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Ingested Medium-Chain Fatty Acids Are Directly Utilized for the Acyl Modification of Ghrelin

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Ghrelin, an acylated brain and gut peptide, is primarily produced by endocrine cells of the gastric mucosa for secretion into the circulation. The major active form of ghrelin is a 28-amino-acid peptide containing an *n*-octanoyl modification at serine that is essential for activity. Studies have identified multiple physiological functions for ghrelin, including GH release, appetite stimulation, and metabolic fuel preference. Until now, there has not been any report detailing the mechanism of ghrelin acyl modification. Here we report that ingestion of either medium-chain fatty acids (MCFAs) or medium-chain triacylglycerols (MCTs) increased the stomach concentrations of acylated ghrelin without changing the total (acyl- and des-acyl-) ghrelin amounts. After ingestion of either MCFAs or MCTs, the carbon chain lengths of the acyl groups attached to nascent ghrelin molecules corresponded to that of the ingested MCFAs or MCTs. Ghrelin peptides modified with *n*-butyryl or *n*-palmitoyl groups, however, could not be detected after ingestion of the corresponding short-chain or long-chain fatty acids, respectively. Moreover, *n*-heptanoyl ghrelin, an unnatural form of ghrelin, could be detected in the stomach of mice after ingestion of either *n*-heptanoic acid or glyceryl triheptanoate. These findings indicate that ingested medium-chain fatty acids are directly used for the acylation of ghrelin. (*Endocrinology* 146: 2255–2264, 2005)

GHRELIN WAS DISCOVERED by our group as an endogenous ligand for the receptor for GH secretagogues, synthetic and unnatural substances with potent GH-releasing activities (1). Whereas initially purified from the stomach, ghrelin is also expressed within the brain, lung, kidney, pancreas, small intestine, and large intestine (2–7). In addition to potent GH-releasing activity (1, 8–10), ghrelin also stimulates appetite, induces adiposity (11–14), improves cardiac function (15–17), and influences metabolic fuel preference (18).

The third amino acid residue of ghrelin, serine (Ser^3), is modified by an acyl group; this modification is essential for ghrelin biological activity (1). Whereas the primary acyl chain-modifying ghrelin molecules in humans and rodents are an *n*-octanoyl group (C8:0, an eight-carbon chain containing no double bonds) (1, 19), additional acyl modifications create a minor population of ghrelin peptides. These acyl groups include *n*-decanoyl (C10:0, a 10-carbon chain lacking double bonds) and *n*-decanoyl (C10:1, a 10-carbon chain containing one double bond) (20–22). Our examination of a variety of synthetic acyl-modified ghrelin peptides determined that the potency of ghrelin biological activity was altered by different modifying acyl groups (23).

To our knowledge, the acyl modification of ghrelin is the first example of the fatty acid modification of a peptide hormone; acylation of a serine hydroxyl group has not been previously reported as a mammalian peptide hormone modification. The enzyme catalyzing the transfer of acyl groups to ghrelin Ser^3, likely a novel acyltransferase, will be important in the regulation of ghrelin production. The nature of this enzyme, however, remains unknown.

We report here that ingested medium-chain fatty acids (MCFAs) and medium-chain triglycerides serve as a source of fatty acids in the acyl modification of ghrelin. Ingestion of MCFAs (*n*-hexanoic, *n*-octanoic, and *n*-decanoic acid) or medium-chain triglycerides (glyceryl trihexanoate, glyceryl trioctanoate, and glyceryl tridecanoate) increased the stomach concentrations of ghrelin bearing an acyl group with the corresponding carbon chain length, *i.e.* *n*-hexanoyl ghrelin, *n*-octanoyl ghrelin, and *n*-decanoyl ghrelin. Ingestion of such lipids, however, did not significantly alter total ghrelin (acyl-modified and des-acyl ghrelin with an intact C-terminal peptide sequence) production. Ingestion by mice of glyceryl triheptanoate, which cannot be naturally synthesized by mammalian cells, resulted in the production of an unnatural ghrelin form incorporating an *n*-heptanoyl modification. These findings indicate that ingested MCFAs and medium-chain triglycerides are likely the direct source of fatty acids destined for acyl modification of ghrelin.

