyrosine phosphorylation of the 175-kDa substrate by 3–5-fold. Since TSH did not increase IGF receptor number or kinase activity, the effect of TSH is assumed to be exerted at a step distal to IGF receptor tyrosine kinase. Surprisingly, IGF-I-independent tyrosine phosphorylation was also increased by pretreatment with TSH. When intact cells were analyzed TSH produced a time- and concentration-dependent increase in tyrosine phosphorylation of a prominent 120–125-kDa substrate and less prominent 100- and 80-kDa substrates. Assays using Triton X-100-soluble extracts incubated with $MgCl_2$, ATP, and orthovanadate demonstrated that TSH pretreatment increased tyrosine phosphorylation over that observed in untreated cells. In this cell-free assay, TSH pretreatment enhanced the phosphorylation of multiple substrates. These studies suggest that a cAMP stimulus that initiates a trophic effect can be propagated indirectly through multiple pathways including enhancement of tyrosine phosphorylation.

The rat thyroid cell line, FRTL-5, has been used by many investigators as a model to study the interactions between TSH[1] and insulin-like growth factors (IGFs) on cell proliferation. Numerous reports have shown that TSH potentiates the IGF-I mitogenic responses of these cells (1–4). The biologic effects of TSH, including its mitogenic actions in FRTL-5 cells, are mediated through a cAMP-dependent pathway, whereas, IGF-I, which has little or no direct effect on cAMP accumulation in these cells (2, 5), is presumed to act via the tyrosine kinase of the IGF-I receptor. We have previously reported that FRTL-5 cell priming with TSH leads to the secretion of an "amplification factor" through a cAMP-dependent mechanism that sensitizes FRTL-5 cells and other cell lines to the mitogenic effects of IGF-I and other growth factors (4).

Tyrosine phosphorylation is now known to play an indispensable role in the actions of many growth factors. Point mutations that abolish activity of the tyrosine kinase domains of growth factor receptors inhibit the ability of these receptors to mediate biological responses in general and cell proliferation in particular (6, 7). On the other hand, the role of the cAMP-dependent pathway in regulation of cell growth is less clear. Although usually regarded as a mediator of differentiated cell functions, in some instances, cAMP levels clearly influence growth factor-dependent cell growth (8, 9). In the present study of tyrosine phosphorylation in IGF-I and TSH-primed FRTL-5 cells, we further define how TSH, acting through the cAMP-dependent pathway, interacts with IGF-I-dependent pathways to potentiate the mitogenic actions of IGF-I.

EXPERIMENTAL PROCEDURES

Materials—Coon's modified Ham's F-12 medium (Coon's F-12) and new born calf serum were purchased from GIBCO; phosphate-free Eagle's minimum essential medium was purchased from Flow Laboratories Inc., McLean, VA. Bovine insulin and human transferrin were purchased from Sigma; bovine TSH (1.23 units/mg) for culture was purchased from Armour; purified bovine TSH (30 units/mg) for biological studies was a generous gift of the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases); recombinant human IGF-I was a generous gift from Dr. William J. Rutter, Chiron Corporation, Emeryville, CA. Polyclonal anti-phosphotyrosine antisera were prepared and affinity purified as previously reported (10); monoclonal anti-phosphotyrosine antibody, PY-20 (11), was purchased from ICN Biomedicals,

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[1] The abbreviations used are: TSH, thyrotropin; IGF, insulin-like growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HBSS, Hanks' balanced salt solution; PNPP, *p*-nitrophenyl phosphate; WGA, wheat germ agglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Inc., Costa Mesa, CA. ^{125}I-Protein A (8.16 μCi/μg), [^{32}P]orthophosphate (8,500 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol)were purchased from Du Pont-New England Nuclear, and [*methyl*-^{3}H]thymidine (6.5 mCi/mmol) and sodium ^{125}I (100 mCi/ml of NaOH solution) were purchased from Amersham Corp. Liquid scintillation counting was carried out in ScintiVerse® BD from Fisher. Except where otherwise designated, all other chemicals were the purest grade available from Sigma.

Cell Cultures—FRTL-5 cells (ATCC No. CRL8305), a line of rat thyroid follicular cells developed by Ambesi-Impiombato *et al.* (12), were generously provided by Dr. Leonard Kohn (Section of Cell Regulation, NIDDK) and the Interthyr Research Foundation, Baltimore, MD. Cells were routinely cultured in Coon's F-12 medium supplemented with 5% new born calf serum, and a mixture of three hormones (3H) including TSH (1 milliunit/ml), insulin (10 μg/ml), and transferrin (5 μg/ml). Cells were cultured in 150-cm flasks (Corning, Corning, NY) at 37 °C in an atmosphere of 95% air, 5% CO_2 in a humidified incubator.

Sample Preparation and Immunoblotting—FRTL-5 cells (4.5×10^5 cells/2 ml) were sparsely seeded in 35-mm dishes (Corning) in Coon's F-12 medium containing 5% new born calf serum and 3H. Five days later, the cells were washed twice with HBSS, and cultures were continued for an additional 24 h in Coon's F-12 medium containing 0.1% bovine serum albumin until the cells became quiescent. The medium was then replaced with the experimental medium without or with TSH, dibutyryl cAMP, or other cAMP generating agents for the indicated times. At the end of the pretreatment, the cells were washed three times with HBSS. The cells were then treated with IGF-I for 1 min unless specifically stated. After this final incubation, the cells were lysed at 0 °C in 0.25 ml of radioimmune precipitation buffer solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM sodium vanadate, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 10 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units/ml of aprotinin, and 10 mg/ml *p*-nitrophenyl phosphate (PNPP) (13). The monolayer was scraped and transferred to a glass test tube. 0.125 ml of concentrated SDS-PAGE sample buffer solution (3 × Laemmli's sample buffer solution containing 9% SDS and 6% 2-mercaptoethanol) was added, and the mixture was incubated for 5 min at 100 °C (14). These samples were then stored at −80 °C until electrophoresis was carried out. A fraction of each sample, kept constant within experiments at approximately 100 μg of protein, was run on 7% SDS-PAGE, and immunoblots with polyclonal or monoclonal anti-phosphotyrosine antibodies were performed as previously described (10). The protein assay was carried out according to the method of Lowry *et al.* (15). Intensity of the bands on the autoradiographs were quantified with an LKB UltroScanXL Laser Densitometer (LKB, Bromma, Sweden). These densitometric data are reported in the figures in arbitrary units of absorbance.

