Biochemical and Biophysical Research Communications **263,** 100–106 (1999)
Article ID bbrc.1999.1314, available online at http://www.idealibrary.com on IDEAL®

Growth Hormone Stimulates Tyrosine Phosphorylation of Focal Adhesion Kinase ($p125^{FAK}$) and Actin Stress Fiber Formation in Human Osteoblast-like Cells, Saos2

Michiko Okazaki Takahashi, Yutaka Takahashi, Keiji Iida, Yasuhiko Okimura, Hidesuke Kaji, Hiromi Abe, and Kazuo Chihara
Third Division, Department of Medicine, Kobe University School of Medicine, Kobe 650-0017, Japan

Received August 4, 1999

Bone is one of the essential target tissues of growth hormone (GH). In bone remodeling, cell-matrix attachment is important where focal adhesion kinase (FAK) is involved. FAK plays a central role in determining the shape and motility of cells in response to the extracellular matrix stimuli. In the present study, we have demonstrated that GH stimulated tyrosine phosphorylation of FAK in human osteoblast-like cells, Saos2. Moreover, GH rapidly enhanced the formation of actin stress fibers. In Saos2, Jak2 was tyrosine phosphorylated by GH stimulation, and AG490, a Jak2 specific inhibitor, inhibited GH-induced tyrosine phosphorylation of FAK and actin stress fiber reorganization. These results suggest that GH activates FAK via Jak2, and stimulates the formation of actin stress fibers in Saos2. Activation of FAK and actin stress fiber formation induced by GH seem to be important for the physiological role of osteoblast. © 1999 Academic Press

Key Words: **focal adhesion kinase; growth hormone; actin stress fiber; janus kinase2; osteoblast-like cell.**

GH plays an important role in the regulation of longitudinal bone growth. Recently, many studies have demonstrated that GH is involved in bone remodeling (1). GH stimulates both bone formation and bone resorption (2, 3). In osteoblast, GH stimulates insulin-like growth factor-I (IGF-I) induction, cell proliferation and differentiation (4). GH increases DNA synthesis, alkaline phosphatase activity and enhances both type I procollagen mRNA expression and collagen synthesis in osteoblast cell, MC3T3-E1 cells (5). Expression of functional GH receptors in osteoblast (6) suggests a presence of direct action of GH.

Abbreviations used: GH, growth hormone; FAK, focal adhesion kinase; Jak, janus kinase; STAT, signal transducers and activators of transcription.

In bone remodeling, an attachment to the bone matrix of osteoblast is an essential step. Tyrosine phosphorylation of focal adhesion kinase (FAK) (7) and paxillin, the cytoskeletal associated proteins (8), has recently been identified as an early event in the action of diverse signaling molecules that affect cell attachment (9). FAK localizes in focal adhesions and is activated not only by β1 and β3 integrins (10, 11) but also by a variety of growth factors or hormones, such as bombesin, vasopressin and endothelin (12), as well as platelet-derived growth factor (13), vascular endothelial growth factor (14), prolactin (15) and GH (16). Although Zhu *et al.* demonstrated that in CHO cells in which GH receptor were expressed artificially, GH induced tyrosine phosphorylation of FAK, whether GH promote activation of FAK and reorganize actin stress fiber in physiological condition is unclear.

FAK-deficient mice are lethal and display severe defects of mesodermal development in embryogenesis (17), indicating that endogenous FAK plays an important physiological role in mesodermal development. Recently, it has been reported that type I collagen-$\alpha 2\beta 1$ integrin interaction facilitated cell differentiation and down-regulated the expression of transforming growth factor-β receptors via the activation of FAK and its diverse downstream signals in murine osteoblast (18). In osteoblastic differentiation, interaction of $\alpha 2\beta 1$ integrin on the cell surface with matrix collagen and that of integrin receptors with fibronectin play essential roles (19).

To investigate the role of GH in regulation of cell attachment in bone remodeling, we have examined the effect of GH on FAK and actin stress fiber in Saos2 cells, an established human osteosarcoma cell line with osteoblastic properties (20). In thisarticle, we demonstrate that GH stimulated tyrosine phosphorylation of FAK with concomitant increases in the length and number of actin stress fibers via Jak signaling pathway in Saos2 cells.