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Abbreviations: AcOH, Acetic acid; $[Ca^{2+}]_i$, intracellular-free calcium concentration; CH_3CN, acetonitrile; C-RIA, radioimmunoassay to C-terminal fragment of ghrelin(13–28); C18 RP-HPLC, reverse-phase HPLC with C18-cartridge; FFA, free fatty acid; GHS-R, GH ghrelin receptor; HF, high-fat (diet); LCT, long-chain triglyceride; -LI, -like immunoreactivity; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCFA, medium-chain fatty acid; MCT, medium-chain triglyceride; N-RIA, radioimmunoassay to N-terminal fragment of *n*-octanoyl ghrelin(1–11); Ser^3, serine; TFA, trifluoroacetic acid.

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Materials and Methods

Animals

Male C57BL/6J mice weighing 20–25 g were used in these experiments. Animals were maintained under controlled temperature (21–23 C) and light conditions (light on 0700–1900 h) with *ad libitum* access to food and water. All experiments were conducted in accordance with the Kurume University Guide for the Care and Use of Experimental Animals.

RIA of ghrelin

RIAs specific for ghrelin were performed as previously described (2). Rabbit polyclonal antibodies were raised against the N terminal [(Gly^1-Lys^{11}) with *O*-*n*-octanoylation at Ser^3] and C-terminal (Gln^{13}-Arg^{28}) fragments of rat ghrelin. RIA incubation mixtures contained 100 μl of either standard ghrelin or an unknown sample with 200 μl of antiserum diluted in RIA buffer [50 mM sodium phosphate buffer (pH 7.4), 0.5% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na, and 0.05% NaN_3] containing 0.5% normal rabbit serum. Antirat ghrelin (1–11) and antirat ghrelin(13–28) antisera were used at final dilutions of 1:3 million and 1:20,000, respectively. After a 12-h incubation at 4 C, 100 μl ^{125}I-labeled ligand (20,000 cpm) was added for an additional 36-h incubation. Then samples were incubated for 24 h at 4 C with 100 μl of antirabbit goat antibody. Free and bound tracers were then separated by centrifugation at 3000 rpm for 30 min. Pellet radioactivity was quantified in a γ-counter (ARC-600, Aloka, Tokyo, Japan). All assays were performed in duplicate.

Both antisera exhibited complete cross-reactivity with human, mouse, and rat ghrelin forms (2). The antirat ghrelin(1–11) antiserum, which specifically recognizes the *n*-octanoylated portion of ghrelin, exhibited 100% cross-reactivity with rat, mouse, and human *n*-octanoyl ghrelin but does not recognize des-acyl ghrelin. The cross-reactivity of N-terminal RIA for *n*-decanoyl and *n*-decanoyl ghrelin was approximately 20 and 25%, respectively. Cross-reactivity to *n*-butyryl, *n*-hexanoyl, *n*-lauryl, and *n*-palmitoyl ghrelin was less than 5%. Antirat ghrelin(13–28) antiserum equally recognizes both des-acyl and all acylated forms of ghrelin peptide including *n*-octanoyl, *n*-decanoyl, or *n*-decanoyl ghrelin (2). The ED_{50} for ghrelin C-terminal and N-terminal RIAs were approximately 32 and 8 fmol/tube, respectively. The minimal detection levels by C-terminal and N-terminal RIAs were 1.0 and 0.25 fmol/tube, respectively. The intraassay coefficients of variation of C-terminal and N-terminal RIAs were 6.0 and 3.0%, respectively. The interassay coefficients of variation were 7.0 and 5.0%, respectively. All samples measured by ghrelin assay were diluted in RIA buffer to fit the range of measurement (between ED_{20} to ED_{80}) for each RIA. Throughout the following sections, the RIA system using the antiserum raised against the N-terminal fragment of rat ghrelin(1–11) is termed N-RIA, whereas the RIA system using the antiserum recognizing the C-terminal fragment(13–28) is termed C-RIA. Ghrelin-like immunoreactivity (-LI) measured by C-RIA is termed ghrelin C-LI, whereas that measured by ghrelin N-RIA is termed ghrelin N-LI.

