(25). Another possibility is that increases in CRP concentrations are related to adipose tissue–derived cytokines (26,27). In this study, there was a strong correlation between BMI and hs-CRP concentration in addition to the correlation with diabetic duration and hs-CRP (data not shown). Schalkwijk et al. (17) and Frohlich et al. (27) also showed a positive correlation between BMI and hs-CRP concentration. However, the role of adipose tissue as a possible cause of the chronic inflammatory condition in youths with type 1 diabetes requires further investigation.

Only a few studies have evaluated the association between CRP concentration and development of carotid atherosclerosis in elderly subjects (14,28). A previous cross-sectional study described an association between CRP concentrations and severity of carotid atherosclerosis; however, the multivariate regression analysis failed to show a correlation between these two factors (14). Also, Folsom et al. (28) reported a weak association between hs-CRP concentration and carotid IMT. In these studies, the subjects were elderly individuals who often had several risk factors including obesity, hyperlipidemia, and hypertension. In our study, however, the subjects were young and did not have any of these risk factors, with the exception of hyperglycemia. Therefore, the slight but significant increase in hs-CRP concentration may affect the early stage of carotid atherosclerosis in these young subjects.

In this study, hs-CRP concentration was correlated with both mean and max IMT in patients with type 1 diabetes. In nondiabetic subjects, however, hs-CRP was correlated with max IMT but was not correlated with mean IMT. The mean IMT was calculated as the average of the thickest point (max IMT) and two adjacent points (located 1 cm away from the max IMT point). Therefore, a focal atheromatous change, such as a single fatty streak, would increase the max IMT but might not substantially increase the mean IMT.

This study clearly shows that elevated hs-CRP concentrations are correlated with the early stage of carotid atherosclerosis in young patients with diabetes. Therefore, low-grade inflammation may be a risk factor for the early stage of carotid atherosclerosis, especially in young patients with type 1 diabetes. To evaluate this possibility, prospective studies are required.

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References

1. Garcia MJ, McNamara PM, Gordon T, Kannel WB: Morbidity and mortality in diabetics in the Framingham population: sixteen year follow-up study. *Diabetes* 23: 105–111, 1974
2. Laing SP, Swerdlow AJ, Slater SD, Botha JL, Burden AC, Waugh NR, Smith AW, Hill RD, Bingley PJ, Patterson CC, Qiao Z, Keen H: The British Diabetic Association Cohort Study II: cause-specific mortality in patients with insulin-treated diabetes mellitus. *Diabet Med* 16:466–471, 1999
3. Pignoli P, Tremoli E, Poli A, Oreste P, Paoletti R: Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. *Circulation* 74: 1399–1406, 1986
4. Pignoli P, Longo T: Evaluation of atherosclerosis with B-mode ultrasound imaging. *J Nucl Med Allied Sci* 32:166–173, 1988
5. Hodis HN, Mack WJ, LaBree L, Selzer RH, Liu CR, Liu CH, Azen SP: The role of carotid arterial intima-media thickness in predicting clinical coronary events. *Ann Intern Med* 128:262–269, 1998
6. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr: Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults: Cardiovascular Health Study Collaborative Research Group. *N Engl J Med* 340: 14–22, 1999
7. Yamasaki Y, Kawamori R, Matsushima H, Nishizawa H, Kodama M, Kajimoto Y, Morishima T, Kamada T: Atherosclerosis in carotid artery of young IDDM patients monitored by ultrasound high-resolution B-mode imaging. *Diabetes* 43:634–639, 1994
8. Peppa-Patrikiou M, Scordili M, Antoniou A, Giannaki M, Dracopoulou M, Dacou-Voutetakis C: Carotid atherosclerosis in adolescents and young adults with IDDM: relation to urinary endothelin, albumin, free cortisol, and other factors. *Diabetes Care* 21:1004–1007, 1998
9. Frost D, Beischer W: Determinants of carotid artery wall thickening in young patients with type 1 diabetes mellitus. *Diabet Med* 15:851–857, 1998
10. Ross R: Atherosclerosis: an inflammatory disease. *N Engl J Med* 340:115–126, 1999
11. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH: Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 336:973–979, 1997
12. Koenig W, Sund M, Frohlich M, Fischer HG, Lowel H, Doring A, Hutchinson WL, Pepys MB: C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation* 99:237–242, 1999
13. Rifai N, Ridker PM: High-sensitivity C-reactive protein: a novel and promising marker of coronary heart disease. *Clin Chem* 47:403–411, 2001
14. Hashimoto H, Kitagawa K, Hougaku H, Shimizu Y, Sakaguchi M, Nagai Y, Iyama S, Yamanishi H, Matsumoto M, Hori M: C-reactive protein is an independent predictor of the rate of increase in early carotid atherosclerosis. *Circulation* 104:63–67, 2001
15. Pickup JC, Mattock MB, Chusney GD, Burt D: NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* 40: 1286–1292, 1997
16. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM: C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 286:327–334, 2001
17. Schalkwijk CG, Poland DC, van Dijk W, Kok A, Emeis JJ, Drager AM, Doni A, van Hinsbergh VW, Stehouwer CD: Plasma concentration of C-reactive protein is increased in type I diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation. *Diabetologia* 42:351–357, 1999
18. Kilpatrick ES, Keevil BG, Jagger C, Spooner RJ, Small M: Determinants of raised C-reactive protein concentration in type 1 diabetes. *Q J Med* 93:231–236, 2000
19. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499–502, 1972
20. Rifai N, Tracy RP, Ridker PM: Clinical efficacy of an automated high-sensitivity C-reactive protein assay. *Clin Chem* 45: 2136–2141, 1999
21. Kawamori R, Yamasaki Y, Matsushima H, Nishizawa H, Nao K, Hougaku H, Maeda H, Handa N, Matsumoto M, Kamada T: Prevalence of carotid atherosclerosis in diabetic patients: ultrasound high-resolution B-mode imaging on carotid arteries. *Diabetes Care* 15:1290–1294, 1992
22. Yamasaki Y, Kawamori R, Matsushima H, Nishizawa H, Kodama M, Kubota M, Ka-

jimoto Y, Kamada T: Asymptomatic hyperglycaemia is associated with increased intimal plus medial thickness of the carotid artery. *Diabetologia* 38:585–591, 1995
23. Kodama M, Yamasaki Y, Sakamoto K, Yoshioka R, Matsuhisa M, Kajimoto Y, Kosugi K, Ueda N, Hori M: Antiplatelet drugs attenuate progression of carotid intima-media thickness in subjects with type 2 diabetes. *Thromb Res* 97:239–245, 2000
24. Yamasaki Y, Kodama M, Nishizawa H, Sakamoto K, Matsuhisa M, Kajimoto Y, Kosugi K, Shimizu Y, Kawamori R, Hori M: Carotid intima-media thickness in Japanese type 2 diabetic subjects: predictors of progression and relationship with incident coronary heart disease. *Diabetes Care* 23:1310–1315, 2000
25. Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A: Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science* 240:1546–1548, 1988
26. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
27. Frohlich M, Imhof A, Berg G, Hutchinson WL, Pepys MB, Boeing H, Muche R, Brenner H, Koenig W: Association between C-reactive protein and features of the metabolic syndrome: a population-based study. *Diabetes Care* 23:1835–1839, 2000
28. Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y, Djousse L, Eckfeldt JH: Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol* 88:112–117, 2001

PDX-1 Induces Differentiation of Intestinal Epithelioid IEC-6 Into Insulin-Producing Cells

Shigeru Yoshida,[1] Yoshitaka Kajimoto,[1] Tetsuyuki Yasuda,[1] Hirotaka Watada,[1] Yoshio Fujitani,[1] Hiroshi Kosaka,[2] Takahiro Gotow,[3] Takeshi Miyatsuka,[1] Yutaka Umayahara,[1] Yoshimitsu Yamasaki,[1] and Masatsugu Hori[1]

A homeodomain containing transcription factor PDX-1 can induce β-cell–specific gene expressions in some non–β-cells and may therefore be useful for future diabetes gene/cell therapy. Among the potential target organs or tissues for transcription factor–mediated induction of β-cell–like differentiation are the intestinal epithelial cells. They have certain merits over other tissues and organs in terms of accessibility for gene delivery and of similarity in developmental background to the pancreatic primordium. In this study, we used an intestinal epithelium–derived cell line, IEC-6 cells, and investigated the possible effects of PDX-1 expression in those cells. By exogenous expression of the PDX-1 gene, IEC-6 cells started expressing multiple β-cell–specific genes such as amylin, glucokinase, and Nkx6.1, which were not found in the original IEC-6 cells. Insulin gene expression, which was missing initially even in the PDX-1–transfected IEC-6 cells, became detectable when the cells were transplanted under the renal capsule of a rat. When the PDX-1$^+$ IEC-6 cells were kept in vitro, treatment with betacellulin could also confer insulin gene expression to them. Although insulin secretory granules became visible by electron microscopy, they were secreted regardless of glucose concentration. The in vivo or in vitro inductions of the insulin gene expression were not observed in the PDX-1$^-$ IEC-6 cells. Thus, our present observations demonstrate the potency of intestinal epithelial cells as a tool for diabetes gene/cell therapy and provide further support for the potency of PDX-1 in driving β-cell–like differentiation in non–β-cells. *Diabetes* **51:2505–2513, 2002**

In recent years, attention has been focused on the possibility of gene or cell therapy of diabetes mellitus using artificially prepared non–β-cell–derived β-cells. Among the potentially useful factors for the induction of β-cell differentiation in non–β-cells is a homeodomain-containing transcription factor, PDX-1. PDX-1, which is also known as IDX-1/IPF-1/STF-1, was originally identified as an insulin gene regulating transcription factor but was later shown to have more extensive roles in regulating pancreas development and maintaining β-cell function (1–4). Gene disruption of PDX-1 inhibits the maturation and outgrowth of the pancreatic bud, resulting in lack of the whole pancreas (4). Also, when this disruption occurs in insulin-producing cells, maturation of the pancreatic β-cells is inhibited, suggesting an essential role for PDX-1 in early and late pancreas development (4,5).

In agreement with the phenotypes of the knockout animals, recent gain-of-function experiments have revealed the potential of PDX-1 in conferring some β-cell–like features to non–β-cells. We and others have recently shown that the exogenous expression of PDX-1 in two lines of glucagon-producing cells can induce the expression of β-cell–specific genes, including the insulin gene, although one cell line required treatment with betacellulin to activate the insulin gene (6,7). Also, more recently, Ferber et al. (8) showed that PDX-1 can endow some cells in the liver with pancreatic β-cell characteristics in vivo using recombinant adenovirus-mediated gene delivery. These data have highlighted the potential usefulness of PDX-1 as a reprogramming factor of non–β-cells toward β-cell–like cells that can be used in diabetes cell/gene therapy.

With relatively good accessibility in terms of gene delivery, the small intestinal epithelium has been considered as a potential target of gene/cell therapy in general (9). This is especially true for the case of diabetes gene/cell therapy, because the intestinal epithelium and pancreatic β-cells have similar developmental backgrounds: after formation of the primitive gut tube, most epithelial cells in the gut tube give rise to the gastrointestinal tract, including the small intestine, and restricted groups of cells located in the posterior foregut give rise to ventral and dorsal pancreatic buds (10,11). Considering the importance of the network formed by multiple transcription factors in β-cell differentiation, the similarity in developmental background is likely to contribute favorably to successful induction of β-cell–like differentiation.

Another positive factor is that small intestinal epithelial cells are composed of both differentiated and undifferentiated cell populations. The latter cells, which are present in the crypt region, are known to serve as stem cells and differentiate into intestinal epithelium cells and endocrine

From the [1]Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan; the [2]Department of Dermatology, Osaka University Graduate School of Medicine, Suita, Japan; and the [3]College of Nutrition, Kohshien University, Takarazuka, Japan.

Address correspondence and reprint requests to Yoshitaka Kajimoto, Department of Internal Medicine and Therapeutics, A8, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. E-mail: kajimoto@medone.med.osaka-u.ac.jp.

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H.W. is currently affiliated with the Department of Medicine, Metabolism and Endocrinology, School of Medicine, Juntendo University, Tokyo, Japan.

ABC, avidin-biotin complex; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; KRH, Krebs-Ringer-HEPES; TGF, transforming growth factor.

cells. Even in the adult intestine, stem cells should remain active and supply enough cells to compensate for a very rapid turnover of intestinal epithelium cells with a mean cell duration time of 2–3 days (12,13). Because some organ-specific stem cells have been shown to retain a transdifferentiation potential, the presence of such stem cells in the intestine makes it a more promising target for diabetes gene/cell therapy (9).

In this study, we show that intestinal crypt-like IEC-6 cells (14) have the potency to induce the expression of multiple β-cell–specific genes such as amylin/islet amyloid polypeptide and glucokinase in response to exogenously introduced PDX-1. Furthermore, the insulin gene expression was switched on in the PDX-1–expressing IEC-6 cells when they were kept under the renal capsule, where the pancreatic bud epithelium can differentiate into mature endocrine cells (15). It is interesting that betacellulin treatment in vitro could mimic the effect of under-renal-capsule transplantation. Together with earlier reports, our results demonstrate the potency of PDX-1 as a β-cell differentiation factor and support the potential usefulness of intestinal epithelial cells for generating artificial β-cells.

RESEARCH DESIGN AND METHODS

Cell culture. The rat intestinal epithelioid cell line, IEC-6 cells, was obtained from Riken Gene Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) heat-inactivated FCS, 1% penicillin, and 1% streptomycin. The rat insulinoma cell line RINm5F (a gift from Dr. K. Yokono, Kobe University, Kobe, Japan) was grown in RPMI-1640 containing 10% (vol/vol) FCS and 1% penicillin-streptomycin. The mouse primitive endoderm-resembling cell line JC44 (16) (a gift from Dr. B.A. Fenderson, Thomas Jefferson University, Philadelphia, PA) was cultured in the same medium as RINm5F. The mouse yolk sac endoderm-derived cell line YSE (17) (a gift from Dr. M.C. Yoder, Indiana University, Bloomington, IN) was maintained in the same medium as IEC-6 cells. MIN6 cells were cultured in DMEM supplemented with 25 mmol/l glucose, 50 mg/l penicillin, 50 mg/l streptomycin, and 15% heat-inactivated FCS.

For examination of the effects of various growth factors, cells were cultured on a Lab-Tek chamber slide (Nalge Nunc International, Rochester, NY) for 4 days in a nonserum medium (DMEM containing 5 μg/ml transferrin, 20 nmol/l progesterone, and 30 mmol/l selenious acid) and 2 nmol/l transforming growth factor (TGF)-α, epidermal growth factor (EGF) (Wakunaga Pharmaceutical, Hiroshima, Japan), IGF-1 (a gift from Fujisawa Pharmaceutical, Osaka, Japan), or betacellulin (a gift from Dr. Reiko Sasada, Takeda Pharmaceutical, Tsukuba, Japan).

For measurement of insulin secretion in response to glucose, arginine, or calcium ionophore A23187 (Sigma, St. Louis, MO), 1×10^5 cells were plated in a six-well plate and kept for 2 days with (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) or without (PDX-1$^+$ IEC-6 and MIN6 cells) 1 nmol/l betacellulin. After washing twice with Krebs-Ringer-HEPES (KRH) containing 128 mmol/l NaCl, 5 mmol/l KCl, 2.7 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l Na_2HPO_4, 20 mmol/l HEPES, and 2.8 mmol/l glucose and 60-min preincubation with the same KRH medium, the insulin secretion was measured by incubation at 37°C for 30 min (MIN6 cells) and 120 min (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) in medium with appropriate secretagogues. After the incubation, supernatant was collected and used for enzyme-linked immunosorbent assay (ELISA) for insulin as described below.

Preparation of stable transfectants. For the generation of stable transfectants, IEC-6 cells were replated in 10-cm plates 24 h before transfection. Ten micrograms of the PDX-1 expression plasmid carrying neomycin phosphotransferase gene, pcDNA3-IPF1 (18), was transfected into cells by a lipofection method using lipofectAMINE reagent (Life Technologies, Tokyo, Japan) under the conditions recommended by the manufacturer. Forty-eight hours after transfection, the cells were transferred to 600 μg/ml G418-containing medium (Sigma). G418-resistant colonies appeared ~4 weeks after transfection, and the resistant clones were subcloned and used for additional analyses.

Northern blot analyses. Northern blot analyses followed a standard protocol using 10 μg of total RNA (18). A mouse PDX-1 cDNA (nucleotide 772-1134) was used as a probe.

Preparation of nuclear extracts, Western blot analyses, and gel mobility shift analyses. Nuclear extracts were prepared following the procedure described by Sadowski and Gilman (19). Western blot analyses were performed as described previously using PDX-1 antibody (7).