Metabolic Labeling with [^{32}P]Orthophosphate, Immunoprecipitation by Anti-phosphotyrosine Antibodies, and Phosphoamino Acid Analysis—FRTL-5 cells were seeded in 60-mm dishes (Corning) and cultured as described above. After cells became quiescent, the medium was replaced with experimental medium without or with TSH (1 nM) for 8 h. The cells were rinsed with 150 mM NaCl and 50 mM HEPES, pH 7.4, three times and incubated with phosphate-free minimum essential medium containing carrier-free [^{32}P]orthophosphate (0.333 mCi/ml) without or with TSH (1 nM) for an additional 16 h. Subsequently, IGF-I (100 ng/ml) was added, and after incubating for 1 min, cells were solublized in 0.3 ml of the lysis buffer solution containing 20 mM HEPES, pH 7.3, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol, 1% Triton X-100, 10 μg/ml phenylmethylsulfonyl fluoride, and 2.5 mM PNPP (16). Cell lysates were boiled in the presence of 1% SDS and 100 nM 2-mercaptoethanol for 5 min. After dilution of the SDS to 0.1%, phosphotyrosyl, proteins were immunoprecipitated by polyclonal or monoclonal anti-phosphotyrosine antibodies. Immunoprecipitated phosphoproteins were subjected to 7% SDS-PAGE and transferred to Immobilon® (Millipore. Bedford, MA). The analysis of acid-hydrolyzed phosphoamino acids was performed by the procedure of Kamps and Sefton (17). Briefly, individual bands visualized by autoradiography were isolated, each ^{32}P-labeled protein was hydrolyzed with 6 N HCl at 110 °C for 2 h, and [^{32}P]phosphoamino acids were determined after two-dimensional electrophoretic analysis.

Specific Activity of the ^{32}P Pool—FRTL-5 cells were seeded in 35-mm dishes and cultured as described above in the presence of [^{32}P] orthophosphate (1 μCi/ml). After treatment, cells were harvested in 1 ml of ice-cold 5% trichloroacetic acid and sonicated. The samples were centrifuged to remove proteins, and the supernatants were used for ATP measurements after being extracted five times with equal volumes of diethylether to remove the trichloroacetic acid. Specific activities of the [^{32}P]ATP pool were measured by the procedure of Ganahan and Glynn (18), using AG1-X4 (Cl form) columns. ATP concentrations were measured by an ATP bioluminescence assay kit (Sigma). In these experiments three dishes were used for each point.

Incorporation of [methyl-^{3}H]Thymidine into DNA—For studies of [*methyl*-^{3}H]Thymidine incorporation into DNA, FRTL-5 cells were seeded in 35-mm dishes and cultured as described above. After becoming quiescent, the cells were incubated without or with TSH for the times indicated. Cells were then washed three times with HBSS and incubated with IGF-I for 24 h. [*methyl*-^{3}H]Thymidine (1 μCi/ml) was added to each dish 4 h before the termination of each experiment. The labeling was stopped by adding 1 M ascorbic acid, and 10% trichloroacetic acid-precipitable materials were measured as thymidine incorporation into DNA (4). In all experiments, each experimental point represents the mean of three replicate dishes.

Binding Assay of IGF Receptor—IGF-I was labeled to a final specific activity of 250 μCi/μg by a gentle chloramine-T method as previously described (19). Binding studies were performed according to the method of Tramontano *et al.* (20). FRTL-5 cells were seeded in 24-well plates (Corning) and cultured as described above. After reaching quiesence, the cells were treated with TSH (1 nM) for 0, 24, or 48 h and then washed three times with binding assay buffer. This buffer consisted of KRB modified by the replacement of NaCl with 280 mM sucrose, a concentration that was sufficient to maintain the tonicity of solution. ^{125}I-Labeled IGF-I and various concentrations of unlabeled IGF-I were added to wells in 250 μl of the binding assay buffer containing 0.1% bovine serum albumin, and cells were incubated for 18 h at 4 °C. At the end of the incubation, cells were washed three times with binding assay buffer. The ^{125}I content of cell lysates was measured after solubilizing the cells in 1 N NaOH. Nonspecific binding was determined in the presence of 500 ng/ml IGF-I.

Autophosphorylation of IGF Type I Receptor—Autophosphorylation of the IGF-I receptor in intact cells was assessed by the method of Momomura *et al.* (21). FRTL-5 cells were seeded in 100-mm dishes (Corning) and cultured as described above. After reaching quiesence, the cells were pretreated without or with TSH (1 nM) for 24 h. At the end of pretreatment, cells were washed three times with HBSS and treated with various concentrations of IGF-I for 1 min. Cells were lysed at 0 °C in 1 ml of Triton X/Tris/lysis buffer (50 mM Tris-HCl, pH 7.4, 500 μM orthovanadate, 1% Triton X-100, 5 mM EDTA, 10 mg/ml PNPP, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin), and the lysates were tumbled for 30 min at 4 °C. After centrifugation at 15,000 × *g* for 30 min, the supernatant was incubated with 100 μl of WGA-agarose (50% (v/v)) for 1 h. The WGA-agarose was then washed three times with lysis buffer and the adsorbed proteins eluted by boiling in Laemmli's sample buffer solution. The eluates were run on 7% SDS-PAGE in preparation for phosphotyrosine immunoblotting.