Calcium mobilization assays of ghrelin

CHO-GHSR62 cells (1) stably expressing rat ghrelin receptor (GHS-R) were plated for 12–15 h in flat-bottom black-walled 96-well plates (Corning Costar Corp., Cambridge, MA) at 4×10^4 cells/well. Cells were then preincubated for 1 h with 4 μM Fluo-4-AM fluorescent indicator dye (Molecular Probes, Inc., Eugene, OR) dissolved in assay buffer [Hanks' balanced salts solution, 10 mM HEPES, and 2.5 mM probenecid] supplemented with 1% fetal calf serum. After washing four times in assay buffer, samples were dissolved in 100 μl assay buffer with 0.01% BSA and added to the prepared cells. We then measured intracellular calcium concentration changes using a FLEX station (Molecular Devices, Sunnyvale, CA).

Preparation of stomach samples for ghrelin assay

All stomach samples, with the exception of those obtained at the 0 h point in the time-course study, were collected during a fed state. After collection from mice, stomachs were washed twice in PBS (pH 7.4). After measuring the wet weight of each sample, whole stomach tissue was diced and boiled for 5 min in a 10-fold volume of water to inactivate intrinsic proteases. After cooling, boiled samples were adjusted to 1 M acetic acid (AcOH)/20 mM HCl. After homogenization with a polytron mixer (PT 6100, Kinematica AG, Littan-Luzern, Switzerland), peptides were extracted and isolated by a 15-min centrifugation at 15,000 rpm (12,000 × *g*), were lyophilized and stored at −80 C. Lyophilized samples were redissolved in either RIA buffer or calcium mobilization assay buffer before ghrelin RIA or calcium mobilization assay, respectively.

Preparation of plasma samples for ghrelin assay

Plasma samples were prepared as previously described (2). Whole blood samples from 10 male mice were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 kallikrein inactivator units per milliliter) and centrifuged at 4 C. Immediately after the isolation of plasma, hydrogen chloride was added to the sample to a final concentration of 0.1 N. Samples were then diluted into an equal volume of saline. Samples were then loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA) preequilibrated in 0.1% trifluoroacetic acid (TFA) and 0.9% NaCl. After washing the cartridges with 0.9% NaCl and 5% acetonitrile (CH_3CN)/0.1% TFA, samples were eluted in 60% CH_3CN/0.1% TFA. The eluates were lyophilized; residual materials were redissolved in 1 M AcOH and adsorbed onto a SP-Sephadex C-25 column (H^+-form, Pharmacia, Uppsala, Sweden) preequilibrated in 1 M AcOH. Successive elution in 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH (pH 5.0) generated three fractions: SP-I, SP-II, and SP-III. The SP-III fraction was first evaporated and redissolved in 1 M AcOH and then separated by reverse-phase HPLC with C18-cartridge (C18 RP-HPLC) (Symmetry 300, 3.9 × 150 mm, Waters) using a linear gradient from 10 to 60% CH_3CN/0.1% TFA at a flow rate of 1.0 ml/min for 40 min, collecting 500-μl fractions. Ghrelin peptide content in each fraction was determined by ghrelin C-RIA as described above.