Gel-shift analyses were performed as described previously using human insulin A3 element as a probe (7). The sequence of the sense strand of the probe was 5′-CCCCTGGTTAAGACTCTAATGACCCGCTGG-3′. In some of the binding assays, 5 μl of preimmune serum or anti–PDX-1 antiserum (18) was added to the 20-μl binding mixture 30 min before the addition of the DNA probes.

RT-PCR. Total RNA extraction and subsequent RT-PCR were performed as described previously (20). One microgram of total RNA was loaded for RT, and 1 μl of the reaction mixture was used for subsequent PCR. The sequence of primers used for PCR of β-actin, insulin, glucokinase, GLUT2, and islet amyloid polypeptide (amylin) mRNAs was described elsewhere (20). For verification of the results for insulin and GLUT2, other sets of primers that were used by Jensen et al. (21) were also used. The primers for somatostatin, Isl1, NeuroD, and Nkx6.1 mRNAs were also as described by Jensen et al. (21). Those for HNF-3β mRNA were as described by Duncan et al. (22), and those for Kir6.2 mRNA were described by Wulfsen et al. (23). The PCR conditions were denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The number of PCR cycles for β-actin cDNA amplifications was 25; that for HNF-3β, amylin, somatostatin, Isl1, and NeuroD was 33; and that for Kir6.2, insulin, glucokinase, and Nkx6.1 was 35. The PCR products were separated on an agarose gel and visualized with ethidium bromide.

Under-renal-capsule transplantation. PDX-1–expressing IEC-6 cells were suspended in prewarmed DMEM medium containing 0.6% agarose and 10% FCS. Droplets (10 μl, 20–30 cells) of the cell suspension solution were placed on a Petri dish (ϕ 60 mm) and allowed to solidify. Liquid medium (DMEM, 10% FCS) was added, and the cells were cultured for 1 week to form aggregates. To calculate cell numbers at this point, we heated a Petri dish containing 5–10 droplets to 42°C to melt the agarose, which was then treated with 0.05% trypsin and 0.02% EDTA, then subjected to cell counting. This revealed that each cell aggregate contained 51 cells on average after 1-week culture in vitro, allowing us, in turn, to estimate the total cell numbers contained in an agarose droplet (1,000–1,400 cells per droplet). Male Sprague-Dawley rats (20–26 weeks old) were anesthetized and subjected to under-renal-capsule transplantation. Through a small midline incision in the abdomen, both kidneys were exposed and a small tear was made in the renal capsule. Five gel droplets containing cell aggregates were inserted and placed on the bare area of a kidney. After 2 or 8 weeks, the rats were killed to harvest the kidney and pancreas for subsequent investigation.

Immunohistochemistry. For the staining of cultured cells, the cells were placed on a Lab-Tek chamber slide and cultured for 3 days in appropriate medium. After rinsing with PBS three times, the cells were fixed with acetone/methanol. For staining of the harvested kidney, the tissues were fixed overnight in 4% paraformaldehyde. Fixed tissues were processed routinely for paraffin embedding, and ~5-μm sections were prepared and mounted on slides. Each slide was treated with the H_2O_2 solution to inactivate endogenous peroxidase. Detection of insulin and PDX-1 was done by the avidin-biotin complex (ABC) method using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). After rehydration and blocking, the mounted sections were incubated for 30 min with guinea pig polyclonal anti-insulin antibody (Dako, Glostrup, Denmark) diluted 1:3,000 or PDX-1 antiserum (7) diluted 1:5,000 in PBS containing 1% bovine serum albumin. They were then incubated for 30 min with biotinylated anti–guinea pig IgG (Vector Laboratories) diluted 1:200 or biotinylated anti-rabbit IgG. The sections were then incubated with ABC reagent for 30 min, and positive reactions were visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride substrate (Zymed Laboratories, San Francisco, CA).

ELISA for insulin. Insulin was extracted from the kidney or pancreas as described elsewhere (24). Insulin concentration was measured using a Lebis insulin ELISA kit (Shibayagi, Gunma, Japan).

Electron microscopic examination for PDX-1$^+$ IEC-6 cells. The cells grown on the culture dish were fixed with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h. After the cells were rinsed with the phosphate buffer, they were postfixed with 2% OsO_4 for 2 h at 4°C, rinsed with distilled water, and stained en bloc with 1% uranyl acetate for 1 h. The cells were then dehydrated in graded concentrations of ethanol and embedded in Epok 812 (Okensyoji, Tokyo, Japan). Thin sections were cut, stained with both 4% uranyl acetate and lead citrate, and examined in an electron microscope (H-300; Hitachi Seisakusyo, Hitachi, Japan).

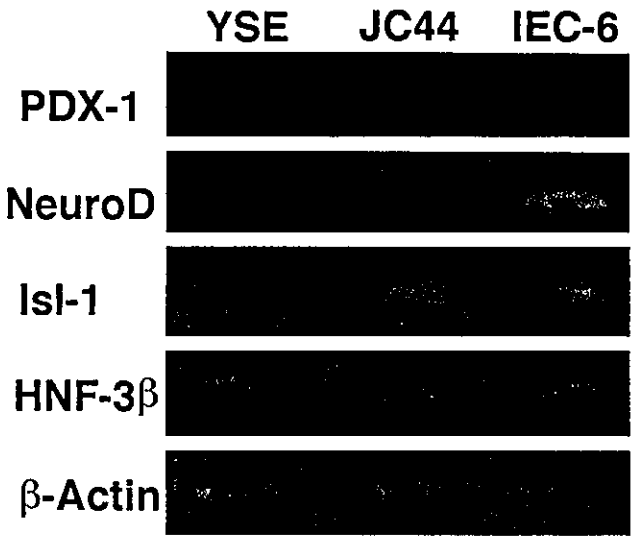

FIG. 1. Expression of islet cell–related transcription factors in IEC-6 cells. Total RNA was isolated from the rat intestinal epithelioid cell line IEC-6, primitive endoderm-resembling cell line JC44, and mouse yolk sac endoderm-derived cell line YSE and used for RT-PCR for the detection of PDX-1, NeuroD, Isl1, and HNF-3β mRNA. β-Actin mRNA was also amplified as a positive control. The sequence information of the primers used for RT and PCR is described in RESEARCH DESIGN AND METHODS. Similar results were obtained in at least three independent experiments.

RESULTS

Preparation of IEC-6 clones stably expressing PDX-1. To evaluate the potential of intestinal crypt cell–like IEC cells to differentiate to β-cells, we first examined the expressions of islet cell–related transcription factors in the cells. As shown in Fig. 1, RT-PCR analyses revealed that IEC-6 cells express HNF-3β (25), Isl1 (26), and NeuroD (27) but lack PDX-1 and Nkx6.1 (28). In contrast, yolk sac endoderm-derived YSE cells (17) and primitive endoderm-derived JC44 cells (16) did not show a comparable expression pattern (Fig. 1). Thus, the expression pattern of IEC-6 cells is consistent with the previous observation obtained with murine intestinal tissues (29) and retains some similarity to pancreatic β-cells.

IEC-6 cells were transfected with PDX-1 expression plasmid, and stable transformants were selected according to G418 resistance. In total, 71 independent clones were obtained, and 28 arbitrarily chosen clones were subjected to Northern blot analyses. As a result, 18 PDX-1–positive clones and 10 PDX-1–negative clones were identified (data not shown). Among them, we randomly chose three positive (PDX-1$^+$ IEC-6; clones A, B, and C) and three negative (PDX-1$^-$ IEC-6; clones D, E, and F) and used them for additional experiments.

As shown in Fig. 2*A*, PDX-1$^+$ IEC-6 cells expressed a 1.8 kb–long PDX-1 mRNA derived from the exogenously introduced gene. Although it was less abundant than the 2.3-kb PDX-1 mRNA originally expressed in β-cell–derived βTC1 cells, the protein amounts of PDX-1 were comparable to those expressed in βTC1 cells (Fig. 2*B*). Immunocytochemical staining with anti–PDX-1 antibody showed that PDX-1 is localized exclusively in the nuclei in PDX-1$^+$ IEC-6 cells (Fig. 2*C*). This observation was confirmed by Western blot analyses using isolated cytosolic fractions (data not shown). Gel mobility shift assays using the A3 region of the insulin gene promoter as a probe identified a specifically binding protein in the nuclear extract of the PDX-1$^+$ IEC-6 cells, and the DNA-protein complex was recognized by a PDX-1 antibody (Fig. 2*D*). In agreement with the similarity in the amounts of PDX-1 protein in the cells, the intensity of the retarded bands was almost comparable to that for βTC1 cells (Fig. 2*D*, *lanes 3* and *7*). These results thus suggested that the exogenously expressed PDX-1 functioned properly in IEC-6 cells in terms of its protein amount, nuclear localization, and DNA binding activity.

Induction of amylin, glucokinase, and Nkx6.1 expressions in PDX-1–positive IEC-6 cells. To investigate the changes of gene expression patterns caused by the exogenous PDX-1 expression in IEC-6 cells, we performed RT-PCR analyses. As presented in Fig. 1, Isl1, NeuroD, and HNF-3β mRNA were detectable even in parental IEC-6 cells and also were expressed in PDX-1$^+$ IEC-6 cells (Fig. 3). In contrast, there was no mRNA for β-cell–specific genes such as insulin, amylin, and glucokinase expressed in parental IEC-6 cells. However, after stable transfection of PDX-1–expressing plasmids, they started expressing amylin, glucokinase, and Nkx6.1 cells (Fig. 3). None of the three clones of PDX-1$^-$ IEC-6 (clones D, E, and F) expressed any of those three genes, suggesting that the phenomenon depends specifically on the introduction of PDX-1 into the cells rather than a nonspecific spontaneous conversion of the cell character during gene manipulation and culturing of the cells in vitro. The gene expressions for insulin (Fig. 3), GLUT2, somatostatin, and Kir6.2 (data not shown) remained undetectable even in PDX-1$^+$ IEC-6 cells.

Induction of insulin production in PDX-1–expressing IEC-6 cells kept under the renal capsule. During embryonic development, the expression of PDX-1 is initiated in the epithelium of the foregut shortly before the evagination of the pancreatic bud and is maintained in the epithelium of the pancreatic bud during early pancreatic development. The PDX-1–expressing pancreatic bud epithelium is known to form islet-like clusters of endocrine cells when separated from the mesenchymal tissue and transplanted under a renal capsule (15). Considering the similarity between the pancreatic bud epithelium and PDX-1–expressing IEC-6 cells, we investigated the possibility of under-renal-capsule transplantation facilitating differentiation toward the endocrine pancreas in PDX-1$^+$ IEC-6 cells.

Figure 4 illustrates the results of immunostaining of a rat kidney into which either PDX-1$^+$ IEC-6 cells or PDX-1$^-$ IEC-6 cells had been transplanted 2 weeks earlier. Both PDX-1–expressing and –nonexpressing IEC-6 cells remained alive, but the cell aggregates did not show any evident increase in volume, suggesting hardly any cell growth under the renal capsule. At 8 weeks after transplantation, the cell aggregates had decreased in mass, indicating enhanced cell death.

Even after transplantation, the PDX-1 expression remained absent in PDX-1$^-$ IEC-6 cells, whereas PDX-1 remained in the nuclei in PDX-1$^+$ IEC-6 cells, suggesting that the under-renal-capsule transplantation did not affect the expression or localization of PDX-1 in IEC-6 cells. It is

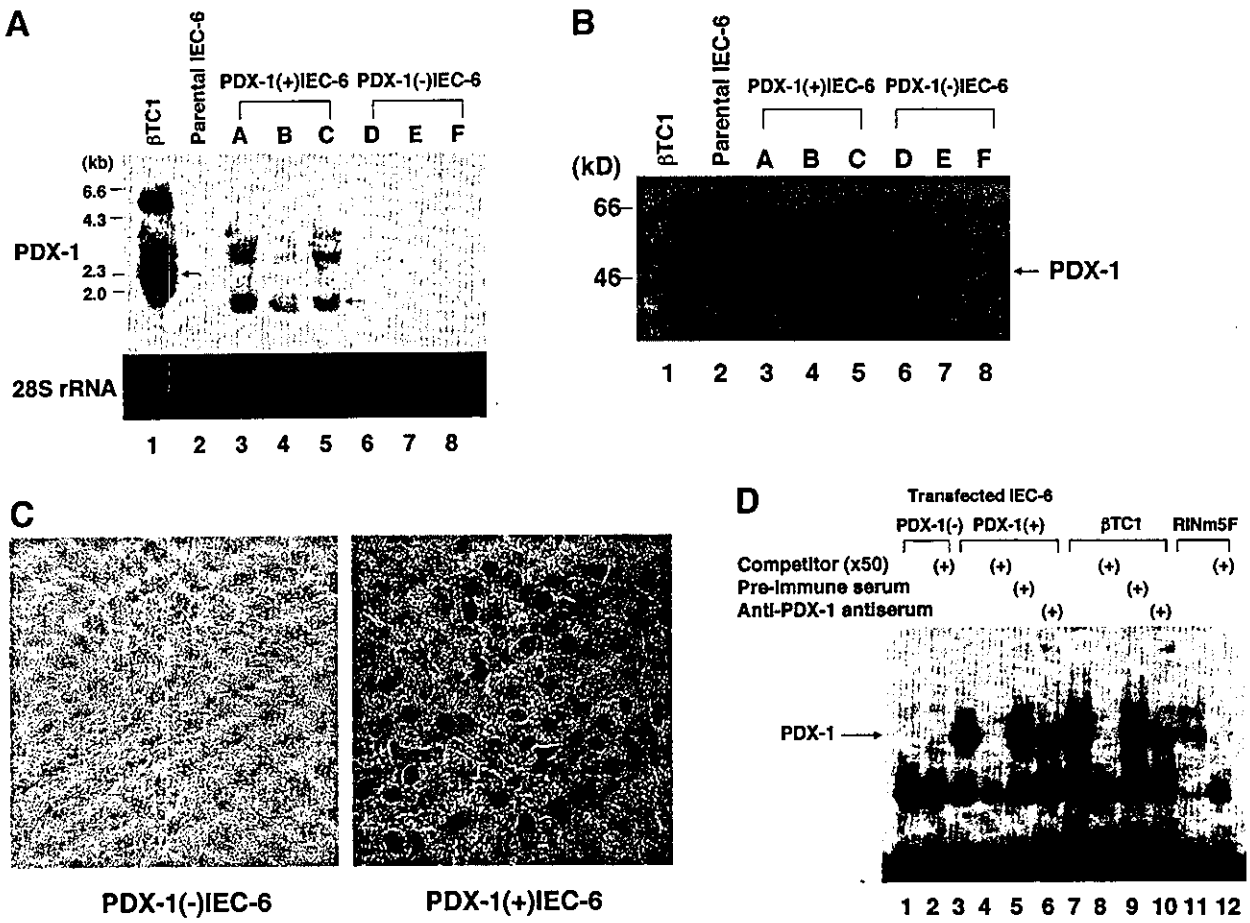

FIG. 2. Expression and function of PDX-1 in stably transfected IEC-6 cells. Three PDX-1$^+$ IEC-6 clones (A, B, and C) and three PDX-1$^-$ IEC-6 clones (D, E, and F) were randomly chosen after an initial screening by Northern blot analyses (data not shown) and subjected to detailed evaluation of PDX-1 expression and function. *A*: Northern blot analysis was performed using 10 μg of total RNA isolated from βTC1 cell (*lane 1*), parental IEC-6 cells (*lane 2*), three independent PDX-1$^+$ IEC-6 clones (clone A, *lane 3*; clone B, *lane 4*; clone C, *lane 5*), and three independent PDX-1$^-$ IEC-6 clones (clone D, *lane 6*; clone E, *lane 7*; clone F, *lane 8*). A PDX-1 cDNA was used as a probe. *B*: Western blot analysis used the same set of cells after isolation of 5 μg of nuclear extracts. The antibody used was an anti–PDX-1 antibody (7). *C*: Immunocytochemical staining for PDX-1 was performed to determine the intracellular localization of exogenously introduced PDX-1. Left and right panels depict PDX-1$^-$ IEC-6 (clone D) and PDX-1$^+$ IEC-6 (clone A) cells, respectively, which were allowed to react with anti–PDX-1 antibody. Similar results were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and PDX-1$^+$ IEC-6 (clones B and C) cells. *D*: Gel mobility shift analysis was performed using nuclear extracts isolated from PDX-1$^-$ IEC-6 cells (clone D; *lanes 1* and *2*) and PDX-1$^+$ IEC-6 cells (clone A; *lanes 3–6*), βTC1 cells (*lanes 7–10*), and RINm5F (*lanes 11* and *12*). The human insulin A3 element was used as a binding probe. Where indicated, 100-fold unlabeled double-strand DNA (*lanes 4* and *8*), 2 μg of preimmune serum (*lanes 5* and *9*), or 2 μg of anti–PDX-1 antiserum (*lanes 6* and *10*) were added to the binding mixtures. Similar results to clone D and to clone A were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and of PDX-1$^+$ IEC-6 cells (clones B and C), respectively.

interesting that intense staining for insulin was observed in >50% of the transplanted PDX-1$^+$ IEC-6 cells (Fig. 4). In contrast, no insulin-expressing cells were found in the transplanted PDX-1$^-$ IEC-6 cells. To estimate the amount of insulin expressed in the transplanted cells, we measured the insulin amount in a whole kidney into which ~5,000–7,000 cells were transplanted. In agreement with the absence of insulin staining, the transplantation of PDX-1$^-$ IEC-6 cells did not increase the insulin content of a kidney (Table 1). In contrast, the kidney that received transplanted PDX-1$^+$ IEC-6 cells revealed a significant increase in the insulin content. These results suggest that the under-renal-capsule transplantation or other treatment that can mimic it may be effective for enhancing β-cell–like function in PDX-1–transfected intestinal epithelioid cells.

