Growth hormone (GH) is the major regulator of postnatal body growth (1). It possesses diverse and pleiotropic effects on the growth, differentiation, and metabolism of cells. GH is thought to initiate its biological actions, including induction of a number of RNA species in mammalian tissues, by interaction with a specific membrane-bound receptor (2). The GH receptor is phosphorylated upon ligand stimulation presumably by the physical association of the nonreceptor tyrosine kinase JAK2 (3). The JAK kinases are linked to transcriptional regulation, and JAK activation results in the phosphorylation of two JAK subunits, paxillin and tensin, and that FAK associates with JAK2 but not JAK1 after GH stimulation of cells. Using FAK-replete and FAK-deficient cells, we also show that FAK is not required for STAT-mediated transcriptional activation by GH. The use of FAK in the signal transduction pathway utilized by GH may be central to many of the pleiotropic effects of GH, including cytoskeletal reorganization, cell migration, chemotaxis, mitogenesis, and/or prevention of apoptosis and gene transcription.

We have demonstrated that growth hormone (GH) activates focal adhesion kinase (FAK), and this activation results in the tyrosine phosphorylation of two FAK substrates, paxillin and tensin. The activation of FAK is time-dependent (maximal activation at 5–15 min) and dose-dependent (maximal activation at 0.05 nM). FAK and paxillin are constitutively associated in the unstimulated state, remain associated during the stimulation phase, and recruit tyrosine-phosphorylated tensin to the complex after GH stimulation. Half of the carboxyl-terminal region of the GH receptor is dispensable for FAK activation, but FAK activation does require the proline-rich box 1 region of the GH receptor, indicative that FAK is downstream of JAK2. FAK associates with JAK2 but not JAK1 after GH stimulation of cells. Using FAK-replete and FAK-deficient cells, we also show that FAK is not required for STAT-mediated transcriptional activation by GH. The use of FAK in the signal transduction pathway utilized by GH may be central to many of the pleiotropic effects of GH, including cytoskeletal reorganization, cell migration, chemotaxis, mitogenesis, and/or prevention of apoptosis and gene transcription.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human growth hormone (hGH) was a generous gift of Novo-Nordisk (Singapore). Anti-phosphotyrosine mAb (PY20), anti-FAK mAb, anti-phosphotyrosine polyclonal antisera, and anti-paxillin mAb were purchased from Transduction Laboratories (Lexington, KY). Anti-tensin antibodies were obtained both from Transduction Laboratories and Chemicon (Temecula, CA). JAK2 antisera was from Upstate Biotechnology (Lake Placid, NY). JAK1 antisera was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG and anti-rabbit IgG were obtained from DAKO (Arhus, Denmark). The enhanced chemiluminescence (ECL) kit was purchased from Amersham (Buckinghamshire, U. K.). Protein G, protein A-agarose, and all other reagents were purchased from Sigma. FAK-replete and FAK-deficient mouse embryo fibroblasts (MEF) were a generous gift of Dr. Dusko Ilic (18).

**Chinese Hamster Ovary (CHO) Cell Lines Stably Transfected with GH Receptor cDNAs**—Rat GH receptor cDNAs were cloned into an expression plasmid containing an SV40 enhancer and a human metallothionein IIA promoter. The cDNAs were transfected into CHO-K1 cells using Lipofectin together with the pIPB-1 plasmid, which contains a neomycin resistance gene fused to the thymidine kinase promoter (19). Stable integrants were selected using 1,000 μg/ml G418. The complete rat GH receptor cDNA (20) coding for amino acids 1–638 was expressed in CHO4–638 or CHO4-638 cells (19) and will be referred to as CHO-GHR1,638. Both cell clones transfected with the full-length receptor cDNA behaved identically for the experiments described here, although by affinity cross-linking the receptor in CHO4–638 cells has an M₅₀ of 84,000 compared with the expected M₅₀ of 120,000 as observed.
in CHO A638 cells (21). The construction of GH receptor cDNA expression plasmids containing a deletion of box 1 (∆297–311) and the individual substitution of proline residues 300, 301, 303, and 305 in box 1 for alanine has been described previously (22). These cDNAs were stably transfected into CHO-K1 cells; the ∆297–311 mutation was expressed in CHO-GHR, ∆297–311 cells, and P300A, P301A, P303A, P305A was expressed in CHO-GHR, ∆329–345 cells and position 455 (expressed in CHO-GHR, ∆455 cells) has also been described previously (19). The level of receptor expression for the individual cell lines is comparable between clones and has been described previously (19, 22).