Concentration and acyl modification of ghrelin after free fatty acid (FFA) or triacylglycerol ingestion

The standard laboratory chow, CE-2 (CLEA Rodent Diet CE-2, CLEA Japan, Osaka, Japan), contained a caloric content of approximately 50.3% carbohydrate, 25.4% protein, and 4.4% fat. MCFAs, such as *n*-hexanoic, *n*-octanoic, and *n*-lauric acid (Sigma-Aldrich Japan Co. Ltd., Tokyo, Japan), were dissolved in water at 5 mg/ml. To equilibrate the total intake of *n*-palmitic acid to the other MCFAs contained in food, this common long-chain fatty acid (Sigma-Aldrich Japan) was mixed into CE-2 chow at a concentration of 1% (wt/wt). Medium- and long-chain triglycerides (MCTs and LCTs), including glyceryl trihexanoate, trioctanoate, tridecanoate, and tripalmitate (Wako Pure Chemical, Osaka, Japan), were mixed into CE-2 chow at a concentration of 5% (wt/wt). Whole-stomach tissues from mice were collected at the indicated times (0–14 d) after the beginning of treatment. To elucidate the forms of ghrelin peptides modified by different acyl groups, stomach peptides, extracted as described above, were collected using a Sep-Pak Plus C18 cartridge (Waters). The recovery of des-acyl, *n*-hexanoyl, *n*-octanoyl, *n*-decanoyl, *n*-lauryl, and *n*-palmitoyl ghrelin from the Sep-Pak C18 cartridges were over 90%. The extracted peptides were subjected to C18 RP-HPLC (Symmetry 300, 3.9 × 150 mm, Waters) using a linear gradient from 10 to 60% CH_3CN/0.1% TFA at a flow rate of 1.0 ml/min for 40 min and collected in 500-μl fractions. The ghrelin peptide content in each fraction was measured by ghrelin C- and N-RIA as described above. No ghrelin degradation was observed during the extraction.

Concentration and acyl modification of ghrelin after high-fat (HF) diet ingestion

To examine the effect of dietary LCTs on the distribution of stomach acyl-modified or des-acyl ghrelin, we fed mice a HF diet enriched in LCTs, in which nearly 50% of the total calories originated from animal fat. This HF diet, modified from an AIN-76A standard chow, derived approximately 35.4% of the total caloric content from carbohydrates, 16.2% from protein, and 48.4% from fat (24). By caloric content, AIN-76A chow contained 69.2% carbohydrate, 18.4% protein, and 12.4% fat. We fed male C57BL/6J mice the HF diet for 2 wk and then compared the distribution of stomach ghrelin with that seen in control mice fed standard AIN-76A chow. The distribution of stomach ghrelin molecules was

measured using ghrelin C-RIA after HPLC fractionation, as described above.

Northern blot analysis

Total RNAs were extracted from the stomachs of male C57BL/6J mice (12 wk old) by acid guanidium thiocyanate-phenol chloroform extraction (25) using TRIzol Reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde and then transferred to a ζ-probe-blotting membrane (Bio-Rad Laboratories, Hercules, CA). A ^{32}P-labeled rat ghrelin cDNA probe was hybridized to the membranes in hybridization buffer, containing 50% formamide, 5× sodium-chloride sodium-phosphate EDTA buffer, 5× Denhardt's solution, 1% sodium dodecyl sulfate, and 100 μg/ml denatured salmon sperm. After overnight hybridization at 37 C, membranes were washed and exposed to BioMax-MS film (Eastman Kodak, Rochester, NY) for 12 h at −80 C. Ghrelin mRNA levels were quantified using a BAS 2000 bioimaging analyzer (Fujix, Tokyo, Japan).

Purification of n-heptanoyl ghrelin

n-Heptanoyl ghrelin was purified as described for the purification of ghrelin using antirat ghrelin(1–11) IgG immunoaffinity chromatography (22). During purification, ghrelin activity was assayed by measuring the changes in intracellular calcium concentrations using a FLEX station (Molecular Devices) in a cell line stably expressing rat GHS-R (CHO-GHSR62). Ghrelin C-RIA was also used to monitor ghrelin immunoreactivity in isolated samples.