Induction of insulin production in PDX-1–expressing IEC-6 cells by betacellulin. The possible involvement of various growth factors in pancreatic differentiation and regeneration has been suggested (30,31). This may also be the case with the differentiation of PDX-1–expressing IEC-6 cells because the space under the renal capsule, where PDX-1$^+$ IEC-6 cells could become insulin-positive, is known to be rich in growth factors (32). Accordingly, we examined the effects of growth factors on the enhancement of β-cell–like differentiation in IEC-6 cells.

As shown in Figs. 5*b* and 6*A*, the addition of betacellulin to the medium induced insulin production in PDX-1$^+$ IEC-6 cells. This induction was not observed when betacellulin was added to PDX-1$^-$ IEC-6 cells (Fig. 5*f*) or parental IEC-6 cells (data not shown). Other growth factors, such as TGF-α, EGF, and IGF-1, had no effect on the gene expression in both PDX-1$^+$ IEC-6 (Fig. 5*c–e*) and PDX-1$^-$ IEC-6 cells. According to the results of Western blot analyses, the cytosolic insulin content of the betacel-

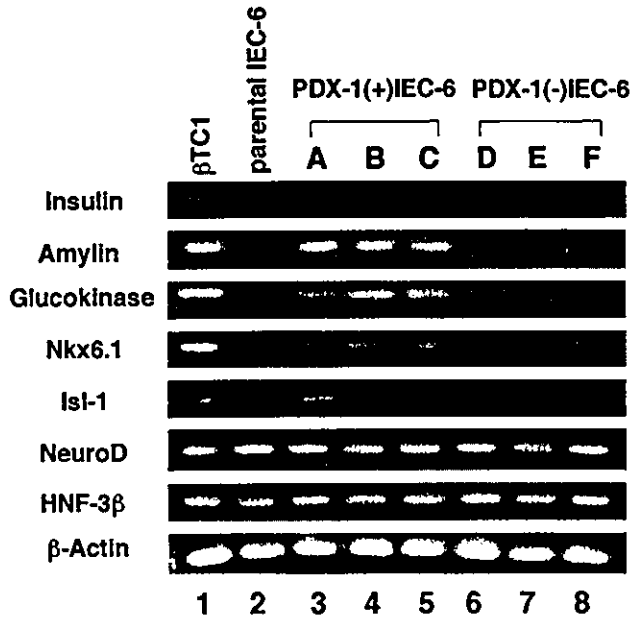

FIG. 3. Expression of β-cell–specific genes in PDX-1–expressing IEC-6 cells. RT-PCR was performed for the detection of insulin, amylin, glucokinase, Nkx6.1, Isl-1, NeuroD, and HNF3β. β-Actin mRNA was also amplified as a positive control. Total RNA isolated from βTC1 cells (*lane 1*), parental IEC-6 cells (*lane 2*), three independent PDX-1$^+$ IEC-6 clones (clone A, *lane 3*; clone B, *lane 4*; clone C, *lane 5*), and three independent PDX-1$^-$ IEC-6 clones (clone D, *lane 6*; clone E, *lane 7*; clone F, *lane 8*) were used. References for the primers are described in RESEARCH DESIGN AND METHODS. Similar results were obtained in at least three independent experiments.

lulin-treated PDX-1$^+$ IEC-6 cells was ~10% of that of βTC1 cells (data not shown).

The betacellulin treatment also induced somatostatin mRNA expression in PDX-1$^+$ IEC-6 cells according to results of RT-PCR analyses (Fig. 6*B*). However, immunocytochemical staining could not detect somatostatin protein in the cells (data not shown), possibly because of its low expression rate and/or constant secretion. Together with previous reports (33,34), our present data support the unique role of betacellulin in enhancing the β/δ-cell–like phenotype in non–β-cells.

Formation of secretory granules in betacellulin-treated PDX-1$^+$ IEC-6 cells. Both the betacellulin-treated PDX-1$^+$ IEC-6 cells and parental IEC-6 cells grew in monolayer and looked similar in appearance (data not shown). For more detailed observation, we performed electron microscopic analyses. Inconsistent with the induction of insulin expression in the cells, there were many small secretory granules in PDX-1$^+$ IEC-6 cells that had been treated with betacellulin (Fig. 7*B*). Some of the secretory granules were located close to or almost in contact with the plasma membrane, suggesting that they were to be secreted by exocytosis. This contrasts with the betacellulin-untreated PDX-1$^+$ IEC-6 cells, which totally lacked secretory granules although they had as well developed rough endoplasmic reticulum and Golgi apparatus (Fig. 7*A*). The parental IEC-6 cells, even when betacellulin was added to them, had no secretory granules (data not shown). In addition to the appearance of secretory gran-

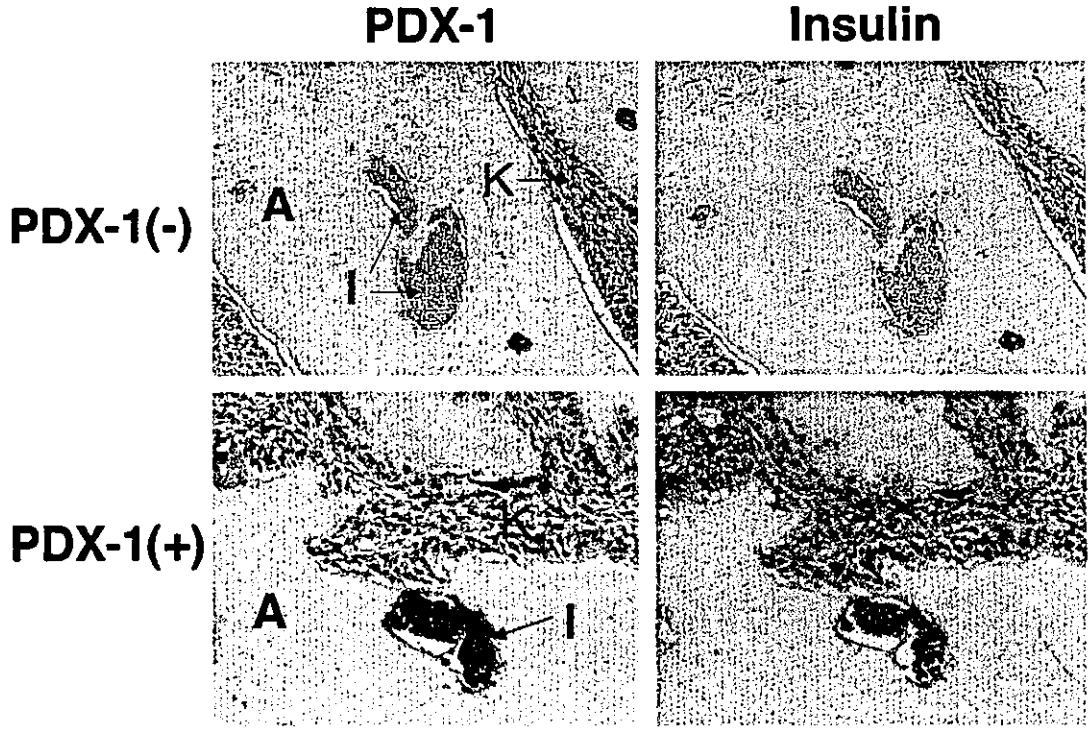

FIG. 4. Insulin staining in PDX-1$^+$ IEC-6 cells transplanted under a renal capsule. PDX-1$^-$ IEC-6 (clone D) and PDX-1$^+$ IEC-6 cells (clone A) were transplanted under a renal capsule of an adult rat and left for 2 weeks. After this period, the kidney was isolated and immunostaining for PDX-1 and insulin was performed. Upper panels represent the results for PDX-1$^-$ IEC-6 cells stained with anti–PDX-1 antibody (left) and anti-insulin antibody (right), and lower panels represent results for PDX-1$^+$ IEC-6 cells stained with anti–PDX-1 antibody (left) and anti-insulin antibody (right). Similar results to clone D and to clone A were obtained with other clones of PDX-1$^-$ IEC-6 (clones E and F) and of PDX-1$^+$ IEC-6 (clones B and C) cells, respectively. I, transplanted IEC-6 cells; K, kidney; A, agarose.

TABLE 1
Insulin content in PDX-1$^+$ IEC-6 cells kept under the renal capsule*

	Agarose	IEC-6	PDX-1$^+$ IEC-6	Pancreas
Immunoreactive insulin (ng/mg protein)	0.03 ± 0.01	0.03 ± 0.01	6.18 ± 1.04	1832 ± 231
n	5	6	6	4

Data are normalized with respect to the protein concentration expressed as means ± SE. *Insulin was extracted from the kidney that had received transplanted cell aggregates of parental IEC-6 cells or PDX-1$^+$ IEC-6 cells or agarose (no cells) as a control.

ules, there was an expansion of lysosomes in the betacellulin-treated cells (Fig. 7*B*). Although its physiological meaning is not known, a similar observation was previously obtained when IEC-6 cells were treated with glucocorticoids (35).

Effects of glucose or other secretagogues on insulin secretion from betacellulin-treated PDX-1$^+$ IEC-6 cells. We investigated whether PDX-1$^+$ IEC-6 cells treated with betacellulin can secrete insulin in response to glucose. As shown in Fig. 8*A*, the betacellulin-treated PDX-1$^+$ IEC-6 cells secrete insulin, whereas the betacellulin-untreated PDX-1$^+$ IEC-6 cells (Fig. 8*A*) or betacellulin-treated PDX-1$^-$ IEC-6 cells (data not shown) did not. However, in contrast to β-cell–derived MIN6 cells (Fig. 8*B*), the betacellulin-treated PDX-1$^+$ IEC-6 cells did not show any increase in insulin secretion even when the cells were exposed to a higher concentration of glucose. Also, neither arginine nor calcium ionophore A23187 enhanced insulin secretion. Thus, the betacellulin-treated PDX-1$^+$ IEC-6 cells expressed and secreted insulin but lacked response to glucose concentration or other secretagogues.

DISCUSSION

In the early developmental process of vertebrates, the definitive endoderm gives rise to all digestive organs, including the pancreas. Whereas most epithelial cells in the gut tube become part of the gastrointestinal tract, including small intestine, some groups of cells located in the posterior foregut give rise to ventral and dorsal pancreatic buds, and finally both buds become fused to give rise to the pancreas (10,11). Considering such developmental similarity between the pancreas and intestine and the high potential for transdifferentiation that has been shown for some tissue/organ-specific stem cells, the intestinal crypt cells may provide a useful tool for diabetes gene/cell therapy. The good accessibility for gene delivery is also an advantage of the intestinal cells.

The IEC-6 cell is derived from neonatal rat ileum and seems to retain its potential to respond to exogenously introduced transcription factors and to differentiate. Suh et al. (36) reported that conditional expression of an intestine-specific homeodomain-containing transcription factor, Cdx2/3 (37–39), in IEC-6 cells led to differentiation

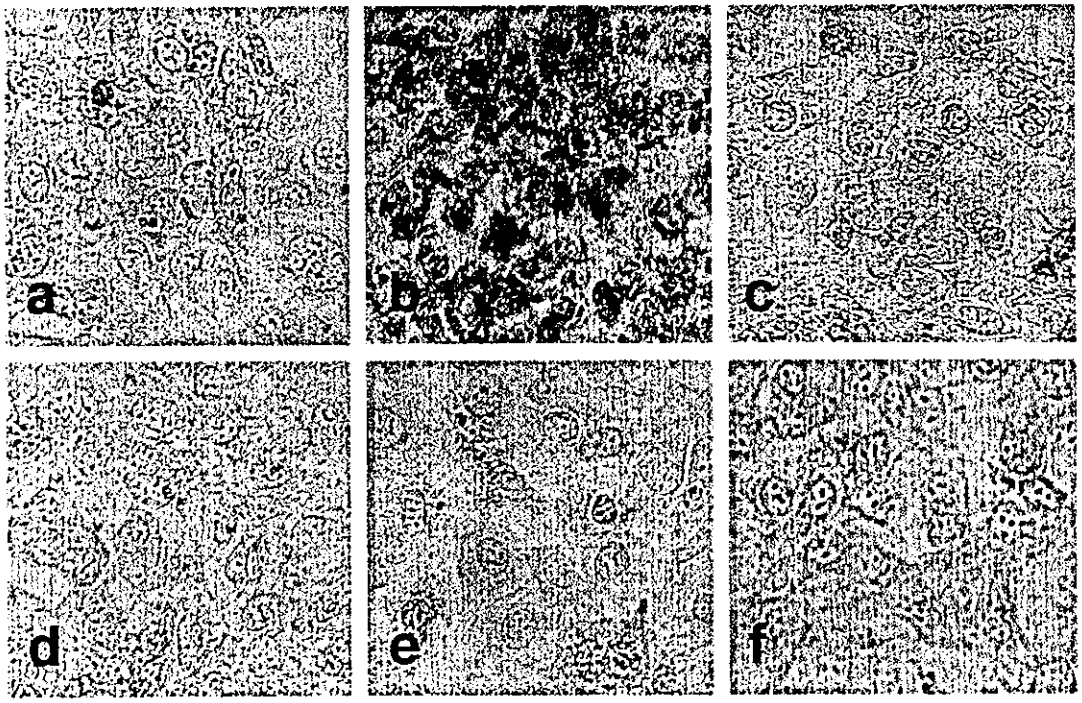

FIG. 5. Immunocytochemistry for insulin in PDX-1$^+$ IEC-6 cells with betacellulin. Insulin immunostaining was performed with PDX-1$^+$ IEC-6 cells (clone A) that were untreated (*a*) or treated for 4 days with 2 nmol/l betacellulin (*b*), 2 nmol/l TGF-α (*c*), 2 nmol/l EGF (*d*), or 2 nmol/l IGF-1 (*e*). Similar results were also obtained with other PDX-1$^+$ IEC-6 clones (clones B and C). As a control, the result obtained with PDX-1$^-$ IEC-6 cells (clone D) treated with betacellulin is shown in *f*. Similar negative results were also obtained with other PDX-1$^-$ IEC-6 clones (clones E and F).

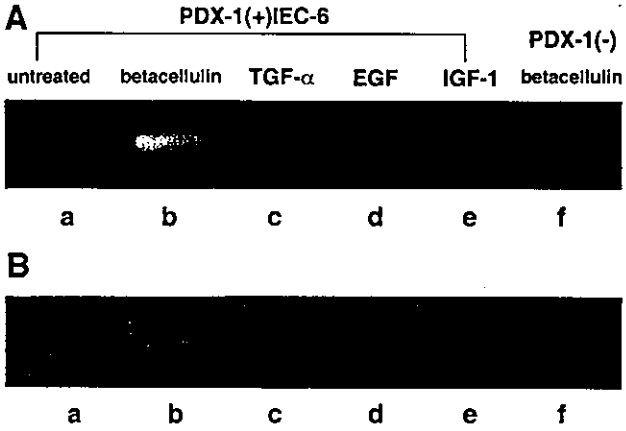

FIG. 6. Effects of growth factors on expression of insulin and somatostatin in PDX-1$^+$ IEC-6 cells. Insulin (*A*) and somatostatin (*B*) gene expression were examined by RT-PCR analyses in the same set of PDX-1$^+$ IEC-6 cells treated with various growth factors. RT-PCR analyses were done using total RNA isolated from PDX-1$^+$ IEC-6 cells (clone A) that were untreated (*lane a*) or treated for 4 days with 2 nmol/l betacellulin (*lane b*), 2 nmol/l TGF-α (*lane c*), 2 nmol/l EGF (*lane d*), or 2 nmol/l IGF-1 (*lane e*). As a control, the result obtained with PDX-1$^-$ IEC-6 cells treated with betacellulin is shown in *lane f*. Similar results were obtained in at least three independent experiments.