Tyrosine Kinase Activity Assay of WGA-adsorbed Fraction—FRTL-5 cells were seeded in 100-mm dishes and treated as described for the autophosphorylation study except that cells were treated without or with IGF-I (100 ng/ml) for 1 min. Cells were lysed at 0 °C in 100 μl of Triton X/HEPES/lysis buffer (50 mM HEPES-NaOH, pH 7.6, 500 μM orthovanadate, 1% Triton X-100, 5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml phenylmethylsulfonyl fluoride, and 100 KIU/ml aprotinin) containing 10 mM PNPP. The lysates were tumbled for 30 min at 4 °C. After centrifugation, (15,000 × *g* for 30 min at 4 °C), the supernatant was incubated with 100 μl of WGA-agarose (50% (v/v)) for 1 h. WGA-agarose was washed three times with the lysis buffer solution, and the adsorbed proteins were eluted with 100 μl of 0.3 M *N*-acetylglucosamine.

Fifty μl of WGA-adsorbed fraction was preincubated without or with IGF-I (100 ng/ml) for 30 min at 25 °C. A 20-min cell-free phosphorylation was initiated with 25 μl of reaction mixture to give final concentration of 50 mM HEPES-NaOH, pH 7.6, 50 mM $MgCl_2$, 500 μM orthovanadate, 1% Triton X-100, 20 μM [γ-^{32}P]ATP (10 Ci/mmol), 2 mg/ml poly(Glu-Tyr) (4:1). The reactions were terminated by applying 30 μl-aliquots to Whatman No. 3MM filter paper. The papers were extensively washed at 4 °C in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate for 20 min followed by two 20-min 5% trichloroacetic acid washes. Filters were rinsed in ethanol, dried, and counted in a liquid scintillation counter. Tyrosine kinase activity was expressed as the phosphate incorporation into the poly(Glu-Tyr) (4:1) during 20 min.

Cell-free Tyrosine Phosphorylation Assay—Cells were grown in 100-mm dishes, pretreated without or with TSH (1 nM) for 24 h, and

treated with IGF-I (100 ng/ml) for 1 min as described above. Cells were lysed in 150 μl of Triton X/HEPES/lysis buffer solution, and cell lysates were centrifuged at 15,000 × *g* for 30 min at 4 °C to obtain a clear supernatant.

Cell-free phosphorylation was initiated by addition of 25 μl of reaction mixture to 50 μl of cell lysates to give final concentration of 50 mM HEPES-NaOH, pH 7.6, 50 mM $MgCl_2$, 500 μM orthovanadate, 1% Triton X-100, and 1 mM ATP. After incubating the mixture for 30 min at 25 °C, the reaction was terminated by adding 37.5 μl of concentrated SDS-PAGE sample buffer solution and boiling the samples for 5 min. These samples were analyzed by 7% SDS-PAGE followed by immunoblotting.

Statistical Analysis—Concentration-response curves were analyzed by fitting the data with the four logistic equations according to the method of De Lean *et al.* (22).

RESULTS

Effect of TSH and Agents That Elevate Intracellular cAMP on Tyrosine Phosphorylation—To test the effect of TSH and other agents that elevate cAMP in FRTL-5 cells on tyrosine phosphorylation, cells were preincubated for 24 h with TSH, forskolin, dibutyryl cAMP, or cholera toxin. At the end of this pretreatment, cells were incubated for 1 min in the absence or presence of IGF-I, and the pattern of tyrosine phosphorylation was evaluated by immunoblot analysis using monospecific anti-phosphotyrosine antibody. TSH pretreatment potentiated IGF-I-dependent tyrosine phosphorylation but also stimulated IGF-I-independent accumulation of tyrosine-phosphorylated substrates (Fig. 1). Tyrosine phosphorylation of the predominant 175-kDa band was dependent on IGF-I concentration, and this IGF-I-dependent phosphorylation increased approximately 3–5-fold after TSH pretreatment (Fig. 1*A*). The phosphotyrosine content of the 120–125-kDa substrates were independent of IGF-I treatment. Surprisingly, the tyrosine phosphorylation of 120–125-kDa substrates was enhanced by TSH pretreatment for 24 h (Fig. 1*A*). Longer exposures of the blot revealed other substrates that followed the two above-mentioned patterns of phosphorylation (Fig. 1*B*). For example, immunoblotting detected a broad band migrating around 90–100 kDa in IGF-I-treated cells; its IGF-I-dependent tyrosine phosphorylation was potentiated by TSH. Conversely, proteins of 100 and 80 kDa were phosphorylated in the absence of IGF-I; their phosphotyrosine content was increased by treatments that raise intracellular cAMP content and was not influenced by IGF-I.

When compared to the effects of TSH, pretreatment of the cells with dibutyryl cAMP, forskolin, or cholera toxin produced identical or even more pronounced increases in the phosphotyrosine content of the same IGF-I-dependent and IGF-I-independent substrates. This suggests that the effect of TSH on tyrosine phosphorylation is mediated by a cAMP-dependent pathway (Fig. 1, *A* and *B*). The same tyrosine phosphorylated substrates were observed when the immunoblots were performed using monoclonal anti-phosphotyrosine antibody, PY-20. These effects were not due to increased cell number or protein (the amount of protein loaded on each lane was control = 102 ± 5 μg, TSH pretreatment = 113 ± 5 μg, dibutyryl cAMP pretreatment = 124 ± 2 μg, forskolin pretreatment = 117 ± 3 μg, cholera toxin pretreatment = 122 ± 2 μg, respectively; mean ± S.E. of triplicate dishes).