Glyceryl triheptanoate (Fluka Chemie GmbH, Buchs, Switzerland) was mixed with standard laboratory chow at a concentration of 5% (wt/wt). Four days after mice (n = 7) were fed glyceryl triheptanoate-containing food, we collected stomachs (total 1000 mg). The total consumption of glyceryl triheptanoate-containing food was approximately 13.5 g/mouse, equivalent to 675 mg total glyceryl triheptanoate ingested by each mouse. Stomachs were prepared and homogenized as described above. After a 30-min centrifugation at 20,000 rpm, homogenization supernatants were loaded onto a Sep-Pak C18 environmental cartridge (Waters) preequilibrated in 0.1% TFA. After washing in 10% CH_3CN/0.1% TFA, peptide fractions were eluted in 60% CH_3CN/0.1% TFA. The eluate was then evaporated and lyophilized. Residual materials were redissolved in 1 M AcOH and fractionated as described above for plasma samples. After application of the lyophilized SP-III fraction to a Sephadex G-50 fine gel-filtration column (1.9 × 145 cm) (Pharmacia), we collected 5-ml fractions. A portion of each fraction was subjected to the ghrelin calcium-mobilization assay. Half of each active fraction (no. 47–51) was collected using a Sep-Pak C18 light cartridge and lyophilized. Samples were then resuspended in 1.0 ml 100 mM phosphate buffer (pH 7.4) and purified by antirat ghrelin(1–11) IgG immunoaffinity chromatography. Adsorbed substances were eluted in 500 μl 10% CH_3CN/0.1% TFA. The eluate was evaporated and separated by RP-HPLC (Symmetry 300, 3.9 × 150 mm, Waters). *n*-Heptanoyl-modified ghrelin was obtained at a retention time of 18.4 min and subjected to a mass spectrometry to confirm the appropriate molecular weight. The amino acid sequences of purified peptides were analyzed using a protein sequencer (494, Applied Biosystems, Foster City, CA).

Mass spectrometric analysis of n-heptanoyl ghrelin

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed using a Voyager DE-Pro spectrometer (Applied Biosystems) (26). Mass spectra were recorded in the reflector mode, with an accelerating voltage of 20 kV. Saturated α-cyano-4-hydroxycinnamic acid in 60% CH_3CN and 0.1% TFA were used as a working matrix solution. Approximately 1 pmol of the final purified sample was mixed with the matrix solution, placed on the sample probe, and dried in the air before analysis. All mass spectra were acquired in positive ion mode, averaged by 100 spectra.

Results

The effect of FFA ingestion for the stomach content of total and n-octanoyl ghrelin measured by ghrelin C- and N-RIA

To examine the effect of daily ingestion of FFAs on the acyl modification of ghrelin, we extracted gastric peptides from mice given *ad libitum* access to water containing *n*-hexanoic acid, *n*-octanoic acid, or *n*-lauric acid or chow containing *n*-palmitic acid. The stomach concentration of *n*-octanoy-modified and total (*n*-octanoylated plus des-acyl) ghrelin forms were measured by ghrelin N- and C-RIA, respectively. The stomach content of *n*-decanoyl, *n*-decanoyl, and *n*-hexanoyl ghrelins in mice fed normal chow was low in comparison to *n*-octanoyl ghrelin (see Fig. 3 and Table 1). The reactivity of N-RIA for *n*-decanoyl-, *n*-decanoyl-, and *n*-hexanoyl-modified ghrelins is low, compared with that seen for *n*-octanoyl ghrelin; thus, the concentration of acyl-modified ghrelin measured by N-RIA primarily reflects *n*-octanoyl ghrelin. During the experimental period (0–14 d), no significant differences between the fatty acid-ingesting and control groups in mouse body weight or total dietary consumption were observed.