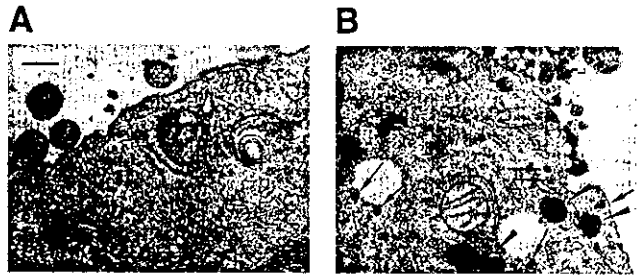

FIG. 7. Electron microscopic examination of PDX-1$^+$ IEC-6 cells. *A*: PDX-1$^+$ IEC-6 cells in the absence of betacellulin. *B*: PDX-1$^+$ IEC-6 cells in the presence of betacellulin. Both cells have ordinary cell organelles, such as rough endoplasmic reticulum and mitochondria. Secretory granules, however, can be seen only in the cells kept in the presence of betacellulin (arrows). Note that some of those granules are located close to plasma membrane. Lysosomes (arrowheads), which are rare in appearance in *A*, are also conspicuous in *B*. Bar = 0.5 μm.

into goblet-like or enterocyte-like cells. In this study, we showed that exogenously introduced PDX-1 conferred some pancreatic β-cell phenotype to the transfected cells. In contrast, whereas developmentally more primitive YSE cells and JC44 cells lack some of the islet-cell–related transcription factors that are expressed in IEC-6 cells, exogenously introduced PDX-1 did not induce any changes in the expressions of the β-cell–specific genes in those two cell lines (S.Y. and Y.K., unpublished results), suggesting that intestinal epithelioid IEC-6 cells retain some feature that facilitates β-cell–like differentiation. Also, being able to differentiate in multiple directions, IEC-6 cells possess some stem cell–like characteristic that is also seen in the crypt-type intestinal cells in vivo (14).

Previous studies showed that PDX-1 is a direct activator of several β-cell–specific genes, such as insulin, amylin, glucokinase, GLUT2, and Nkx6.1 (18,40–42). In this study, we found that the PDX-1–transfected IEC-6 cells expressed glucokinase, amylin, and Nkx6.1 but not GLUT2. This agrees with our previous observation with glucagon-producing αTC1 cells showing that PDX-1 can also induce glucokinase gene expression but not GLUT2 (7). However, apart from these gain-of-function experiments, the loss-of-function experiment done by Ahlgren et al. (5) showed that β-cell–specific gene disruption of PDX-1 caused the decrease of GLUT2, Nkx6.1, and amylin but not glucokinase. These observations tempt us to assume that PDX-1 is necessary but not sufficient for GLUT2 gene expression, whereas it is sufficient but not necessary for the glucokinase gene expression. This is possible because a family of homeodomain-containing transcription factors is known to be expressed in pancreatic β-cells, and they may bind to

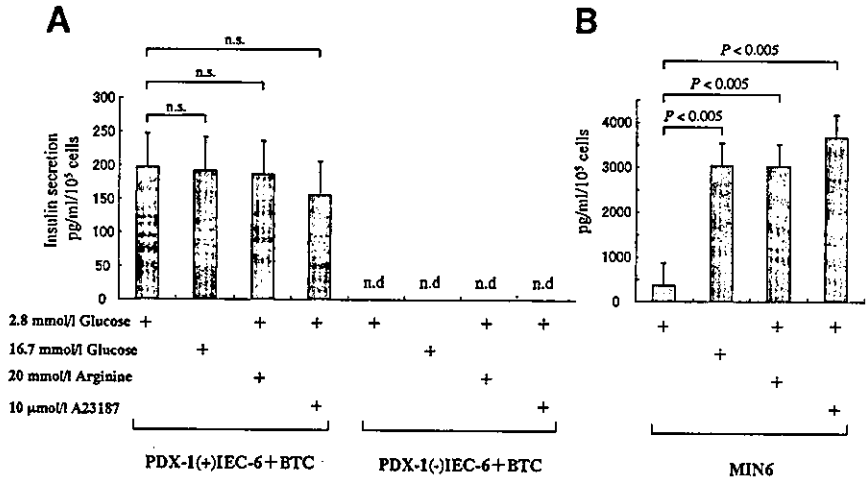

FIG. 8. Evaluation of glucose-responsive insulin secretion. Insulin secretion in response to glucose, arginine, or calcium ionophore A 23187 was investigated in betacellulin-treated PDX-1$^+$ IEC-6 cells (clone A) and betacellulin-treated PDX-1$^-$ IEC-6 cells (clone D) (*A*). As control, data were also obtained with MIN6 cells (*B*). The cells were kept for 2 h (PDX-1$^+$ IEC-6 and PDX-1$^-$ IEC-6 cells) or 30 min (MIN6 cells) in medium containing 2.8 or 16.7 mmol/l glucose. Where indicated, 20 mmol/l arginine or 10 μmol/l calcium ionophore A23187 was added to medium containing 2.8 mmol/l glucose. The insulin concentrations in the medium were expressed as means ± SE (pg/ml per 10^5 cells) of at least four experiments. Another PDX-1$^+$ IEC-6 clone (clone B) gave similar results to clone A. BTC, betacellulin; n.d., not detectable; n.s., not significant.

the AT-rich motif, to which PDX-1 binds, and compensate for some of the PDX-1 function in its absence. Although speculative, it is possible that no other homeodomain factors can substitute for PDX-1 in terms of the activation of the GLUT2 gene, for example, as a result of the involvement of a co-factor that can associate with PDX-1 but not with other homeodomain transcription factors.

Gittes et al. (15) previously showed that the epithelium of isolated pancreatic bud in the embryo forms mature islets when kept under the renal capsule. It was this observation that prompted us to do a similar trial using the PDX-1–transfected IEC-6 cells in the present study. Because the epithelial cells of the pancreatic bud share some common features with the PDX-1–transfected IEC-6 cells, such as the developmental origin being the gut and the expression of PDX-1, we considered that the under-renal-capsule transplantation may also work for the PDX-1–expressing IEC-6 cells. Our present results proved this hypothesis, supporting the potential usefulness of the space as a place for in vivo induction of β-cell differentiation. The detailed mechanism underlying the phenomenon needs to be examined, especially focusing on the possible involvement of diffusible mediators such as growth factors.

An ELISA experiment revealed that the insulin amount detected in the kidney after transplantation of PDX-1$^+$ IEC-6 cells was still far less than that in the pancreas (Table 1). However, there is a great difference in numbers of insulin-producing cells between the kidney, to which PDX-1$^+$ IEC-6 cells were transplanted, and the pancreas: 5,000–7,000 transplanted PDX-1$^+$ IEC-6 cells in the kidney versus roughly 1 million β-cells in the rat pancreas. This indicates that the insulin content per single PDX-1$^+$ IEC-6 cell (0.58–0.81 pg) was comparable to that of a β-cell (1.2–2.0 pg).

As a clue to identifying the factors that induce the insulin expression in the PDX-1–expressing IEC-6 cells, we screened the possible effects of various growth factors that may exert some roles in the pancreas. Among them, betacellulin was originally identified from the conditioned medium of β-cell–derived βTC3 cells as a growth-promoting factor having structural characteristics of the EGF family members (31). We previously reported that exogenously expressed PDX-1 induces glucokinase and insulin gene expressions in α-cell–derived αTC1.6 cells in the presence of betacellulin (7). In addition, betacellulin with the aid of activin A could convert the amylase-secreting pancreatic acinar cell–like AR42J cells into insulin-secreting cells (33). Together with the present results revealing the potential of betacellulin in inducing endogenous insulin gene expression in the intestinal cell–derived IEC-6 cells, these data indicate that betacellulin is likely to be a potent and widely functional inducer of β-cell differentiation. A recent study that demonstrated the in vivo effect of betacellulin on promotion of the islet neogenesis in alloxan-perfused pancreas supports this idea (34).

In electron microscopy, secretory granules were visible in the cytoplasm of the betacellulin-treated PDX-1$^+$ IEC-6 cells, supporting the idea that the cells acquired some neuroendocrine cell characteristics. However, the granules seemed to be secreted even when the cells were kept under low glucose concentrations. One possible cause for this is the lack of Kir6.2 expression in the cells. Kir6.2 is an essential component of ATP-sensitive potassium channel, and disruption of its gene in mice causes total loss of glucose responsiveness in insulin secretion (43). Although other factors involved in glucose-responsive insulin secretion might also be in effect, it may be worthwhile to restore Kir6.2 expression in PDX-1$^+$ IEC-6 cells to see whether it can confer glucose responsiveness.

During preparation of this manuscript, Yamada et al. (44) reported a similar observation using the same set of intestinal cells and the same transcription factor. In that study, they grew the PDX-1$^+$ IEC-6 cells on micropore filters and found that two types of cells developed: trabecular-shaped and non–trabecular-shaped. According to them, the trabecular-shaped cells showed strong positive staining for PDX-1 in the nucleus, whereas the non–trabecular-shaped cells showed only weak cytoplasm staining for PDX-1. This obviously contrasts with our present results that found PDX-1 localized exclusively in the nuclei of PDX-1$^+$ IEC-6 cells (Fig. 2*C*). This difference may depend on the difference in culture condition or on possible clonal variation of the parental IEC-6 cells used in each experiment. However, apart from that, their study proved the potential of PDX-1 in inducing some enteroendocrine cell–like characteristics but no insulin expression (44). Therefore, their observation is largely consistent with the phenotype of our PDX-1$^+$ IEC-6 cells, which was observed before the under-renal-capsule transplantation or the betacellulin treatment. Indeed, our PDX-1$^+$ IEC-6 cells also expressed some enteroendocrine markers, such as cholecystokinin and gastrin (S.Y. and Y.K., unpublished observations).

In conclusion, our present observations demonstrated the potency of PDX-1 in inducing the expression of β-cell–characteristic genes in intestinal crypt cell–like IEC-6 cells. Because of the accessibility of intestinal tissues from brain-death donors, ex vivo transfection of a transcription factor would be a reasonable step to try toward diabetes cell therapy. Also, with a relatively facile in vivo gene delivery, the intestinal epithelium may be a good target for transcription factor–mediated in vivo induction of β-cell differentiation. Toward the long-term goal of diabetes gene/cell therapy, however, efforts need to be focused on acquiring stable glucose-responsive insulin secretion in engineered intestinal epithelium cells. Establishment of an efficient system for gene delivery to the intestine would also be necessary for future clinical use.

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REFERENCES

1. Leonard J, Peers B, Johnson T, Ferreri K, Lee S, Montminy MR: Characterization of somatostatin transactivating factor-1, a novel homeobox

factor that stimulates somatostatin expression in pancreatic islet cells. *Mol Endocrinol* 7:1275–1283, 1993
2. Ohlsson H, Karlsson K, Edlund T: IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J* 12:4251–4259, 1993
3. Miller CP, McGehee RE, Habener JF: IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. *EMBO J* 13:1145–1156, 1994
4. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
5. Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H: β-Cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the β-cell phenotype and maturity onset diabetes. *Genes Dev* 12:1763–1768, 1998
6. Serup P, Jensen J, Andersen FG, Jorgensen MC, Blume N, Holst JJ, Madsen OD: Induction of insulin and islet amyloid polypeptide production in pancreatic islet glucagonoma cells by insulin promoter factor 1. *Proc Natl Acad Sci U S A* 93:9015–9020, 1996
7. Watada H, Kajimoto Y, Miyagawa J, Hanafusa T, Hamaguchi K, Matsuoka T, Yamamoto K, Matsuzawa Y, Kawamori R, Yamasaki Y: PDX-1 induces insulin and glucokinase gene expressions in αTC1 clone 6 cells in the presence of betacellulin. *Diabetes* 45:1826–1831, 1996
8. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A: Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat Med* 6:568–572, 2000
9. Cairnie AB, Lamerton LF, Steel GG: Cell proliferation studies in the intestinal epithelium of the rat. II. Theoretical aspects. *Exp Cell Res* 39:539–553, 1965
10. Cairnie AB, Lamerton LF, Steel GG: Cell proliferation studies in the intestinal epithelium of the rat. I. Determination of the kinetic parameters. *Exp Cell Res* 39:528–538, 1965
11. Grapin-Botton A, Melton DA: Endoderm development: from patterning to organogenesis. *Trends Genet* 16:124–130, 2000
12. Wells JM, Melton DA: Vertebrate endoderm development. *Annu Rev Cell Dev Biol* 15:393–410, 1999
13. Lau C, Soriano HE, Ledley FD, Finegold MJ, Wolfe JH, Birkenmeier EH, Henning SJ: Retroviral gene transfer into the intestinal epithelium. *Hum Gene Ther* 6:1145–1151, 1995
14. Quaroni A, Wands J, Trelstad RL, Isselbacher KJ: Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J Cell Biol* 80:248–265, 1979
15. Gittes GK, Galante PE, Hanahan D, Rutter WJ, Debase HT: Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. *Development* 122:439–447, 1996
16. Krupnick JG, Damjanov I, Damjanov A, Zhu ZM, Fenderson BA: Globo-series carbohydrate antigens are expressed in different forms on human and murine teratocarcinoma-derived cells. *Int J Cancer* 59:692–698, 1994
17. Yoder MC, King B, Hiatt K, Williams DA: Murine embryonic yolk sac cells promote in vitro proliferation of bone marrow high proliferative potential colony-forming cells. *Blood* 86:1322–1330, 1995
18. Watada H, Kajimoto Y, Umayahara Y, Matsuoka T, Kaneto H, Fujitani Y, Kamada T, Kawamori R, Yamasaki Y: The human glucokinase gene β-cell-type promoter: an essential role of insulin promoter factor 1/PDX-1 in its activation in HIT-T15 cells. *Diabetes* 45:1478–1488, 1996
19. Sadowski HB, Gilman MZ: Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature* 362:79–83, 1993
20. Matsuoka T, Kajimoto Y, Watada H, Umayahara Y, Kubota M, Kawamori R, Yamasaki Y, Kamada T: Expression of CD38 gene, but not of mitochondrial glycerol-3-phosphate dehydrogenase gene, is impaired in pancreatic islets of GK rats. *Biochem Biophys Res Commun* 214:239–246, 1995
21. Jensen J, Serup P, Karlsen C, Nielsen TF, Madsen OD: mRNA profiling of rat islet tumors reveals nkx 6.1 as a β-cell-specific homeodomain transcription factor. *J Biol Chem* 271:18749–18758, 1996
22. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M: Regulation of a transcription factor network required for differentiation and metabolism. *Science* 281:692–695, 1998
23. Wulfsen I, Hauber HP, Schiemann D, Bauer CK, Schwarz JR: Expression of mRNA for voltage-dependent and inward-rectifying K channels in GH3/B6 cells and rat pituitary. *J Neuroendocrinol* 12:263–272, 2000
24. Kenny AJ: Extractable glucagon of the human pancreas. *J Clin Endocrinol Metab* 15:1089–1105, 1955
25. Gerrish K, Gannon M, Shih D, Henderson E, Stoffel M, Wright CV, Stein R: Pancreatic β cell-specific transcription of the pdx-1 gene. The role of conserved upstream control regions and their hepatic nuclear factor 3β sites. *J Biol Chem* 275:3485–3492, 2000
26. Karlsson O, Thor S, Norberg T, Ohlsson H, Edlund T: Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature* 344:879–882, 1990
27. Naya FJ, Stellrecht CM, Tsai MJ: Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* 9:1009–1019, 1995
28. Mirmira RG, Watada H, German MS: β-Cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. *J Biol Chem* 275:14743–14751, 2000
29. Mutoh H, Fung BP, Naya FJ, Tai M-J, Nishitani J, Leiter AB: The basic helix-loop-helix transcription factor BETA2/NeuroD is expressed in mammalian enteroendocrine cells and activates secretin gene expression. *Proc Natl Acad Sci U S A* 94:3560–3564, 1997
30. Otonkoski T, Beattie GM, Rubin JS, Lopez AD, Baird A, Hayek A: Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. *Diabetes* 43:947–953, 1994
31. Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K, Folkman J: Betacellulin: a mitogen from pancreatic β cell tumors. *Science* 259:1604–1607, 1993
32. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG: Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med* 6:278–282, 2000
33. Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I: Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest* 97:1647–1654, 1996
34. Yamamoto K, Miyagawa J, Waguri M, Sasada R, Igarashi K, Li M, Nammo T, Moriwaki M, Imagawa A, Yamagata K, Nakajima H, Namba M, Tochino Y, Hanafusa T, Matsuzawa Y: Recombinant human betacellulin promotes the neogenesis of β-cells and ameliorates glucose intolerance in mice with diabetes induced by selective alloxan perfusion. *Diabetes* 49:2021–2027, 2000
35. Quaroni A, Tian JQ, Goke M, Podolsky DK: Glucocorticoids have pleiotropic effects on small intestinal crypt cells. *Am J Physiol Gastrointest Liver Physiol* 277:G1027–G1040, 1999
36. Suh E, Chen L, Taylor J, Traber PG: A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol Cell Biol* 14:7340–7351, 1994
37. German MS, Wang J, Chadwick RB, Rutter WJ: Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. *Genes Dev* 6:2165–2176, 1992
38. James R, Kazenwadel J: Homeobox gene expression in the intestinal epithelium of adult mice. *J Biol Chem* 266:3246–3251, 1991
39. James R, Erler T, Kazenwadel J: Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. *J Biol Chem* 269:15229–15237, 1994
40. Waeber G, Thompson N, Nicod P, Bonny C: Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. *Mol Endocrinol* 10:1327–1334, 1996
41. Watada H, Kajimoto Y, Kaneto H, Matsuoka T, Fujitani Y, Miyazaki J, Yamasaki Y: Involvement of the homeodomain-containing transcription factor PDX-1 in islet amyloid polypeptide gene transcription. *Biochem Biophys Res Commun* 229:746–751, 1996
42. Watada H, Mirmira RG, Leung J, German MS: Transcriptional and translational regulation of β-cell differentiation factor Nkx6.1. *J Biol Chem* 275:34224–34230, 2000
43. Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A, Gonoi T, Iwanaga T, Miyazaki J, Seino S: Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. *Proc Natl Acad Sci U S A* 95:10402–10406, 1998
44. Yamada S, Kojima H, Fujimiya M, Nakamura T, Kashiwagi A, Kikkawa R: Differentiation of immature enterocytes into enteroendocrine cells by Pdx1 overexpression. *Am J Physiol Gastrointest Liver Physiol* 281:G229–G236, 2001