0006-291X/99 $30.00

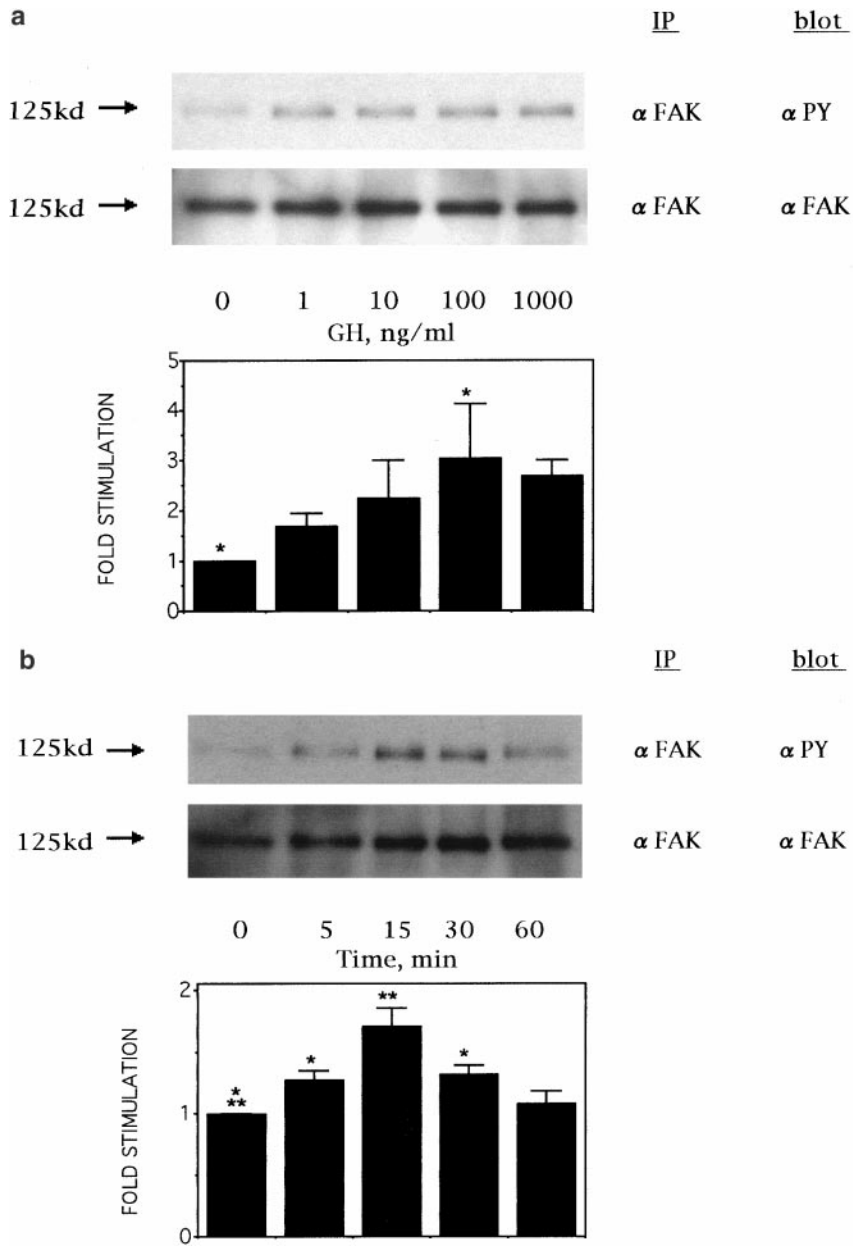


FIG. 1. Concentration dependence (a) and time course (b) of FAK tyrosine phosphorylation induced by GH in Saos2 cells. Cells were treated with the indicated concentrations of GH for 15 min (a) and with 100 ng/ml GH for indicated times (b) and lysed. Whole cell lysates were immunoprecipitated with polyclonal antiserum against FAK, respectively. Immunoprecipitates were analyzed by SDS–PAGE followed by transfer of proteins to PVDF membranes and anti-phosphotyrosine immunoblotting. The membrane was stripped and reblotted by anti-FAK antiserum, respectively. These results shown are the representatives of several independent experiments. Bar graphs are expressed as the ratio to the value of untreated Saos2 cells' tyrosine phosphorylation of FAK. Each bar represents the mean ± s.e. of three independent experiments (*$p < 0.05$, **$p < 0.005$).

MATERIALS AND METHODS

Reagents. Recombinant human GH was purchased from Eli Lilly Japan (Kobe Japan). FAK polyclonal antiserum (C-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Protein A agarose from Boehringer Mannheim (Mannheim, Germany). Anti phosphotyrosine monoclonal antibody (RC20) conjugated to horseradish peroxidase (HRP) were obtained from Transduction Laboratories (Lexington, KY), Anti-rabbit Ig, horseradish peroxidase linked antibody from Amersham Life Science (Buckingham Shire, England), and rhodamine-phalloidin from Molecular Probes (Eugene, OR). AG490 (Tyrphostin B42) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA) and Cytochalasin D from sigma Chemical Co. (St. Louis, MO).