After a 14-d administration of *n*-hexanoic acid, *n*-octanoic acid, *n*-lauric acid, or *n*-palmitic acid, we compared the gastric concentrations of *n*-octanoyl and total ghrelin with concentrations seen in control mice fed normal chow and water. The gastric concentrations of *n*-octanoyl ghrelin increased significantly in mice fed *n*-octanoic acid (Fig. 1A). The mean stomach concentrations of *n*-octanoyl ghrelin were 1795 fmol/mg wet weight in control rats fed normal food (n = 8) and 2455 fmol/mg wet weight in mice fed *n*-octanoic acid-containing food (n = 8). No significant changes were observed in the total ghrelin concentrations measured by C-RIA (Fig. 1B). Therefore, the ratio of *n*-octanoyl ghrelin/total ghrelin increased significantly in mice fed *n*-octanoic acid (Fig. 1C). No significant changes in the stomach contents of total

TABLE 1. Concentrations of des-acyl and acyl-modified ghrelins in the stomachs of mice after ingestion of medium-chain (C6:0-C10:0) triglycerides

	des-acyl Ghrelin	C6:0-ghrelin	C8:0-ghrelin	C10:0-ghrelin	Total ghrelin
Control	301.7 ± 19.0	28.6 ± 3.5	531.1 ± 27.3	30.8 ± 5.5	1146.6 ± 75.4
C6:0-MCT	253.7 ± 4.4	237.8 ± 34.8[a]	360.8 ± 33.3[a]	25.8 ± 6.0	1075.4 ± 46.0
C8:0-MCT	227.2 ± 34.9[b]	12.3 ± 4.5	788.8 ± 82.6[a]	8.8 ± 5.7[a]	1145.1 ± 95.8
C10:0-MCT	207.5 ± 27.0[c]	24.6 ± 4.4	516.9 ± 42.3	108.4 ± 12.0[a]	958.7 ± 84.2[b]

Male C57BL/6J mice were fed chow mixed with 5% (wt/wt) glyceryl trihexanoate (C6:0-MCT), glyceryl trioctanoate (C8:0-MCT), or glyceryl tridecanoate (C10:0-MCT) for 14 d. The concentrations of des-acyl ghrelin, *n*-hexanoyl ghrelin (C6:0-ghrelin), *n*-octanoyl ghrelin (C8:0-ghrelin), *n*-decanoyl ghrelin (C10:0-ghrelin), and total ghrelin in stomach samples (from 0.2 mg wet weight) were measured by ghrelin C-RIA after HPLC fractionation. Data represent mean ± SD of six samples.

[a] $P < 0.001$.

[b] $P < 0.05$.

[c] $P < 0.01$ *vs.* control.