IGF-I-dependent Tyrosine Phosphorylation—TSH increased the phosphotyrosine content of the IGF-I-dependent 175-kDa band without altering the time course or the concentration dependence of the response to IGF-I (Fig. 2, *A* and *B*, respectively). The EC_{50} for IGF-I after 24 h was 15.3 ± 1.9 ng/ml in the absence of TSH and 22.9 ± 6.9 ng/ml in the presence of 1 nM TSH (Fig. 2*B*). TSH pretreatment caused some broadening of the 175-kDa band, and in some experiments doublet formation was observed in this band. However, the resolution was insufficient to determine whether TSH affected the upper and/or lower components. The 175-kDa

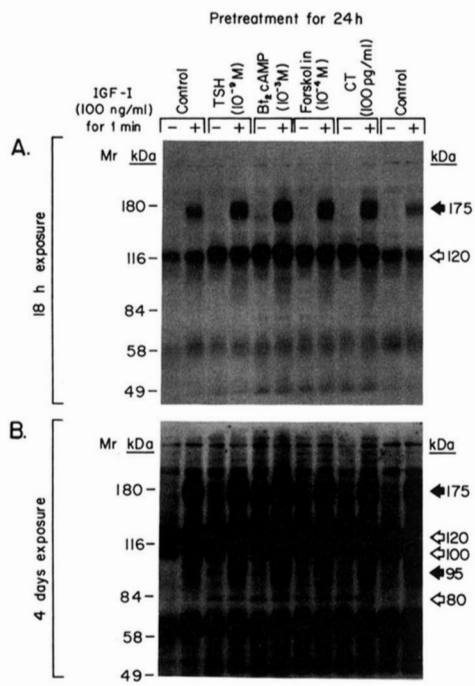

FIG. 1. **Effect of TSH and other cAMP-generating agents on tyrosine phosphorylation in FRTL-5 cells.** Quiescent FRTL-5 cells were pretreated with 1 nM TSH, 1 mM dibutyryl cAMP, 100 μM forskolin, and 100 pg/ml cholera toxin for 24 h, washed three times with HBSS, and incubated without or with 100 ng/ml IGF-I for 1 min. After treatment, cells were harvested with radioimmune precipitation buffer solution and immunoblot analysis was performed with anti-phosphotyrosine antibodies as described under "Experimental Procedures." 18 h of exposure (*A*) and 4 days of exposure (*B*).

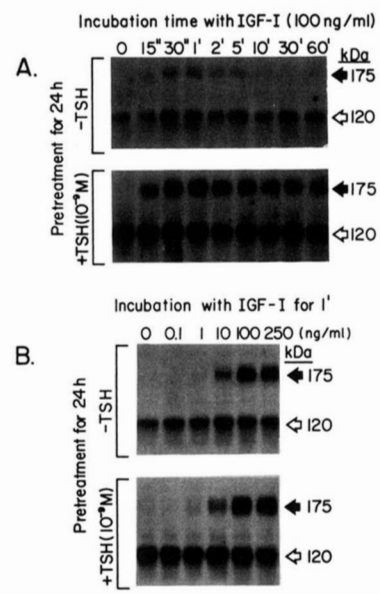

FIG. 2. **Effect of IGF-I on tyrosine phosphorylation in FRTL-5 cells.** *A*, time course of IGF-I effect on tyrosine phosphorylation. Quiescent cells were incubated without or with 1 nM TSH for 24 h, washed, and incubated with 100 ng/ml IGF-I for the times indicated. Cells were harvested and immunoblot analysis was performed. *B*, Effect of IGF-I concentration on tyrosine phosphorylation. Quiescent cells were incubated without or with 1 nM TSH for 24 h, washed, and incubated with various concentration of IGF-I for 1 min. After treatment, cells were harvested and immunoblot analysis was performed.

band was not tyrosine phosphorylated in the absence of IGF-I whether or not the cells had been pretreated with TSH. The effect of TSH to increase IGF-I-dependent phosphorylation was consistently present after 1 h and was maximal after 24 h (*e.g.* see Fig. 4*C*).

IGF-I-dependent increases in the phosphotyrosine content were similar whether or not the cells had been preincubated with 200 μM sodium orthovanadate for 15 min prior to the addition of IGF-I (data not shown). This suggested that the effects of IGF-I were not due to inhibition of a vanadate-sensitive tyrosine phosphatase.

An IGF-I-dependent increase in the phosphotyrosine content of a 175-kDa substrate was confirmed by immunoprecipitating phosphoproteins from cells labeled with [^{32}P]orthophosphate using anti-phosphotyrosine antibodies followed by phosphoamino acid analysis. The addition of IGF-I (100 ng/ml) to TSH-pretreated cells increased [^{32}P]phosphotyrosine of a 175-kDa substrate by 11.3-fold (Table I). IGF-I had no significant effect on the specific activity of the [^{32}P]ATP pool or on the intracellular ATP pool size (data not shown).

TSH-dependent Tyrosine Phosphorylation—With short exposure times the 120–125-kDa IGF-I-independent band could be resolved into two bands, as reported by others (23). The predominant effect of TSH was an increase in the phosphotyrosine content of the more slowly migrating band labeled *120 H* in Fig. 3*A*. In the absence of TSH, the lower component of the 120-kDa substrate (*120 L*) was clearly evident, while the upper component was usually at the limit of detection. As indicated before, TSH pretreatment increased tyrosine phosphorylation of at least two other substrates, 100- and 80-kDa proteins, which were resolved as single bands.