Western blotting analysis. Saos2 cells were kindly provided by Dr. Masamichi Nasu (Kobe University) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

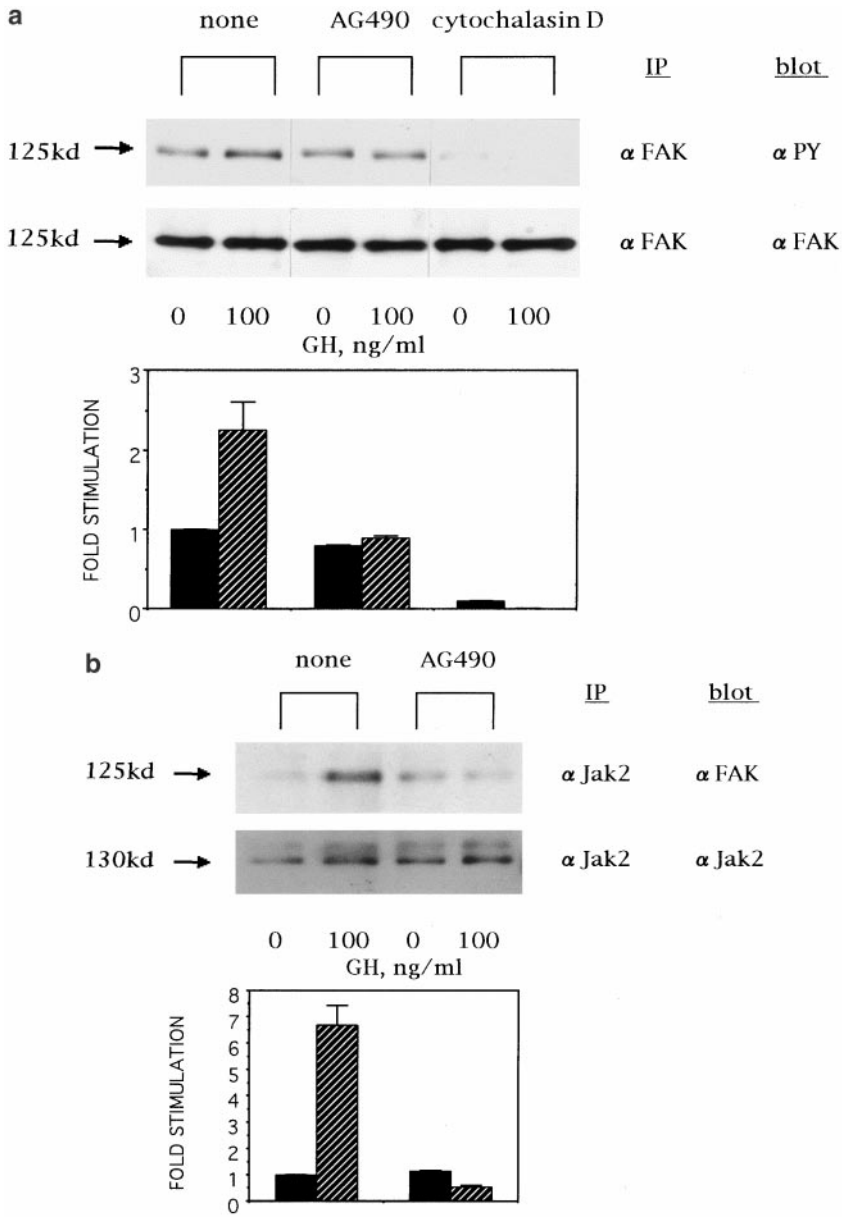


FIG. 2. Effect of AG490 and cytochalasin D on tyrosine phosphorylation of FAK induced by GH in Saos2 cells (a). Cells were pretreated in the presence of 1 μM cytochalasin D for 2 h or 10 μM AG490 for 16 h. Saos2 cells were stimulated for 15 min by 100 ng/ml GH. Effect of AG490 on GH-induced association of Jak2 and FAK in Saos2 cells (b). Bar graphs are expressed as the ratio to the value of untreated Saos2 cells' tyrosine phosphorylation of FAK (a), and association of Jak2 and FAK (b). Each bar represents the mean ± s.e. of three independent experiments.

fetal bovine serum in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. For experimental purposes, cells were plated either in 60 mm/tissue culture dishes at 6×10^5 cells/dish, or in 35 mm dishes at 1×10^5 cells/dish, in DMEM containing 10% fetal bovine serum and used when the cells were subconfluent. Whole cell extracts were prepared by detergent solubilization in 1 ml of lysis buffer (20 mM Tris–HCl, pH 7.4, 0.1% SDS, 1% Triton X, 1% Sodium deoxycholate, 0.5% Aprotinin, 1 mM PMSF, 1 mM Orthovanadate) for 1 h at room temperature. The cell extracts were clarified by centrifugation at 15,000*g* for 20 min and transferred to fresh tubes for immunoprecipitation. Immunoprecipitation was performed by incubating lysates with anti-FAK polyclonal antiserum and collected by incubating lysates with Protein A agarose beads. Immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis and transferred to the PVDF membrane. The membrane was analyzed by immunoblotting with the corresponding antibody.