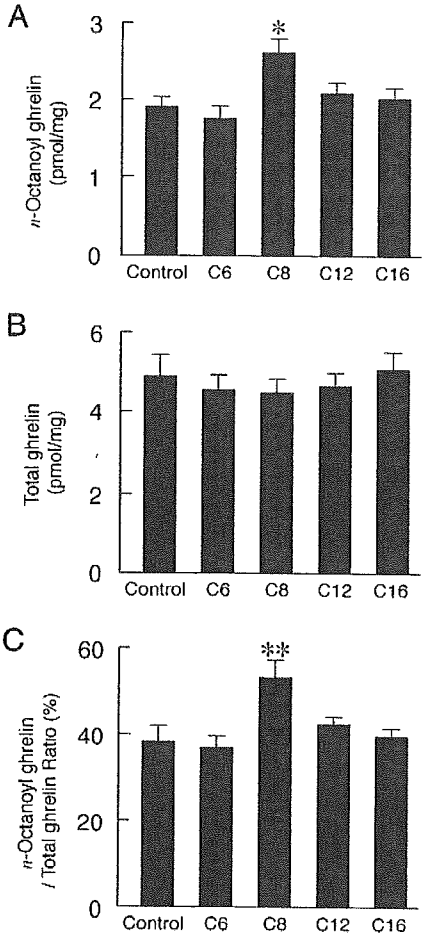

FIG. 1. Ghrelin concentrations in the stomachs of normal control animals (control) fed standard chow and water and mice fed *n*-hexanoic acid (C6), *n*-octanoic acid (C8), *n*-lauric acid (C12), or *n*-palmitic acid (C16). A, *n*-Octanoyl ghrelin concentrations measured by ghrelin N-RIA (n = 8). Because N-RIA is highly specific for *n*-octanoyl ghrelin, exhibiting low cross-reactivity to other acylated forms of ghrelin such as *n*-hexanoyl, *n*-lauryl, or *n*-palmitoyl ghrelin, the concentration of acyl-modified ghrelin measured by N-RIA primarily reflects *n*-octanoyl ghrelin. B, Total ghrelin concentrations measured by ghrelin C-RIA (n = 8), including both acylated and des-acyl ghrelin. The C-RIA equally recognizes all des-acyl and acylated forms of ghrelin containing an intact C-terminal sequence. C, Ratios of acyl-modified to total ghrelin. Data represent mean ± SD of ghrelin concentrations in stomach extracts (from 1 mg wet weight). Statistical significance is indicated by *asterisks*. *, $P < 0.01$; **, $P < 0.001$ *vs.* control.

ghrelin measured by C-RIA could be observed after treatment with *n*-hexanoic, *n*-decanoic, or *n*-palmitic acids. After this treatment, no significant differences were detected in the stomach content of *n*-octanoyl ghrelin. Thus, the exogenously supplied *n*-octanoic acid specifically increased gastric concentrations of *n*-octanoyl ghrelin without altering the total quantities of ghrelin peptide.

The effect of triacylglycerol ingestion for the stomach content of total and n-octanoyl ghrelin measured by ghrelin C- and N-RIAs

Orally ingested triacylglycerols are intraluminally hydrolyzed and absorbed through the gastrointestinal mucosa as FFAs or monoglycerides. Thus, ingested triacylglycerols may serve as a source of FFAs (27). To examine whether ingested triacylglycerols are used for acyl modification of ghrelin, mice were fed chow mixed with 5% (wt/wt) glyceryl trihexanoate, trioctanoate, tridecanoate, and tripalmitate. All mice were given *ad libitum* access to experimental food and water. After 2 wk, we measured the content of *n*-octanoyl and total ghrelin in extracted gastric peptides by N- and C-RIAs. Glyceryl trioctanoate ingestion increased stomach concentrations of *n*-octanoyl ghrelin (Fig. 2A). In contrast, glyceryl trihexanoate ingestion decreased the stomach contents of *n*-octanoyl ghrelin identified by ghrelin N-RIA. Mice fed glyceryl trihexanoate, however, exhibited increased concentrations of *n*-hexanoyl ghrelin (Fig. 3 and Table 1). Ingestion of glyceryl tridecanoate and glyceryl tripalmitate had no effect on the production of *n*-octanoylated ghrelin (Fig. 2A)