When the pattern of hormonally induced tyrosine phosphorylation was compared with the effect of similar hormonal treatments on thymidine incorporation into DNA, it was found that the optimal concentration of TSH on IGF-I-dependent DNA synthesis is similar to that of TSH on IGF-I-dependent tyrosine phosphorylation in these cells (Fig. 3*B*). The calculated EC_{50} of TSH was approximately 290 ± 107 pM for the 175-kDa substrate and 189 ± 87 pM for the 120-kDa substrate. This is in the same range as the potentiation of DNA synthesis in FRTL-5 cells (EC_{50}= 150 ± 25 pM).

Phosphoamino acid analysis of immunoprecipitated phosphotyrosyl proteins from ^{32}P-labeled cells revealed labeled phosphotyrosine in the 120-kDa substrates. The absolute

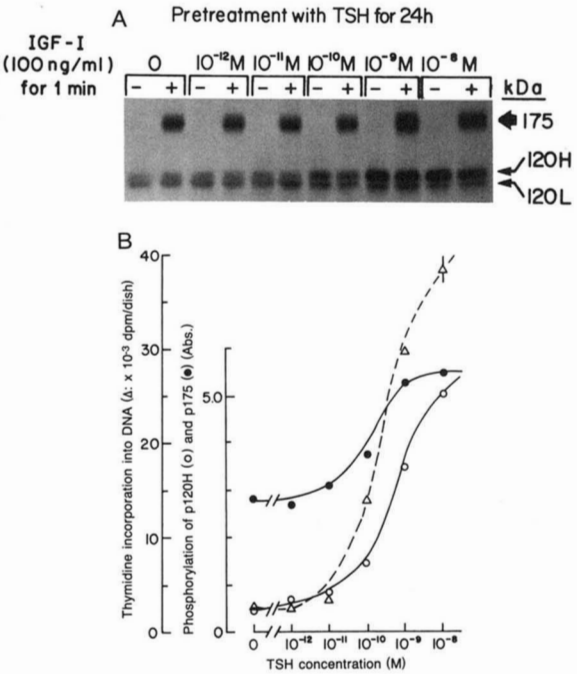

FIG. 3. **Effect of TSH concentration on tyrosine phosphorylation and DNA synthesis in FRTL-5 cells.** *A*, quiescent FRTL-5 cells were incubated with various concentrations of TSH for 24 h, washed, and incubated without or with 100 ng/ml IGF-I for 1 min. Cells were harvested and immunoblot analysis was performed. *B*, for the DNA synthesis assay, cells were pretreated with various concentrations of TSH for 24 h, and incubated with 100 ng/ml IGF-I for an additional 24 h. [*methyl*-^{3}H]Thymidine incorporation into DNA was measured for the last 4 h of incubation (△). The regions of the autoradiogram of pp120-H (○) and pp175 (●) of the cells pretreated with 1 nM TSH for 24 h and treated with 100 ng/ml IGF-I for 1 min, were quantitated by densitometric scanning.

counts of [^{32}P]phosphotyrosine in this band from cells that had been pretreated with TSH were 4.2-fold greater than in control cells (Table I). The interpretation of this result was complicated, however, by finding that TSH pretreatment increased the specific activity of [^{32}P]ATP by 2.2-fold (control, 8,437 ± 343; TSH treated, 19,623 + 317 cpm/nmol). This change in specific activity may contribute to our observation of increased [^{32}P]orthophosphate labeling of cell substrates. Time course studies showed that this effect of TSH in quiescent cells became evident over an 8-h period of TSH incubation and did not decline until 16 h after TSH withdrawal (data not shown). Although these effects of TSH must be considered when interpreting tyrosine phosphorylation studies, they clearly do not account for the selective TSH-dependent increase in tyrosine phosphorylation of specific substrates, *e.g.* the more slowly migrating 120-kDa substrate and the 100- and 80-kDa substrates, which were clearly evident with the immunoblot technique.

TSH stimulation of the 120-kDa protein was dependent on the time of TSH pretreatment (Fig. 4*A*). In four different experiments, densitometric analysis revealed a small increase in the phosphotyrosine content of this substrate after pretreatment with TSH for 10 min (Fig. 4*B*). Because of difficulties in resolving the two 120-kDa region (*120 H*), the significance of this early increase remains unclear. However, TSH pretreatment times of 6–24 h consistently produced a substantial increase in the upper component of the 120-kDa region (*120 H*). In those experiments in which the 120 H could be clearly resolved, TSH pretreatment produced little or no change in the lower component (Fig. 4, *A* and *B*). The time required for TSH to induce changes in tyrosine phos-

TABLE I

Phosphoamino acid analysis of immunoprecipitated phosphotyrosyl proteins, 175- and 120-kDa substrates, from ^{32}P-labeled cells

Quiescent FRTL-5 cells were pretreated without or with TSH (1 nM) for 8 h and were incubated with phosphate-free minimal essential medium containing carrier-free [^{32}P]orthophosphate (0.333 mCi/ml) without or with TSH (1 nM) for an additional 16 h. Subsequently, IGF-I (100 ng/ml) was added after incubating for 1 min, and cells were solubilized in the lysis buffer solution. Phosphotyrosyl proteins were immunoprecipitated by anti-phosphotyrosine antibodies, and phosphoamino acids of pp175 and the entire pp120 doublet complex were analyzed as described under "Experimental Procedures."