Actin cytoskeleton staining. Cells grown on glass coverslips were washed with PBS and then fixed in 3.7% formaldehyde/PBS for 20 min at room temperature. Fixed cells were incubated with 200 μl of rhodamine-phalloidin (0.1 mg/ml) in a humidified chamber for 40 min at room temperature. After washing with PBS cells were mounted in 20% glycerol/PBS containing 1 mg/ml *p*-phenylenediamine. The actin reorganization of stress fibers or actin in membrane ruffles was observed under a fluorescence microscope (model Axiophot, Carl Zeiss, Oberkochen, Germany).

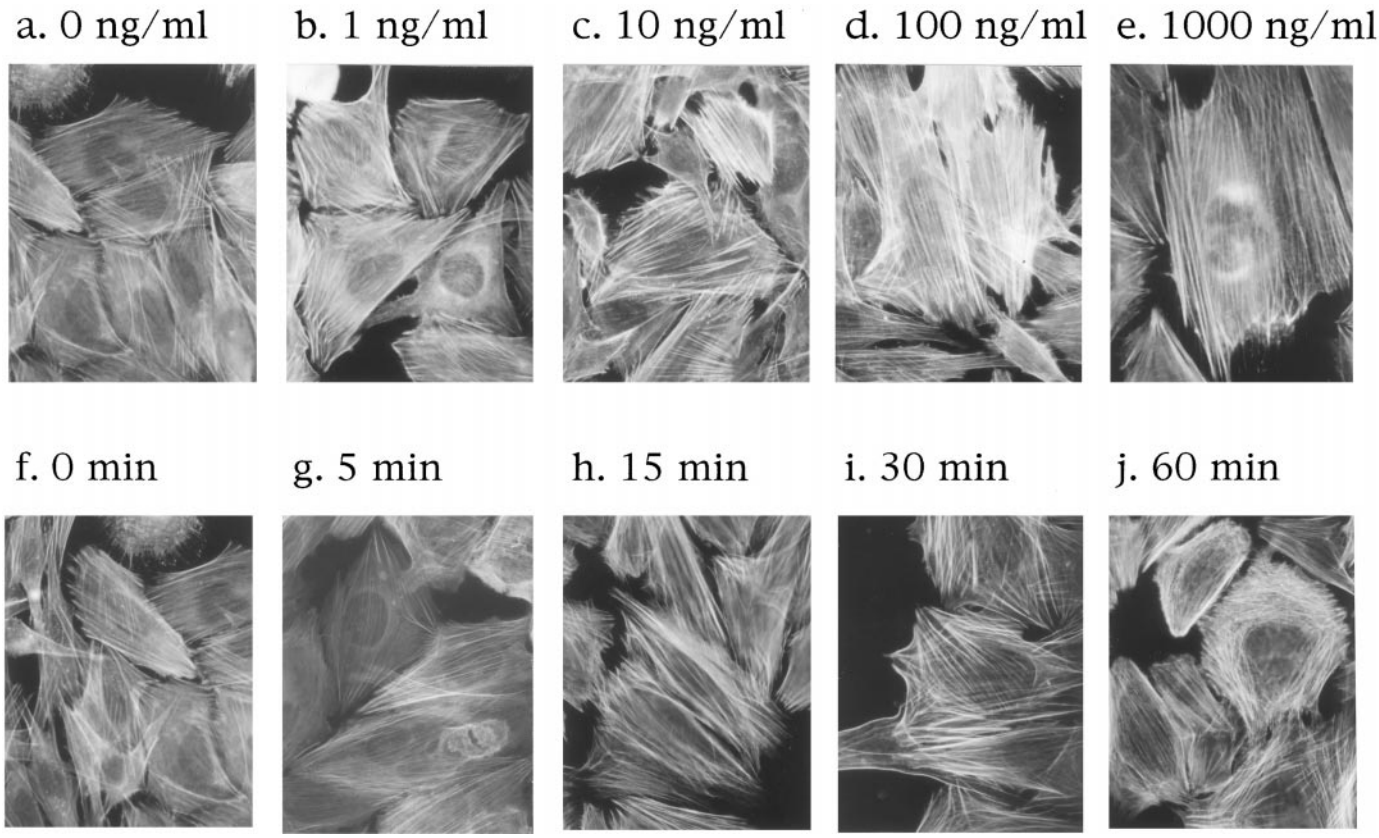


FIG. 3. Representative microphotographs of GH-induced reorganization of actin cytoskeleton in Saos2 cells. Actin stress fibers were visualized by rhodamine-phalloidin. Concentration dependence (a–e) and time course (f–j) of GH stimulation were shown.