Cell Culture—CHO cells stably transfected with rat GH receptor cDNA were maintained in Ham’s F-12 medium plus 10% v/v fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO2 and 95% air at 37 °C as described previously (19). MEF were grown in Dulbecco’s modified Eagle’s medium and supplemented as above.

Cell Stimulation and Immunoprecipitation—CHO cells were grown to confluence, incubated for 16 h in serum-free medium, washed twice in serum-free medium, and supplemented as above.

Western Blot Analysis—Nitrocellulose membranes were blocked with 5% nonfat dry milk (for phosphotyrosine immunoblotting) or by incubating with 5 μg of anti-mouse IgG for 1 h (polyclonal antiserum) or by incubating with 5 μg of mouse IgG for 1 h (monoclonal antibodies). Immunoblots were washed three times with ice-cold 3% saline. The pellet was resuspended in 2–4 μg/ml of the respective antibody for 2 h at 4 °C. Immunocomplexes were collected either by incubating with 50 μl of protein G-agarose for 1 h (polyclonal antiserum) or by incubating with 5 μg of anti-mouse IgG for 1 h followed by 1-h incubation with protein G-agarose (monoclonal antibodies). Immunoprecipitations were washed three times with ice-cold 1× saline.

Statistics and Presentation of Data—All numerical data are expressed as mean ± S.D. Data were analyzed using the two-tailed t test or analysis of variance.
again revealed GH-stimulated tyrosine phosphorylation of p125 and p215. Human GH-stimulated tyrosine phosphorylation was first observed at 0.005 nM hGH, and maximal tyrosine phosphorylation was observed at 0.05–0.5 nM hGH (Fig. 2C). Some diminution of the hGH response was observed at higher doses presumably concordant with the receptor dimerization theory of GH signal transduction (24). We continued to use 50 nM hGH for consistency of cellular responses.

FAK has been reported to be constitutively associated with paxillin, and paxillin is a major substrate for FAK both in vitro and in vivo (25, 26). To first determine if GH could stimulate the tyrosine phosphorylation of paxillin we treated CHO-GHR1–638 cells for 0–60 min with 50 nM hGH, prepared cell extracts, and immunoprecipitated tyrosine-phosphorylated proteins with PY20. SDS-PAGE and subsequent Western blotting for paxillin revealed a constitutive association between FAK and paxillin (66–68 kDa) which was not dependent upon hormonal stimulation (Fig. 4). In the above experiments hGH stimulated the tyrosine phosphorylation of a protein band of 215 kDa, observed in both FAK and paxillin immunoprecipitates. This protein is likely to be tensin because tensin is a 215-kDa protein substrate of FAK (27). To determine first if hGH could stimulate the tyrosine phosphorylation of tensin we treated CHO-GHR1–638 cells for 0–60 min with 50 nM hGH, prepared cell extracts, and immunoprecipitated tyrosine-phosphorylated proteins with PY20. SDS-PAGE and subsequent Western blotting for tensin revealed a time- and hGH-dependent increase in the level of tensin protein in the phosphotyrosine immunoprecipitates (Fig. 1B). Tensin protein was identified as a band of between 66 and 68 kDa. Some basal level of tensin tyrosine phosphorylation was observed in serum-deprived cells. The maximal hGH-stimulated tyrosine phosphorylation of tensin was observed between 10 and 30 min after
stimulation with the relative level of tensin tyrosine phosphorylation decreasing at 60 min. We next immunoprecipitated tensin from cell extracts of CHO-GHR1–638 cells stimulated for 0–60 min with 50 nm hGH and examined the level of tyrosine phosphorylation by Western blot analysis with PY20. We observed the presence of two time- and hGH-dependent tyrosine-phosphorylated bands of 125 and 215 kDa, respectively (Fig. 5A). The 125-kDa band co-migrated with FAK protein as expected, and the 215-kDa protein co-migrated with tensin protein. Paxillin could not be identified definitively because of its proximity to the IgG heavy chain. Increased hGH-dependent tyrosine phosphorylation of FAK was observed first at 5 min, was maximal 10 min after stimulation, and then declined to 60 min. We next proceeded to determine the association state between FAK and tensin. We therefore treated CHO-GHR1–638 cells for 0–60 min with 50 nm hGH, prepared cell extracts, and immunoprecipitated FAK from the cell extracts. Subsequent SDS-PAGE and Western blotting analysis for tensin revealed a time- and hGH-dependent association of tensin with FAK. Tensin was minimally associated with FAK in the unstimulated state, and maximal hGH-dependent association of tensin with FAK was observed between 10 and 30 min (Fig. 5C).