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Protein Kinase C-dependent, CCAAT/Enhancer-binding Protein β-mediated Expression of Insulin-like Growth Factor I Gene*

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Yutaka Umayahara, Yoshitaka Kajimoto‡, Yoshio Fujitani, Shin-ichi Gorogawa, Tetsuyuki Yasuda, Akio Kuroda, Kentaro Ohtoshi, Shigeru Yoshida, Dan Kawamori, Yoshimitsu Yamasaki, and Masatsugu Hori

From the Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka Pref. 565-0871, Japan

The possible involvement of the protein kinase C (PKC) pathway in transcriptional regulation of the human insulin-like growth factor-I (IGF-I) gene has been suggested. In this study, we sought to determine whether a PKC-dependent pathway is implicated in the transcriptional control, and if it is, how this occurs. Treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) caused an increase in the activity of the human IGF-I gene major promoter in HepG2 cells. A CCAAT/enhancer-binding protein (C/EBP) binding site located at +22 to +30 was bound by C/EBPβ in a TPA-dependent manner and was solely responsible for the TPA responsiveness. This increase in C/EBPβ activity occurs through transcriptional and posttranslational regulation, and the latter is mediated by activation of p90 ribosomal S6 kinase (RSK): co-expression of dominant negative RSK abolished the TPA-responsive and C/EBPβ-dependent transactivation. Also, TPA-responsive activation of GAL4-C/EBPβ chimera required the Ser residue known as the RSK target. In SK-N-MC cells, which display constitutive, high expression of IGF-I on use of the major promoter, a large amount of C/EBPβ binding was observed with the C/EBP site in the basal state. Treatment with PKC inhibitors substantially reduced the promoter activity and mRNA amounts of IGF-I, with the binding of C/EBPβ to the C/EBP site also being reduced. When the C/EBP site was disrupted, the basal promoter activity was reduced, but the reduction by the PKC inhibitor was no longer observed. These observations suggest that the increase of C/EBPβ binding to the C/EBP site, which is in part mediated via activation of RSK, can primarily explain the TPA responsiveness of the IGF-I gene promoter. The intrinsic PKC activity in SK-N-MC cells should play a major role in the constitutive, high expression of IGF-I and may therefore contribute in part to the maintenance of the tumor phenotype of the cells.

Insulin-like growth factor I (IGF-I),[1] a 70-residue single-chain growth-promoting polypeptide, is produced in many organs and tissues and plays a major role in somatic growth, cell survival, tissue differentiation, and intermediary metabolism (1–3). Although various tissue-dependent factors as well as endocrine hormones seem to regulate the IGF-I gene expression, their mechanisms, except for those involved in prostaglandin E_2 or cAMP (4–6), are poorly understood.

The protein kinase-C (PKC) pathway is among the few that have been suggested to be involved in IGF-I gene regulation. The result of a nuclear run-on assay indicated that treatment of human macrophage-like cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) increased the transcription rate of the IGF-I gene 4- to 5-fold (7), suggesting that the human IGF-I gene regulatory sequences contain something that responds to PKC. Support for this also comes from our recent observations with the chicken IGF-I gene, *i.e.* that the gene promoter can be activated by TPA through an AP-1 binding site located in it (8). However, it is unknown whether the mammalian IGF-I genes are activated by PKC, and if they are, how this occurs.

In contrast to protein kinase A, which seems to be involved in parathyroid hormone or prostaglandin E_2-induced IGF-I gene activation, PKC has been often discussed in correlation with tumorigenesis. Indeed, the best-known activator of PKC, TPA, is a strong tumor promoter (9, 10). *In vitro* overexpression studies have suggested that individual PKC isozymes control cell proliferation and malignant transformation. For example, when PKCβI was overexpressed in rat fibroblasts, the cells were partially transformed and could form tumors in nude mice (11). Overexpression of PKCα also occasionally leads to transformation of fibroblasts (12). Because the IGF system is known to play an essential role in inducing transformation (13) or maintaining the tumor phenotype in some cells, such as a human neuroblastoma cell line SK-N-MC (14), it is likely that PKC-dependent activation of the IGF-I gene, if it occurs in mammals, may be partially involved in the tumorigenesis.

As a step toward elucidating the molecular basis of IGF-I gene regulation, we examined whether the human IGF-I gene promoter is a target of PKC regulation and sought to elucidate the physiological roles of the PKC pathway in the gene expression. Here we report that the major promoter of the gene, which is located within the 5′-flanking region and untranslated region of exon 1, is indeed a target of TPA stimulation and a CCAAT/enhancer binding protein (C/EBP) site within the promoter is responsible for the phenomenon. C/EBPβ, which is activated by PKC both at the level of transcription and of posttranslation, binds to the C/EBP site and thereby mediates the phenomenon. Interestingly, the posttranslational activa-

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‡ To whom correspondence should be addressed. Tel.: 81-6-6879-3633; Fax: 81-6-6879-3639; E-mail: kajimoto@medone.med.osaka-u.ac.jp.

[1] The abbreviations used are: IGF-I, insulin-like growth factor-I; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; C/EBP, CCAAT/enhancer-binding protein; RSK, p90 ribosomal S6 kinase; HA, hemagglutinin; DN, dominant negative; PBS, phosphate-buffered saline; aa, amino acids; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IGF-IR, type-I insulin-like growth factor receptor.

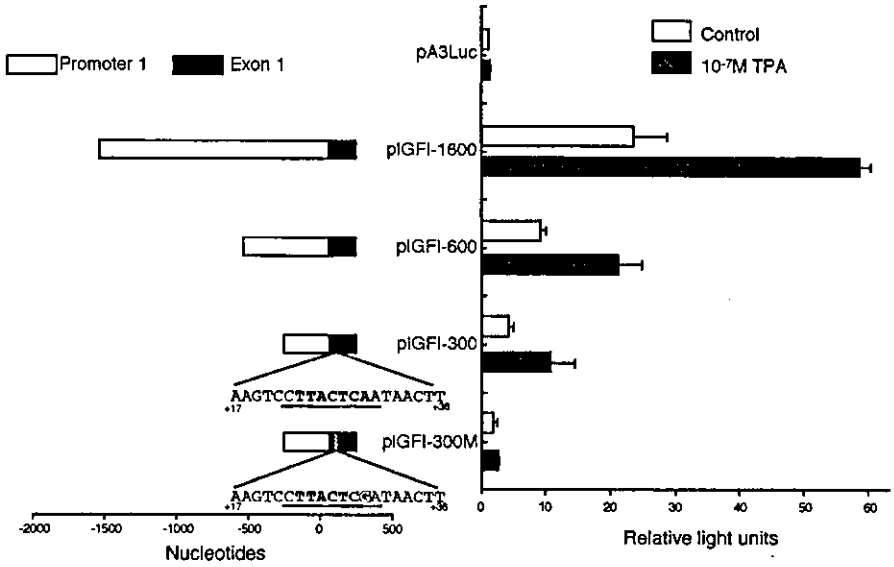

FIG. 1. **Putative TPA-responsive site is located in the 5′-untranslated region of exon 1 of human IGF-I gene.** *Left panel*, diagrammatic representation of IGF-I promoter-1-luciferase reporter plasmids. The nucleotide sequence is shown for the region containing AP-1-like sequence (*bold*) and C/EBP binding site (*underlined*). pIGFI-300M has a point mutation, and the altered base is in *outline lettering*. *Right panel*, IGF-I promoter-1-luciferase reporter plasmids were cotransfected with a pMSV-β-galactosidase control vector into HepG2 cell using LipofectAMINE. After a 24-h incubation, the cultures were exposed to control medium (containing vehicle) or TPA (100 nM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency (β-galactosidase expression) and presented as means ± S.D. of at least three independent experiments performed in duplicate.

tion of C/EBPβ occurs primarily through activation of p90 ribosomal S6 kinase (RSK). Moreover, as support for the pathophysiological significance of these findings, we found that the constitutive IGF-I gene expression in the human neuroblastoma-derived SK-N-MC cells depends on the intrinsically activated PKC.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies to C/EBPα (14AA), C/EBPβ (C-19), C/EBPδ (C-22), HNF-1α (C-19), and to c-Myb (M-19) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Antibodies to c-Fos and to c-Jun were purchased from Oncogene Science (Uniondale, NY). Antibody to HA tag was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture—HepG2 cells (Riken Cell Bank, Tsukuba, Japan, catalog no. RCB459) were maintained as previously described (15). SK-N-MC cells (ATCC catalog no. HTB10) were maintained in Earle's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, non-essential amino acids, penicillin, and streptomycin (basal condition medium). 293T cells were maintained as previously described (16).

Plasmids—Human IGF-I promoter-1-luciferase fusion genes were constructed as follows. A phage clone containing the 5′-flanking region and exon 1 of the human IGF-I gene was isolated from a human genomic library and used as a template to make the reporter gene plasmids. A series of PCR was performed using the phage DNA as a template to amplify promoter-1 DNA fragments, which were comprised of either 1600 bp or 300 bp of the human IGF-I gene 5′-flanking region and the 197 bp of the exon 1 untranslated region. The PCR primers used were 5′-GCGGTACCGCCTCTCAATGACACAATCTG-3′(for the 1600-bp fragment), 5′-GCGGTACCGAGTTTGCTGGAGAGG-GTCT-3′(for the 300-bp fragment) and 5′-GGCAAGCTTGCGCAGGCT-CTATCTGCT-3′ (for both). To make the plasmid pIGFI-1600 (Fig. 1), the PCR-amplified 1600-bp fragment was made blunt-ended using the DNA Blunting Kit (Takara, Kyoto, Japan), digested with *Hin*dIII, and ligated into *Sma*I/*Hin*dIII-digested pA3Luc (a kind gift from I. H. Maxwell, University of Colorado Health Science Center, Denver, CO) (17). The plasmid pIGFI-600 (Fig. 1) was constructed by digesting the 1600-bp fragment with *Kpn*I and *Hin*dIII and subcloning the resulting 600-bp fragment into the *Kpn*I/*Hin*dIII-digested pA3Luc. The 300-bp fragment was digested with *Kpn*I and *Hin*dIII and ligated into *Kpn*I/*Hin*dIII-digested pA3Luc to construct the plasmid pIGFI-300 (Fig. 1). Site-directed mutagenesis was performed as described previously (8).

C/EBPβ expression vector pcDNA3C/EBPβ has been described previously (5). Wild type and dominant negative (DN) type RSK expression vectors were gifts from Dr. J. Blenis (Boston, MA). The plasmid pMSV β-gal is an expression plasmid of the β-galactosidase gene driven by the murine sarcoma virus long terminal repeat (18). The plasmid pRL-CMV was purchased from Promega Corp (Madison, WI).

Gene Transfer Experiments—Transfection studies using HepG2 cells were performed as follows. One microgram of IGF-I promoter-1-luciferase fusion genes were cotransfected with 500 ng of pMSV β-gal to normalize for transfection efficiency. Cultures at 50% confluent density were rinsed in serum-free medium and exposed to plasmids in the presence of LipofectAMINE™ for 5 h. After washing the plates two times with PBS, the solution was then replaced with serum-free medium (15), and the cells were incubated for 24 h. Next, the cells were treated for 24 h with vehicle (Me_2SO) or 10^{-7} M TPA. After incubation, the medium was aspirated, the cultures were rinsed with PBS twice and lysed in cell lysis buffer (Promega), and luciferase activity was measured as described previously (15).

Transfection studies using SK-N-MC cells were performed as follows. One microgram of IGF-I promoter-1-lucferase fusion genes were cotransfected with 5 ng of pRL-CMV to normalize for transfection efficiency. Cultures at 50% confluent density were rinsed in serum-free medium and exposed to plasmids in the presence of LipofectAMINE™ for 5 h. After washing the plates two times with PBS, the solution was replaced with culture medium containing 10% fetal bovine serum, and the cells were incubated for 24 h. Next, the cells were treated for 24 h with vehicle (Me_2SO) or 10^{-7} M GF109203X. After incubation, the medium was aspirated, the cultures were rinsed with PBS twice and lysed in cell lysis buffer, and dual-luciferase assay was performed according to the manufacturer's instructions (Promega).

Transfection studies using 293T cells were performed basically in the same way as the SK-N-MC cells described above. One microgram of IGF-I promoter-1-luciferase fusion genes were cotransfected with the indicated amount of C/EBPβ expression vector, 1 μg of wild type or dominant negative type RSK expression vector, when required, and 5 ng of pRL-CMV. After transfection, the cells were incubated for 24 h and dual-luciferase assay (Promega) was performed following the manufacturer's directions.

Gal4 Fusion Protein Reporter Gene Analyses—The Gal4 fusion constructs (Gal4C/EBPβ118 and Gal4C/EBPβ166) were generated by isolating (by PCR) and introducing appropriate DNA fragments of C/EBPβ into the *Eco*RI-*Bgl*II site of pFACMV plasmid (Stratagene), which contained the DNA-binding domain (positions 1–147) of Gal4. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) using two synthetic complementary oligonucleotides, 5′-CCGAGCAAGAAGCCGGCCGACTACGGTTACG-3′ and 5′-CGTAACCGTAGTCGGCCGGCTTCTTGCTCGG-3′ (mutated sequence is underlined), to generate Gal4C/EBPβ118mutAla105 and Gal4C/EBPβ166mut105Ala. The Gal4-responsive reporter plasmid pFR-Luc plasmid containing five copies of Gal4-binding element upstream of the basic promoter element (TATA box) linked to luciferase structural gene was purchased from Stratagene. By lipofection, 1 μg of each Gal4 fusion plasmid was cotransfected into the host cell with 1 μg of pFR-Luc and 5 ng of pRL-CMV. The cells were then incubated for 48 h, followed by a dual-luciferase assay (Promega) performed according to the manufacturer's directions.

Nuclear Protein Extracts—HepG2 and SK-N-MC cell nuclear extracts were prepared by the method of Lee *et al.* (19) with minor modifications. Cells were harvested with a cell scraper and gently pelleted, and the pellets were washed with phosphate-buffered saline.

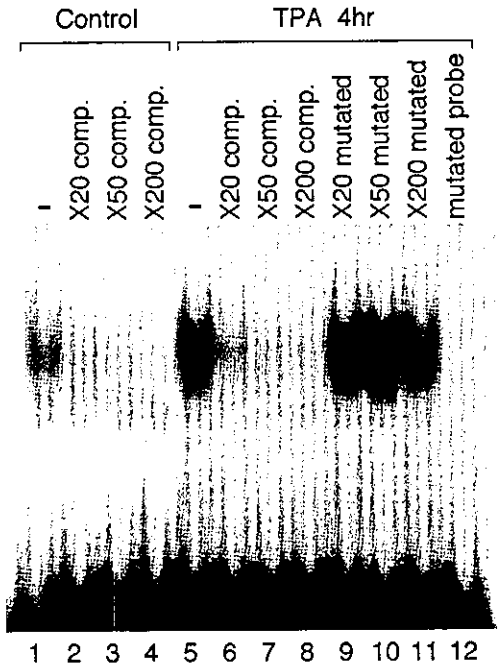

FIG. 2. **TPA induced specific nuclear protein binding to the putative TPA responsive site.** Gel mobility shift experiments were performed as described under "Experimental Procedures" with nuclear protein extracts isolated from HepG2 cell after incubation with vehicle or 100 nM TPA for 4 h. *Lanes 1–11* show protein binding to the ^{32}P-labeled wild type probe, and *lane 12* shows protein binding to the ^{32}P-labeled mutated type probe. The sense strand sequence of probes are as follows: wild type probe, 5′-AAGTCCTTACTCAATAACTT and mutated type probe, 5′-AAGTCCTTACTCGATAACTT (mutated nucleotide is *underlined*). Unlabeled 20- to 200-fold molar excess competitor DNAs were added to the binding reaction as indicated. Similar results were obtained in three independent experiments.