RESULTS

GH-Induced Tyrosine Phosphorylation of FAK

We examined tyrosine phosphorylation of FAK following GH treatment of Saos2 cells (Figs. 1a and 1b). As shown in Fig. 1a, GH increased tyrosine phosphorylation of FAK in a concentration-dependent manner with the maximum effect at 100 ng/ml. The effect of GH on FAK tyrosine phosphorylation was also time-dependent with detectable increases at 5 min and the maximum effect at 15 min after GH stimulation (Fig. 1b).

Jak2 Was Involved in FAK Activation by GH

Jak2 plays an essential role in GH signal transduction. Then, we confirmed whether Jak2 is activated by GH in Saos2 cells.

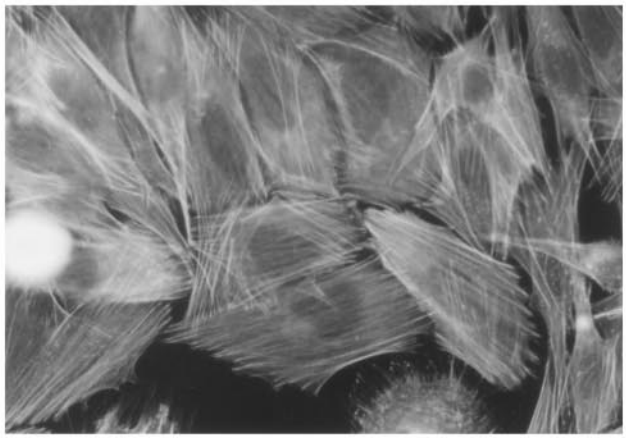
GH stimulated Jak2 tyrosine phosphorylation in the cells. Jak2 was tyrosine phosphorylated by GH with the maximum effect at 5 min in dose-dependent manner (data not shown). GH also caused the association of Jak2 with FAK (Fig. 2b) suggesting that FAK is directly activated by Jak2.

AG490, a Jak2 specific inhibitor (21, 22), inhibited GH-induced tyrosine phosphorylation of FAK (Fig. 2a). AG490 also inhibited GH-induced association of Jak2 and FAK (Fig. 2b). Cytochalasin D, a selective inhibitor of actin filament network formation, significantly diminished both basal- and GH-induced tyrosine phosphorylation of FAK (Fig. 2a).

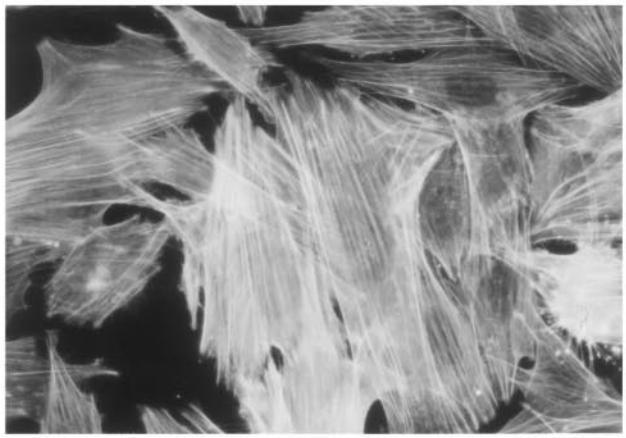
GH Stimulated Actin Stress Fiber Formation in Saos2 Cells

To investigate whether GH affects the structure of cytoskeleton, we examined the effect of GH on the formation of actin stress fibers in Saos2 cells, which were visualized with rhodamine-phalloidin staining. As shown in Figs. 3a–3e), quiescent Saos2 cells contained a few and fine actin stress fibers. After GH stimulation, numbers and length of actin stress fibers increased in a concentration-dependent manner with the maximum effect at 100 ng/ml. The effect of GH on actin stress fiber formation was also time-dependent with the maximum effect at 15–60 min (Figs. 3f–3j). The changes in actin stress fibers appeared to be comparable to the strength of tyrosine phosphorylation of FAK (Figs. 1a and 1b). We also examined the effects of inhibitors on actin stress fiber formations induced by GH. Cytochalasin D disrupted the basal actin fiber formation (Fig. 4c), and GH failed to induce any fiber