We proceeded to determine the GH receptor regions required for FAK activation. We therefore utilized well characterized CHO cell clones stably expressing the wild type receptor (CHO-GHR1–638), a receptor truncation containing only 5 of 349 amino acids in the intracellular domain (CHO-GHR1–294), a receptor truncation containing 184 of 349 amino acids in the intracellular domain (CHO-GHR1–454), a receptor mutation in which the proline-rich box 1 region had been deleted (CHO-GHR1–638P300,301,303,305A) (Fig. 6A). We therefore stimulated each cell line with 50 nm hGH, prepared cell extracts, and examined FAK tyrosine phosphorylation by Western blot analysis. Of these receptor mutations, only CHO-GHR1–638 cells were stimulated with 50 nm hGH for the indicated time periods, cell extracts prepared, and FAK protein immunoprecipitated as described under "Experimental Procedures." Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for paxillin protein. The loading control for FAK is shown in B. Paxillin is indicated as a broad protein band of 66–68 kDa. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments.

Fig. 3. Time and dose dependence of the hGH-stimulated tyrosine phosphorylation of paxillin and paxillin-associated proteins in CHO cells stably transfected with GH receptor cDNA (CHO-GHR1–638). A, CHO-GHR1–638 cells were stimulated with 50 nm hGH for the indicated time periods, cell extracts prepared, and paxillin protein immunoprecipitated (lanes 1, 2, 5, and 6) or a control antibody (c-jun) used (lanes 3 and 4) as described under "Experimental Procedures." Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for tyrosine phosphorylation with the use of PY20 (lanes 1–4) or paxillin protein (lanes 5 and 6). The arrowheads mark the positions of the tyrosine-phosphorylated band detected at 66–68 kDa and the IgG heavy chain. The position of the molecular mass standard is indicated on the right. The data are representative of at least three separate experiments. B, CHO-GHR1–638 cells were stimulated with 50 nm hGH for the indicated time periods, cell extracts prepared, and paxillin protein immunoprecipitated as described under "Experimental Procedures." Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for tyrosine phosphorylation with the use of PY20. The loading control for paxillin is shown in C. The arrowhead marks the position of the tyrosine-phosphorylated band detected at 66–68 kDa. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments. D, Western blot analysis performed similarly to B but run such that large molecular mass proteins can enter the SDS-PAGE running gel easily. Arrowheads mark the positions of the two major tyrosine-phosphorylated bands detected at 125 and 215 kDa. The loading control for paxillin is shown in E. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments. E, CHO-GHR1–638 cells were stimulated with the indicated concentrations of hGH for 10 min, cell extracts prepared, and paxillin-associated proteins immunoprecipitated as described under "Experimental Procedures." Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for tyrosine phosphorylation with the use of PY20. The loading control for paxillin is shown in G. Arrowheads mark the positions of the two major tyrosine-phosphorylated bands detected at 125 and 215 kDa. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments.
The immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for tyrosine phosphorylation with the use of PY20. The loading control for tensin is shown in Fig. 5. Arrowheads mark the positions of the two major tyrosine-phosphorylated bands detected at 125 and 215 kDa. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments.