Pretreatment with TSH	−	−	+	+
Treatment with IGF-I	−	+	−	+
pp175 (cpm)				
[^{32}P]Ser	111.5	131.4	434.6	571.5
[^{32}P]Thr	7.7	12.4	38.3	62.3
[^{32}P]Tyr	0.0	14.1	9.1	102.4
pp120 complex (cpm)				
[^{32}P]Ser	142.4	170.7	606.7	676.7
[^{32}P]Thr	15.7	12.9	53.8	55.1
[^{32}P]Tyr	3.9	6.1	16.5	41.2

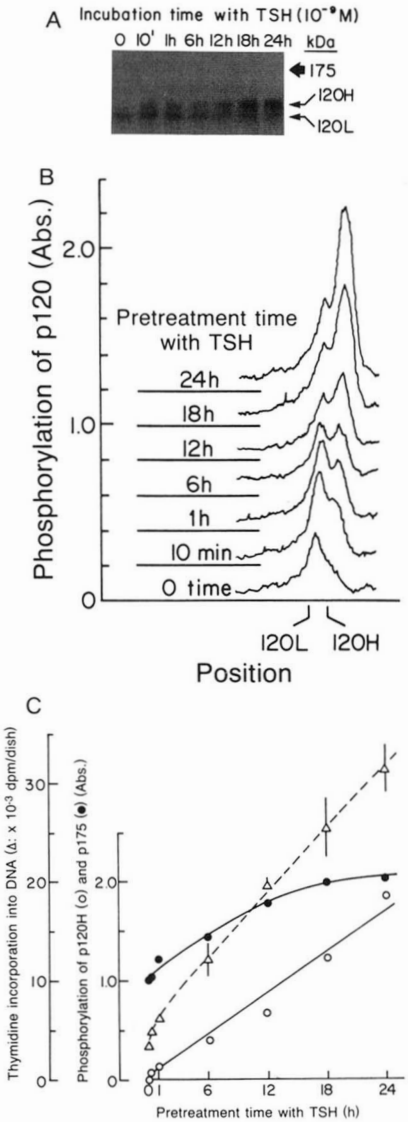

FIG. 4. **Time course of TSH effect on tyrosine phosphorylation in FRTL-5 cells.** *A*, quiescent FRTL-5 cells were pretreated with 1 nM TSH for the times indicated and then harvested and analyzed by immunoblotting. *B*, the region of the autoradiogram (*A*) corresponding to pp120 was quantitated by densitometric scanning. *C*, for the DNA synthesis assay, after pretreatment with 1 nM TSH for the indicated times, cells were incubated with 100 ng/ml IGF-I for an additional 24 h, and [*methyl*-^{3}H]thymidine incorporation into DNA was measured for the last 4 h (△). The region of the autoradiogram of pp120-H (○) and pp175 (●) was quantitated by densitometric scanning after cells were pretreated with 1 nM TSH for the indicated times, washed, and treated with 100 ng/ml IGF-I for 1 min.

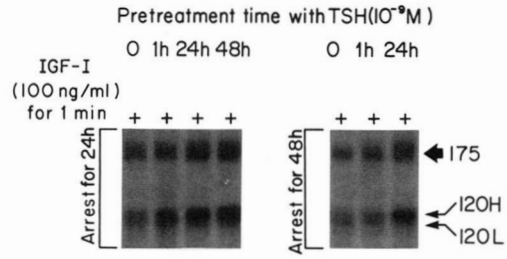

FIG. 5. **Phosphorylation of the 120- and 175-kDa substrate in FRTL-5 cells after different times of growth factors deprivation.** FRTL-5 cells were incubated in Coon's F-12 medium containing 0.1% bovine serum album for 24 or 48 h, pretreated with 1 nM TSH for 24 h, washed, and incubated with 100 ng/ml IGF-I for 1 min. Cells were harvested and immunoblot analysis was performed.

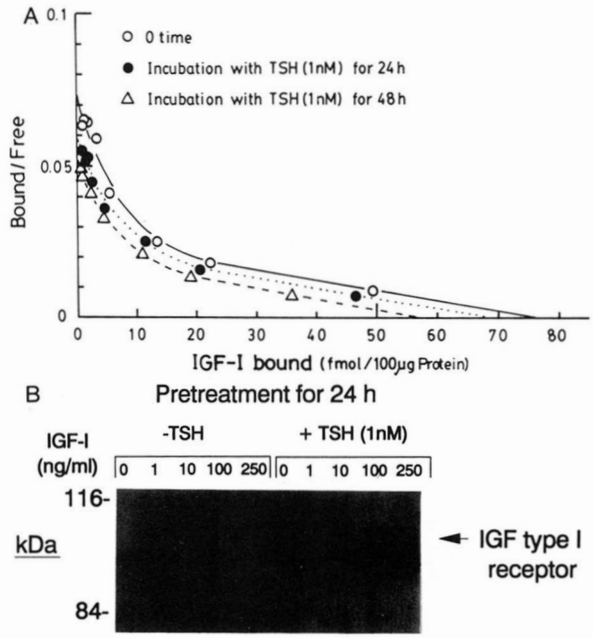

FIG. 6. **Effect of TSH on IGF-I binding and IGF receptor autophosphorylation.** *A*, Scatchard analysis of IGF binding 0, 24, or 48 h after TSH treatment. Quiescent FRTL-5 cells were treated with TSH (1 nM) for 0 (○), 24 (●), or 48 h (*D*). After treatment, binding assays were performed at 4 °C for 18 h, and the specific binding of IGF-I to receptor was plotted by the method of Scatchard. *B*, effect of IGF-I concentration on tyrosine phosphorylation of proteins isolated by WGA-adsorption. Quiescent FRTL-5 cells were preincubated without or with TSH (1 nM) for 24 h, washed, and incubated with various concentrations of IGF-I for 1 min. After treatment, WGA-adsorbed proteins were prepared from cell lysates, run on a 7% SDS-PAGE, and subjected to immunoblot analysis.

phorylation of the 175- and 120-kDa substrates (6–12 h) was similar to that required to clearly induce increased responsivity to IGF-I in terms of thymidine incorporation into DNA (Fig. 4*C*).

The effect of TSH on both the 175- and 120-kDa substrates was evident whether the hormone was added 24, 48 (Fig. 5), or 72 h (data not shown) after the cells had been changed to nonpermissive medium. After longer periods of quiescence, however, tyrosine phosphorylation in response to TSH pretreatment was less intense, again paralleling the degree of thymidine incorporation into DNA (data not shown).