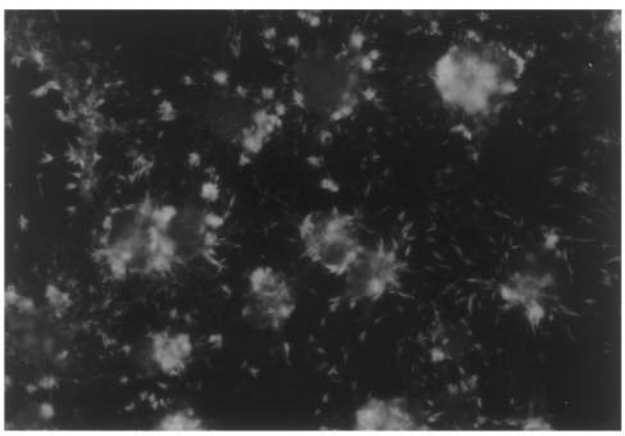
a. GH 0 ng/ml



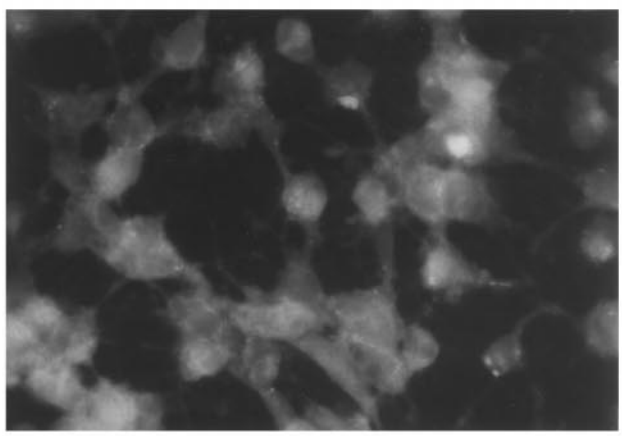
b. GH 100 ng/ml



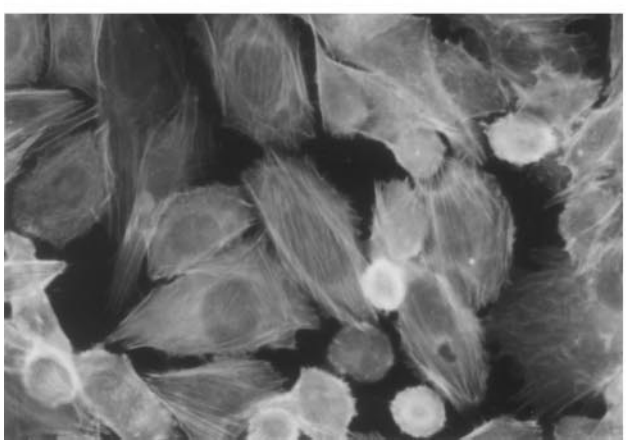
c. CD 1 μM + GH 0 ng/ml



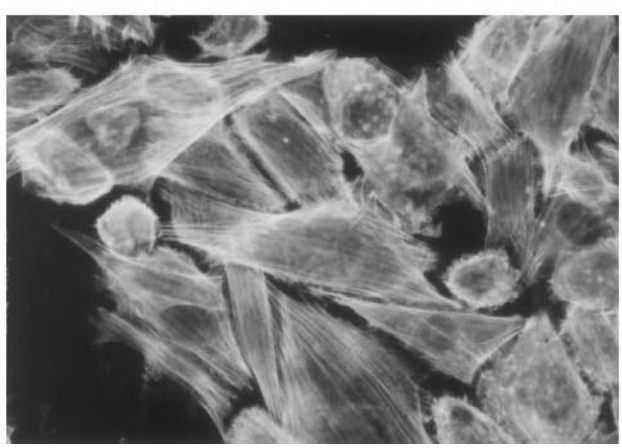
d. CD 1 μM + GH 100 ng/ml



e. AG490 10 μM + GH 0 ng/ml



f. AG490 10 μM + GH 100 ng/ml



formation (Fig. 4d). Compared with Fig. 4b, AG490 partially inhibited GH-induced actin stress fiber formation (Fig. 4f).

DISCUSSION

We have demonstrated here that GH induces tyrosine phosphorylation of FAK and subsequent reinforcement of actin stress fiber in human osteoblast-like cells, Saos2. Our findings are consistent with the results using stable transfected CHO cells with GH receptor cDNA, in which GH stimulates tyrosine phosphorylation and association of FAK with Jak2 (16) and enhances actin stress fiber formation (23). Besides, we have first demonstrated here that in a natural cell line, osteoblast-like cells, Saos2, in which GH plays a key role. GH induced an activation of FAK and subsequent actin stress fiber formation via an activation of Jak2. It is known that GH induces cell proliferation and differentiation in Saos2 (24). FAK induces cell proliferation (25, 26) and/or differentiation (18, 27) in several cell lines. In Saos2 cells, therefore, GH-induced activation of FAK might be involved in the cell proliferation and differentiation. As well, sequential formation of actin stress fiber induced by GH appears to be responsible for the functional property of osteoblast-like cells. GH exerts a strong stimulatory effect on osteoblast progenitor cell proliferation, cartilage differentiation, and extracellular mineralization, which supports the role of GH in *de novo* bone formation *in vitro* (28). The activation of FAK and actin stress fiber formation induced by GH is possibly involved in these effects.