CHO-GHR1–638 cells were stimulated with 50 nM hGH for 10 min, cell extracts prepared, and FAK protein immunoprecipitated as described under “Experimental Procedures.” Immunoprecipitated proteins were subjected to SDS-PAGE and Western blot analysis for tensin protein. The loading control for FAK is shown in Fig. 5. Arrowheads mark the positions of tensin at 215 kDa. The positions of the molecular mass standards are indicated on the right. The data are representative of at least three separate experiments.

GHR1–638 and CHO-GHR1–454 displayed a hormone-dependent activation of FAK tyrosine phosphorylation (Fig. 6B). Parental untransfected CHO cells also did not display hGH-dependent activation of FAK. The proline-rich box 1 region of the receptor is required for the association and activation of JAK1 and JAK2 by GH (3). This association is direct and is the initial event in GH signal transduction into the cell; no GH-dependent tyrosine phosphorylation in the cell is achieved with receptor mutations lacking box 1 (22 and data not shown). Thus it is apparent that the GH activation of FAK requires the proline-rich box 1 region of the GH receptor.

Because the box 1 region of the receptor is required for GH-dependent JAK association and activation, we examined a possible association between FAK and either JAK1 or JAK2. JAK2 is the major GH-activated member of the JAK family. We therefore treated CHO-GHR1–638 cells for 0–60 min with 50 nM hGH, prepared cell extracts, and immunoprecipitated FAK from the cell extracts. Subsequent SDS-PAGE and Western blotting analysis for JAK2 revealed a time- and hGH-dependent association of FAK with JAK2 (Fig. 6A). Association between FAK and JAK2 was apparent at 5 min, maximal at 10–30 min after hGH stimulation, and diminished 60 min after hGH stimulation. GH has also been demonstrated to activate JAK1, but the activation of JAK1 is minimal compared with the GH-stimulated activation of JAK2 (28, 29). No apparent association between JAK1 and FAK existed because JAK1 could not be detected in FAK immunoprecipitates despite the ability of the JAK1 antibody to recognize JAK1 by Western blot analysis in JAK1 immunoprecipitates of the cell lysates (data not shown). Conversely, we also showed the JAK2-FAK association by immunoprecipitation with JAK2 and immunoblotting with FAK (Fig. 7C).

GH utilizes the JAK-STAT pathway for transcriptional regulation. To determine if the GH-dependent tyrosine phosphorylation of FAK is required for GH-dependent STAT-mediated transcription we examined the transcriptional response in FAK-deficient and FAK-replete cells. For this we utilized MEF from normal and FAK knockout mice and measured the transcriptional response mediated by STAT1 and STAT3 (c-fos-SIE-CAT) and STAT5 (SPI-GLE1-CAT) by reporter assay. Pooled MEF did not possess a response to hGH either through c-fos-SIE-CAT or SPI-GLE1-CAT in the absence of transfected GH receptor cDNA (Fig. 8A). Concomitant transient transfection of rat GH receptor cDNA enabled a hGH-dependent STAT5-mediated response through SPI-GLE1-CAT to an extent similar to that observed in other cell lines. No significant hGH stimulation of reporter activity from c-fos-SIE-CAT was
observed in MEF even upon co-transfection of the GH receptor cDNA. Pooled MEF from FAK-deficient mice transiently transfected with GH receptor cDNA also responded to hGH stimulation with a response through SPI-GLE1-CAT similar to that observed with the wild type MEF. Such results were also observed with individual cell clones from FAK-replete and FAK-deficient mice (Fig. 8), indicative that FAK is not required for GH-stimulated STAT-mediated transcriptional activation. No significant response of c-fos-SIE-CAT to GH was obtained in MEF derived from FAK-deficient mice.

**DISCUSSION**

We have demonstrated that p125 FAK and at least two of its substrates are tyrosine-phosphorylated in response to cellular stimulation by GH. We have also placed FAK as a component of the JAK pathway and have therefore described a novel mechanism for the cross-talk between members of the cytokine receptor superfamily and signal transduction by the ECM.