TSH-dependent Tyrosine Phosphorylation: Studies of Potential Mechanisms—Several exploratory experiments were performed to elucidate the mechanisms by which TSH potentiates IGF-I-dependent and stimulates IGF-I-independent phosphorylation. In agreement with other workers (24), we found that TSH treatment of FRTL-5 cells for 24 h did not significantly alter the number of IGF-I receptors nor did it alter their affinity for IGF-I (Fig. 6*A*). We used preparations for IGF receptor that had been partially purified by adsorption to WGA-agarose to test the possibility that TSH may have induced an increase in phosphorylation of IGF-type I receptor or its intrinsic tyrosine kinase activity. Autophosphorylation was determined by immunoblotting (Fig. 6*B*), and tyrosine kinase activity of the WGA-adsorbed fraction was measured by using an exogenous substrate (Table II). We found that TSH changed neither autophosphorylation nor the IGF-dependent tyrosine kinase activity of the IGF-type I receptor.

In addition, tyrosine phosphorylation was assessed in a cell-free assay after 24 h of incubation in the absence or presence of TSH followed by 1 min of incubation with IGF-I (Fig. 7). When cell homogenates were treated with detergent and

TABLE II

Tyrosine kinase activity of the WGA-agarose adsorbed fraction

Quiescent FRTL-5 cells were pretreated without or with TSH (1 nM) for 24 h, washed, and incubated without or with IGF-I (100 ng/ml) for 1 min. Cells were solubilized in the lysis buffer. After centrifugation, the clear supernatant was incubated with WGA-agarose, and the adsorbed proteins were eluted with the lysis buffer containing 0.3 M *N*-acetylglucosamine. Tyrosine kinase activity of this fraction was measured using poly(Glu/Tyr) (1:4) and [γ-^{32}P] ATP in the absence or presence of IGF-I (100 ng/ml) as described under "Experimental Procedures."

Pretreatment with TSH	−	+	−	+
Treatment with IGF-I	−	−	+	+
Cell-free assay (pmol/μg protein/20 min)				
−IGF-I	0.25 ± 0.02	0.31 ± 0.01	6.75 ± 0.20	4.65 ± 0.07
+IGF-I	8.57 ± 0.45	11.41 ± 0.06	18.44 ± 0.50	15.35 ± 0.51

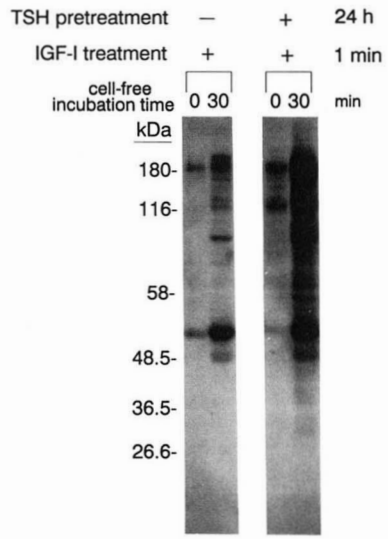

FIG. 7. **Cell-free tyrosine phosphorylation in detergent-soluble extracts.** Quiescent FRTL-5 cells were preincubated without or with TSH (1 nM) for 24 h, washed, and incubated with IGF-I (100 ng/ml) for 1 min. After treatment, cells were lysed and homogenized in Triton X-100/HEPES/lysis buffer. The zero time indicates those samples that were immediately boiled with SDS-PAGE sample buffer. Duplicate samples were incubated at 25 °C for 30 min in the presence of 500 μM orthovanadate, 50 mM $MgCl_2$ and 1 mM ATP. At the end of cell-free phosphorylation reaction, samples were boiled in SDS-PAGE sample buffer and both initial (*0 time*) and cell-free phosphorylated sample (*30 min*) were run on 7% SDS-PAGE and subjected to immunoblotting. Similar results were observed in three experiments.

phosphorylated in the presence of 1 mM cold ATP and 500 mM orthovanadate, we found increases in tyrosine phosphorylated substrates compared to the initial level, *i.e.* in lysates of intact cells. This provided evidence of continued tyrosine kinase activity in the broken cell preparations. In addition, cell-free extracts from TSH-treated cells exhibited increased tyrosine phosphorylation of multiple substrates (including p175 and p120 H) when compared to extracts from control cells.

DISCUSSION

The role of cAMP-dependent pathways in the regulation of mitosis and cell cycle is poorly understood. A number of hormones that increase cAMP have also been shown to affect entry in the cell cycle (8), whereas other reports clearly link an increase in intracellular cAMP with exit from the cell cycle (25). Thus, there is reason to believe that cAMP-mediated events might influence cell entry and exit from the cell cycle at defined restriction points. The effect of cAMP may also vary with cell type. In this study, we tested the hypothesis that in FRTL-5 cells the TSH/cAMP-dependent pathway affects growth factor action by altering the phosphorylation of tyrosine kinase substrates. Our findings indicate that TSH, through activation of the cAMP-dependent pathway, potentiates IGF-I-dependent tyrosine phosphorylation and stimulates IGF-I-independent tyrosine phosphorylation. The fact that we could observe at least two subsets of tyrosine kinase substrates, *i.e.* IGF-I dependent or IGF-I independent, indicates that more than one mechanism may be responsible for these events. This observation was confirmed on immunoblots using two polyclonal and one monoclonal anti-phosphotyrosine antibodies. Immunoprecipitation of phosphotyrosyl proteins from ^{32}P-labeled cells using these antibodies also confirmed this observation. Since each of these experiments involved recognition of phosphotyrosine by these antibodies after the proteins had been treated with the buffer solution containing SDS and boiled, it is unlikely that a phosphorylation-dependent change in conformation could explain our results, although this possibility could not be excluded by direct testing.