The precise mechanism of activation of FAK by GH remains unclear. Cytochalasin D, causing selective disruption of actin microfilaments, completely inhibited GH-induced actin stress fiber formation and tyrosine phosphorylation of FAK, suggesting that the presence of actin microfilament is mandatory to FAK activation. AG490, a selective inhibitor of Jak2, inhibited GH-induced tyrosine phosphorylation of FAK and partially impaired the actin stress fiber formation. Moreover, it is demonstrated that Jak2 was associated with FAK by GH stimulation, it is plausible to consider that GH-induced FAK activation and subsequent actin stress fiber formation are at least in part dependent on the Jak2 activation.

It is demonstrated that a number of hormones such as parathyroid hormone (29), sex steroids (30), corticosteroids (31) and GH (16, 32) as well as growth factors like transforming growth factor-β, insulin-like growth factors (33) and morphogenetic proteins (34) play an important role in regulating the biochemical activities of osteoblast. However, little is known about the influence of extracellular matrix components on the function of osteoblast (35). Bone formation requires close contact with extracellular matrix (36). In calvarial osteoblast differentiation, interactions between integrin receptors and fibronectin are important. As a ligand, fibronectin activates FAK via integrin receptors (37). Type I collagen, which is secreted by osteoblasts onto bone surface, is the major structural component of bone. Thus osteoblasts are continuously adherent to a substratum of type I collagen. The adhesion is essential process for osteoblast differentiation (38). It has been reported that type I collagen-$\alpha 2\beta 1$ integrin interaction facilitates the differentiation of murine osteoblastic cells and down-regulates TGF-β receptors via activation of FAK and its diverse downstream signals. These signaling pathways may play an important role in the sequential differentiation of osteoblasts during bone formation (18, 19). Type I collagen has been shown to increase alkaline phosphatase activity of Saos2 cells (20). Cross talk between signaling from the bone matrix and various growth factor or cytokine including GH, might be important for osteoblast proliferation and differentiation.

Recently, it is demonstrated that Src-dependent tyrosine phosphorylation of proline-rich tyrosine kinase 2 (PYK2), a cytoplasmic kinase related to FAK, is involved in the adhesion-induced formation of the sealing zone, required for osteoclastic bone resorption (39). PYK2 is a member of the FAK family (40). In osteoblast, FAK might be important for physiological function.

In conclusion, we have demonstrated that in Saos2 cells, GH induced tyrosine phosphorylation of FAK and actin stress fiber formation via Jak2. These results indicated that FAK played an important role in down stream of Jak2 in GH signal transduction. Various action in bone remodeling including cell proliferation and differentiation by GH might be involved by FAK activation and actin fiber reorganization.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Toshimitsu Matsui, Dr. Tohru Murayama, and Dr. Kazuhiro Yagita for technical suggestions and Dr. Toshitsugu Sugimoto for valuable discussion. We are also grateful to Miss Chika Ogata for her excellent technical assistance. This study was supported in part by a grant-in-aid for scientific research (c) 09877199, 11770630 from the Japanese Ministry of Education, Science, Sports and Culture, and Growth Science Foundation in 1997 and 1998.

FIG. 4. Inhibition by cytochalasin D (CD) (c, d) and AG490 (e, f) of GH-induced reorganization of actin filament in Saos2 cells. Saos2 cells were grown on grass coverslips, deprived of serum and in the presence of 1μ]M cytochalasin D or 10 μM of AG490, then stimulated for the indicated concentrations of GH at 15 min.