We have provided a possible mechanism for the interaction of cytokine signaling pathways and those utilized by the ECM. Exactly how these two pathways interact is an interesting question. For example, expression of β-casein in mammary epithelial cells is regulated by lactogenic hormones (prolactin, GH) and the ECM (30). In the absence of the ECM, cells are flat and do not produce β-casein. Use of ECM results in cell rounding and clustering, which are apparently necessary for the cell to respond to prolactin. Both prolactin and GH regulate the expression of β-casein (31) and β-lactoglobulin through STAT5 (11), and STAT5 is required for the ECM dependence of β-lactoglobulin expression (11). In this regard it is interesting that FAK is not required for STAT5 activation as it may have provided the common link for hormone and ECM activation of certain transcriptional events such as those required for milk protein production. Further evidence that FAK or a FAK-related protein is not required for STAT5 activation is the ability...
of GH to activate STAT5 normally in the presence of cytochalasin B or cytochalasin D (13). Cytochalasins destroy the integrity of the actin cytoskeleton, which is required for FAK activation and function (32, 33).

The use of FAK by GH for signal transduction permits the GH signal to be propagated through multiple alternate transduction pathways. Tyrosine phosphorylation of the p85 subunit of PI-3 kinase is regulated by cell adhesion in vivo, and it can be phosphorylated by FAK in vitro (23) suggestive that PI-3 kinase may be a substrate of FAK in vivo. The association of FAK and PI-3 kinase is direct and dependent on FAK autophosphorylation (23, 34). GH has been demonstrated to promote the association of the p85 subunit of PI-3 kinase with IRS-1 (35, 36), IRS-2 (37), and JAK2 (38) and to increase the PI-3 kinase activity associated with IRS-1 (35). It is therefore possible that GH may utilize the FAK-PI-3 kinase pathway to increase phosphatidylinositol 3,4,5-trisphosphate levels within the cell as well as the IRS-PI-3 kinase pathway. Such potential utilization of two alternate pathways to activate the same kinase may permit the use of PI-3 kinase for distinct cellular purposes. For example, the activation of PI-3 kinase via IRS may be involved in GH stimulation of metabolic events such as lipogenesis (39), whereas activation of PI-3 kinase via FAK may regulate GH-stimulated cytoskeletal reorganization (13). Such alternate use of pathways would allow the cell to respond precisely to hormonal stimuli dependent on cell type and differentiation status. The use of cells specifically deficient in either IRS-1/IRS-2 and FAK will be instrumental in resolving these hypotheses. GH has also been demonstrated to stimulate mitogen-activated protein kinase activity (19, 40, 41) and the association of SHC and Grb2 with JAK2 (42, 43). JAK2 is required for GH stimulation of mitogen-activated protein kinase activity (22, 44) and also for Ras and Raf activation, which mediates GH activation of mitogen-activated protein kinase (44). Tyr295 of FAK is phosphorylated by c-Src and serves as a binding site for the Grb2-Sos complex both in vivo and in vitro (34). It is therefore possible that FAK is an upstream intermediary in the GH stimulation of the mitogen-activated protein kinase pathway. Similarly, platelet-derived growth factor stimulation of FAK has been reported to be PI-3 kinase-dependent (45), and therefore FAK may complex with JAK via Tec and PI-3 kinase (46). Studies using FAK-deficient cells stably transfected with GH receptor cDNA should be useful in delineating the role of FAK in the GH activation of various signal transduction pathways.

The GH-stimulated tyrosine phosphorylation of the actin-binding protein tensin (27) may provide a possible explanation for the observed effects of GH on the actin cytoskeleton (13). Actin filament polymerization involves exchange of subunits of filament ends, which can be controlled by other proteins that bind actin filaments and inhibit subunit addition or loss at the ends (47). Purified tensin cross-links actin filaments and reseals actin filament ends (47). Purified tensin cross-links actin filaments and reseals actin filament ends, which can be controlled by other proteins that bind actin filaments and inhibit subunit addition or loss at the ends (47).

REFERENCES
Growth Hormone Stimulates the Tyrosine Phosphorylation and Association of p125 Focal Adhesion Kinase (FAK) with JAK2: FAK IS NOT REQUIRED FOR STAT-MEDIATED TRANSCRIPTION
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