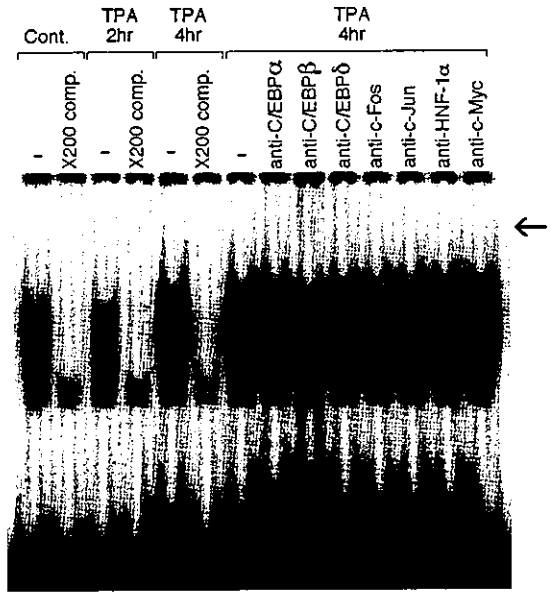

FIG. 3. **The TPA-induced-binding protein is C/EBPβ and the TPA responsive site works as a C/EBP site.** Gel mobility shift experiments were performed as described under "Experimental Procedures" with nuclear protein extracts isolated from HepG2 cell after incubation with vehicle (*lanes 1,2*), 100 nM TPA for 2 h (*lanes 3,4*) or for 4 h (*lanes 5–14*). ^{32}P-labeled wild type probe whose sequence is shown in Fig. 2 was used in all lanes. Unlabeled 200-fold molar excess competitor DNAs were added to the binding reaction as indicated (*lanes 2,4,6*). Specific antibodies (1 μg) to C/EBPα (*lane 8*), C/EBPβ (*lane 9*), C/EBPδ (*lane 10*), c-Fos (*lane 11*), c-Jun (*lane 12*), HNF-1α (*lane 13*), or c-Myc (*lane 14*) were added to the binding reaction as described in "Experimental Procedures". An arrow shows the supershift band which appeared only when specific antibody to C/EBPβ was added. Similar results were obtained in three independent experiments.

The cells were then lysed in hypotonic buffer (10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol). Nuclei were pelleted and resuspended in hypertonic buffer containing 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Soluble proteins released by a 30-min incubation at 4 °C were collected by centrifugation at 12,000 × *g* for 20 min, and the supernatant was collected. The protein concentration was measured using a modified Bradford assay (Bio-Rad).

DNA-Protein Binding Studies—Gel mobility shift experiments followed previously published methods (4). Radiolabeled double-stranded DNA probes were synthesized by annealing complementary end-labeled oligonucleotides. Nuclear protein extracts (5 μg) were preincubated for 20 min on ice with 2 mg of poly(dI-dC) with or without unlabeled specific or nonspecific DNA competitor or antibodies in 25 mM HEPES, pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.025% bovine serum albumin. After the addition of 5 × 10^4 cpm of DNA probe for 30 min on ice, the samples were applied to 12% nondenaturing polyacrylamide gel that had been pre-electrophoresed for 30 min at 12.5 V/cm at 25 °C in 45 mM Tris, 45 mM boric acid, and 1 mM EDTA. Electrophoresis was conducted for 2.5 h under identical conditions. The dried gels were exposed to x-ray film at −80 °C with an intensifying screen.

Deoxynuclease I (DNase) footprinting was performed as described elsewhere (6). End-labeled double-stranded DNA probes flanking the C/EBP site in human IGF-I promoter-1, which corresponds to −46 to +96 bp (relative to transcriptional start site) of the human IGF-I gene, were generated by polymerase chain reaction using one end-labeled oligonucleotide primer (5′-ATGCTCTGTCTCTAGTT-3′) and one unlabeled primer (5′-ACTGTAGACAGGAAACAGCT-3′). Nuclear protein (10 μg) was preincubated for 15 min with poly(dI-dC) in 25 mM HEPES, pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.05% bovine serum albumin, followed by the addition of labeled probe (5.0 × 10^5 cpm/sample) and incubation for 60 min on ice. The reaction mixture was then treated with DNase I (final concentration 1.15 mg/ml, Worthington Biochemical Corp., Freehold, NJ) in 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ for 1 min at 25 C. Nuclease treatment was terminated by addition of 20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, and 10 mg of yeast transfer RNA followed by phenol-chloroform extraction and ethanol precipitation. Samples were analyzed after electrophoresis on 8% polyacrylamide, 8 M urea gel, and autoradiography for 16 h at −80 °C with an intensifying screen.

RNA Isolation and Analyses—Total RNA was extracted from HepG2 cells or SK-N-MC cells by homogenization in guanidine thiocyanate. Northern blots followed standard procedures using 10 μg of total RNA, and the buffer conditions were as described. The hybridization probes were 7 × 10^6 cpm of ^{32}P-labeled rat C/EBPβ cDNA probe and human IGF-I cDNA probe. Reverse transcription-PCR were performed using primers 5′-ATCAGCGTCTTCCAACCCAATTA-3′ and 5′-TGGCGCTGGGCACGGACAGA-3′ (for human IGF-I), and 5′-AAGGCCGGCTTCGCGGCGA-3′ and 5′-CCGGCCAGCCAGGTCCAGAC-3′ (for β-actin).

Western Blot Analysis—293T cell nuclear proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After the membranes were blocked with 5% nonfat dry milk and 2% fetal bovine serum in 20 mM Tris-Cl, pH 7.6, and 137 mM NaCl for 1 h at 25 °C, they were incubated with an antibody to HA for 1 h at 25 °C. Subsequent steps were performed as described elsewhere (5).

RESULTS

Human IGF-I Gene Promoter-1 Is Activated by Phorbol Ester—First we examined whether the major promoter (promoter-1) of the human IGF-I gene is a target of PKC activation. A series of gene transfer studies were performed with human hepatocellular carcinoma-derived HepG2 cells. Under the basal condition, HepG2 cells barely express the IGF-I gene according to reverse transcription-PCR results, but the IGF-I mRNA derived from the promoter 1 was induced when the cells were treated with 10^{-7} M TPA for 4 h (data not shown). Each reporter gene plasmid contained various lengths of IGF-I gene 5′-flanking sequences and 197 bp of the exon 1 untranslated

region linked to the firefly luciferase reporter because this portion of exon 1 untranslated region appeared to be important for basal promoter activity of promoter-1 in SK-N-MC cells (20).

As shown in Fig. 1, despite differences in the basal promoter activities, the promoter activities of the 1600 bp (pIGFI-1600), 600 bp (pIGFI-600), and 300 bp (pIGFI-300) were activated to a similar extent after treatment with TPA, about 2.5-fold. This result showed that the human IGF-I gene promoter-1 could be activated by TPA treatment of the cells and that the major portion of the cis-active elements mediating this phenomenon is located within the 300 bp of the 5′-flanking sequence and/or the 197 bp of the untranslated region of exon 1.

Identification of a Putative TPA-responsive Element—There was no region that perfectly matched the consensus AP-1 motif (T(G/T)AGTCA) within the region of the IGF-I gene which revealed the TPA responsiveness (−300 to ~+197). However, a region of high similarity to the AP-1 consensus was seen within the exon 1 untranslated region (+23 to ~+29; TTACTCA); indeed, the same sequence in the JE-1 gene was shown to be a target for TPA-responsive activation in MC3T3-E1 cells (21).

To find whether this portion is involved in the TPA responsiveness, we performed a mutation analysis. Because this portion was located within a region where multiple transcription initiation sites are clustered, it seemed possible that a mutation in this region could cause unpredictable, nonspecific damage to the promoter activity. To avoid this, we changed one sequence of the possible TPA-responsive region (A (+29) to G) so that the sequence became the same as the homologous regions of the chicken and rat IGF-I genes (22, 23).

As shown in Fig. 1, when one nucleotide mutation was introduced into the portion (pIGFI-300M), TPA-induced promoter activation was completely abolished, showing that this portion does play an essential role in mediating TPA effects on the IGF-I gene promoter. The mutated promoter also caused a decrease in the basal promoter activity (Fig. 1).

C/EBPβ Binds to the Putative TPA-responsive Region—Interestingly, the putative TPA-responsive region in the human IGF-I gene also contains the consensus for the C/EBP binding motif, CTTACTCAA. Indeed, Nolten *et al.* previously demonstrated *in vitro* that C/EBPα and C/EBPβ, when overexpressed, can bind to this region (24).

To characterize the factors involved in the TPA activation of the human IGF-I gene, we performed gel-mobility shift analyses. As shown in Fig. 2, TPA enhanced specific protein bindings to the putative TPA-responsive region (*lanes 1* and *5*). The unlabeled wild type competitor, but not the mutated competitor to which the same point mutation was introduced as in the reporter gene construct (A (+29) to G), inhibited the DNA-protein bindings (*lanes 6–11*). Also, when the mutation was introduced to the labeled probe, no binding was observed at all (*lane 12*). Thus, a certain factor or factors bind to the putative TPA-responsive region in the human IGF-I gene promoter in a TPA-responsive manner, and this mediates the TPA responsiveness of the gene transcription.

As mentioned above, the putative TPA-responsive region reveals similarity to the AP-1 motif but also is a potential C/EBP binding site. To identify the factor that mediates the TPA responsiveness by binding to the region, we next performed a gel mobility supershift assay using specific antibodies against C/EBPα, C/EBPβ, C/EBPδ, c-Fos, c-Jun, HNF-1α, and c-Myc, respectively. Each antibody was added to a tube prior to the binding reaction. As shown in Fig. 3, a supershifted band was observed together with a reduction of the gel-shift complex only when the C/EBPβ antibody was added (*lane 9*). Thus, the results clearly indicated that the TPA-responsive binding protein includes C/EBPβ, and the TPA-responsive region works as a C/EBP binding site.

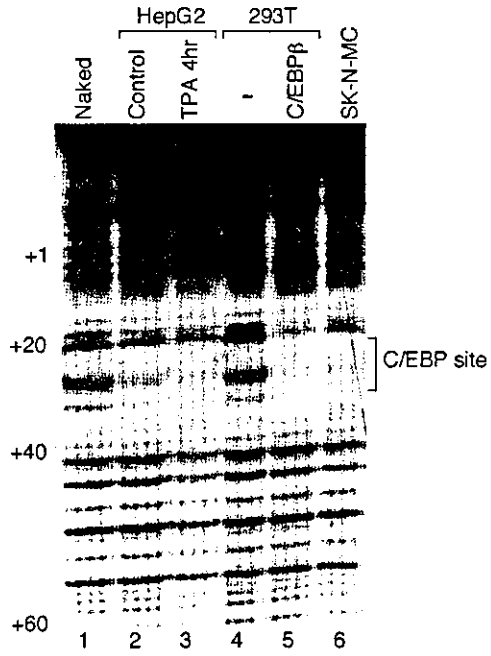

FIG. 4. **DNase footprinting assays confirm that the TPA inducible-binding protein is C/EBPβ and that the C/EBP site is protected in SK-N-MC cell nuclear extract.** DNase footprinting assays were performed as described under "Experimental Procedures" without (*lane 1*), with nuclear protein extracts isolated from HepG2 cell after incubation with vehicle (*lane 2*), with 100 nM TPA for 4 h (*lane 3*), with nuclear protein from 293T cells transfected with control vector (pcDNA3, *lane 4*), with C/EBPβ expression vector (pcDNA3C/EBPβ, *lane 5*), or with nuclear protein from SK-N-MC cells (*lane 6*). Similar results were obtained in three independent experiments.

Next, we performed a DNase footprinting assay with end-labeled double-stranded DNA probes derived from human IGF-I promoter-1 and nuclear extracts from HepG2 cell or C/EBPβ overexpressing 293T cells (Fig. 4). The results indicated that overexpressed C/EBPβ protected the C/EBP site from nuclease digestion (*lanes 4* and *5*). With the nuclear extract of HepG2 cell, the same site was protected (*lane 2*), and this protection was enhanced by 4 h of incubation with TPA (*lane 3*), confirming that C/EBPβ binds to the C/EBP site in a TPA-dependent manner in HepG2 cells. Thus, C/EBPβ binds to the C/EBP site in IGF-I gene promoter-1 and mediates the TPA effects on the IGF-I gene promoter.

C/EBPβ Transactivates IGF-I Gene Promoter-1—To investigate whether C/EBPβ that binds to the C/EBP site can activate the IGF-I gene transcription, we overexpressed C/EBPβ in 293T cells and evaluated the effects on the IGF-I gene promoter activity. 293T cells were chosen because they lack intrinsic expression of C/EBPβ (data not shown). As shown in Fig. 5, overexpression of C/EBPβ transactivates the IGF-I promoter-1 in a dose-dependent manner. When a point mutation was introduced to the C/EBP site, the transactivation effect of C/EBPβ disappeared, suggesting that C/EBPβ transactivates human IGF-I promoter-1 through the C/EBP site. Taken together, these results indicate that PKC activation induces human IGF-I gene transcription through enhancement of the C/EBPβ binding to promoter 1.

PKC Transcriptionally Activates C/EBPβ in HepG2 Cells—Next we investigated the mechanism that underlies the PKC-dependent activation of C/EBPβ. First, the effects of TPA on C/EBPβ mRNA were evaluated in HepG2 cells. The results of Northern blot analysis (Fig. 6) revealed that TPA stimulation

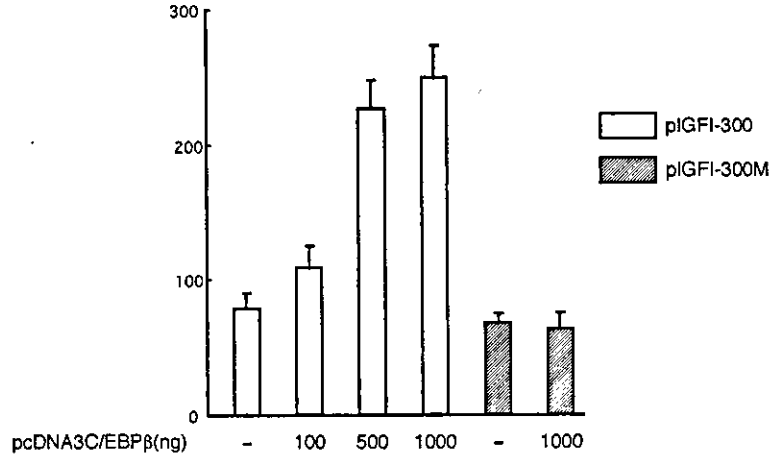

FIG. 5. **Overexpressed C/EBPβ transactivates the human IGF-I promoter-1 through the C/EBP site in 293T cells.** Wild type IGF-I promoter-1-luciferase reporter plasmid (pIGFI-300) or mutated type IGF-I promoter-1-luciferase reporter plasmid (pIGFI-300M) was cotransfected with 1 μg of control expression vector (indicated as −) or indicated amount of C/EBPβ expression vector (pcDNA3C/EBPβ) with a pMSV-β-galactosidase control vector into 293T cell using LipofectAMINE. After 48 h of incubation, cytoplasmic extracts were prepared, and luciferase activity was measured. Data were normalized for transfection efficiency (β-galactosidase expression) and presented as means ± S.D. of at least three independent experiments performed in duplicate.

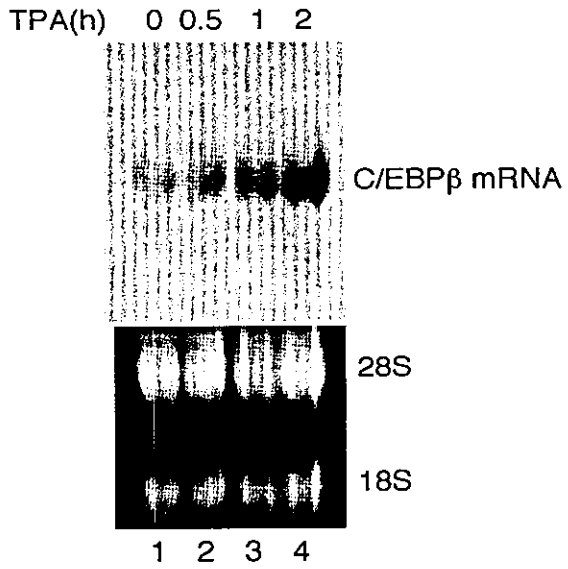

FIG. 6. **TPA stimulates C/EBPβ gene transcription in HepG2 cells.** Northern blotting analyses were performed with 10 μg of total RNA isolated from HepG2 cells after incubation with 100 nM of TPA for the times indicated. The probe was ^{32}P-labeled rat C/EBPβ probe. Similar results were obtained in three independent experiments.