REFERENCES

1. Ohlsson, C., Bengtsson, B. A., Isaksson, O. G., Andreassen, T. T., and Slootweg, M. C. (1998) *Endocr. Rev.* **19,** 55–79.
2. Parfitt, A. M. (1991) *Clin. Endocrinol.* **35,** 467–470.
3. Wuster, C. (1993) *Acta Endocrinol.* **128,** 14–18.
4. 4. Saggese, G., Federico, G., and Cinquanta, L. (1993) *Acta Paediatr.* **82,** 54–59.
5. Chihara, K., and Sugimoto, T. (1997) *Horm. Res.* **48,** 45–49.
6. Lehmann, E. D., Hopkins, K. D., Weissberger, A. J., Gosling, R. G., and Sonksen, P. H. (1993) *Lancet* **341,** 309.
7. Schaller, M. D., and Parsons, J. T. (1994) *Curr. Opin. Cell. Biol.* **6,** 705–710.
8. Tachibana, K., Sato, T., D'Avirro, N., and Morimoto, C. (1995) *J. Exp. Med.* **182,** 1089–1099.
9. Hitt, A. L., and Luna, E. J. (1994) *Curr. Opin. Cell. Biol.* **6,** 120–130.
10. Guan, J. L., and Shalloway, D. (1992) *Nature* **358,** 690–692.
11. Huang, M. M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (1993) *J. Cell Biol.* **122,** 473–483.
12. Sinnet-Smith, J., Zachary, I., Valverde, A., and Rozengurt, E. (1993) *J. Biol. Chem.* **268,** 14261–14268.
13. Rankin, S., and Rozengurt, E. (1994) *J. Biol. Chem.* **269,** 704–710.
14. Abedi, H., and Zachary, I. (1997) *J. Biol. Chem.* **272,** 15442–15451.
15. Canbay, E., Norman, M., Kilic, E., Goffin, V., and Zachary, I. (1997) *Biochem. J.* **324,** 231–236.
16. Zhu, T., Goh, E. L. K., and Lobie, P. E. (1998) *J. Biol. Chem.* **273,** 10682–10689.
17. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539–544.
18. Takeuchi, Y., Suzawa, M., Kikuchi, T., Nishida, E., Fujita, T., and Matsumoto, T. (1997) *J. Biol. Chem.* **272,** 29309–29316.
19. Takeuchi, Y., Nakayama, K., and Matsumoto, T. (1996) *J. Biol. Chem.* **271,** 3938–3944.
20. Rodan, S. B., Imai, Y., Thiede, M. A., Wesolowski, G., Thompson, *Cancer Res.* **47,** 4961–4966.
21. Chang, N. S., Joki, N., Mattison, J., Dinh, T., and John, S. (1997) *Cell Death Differ.* **4,** 779–786.
22. Meydan, N., Grunberger, T., Dadi, H., Shahar, M., Arpaia, E., Lapidot, Z., Leeder, J. S., Freedman, M., Cohen, A., Gazit, A., Levitzki, A., and Roifman, C. M. (1996) *Nature* **379,** 645–648.
23. Goh, E. L. K., Pircher, T. J., Wood, T. J. J., Norstedt, G., Graichen, R., and Lobie, P. E. (1997) *Endocrinology* **138,** 3207–3215.
24. Saggese, G., Federico, G., and Cinquanta, L. (1993) *Acta Paediatr.* **82,** 54–59.
25. Cary, L. A., Chang, J. F., and Guan, J. L. (1996) *J. Cell. Sci.* **109,** 1787–1794.
26. Guan, J. L. (1997) *Int. J. Biochem. Cell. Biol.* **29,** 1085–1096.
27. Tremblay, L., Hauck, W., Nguyen, L. T., Allard, P., Landry, F., Chapdelaine, A., and Chevalier, S. (1996) *Mol. Endocrinol.* **10,** 1010–1020.
28. Maor, G., Hochberg, Z., von der Mark, K., Heinegard, D., and Silbermann, M. (1989) *Endocrinology* **125,** 1239–1245.
29. Tam, V. K., Clemens, T. L., and Green, J. (1998) *Endocrinology* **139,** 3072–3080.
30. Purohit, A., Flanagan, A. M., and Reed, M. J. (1992) *Biochem. Biophys. Res. Commun.* **186,** 54–60.
31. Centrella, M., Rosen, V., Wozney, J. M., Casinghino, S. R., and McCarthy, T. L. (1997) *J. Cell. Biochem.* **67,** 528–540.
32. Kassem, M. (1997) *APMIS* **71,** 1–30.
33. Rydziel, S., Delany, A. M., and Canalis, E. (1997) *J. Cell. Biochem.* **67,** 176–183.
34. Yeh, L. C., Adamo, M. L., Olson, M. S., and Lee, J. C. (1997) *Endocrinology* **138,** 4181–4190.
35. Mackie, E. J., and Ramsey, S. (1996) *J. Cell. Sci.* **109,** 1597–1604.
36. Garcia, A. J., Ducheyne, P., and Boettiger, D. (1998) *J. Biomed. Mater. Res.* **40,** 48–56.
37. Akiyama, S. K. (1996) *Hum. Cell.* **9,** 181–186.
38. Masi, L., Franchi, A., Santucci, M., Danielli, D., Arganini, L., Giannone, V., Formigli, L., Benvenuti, S., Tanini, A., and Beghe, F. (1992) *Calcif. Tissue Int.* **51,** 202–212.
39. Duong, L. T., Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M., and Rodan, G. A. (1998) *J. Clin. Invest.* **102,** 881–892.
40. Diamond, G., Jones, D. E., and Bevins, C. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4596–4600.