The finding that TSH potentiated IGF-I-dependent tyrosine phosphorylation of some substrates (*e.g.* p175 and p90–100), suggests that this enhancement may be included in the chain of events by which the cAMP-dependent pathway potentiates the mitogenic responses mediated through the growth factor-dependent pathway. This hypothesis is supported by the finding that the TSH concentration dependence of these phosphorylation changes is similar to that of the TSH potentiation of IGF-I-stimulated DNA synthesis and by the finding that these different effects of TSH have similar time courses.

We used several approaches to determine whether 24 h of preincubation with TSH may have induced changes in the IGF receptor. We first confirmed the findings of others (24) that exposure of FRTL-5 cells to TSH did not increase IGF receptor number nor was there any change in affinity (Fig. 6*A*). We then showed that in intact cells preincubation with TSH for 24 h had no effect on the autophosphorylation of the IGF receptor induced by IGF-I (Fig. 6*B*). Furthermore, using the WGA-adsorbed fraction of these cells after exposure to IGF-I, we found that TSH had no effect on IGF receptor kinase activity (Table II). Stadtmauer *et al.* (26) and Roth *et al.* (27) found that although the insulin receptor can be phosphorylated by cAMP-dependent kinase, this phosphorylation decreases rather than increases the insulin-dependent activation of tyrosine kinase. From these results, we conclude that the effect of TSH is exerted at a step distal to IGF receptor tyrosine kinase.

The finding that TSH stimulates tyrosine phosphorylation of proteins whose phosphorylation is independent of IGF-I (*e.g.* p120–125, p100, and p80), indicates that the initial cAMP stimulus can be propagated through multiple pathways including those dependent on tyrosine kinases. Immunoblots

demonstrated that TSH increases the phosphotyrosine content of the IGF-I-independent 120-kDa substrate in a time-dependent manner. The TSH-dependent phosphorylation is largely confined to the upper band of a potential 120–125-kDa doublet. In preliminary experiments, we found that a monoclonal antibody that recognizes a pp125 phosphotyrosyl protein in v-*src*-transfected chicken embryo fibroblasts (28) recognized pp120L but not pp120H.[2] This suggests that pp120L and pp120H in FRTL-5 cells may be distinct proteins. However, we cannot rule out the possibility that this doublet may have arisen as subsets of a single substrate, the electrophoretic mobility of which had been altered by phosphorylation at multiple sites.

The long time required for substantial TSH-dependent potentiation of tyrosine phosphorylation of p175 induced by IGF-I and for the stimulation of tyrosine phosphorylation of p120 is an indication of the complexity of these changes. These changes might involve 1) increased synthesis or activation of tyrosine-specific protein kinases; 2) increased synthesis of substrates or alterations in substrate conformation; 3) inhibition of phosphatases; or 4) a combination of the above. Each of these mechanisms could be a direct effect of TSH or could be due to a secondary process such as increased production of intermediate autocrine factors such as the putative TSH-dependent amplification factor that we have described (4). The findings using the cell-free phosphorylation assay (Fig. 7) demonstrated that TSH greatly potentiated IGF-I-dependent tyrosine phosphorylation of substrates such as pp175 and stimulated IGF-I-independent tyrosine phosphorylation of many substrates. While all of the multiple phosphotyrosyl proteins seen in the cell-free assay may not be physiological substrates in the intact cell, the broader range of enhanced tyrosine phosphorylation of substrates in the detergent-soluble extracts from cells pretreated with TSH is most consistent with an increase in tyrosine kinase activity. However, we have not eliminated the possibility that some of the increased tyrosine phosphorylation is due to a TSH-dependent increase in specific substrates, a TSH-induced alteration in substrate conformation, or a TSH-induced decrease in phosphatase activity.

In 3T3 mouse fibroblasts, serum produces short term, reversible changes in the pattern of tyrosine phosphorylation. However, these short term effects are followed by a delayed increase in tyrosine phosphorylation of additional substrates such as cdc2, which are key regulators of mitosis (29, 30). These events are reminiscent of what we have shown in FRTL-5 cells. Here, TSH produces delayed changes in tyrosine phosphorylation that are ultimately correlated with increased DNA synthesis. It is thus possible that these TSH-dependent changes in pattern of phosphorylation play an important role in promoting entry of thyroid cells into the cell cycle and represent a novel gateway to a common path of increased intracellular tyrosine phosphorylation.

The 175-kDa substrate(s) of tyrosine kinase is probably the same as that observed in L6 skeletal muscle cells (31, 32) and in FRTL-5 cells (23). The time course of substrate phosphorylation is identical to that shown by Condorelli *et al.* (23) in FRTL-5 cells and by Izumi *et al.* (33) in NRK cells. Condorelli *et al.* (23) have published that pp175 is an insoluble protein that may be a component of the cell cytoskeleton. They proposed that this is a means by which insulin and IGF-I regulate cytoskeletal function (32). There is also a report describing insulin-dependent and IGF-I-dependent phosphorylation of a 120-kDa protein (34, 35). However, under our experimental conditions, IGF-I-dependent phosphorylation of 120-kDa proteins was not observed.

In summary, our data show that TSH, through the cAMP-dependent pathway, regulates the steady-state level of tyrosine phosphorylation of several substrates in FRTL-5 cells. Phosphorylation of some of these substrates is regulated also by IGF-I, demonstrating that TSH and IGF-I-dependent signals converge into a common pathway. In addition, these hormones also stimulate serine/threonine phosphorylation. These results support the concept that a cAMP stimulus initiates a prolonged or trophic effect that can be propagated indirectly through multiple pathways including tyrosine phosphorylation. This interaction between cAMP-dependent and growth factor-dependent pathways may play a fundamental role in biological actions induced by TSH and IGF-I in this system.

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