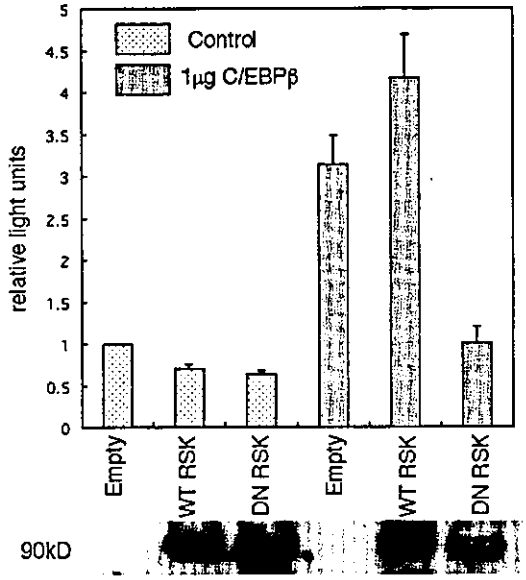

FIG. 7. **Overexpressed dominant negative type RSK inhibits C/EBPβ transactivation of IGF-I promoter-1.** *Upper panel*, IGF-I promoter-1-luciferase reporter plasmids (pIGFI-300) were cotransfected with 1 μg of control expression vector (pcDNA3, *lanes 1–3*) or 1 μg of C/EBPβ expression vector (pcDNA3C/EBPβ, *lanes 4–6*), and 1 mg of another control expression vector (pKH3. *lanes 1, 4*) or 1 μg of HA tagged wild type RSK expression vector (WT RSK, *lanes 2, 5*) or HA tagged dominant negative type RSK expression vector (DN RSK, *lanes 3, 6*), and *Renilla* luciferase (pRLCMV) control vector into 293T cell using LipofectAMINE. After 48 h of incubation, cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency (*Renilla* luciferase expression) and presented as means ± S.D. of at least three independent experiments performed in duplicate. *Lower panel*, Western blotting with cytoplasmic extracts using anti-HA antibody was performed.

increases the C/EBPβ mRNA amount by ~3-fold, suggesting that PKC can stimulate C/EBPβ gene transcription, and this may in part explain the PKC activation of the human IGF gene promoter.

PKC Posttranslationally Activates C/EBPβ via Activation of RSK—Recently, RSK was shown to stimulate C/EBPβ activity, and this facilitates TGFβ-induced hepatocyte proliferation (25). Because RSK is known to be a downstream target of PKC, we investigated the possibility of RSK also being involved in the PKC-dependent activation of C/EBPβ and IGF-I gene activation. For this purpose, we used a DN mutant of RSK.

As shown in Fig. 7, the DN RSK mutant, when co-overexpressed in the 293T cells with C/EBPβ, significantly suppressed the transactivation potential of C/EBPβ in terms of the activation of the human IGF-I gene promoter. This effect was not observed when C/EBPβ was absent (Fig. 7). These results suggest that the transactivation potential of C/EBPβ depends on the RSK activity in 293T cells.

To further clarify the mechanism underlying this RSK-dependent activation of C/EBPβ, we employed the *Saccharomyces cerevisiae* GAL4 fusion protein reporter system (Fig 8). It is known that the N-terminal region of C/EBPβ contains a transcription activation domain and a transrepression domain (Fig. 8*b*, (26)). It also includes a Ser residue at 105, which was previously shown to be critical for the C/EBPβ activation by TPA (27) and was also identified recently as the phosphorylated site by RSK (25).

Accordingly, we prepared GAL4 fusion constructs containing either 118 or 166 amino acids (aa) of N-terminal region of C/EBPβ fused to the heterologous DNA-binding domain of the GAL4 transcription factor (Fig. 8*b*). These chimeric GAL4-C/EBPβ fusion proteins were expressed in HepG2 cells, and effects on the GAL4 reporter were evaluated. HepG2 cells were used for this experiment because they show a very good response to TPA and have intrinsic C/EBPβ.

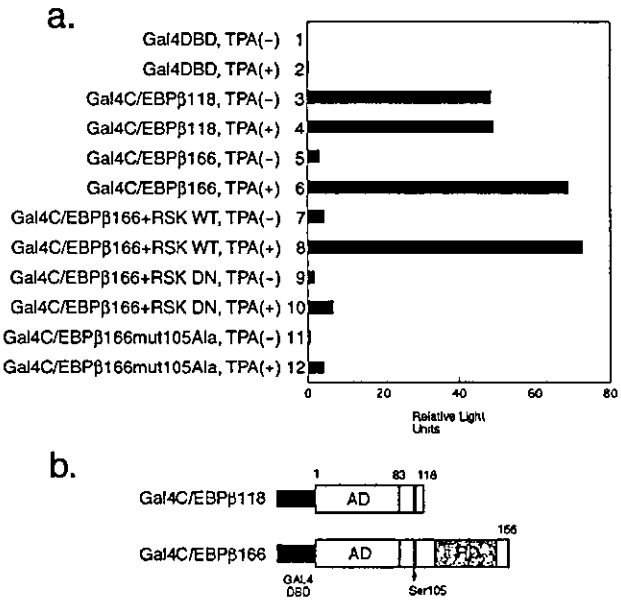

FIG. 8. **TPA-induced activation function of C/EBPβ depends on RSK.** The *bar graph* (*a*) depicts the transactivation potential of GAL4-C/EBPβ chimeras. The fusion proteins were obtained by fusing the DNA-binding domain of GAL4 (aa 1–147) to the 118-aa (Gal4C/EBPβ118) or 166-aa (Gal4C/EBPβ166) N-terminal region of rat C/EBPβ (*b*). A single aa substitution (Ser-105 to Ala) was introduced to Gal4C/EBPβ166 plasmid to produce Gal4C/EBPβ166mut105Ala plasmid. One microgram of each GAL4-C/EBPβ fusion plasmid was cotransfected into HepG2 cells with 1 μg of Gal4-responsive reporter plasmid (pFR-Luc) and 5 ng of an internal control, pRL-CMV. Where indicated, 1 μg of wild type or dominant negative type RSK expression plasmid was also cotransfected. After transfection, cells were incubated for 24 h, and then 10^{-7} M TPA or vehicle was added. After another 24-h incubation, dual-luciferase assays were performed. The firefly luciferase results were normalized with respect to transfection efficiency assessed by *Renilla* luciferase results. The data were presented as means of at least three independent experiments performed in duplicate. *AD*, activation domain. *RD*, repression domain.

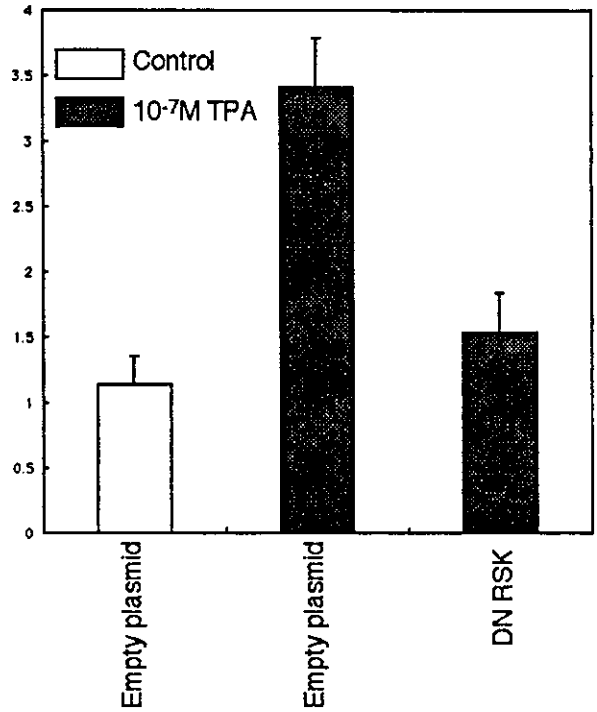

FIG. 9. **Overexpressed dominant negative type RSK inhibits TPA stimulation of IGF-I promoter activity.** IGF-I promoter-1-luciferase reporter plasmids (pIGFI-300) were cotransfected with 1 mg of control expression vector or 1 mg of dominant negative type RSK expression vector as indicated and *Renilla* luciferase (pRLCMV) control plasmid into 293T cell using LipofectAMINE. After 24 h of incubation, cultures were exposed to control medium (containing vehicle) or TPA (100 nM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency assessed by *Renilla* luciferase results and presented as means ± S.D. of at least three independent experiments performed in duplicate.

As shown in Fig. 8*a* (*lane 3*), the Gal4C/EBPβ118 construct transactivated the GAL4 reporter in serum-free medium. On the other hand, the Gal4C/EBPβ166 construct did not activate the GAL4 reporter in the basal state (*lane 5*). This observation is consistent with former report by Williams *et al.* (26) and provides further support for the idea that the transrepression domain was potent enough to almost totally suppress the transactivation potential. Unlike the Gal4C/EBPβ118 construct revealing no further activation by TPA (Fig. 8*a*, *lanes 3* and *4*), the Gal4C/EBPβ166 construct was converted to a transcriptional activator by the TPA treatment (*lanes 5* and *6*). This clearly indicated that TPA activates C/EBPβ by inhibiting the activity of transrepression domain rather than activating the transactivating domain.

In support of the implication of RSK, overexpression of DN RSK mutant abolished the TPA-responsive activation of Gal4C/EBPβ166 (Fig. 8*a*, *lanes 9* and *10*). Moreover, when the previously identified phosphorylation site for RSK within C/EBPβ (Ser-105; Ref. 25) was disrupted (substituted by Ala) in the GAL4 fusion protein (Gal4C/EBPβ166mut105Ala), it could not transactivate the GAL4 reporter in response to the TPA stimulation (*lanes 11* and *12*). These observations thus suggest that TPA stimulates activation function of C/EBPβ via RSK activation followed by the phosphorylation of Ser-105 of C/EBPβ.

We next investigated the implication of the RSK-C/EBPβ axis in the IGF-I gene regulation. As shown in Fig. 9, the coexpression of DN RSK mutant in TPA-stimulated HepG2 cells suppressed the promoter activity of the IGF-I gene in the cells. This agrees with the idea that RSK plays a primary role in mediating the TPA-dependent activation of C/EBPβ and, subsequently, the activation of the IGF-I gene transcription. Taken together, these data demonstrated that PKC activates C/EBPβ both at the level of its transcription (Fig. 6) and of its posttranslation, which is mediated by the activation of RSK (Figs. 7–9).

IGF-I Gene Expression in SK-N-MC Cells Depends on PKC-dependent, Transcriptional, and Posttranslational Regulation of C/EBPβ—SK-N-MC is a human neuroblastoma cell line that displays constitutive expression of the IGF-I gene (22). It was previously shown that IGF-I mRNAs expressed in SK-N-MC cells contained exon 1 sequences but lacked exon 2 sequences and that promoter-1, but not promoter-2, was active in the cells (28). As support for physiological implication of the PKC-dependent pathway in the IGF-I gene regulation, we found that the IGF-I mRNA amount was significantly decreased in SK-N-MC cells when the cells were incubated for 24 h in the presence of the PKC inhibitor staurosporine (Fig. 10).

To clarify the factors involved in the intrinsic IGF-I gene expression in SK-N-MC cells, we investigated whether a certain nuclear factor binds to the C/EBP site of the human IGF-I gene in SK-N-MC cells. According to results of gel mobility shift assays (Fig. 11), there was a nuclear protein that specifically binds to the C/EBP site. This agreed with the results of the DNase footprinting assay shown in Fig. 4 (*lane 6*). A group of specific antibodies were added to identify the binding protein, and the results clearly demonstrated that only C/EBPβ occupies the C/EBP site in SK-N-MC cells (Fig. 11; *lanes 8–14*).

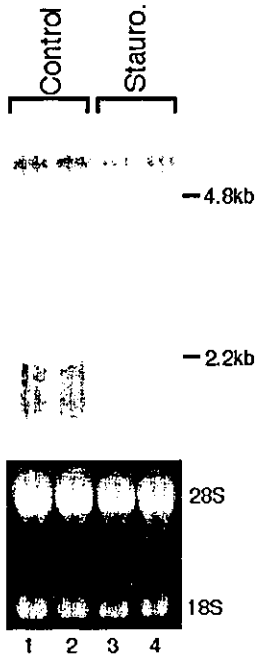

FIG. 10. **PKC inhibitor decreases IGF-I mRNA in SK-N-MC cells.** Northern blotting analyses were performed with 10 μg of total RNA isolated from SK-N-MC cells after incubation with vehicle (*lanes 1, 2*) or 100 nM of staurosporine (*lanes 3, 4*) for 24 h. The probe was ^{32}P-labeled human IGF-I cDNA probe. Similar results were obtained in three independent experiments performed in duplicate.

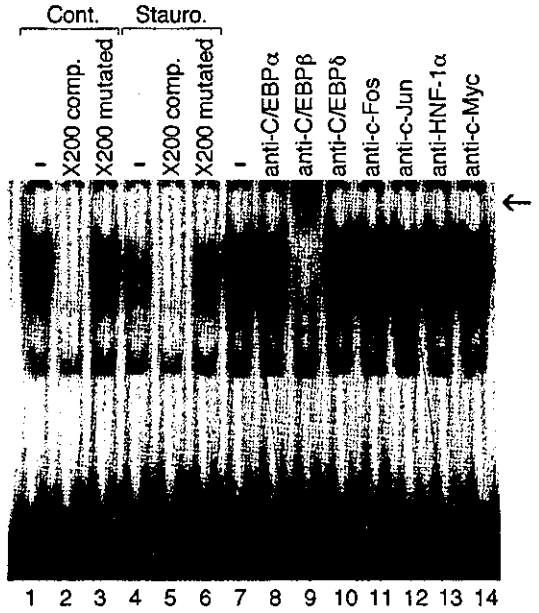

FIG. 11. **C/EBPβ binds to the C/EBP site of IGF-I promoter-1 in SK-N-MC cells in a PKC-dependent manner.** Gel mobility shift experiments were performed as described under "Experimental Procedures" with nuclear protein extracts isolated from SK-N-MC cell after incubation with vehicle (*lanes 1–3, 7–14*) or 100 nM TPA for 4 h. ^{32}P-labeled wild type probe, whose sequence is shown in Fig. 2, was used in all lanes. Unlabeled 200-fold molar excess wild type competitor (*lanes 2, 5*) or mutated type competitor (*lanes 3, 6*) was added to the binding. Specific antibodies (1 μg) to C/EBPα (*lane 8*), C/EBPβ (*lane 9*), C/EBPδ (*lane 10*), c-Fos (*lane 11*), c-Jun (*lane 12*), HNF-1α (*lane 13*), or c-Myc (*lane 14*) was added to binding reaction as described under "Experimental Procedures". The *arrow* shows the supershift band that appeared only when a specific antibody to C/EBPβ was added. Similar results were obtained in three independent experiments.

Because the intensity of the retarded band (C/EBPβ) was weakened when a PKC inhibitor staurosporine was added to the cells (*lanes 4–6*), we concluded that PKC, which seems readily activated even in the basal state (without any stimulation) in SK-N-MC cells, is involved in the activation of the nuclear protein binding to the C/EBP site in these cells.

We next sought to determine whether this PKC-dependent binding of C/EBPβ to the C/EBP site is required for the human IGF-I gene expression in SK-N-MC cells. Results of reporter gene analyses revealed that incubation of SK-N-MC cells with a specific PKC inhibitor GF109203X decreased the basal promoter activity of 1600-bp (pIGFI-1600), 600-bp (pIGFI-600), and 300-bp (pIGFI-300) human IGF-I promoter-1 by more than 50% (Fig. 12). This suppressive effect of the PKC inhibitor was no longer observed with pIGFI-300M in which the C/EBP site was disrupted (Fig. 12), suggesting that intrinsically activated PKC enhances the C/EBPβ-binding to the C/EBP site and thus is responsible for the human IGF-I gene transcription.

To investigate whether both transcriptional and posttranscriptional regulation of C/EBPβ by PKC is involved in IGF-I gene expression in SK-N-MC cells, we performed Northern blot analyses and GAL4 fusion protein reporter analyses. As shown in Fig. 13*A*, the amount of C/EBPβ mRNA was decreased in SK-N-MC cells after a 24-h incubation with staurosporine, suggesting that the transcriptional control of C/EBPβ by intrinsically activated PKC can in part be involved in the regulation of IGF-I gene expression in SK-N-MC cells.

On the other hand, the GAL4 fusion protein reporter assays in SK-N-MC cells revealed that the Gal4C/EBPβ166 construct transactivated the GAL4 reporter in SK-N-MC cells kept under the basal condition (Fig. 13*B*, *lane 2*). This contrasted with the observation obtained with HepG2 cells, in which TPA was required for the transactivation potential activated (Fig. 8, *lanes 5* and *6*). The transactivation potential was dramatically suppressed by addition of PKC inhibitor GF10923X (Fig. 13*B*, *lane 3*), suggesting that intrinsically activated PKC readily activated C/EBPβ in SK-N-MC cells even without any stimuli. Substitution of Ser-105 by Ala (Gal4C/EBPβ166mut105Ala) or overexpression of the DN RSK mutant also suppressed the activation function of C/EBPβ (*lane 4-6*). Thus it was demonstrated that the IGF-I gene expression in SK-N-MC cells depends on the intrinsically activated PKC, which is involved in the activation of C/EBPβ probably through two different mechanisms: the increment of its transcription and the RSK-mediated posttranslational activation of its transactivation potential.

DISCUSSION

In the present study, we showed that the major promoter of the human IGF-I gene is a target of TPA stimulation (Fig. 1) and that a C/EBP site within the promoter is responsible for the phenomenon (Figs. 2–5). C/EBPβ, which is activated by PKC both at the level of transcription (Figs. 6 and 13*a*) and of posttranslation (Figs. 8 and 13*b*), binds to the C/EBP site and thereby mediates the phenomenon. In non-C/EBPβ-expressing 293T cells, transiently expressed ectopic C/EBPβ transactivated the human IGF-I gene promoter (Figs. 5 and 7) and indeed induced intrinsic IGF-I gene expression in the cells,[2] providing support for the physiological implication of C/EBPβ in the IGF-I gene expression.

The C/EBP family proteins comprise a diverse group of transcriptional regulators active in tissue development and regeneration, inflammation, and intermediary metabolism (29, 30). They are members of the basic leucine zipper family of transcription factors (29–31) and also reveal strong amino acid similarity in

[2] Y. Umayahara and Y. Kajimoto, unpublished observation.

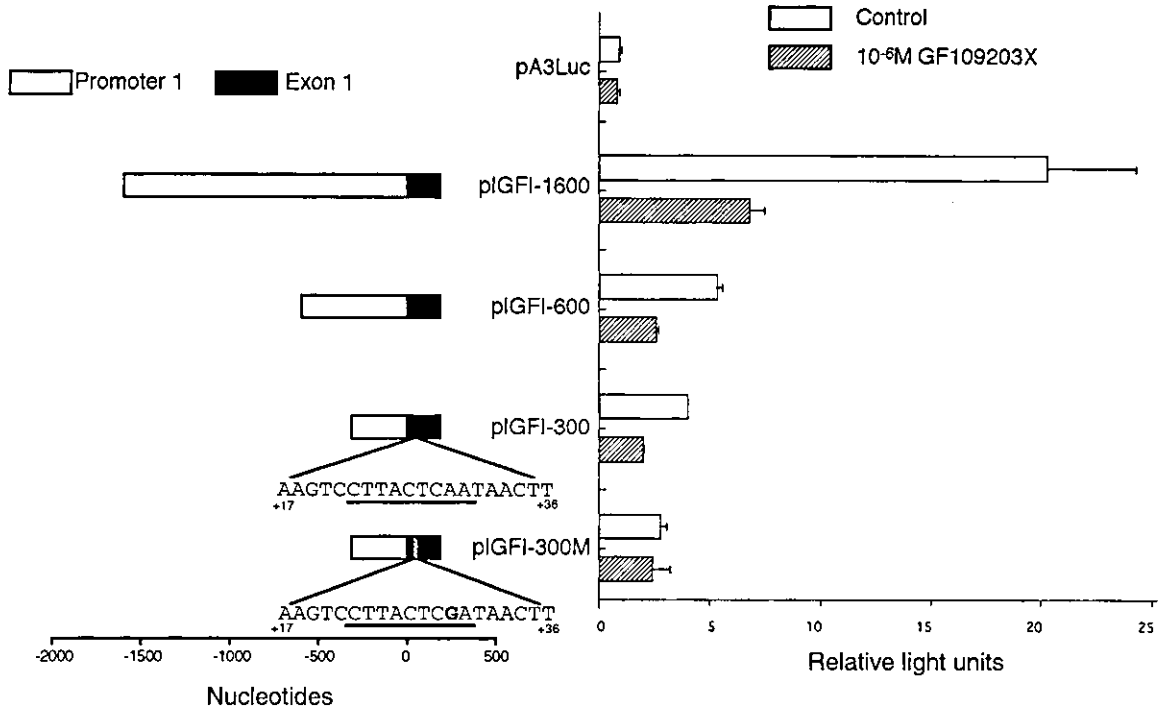

FIG. 12. **PKC inhibitor decreases basal promoter activity of human IGF-I promoter-1 in SK-N-MC cells.** *Left panel*, diagrammatic representation of IGF-I promoter-1-luciferase reporter plasmids. The nucleotide sequence is shown for the region containing the C/EBP site (*underlined*). pIGFI-300M has a point mutation, and the altered base is in *bold*. *Right panel*, IGF-I promoter-1-luciferase reporter plasmids were cotransfected with a *Renilla* luciferase control plasmid into SK-N-MC cell using LipofectAMINE. After 24-h of incubation, cultures were exposed to control medium (containing vehicle) or GF109203X (1 μM) for 24 h. Cytoplasmic extracts were prepared, and luciferase activity was determined. Data were normalized for transfection efficiency assessed by *Renilla* luciferase results and presented as means ± S.D. of at least three independent experiments performed in duplicate.

their COOH domains, which have been shown to be responsible for protein dimerization and DNA binding (29, 30).

Nolten *et al.* (24) previously reported that overexpressed C/EBPα and C/EBPβ can bind to the region that we found to be the putative TPA-responsive region and can stimulate the promoter activity of human IGF-I promoter-1. Our data showed that, at least in the case of HepG2 and SK-N-MC cells, C/EBPβ is the major binding factor for the C/EBP site and that no other C/EBP family proteins such as C/EBPα and C/EBPδ are included in the binding complexes formed with the C/EBP site of the human IGF-I gene promoter. Whereas the C/EBP family proteins share a common binding preference, this may be because the C/EBP family proteins other than C/EBPβ are not expressed in those cells or because they are inactive even when expressed.

The human neuroblastoma cell line SK-N-MC reveals relatively high expression of IGF-I under the basal condition; the amount of IGF-I mRNA was even more than that expressed in the human liver (22). The fact that the addition of neutralized antibody to IGF-IR prevents the cell from proliferating (14) suggests that the IGF-I autocrine loop is essential for the tumor phenotype of SK-N-MC cells. As a step toward elucidating the mechanism underlying the constitutive expression of the IGF-I gene in SK-N-MC cells, we sought to clarify the physiological role of the C/EBP site in the IGF-I gene expression. The results of the DNase footprinting assay (Fig. 4) and supershift assay (Fig. 11) clearly showed that C/EBPβ binds to the C/EBP site in SK-N-MC cells under basal condition, and reporter gene analysis (Fig. 12) showed that the C/EBPβ binding to the C/EBP site is crucial for strong activity of the promoter-1 in SK-N-MC cells. Support for this comes from the observation by Mittanck *et al.* (19) showing that the initial 50 base pairs of the exon 1 untranslated region are essential for the promoter-1 of human IGF-I gene being active in the SK-N-MC cell line; although, for some unknown reason, they failed to detect any protein binding with a probe containing the C/EBP site.

The fact that the mutated human C/EBP site we used in this study, whose sequence completely matches the sequence of the corresponding region of rat IGF-I promoter-1, could not be bound by C/EBPβ clearly showed that this C/EBP site is not conserved in the rat or mouse IGF-I gene. However, we previously identified another high affinity C/EBP site, termed the HS3D site, in rat IGF-I promoter-1 located 5′-untranslated region of exon 1 (4–6). As support for the extensive implication of C/EBP family proteins in IGF-I gene regulation, another C/EBP family protein, C/EBPδ, was shown to mediate cAMP responsiveness of rat IGF-I promoter-1 through this site (5, 6). Considering the conserved binding preference for the C/EBP family proteins, it is possible that C/EBP family proteins are involved in various regulation of the IGF-I gene through the same C/EBP binding site with their activity being posttranslationally modulated by various kinases.

A serine/threonine kinase RSK has been shown to function as a downstream target of PKC (32, 33). Tan *et al.* (33) showed that RSK mediates the PKC-dependent prevention of Bad-mediated apoptosis by directly phosphorylating Bad protein. In terms of the substrates of RSK, only a few have been identified to date including transcription factors cAMP-response element-binding protein and Fos (34, 35). Recently, C/EBPβ was added to the list of direct substrates of RSK; activated RSK phosphorylates Ser-105 of C/EBPβ, and this activation is critical for hepatocyte proliferation (25). These reports prompted us to consider that RSK may mediate the present phenomenon; using a dominant negative mutant of RSK, we were able to show that RSK plays a key role in the PKC-dependent, C/EBPβ-mediated activation of the IGF-I gene promoter (Figs. 7–9).

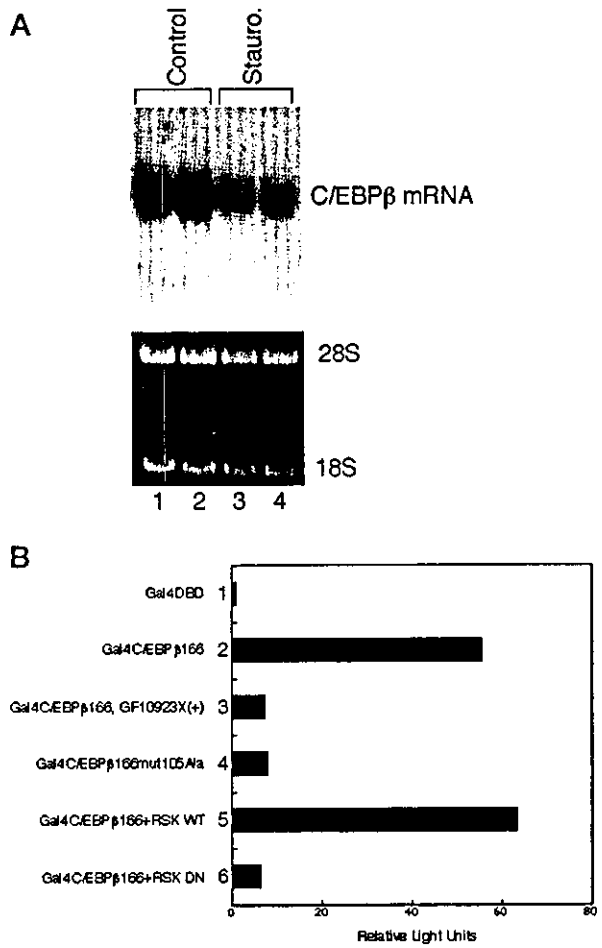

FIG. 13. **Dual control of C/EBPβ activity by intrinsically activated PKC in SK-N-MC cells.** *A*, PKC inhibitor decreases C/EBPβ mRNA in SK-N-MC cells. Northern blotting analyses were performed using ^{32}P-labeled rat C/EBPβ cDNA as a probe. Ten micrograms of total RNA isolated from SK-N-MC cells after incubation with vehicle (*lanes 1, 2*) or 100 nM of staurosporine (*lanes 3, 4*) for 24 h were loaded. Similar results were obtained in three independent experiments performed in duplicate. *B*, intrinsically activated PKC stimulates the activation function of C/EBPβ through RSK activation. The *bar graph* depicts the transactivation potential of GAL4-C/EBPβ chimeras. The fusion protein (Gal4C/EBPβ166) was obtained by fusing the DNA-binding domain (*DBD*) of GAL4 (aa 1–147) to the 166-aa N-terminal region of rat C/EBPβ. A single aa substitution (Ser-105 to Ala) was introduced to Gal4C/EBPβ166 plasmid to produce Gal4C/EBPβ166mut105Ala plasmid. One microgram of each GAL4-C/EBPβ fusion plasmid was cotransfected into SK-N-MC cells with 1 μg of Gal4-responsive reporter plasmid (pFR-Luc) and 5 ng of an internal control, pRL-CMV. Where indicated, 1 μg of wild type (*RSK-WT*) or dominant negative type RSK (*RSK-DN*) expression plasmid was also cotransfected. After transfection, cells were incubated in the basal condition medium for 24 h, and then 1 μM GF109203X or vehicle was added. After another 24-h incubation, dual-luciferase assays were performed. The firefly luciferase results were normalized with respect to transfection efficiency assessed by *Renilla* luciferase results. The data were presented as means of at least three independent experiments performed in duplicate.

Moreover, the data obtained with *S. cerevisiae* GAL4 fusion protein reporter system revealed that the TPA-responsive activation of the GAL4-C/EBPβ chimera depends on a Ser residue at position 105 (Ser-105) of C/EBPβ (Fig. 8). Because Ser-105 is known as a direct phosphorylation site by RSK (25), it is likely that the TPA-induced transactivation of C/EBPβ is also mediated by direct phosphorylation/posttranslational activation by RSK.

Although PKC seems to act as an essential upstream factor for the RSK-dependent, C/EBPβ-mediated IGF-I gene activation in SK-N-MC cells, this may not be the case for all cell types. For example, despite the implication of RSK activation in the C/EBPβ-mediated IGF-I gene activation in 293T cells (Fig. 7), there has been no evidence reported to date that supports the constitutive PKC activation in the cells. Rather, according to a report by Arould *et al.* (36), they directly measured PKC activity in 293T cells but detected no significant PKC activity, thus disputing the implication of PKC in the RSK activation in 293T cells. Although speculative, mitogen-activated protein kinases (MAPK), instead of PKC, may play a key role in the RSK activation in 293T cells. It was shown previously that MAPK can directly activate RSK (37). Also, the 293T cell was transformed with SV40 large T antigen and E1A, both of which are well known activators for the MAPK cascade (38). Indeed, 10% serum stimulation was shown to cause a partial nuclear translocation of MEK in 293T cells (39). Because our 293T cells were always maintained in serum-containing (10%) medium, it is possible that the MAPK cascade was kept activated in the cells and thereby involved in the RSK activation. Thus, the RSK-C/EBPβ-IGF-I axis, which is probably activated not only by PKC but also by some growth factors that can activate MAPK, may operate in various cell types and contribute to the expression of the IGF-I gene in those cells.

Apart from this RSK-mediated effect on the activation function of C/EBPβ, our present study failed to clarify whether the TPA treatment enhances the DNA-binding affinity of C/EBPβ. Although we found that TPA stimulation enhanced the C/EBPβ binding to its target DNA in HepG2 cells (Figs. 2 and 3), this does not necessarily mean that the DNA-binding affinity was increased in response to TPA as the increment of the C/EBPβ gene expression can by itself explain the phenomenon (Fig. 6). Indeed, Trautwein *et al.* (27) previously reported that TPA stimulates transactivation potential of C/EBPβ without changing its DNA-binding affinity in HepG2 cells. Thus we assume that the RSK-mediated regulation of C/EBPβ activity takes place probably independent of controlling its DNA-binding affinity.

IGF-I is known to function as an autocrine or paracrine growth factor in a variety of mesenchymal and epithelial tumors (40). Recent studies have demonstrated that expression of the type-I insulin-like growth factor receptor (IGF-IR), which mediates most of the IGF-I action, is required for the establishment and maintenance of the transformed phenotype in some cell lines (13). A gene-targeting study revealed that cells derived from IGF-IR(−/−) mouse embryos cannot be transformed by the SV40 large T antigen or by an activated and overexpressed Ha-Ras, while stable transformation of cells with human IGF-IR expression plasmid restored the ability to be transformed (13). These findings suggest that the IGF system (including IGF-I and -II and IGF-IR) plays an essential role in, at least, some cases of transformation or tumorigenesis. On the other hand, it is well known that the PKC activator TPA is a strong carcinogen. Because the IGF-I gene expressed in SK-N-MC cells was shown to play a major role in the proliferation of SK-N-MC, it is likely that the constitutive active PKC subtypes contribute toward keeping the IGF system active and thereby maintaining the tumor phenotype of the cells.

In conclusion, our present study revealed that PKC can activate the IGF-I gene expression through the RSK and C/EBPβ-mediated pathway. Whereas the IGF-I system is essential for proliferation of SK-N-MC cells (14), constitutively active PKC may contribute to determining tumor phenotype in some cancer cells by keeping the IGF-I gene expression active.

Acknowledgments—We thank Dr. Peter Rotwein of Washington University School of Medicine for the human IGF-I cDNA, Drs. Ian H. Maxwell and William M. Wood of University of Colorado Health Science Center for the pA3Luc plasmid, Dr. John Blenis of Harvard Medical School for the wild type and dominant negative type RSK expression