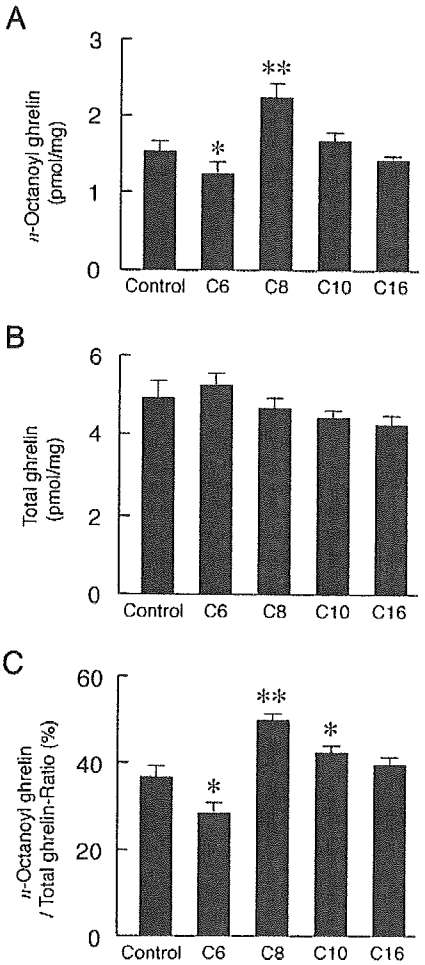

FIG. 2. Ghrelin concentration in the stomachs of mice fed standard laboratory chow (control) (n = 8) and mice fed chow containing glyceryl trihexanoate (C6), trioctanoate (C8), tridecanoate (C10), or tripalmitate (C16). A, *n*-Octanoyl ghrelin concentrations were measured by ghrelin N-RIA. B, Total ghrelin concentrations were measured by ghrelin C-RIA. Data represent the mean ± SD of ghrelin concentrations in stomach extracts (from 1 mg wet weight) (n = 5). C, The ratio of *n*-octanoyl to total ghrelin. Data represent mean ± SD of calculated ratios (n = 5). Statistical significance is indicated by *asterisks*. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

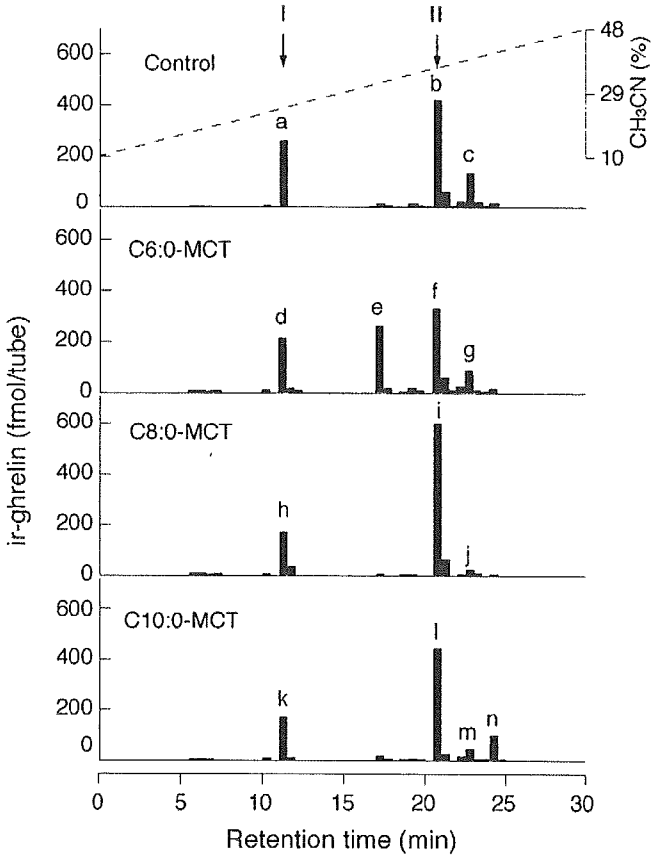

FIG. 3. The molecular forms of ghrelin peptides isolated from the stomachs of mice fed standard laboratory chow (control) or chow containing glyceryl trihexanoate (C6:0-MCT), glyceryl trioctanoate (C8:0-MCT), or glyceryl tridecanoate (C10:0-MCT). Ghrelin immunoreactivity in peptide extracts from mouse stomachs, fractionated by HPLC, was quantitated by C-RIA. Assay tubes contained equivalent quantities of peptide extract derived from 0.2 mg of stomach tissue. *Black bars* represent immunoreactive ghrelin (ir-ghrelin) concentrations determined by ghrelin C-RIA. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl ghrelin (II). Based on the retention times of synthetic ghrelin forms, peaks a, d, h, and k correspond to des-acyl ghrelin, whereas peaks b, f, i, and l correspond to *n*-octanoyl ghrelin. Peaks c, g, j, and m correspond to *n*-decanoyl (C10:1) ghrelin. Peak n corresponds to *n*-decanoyl (C10:0) ghrelin.

or the total stomach concentrations of ghrelin (des-acyl and acyl-modified ghrelins) in five independent groups of mice (Fig. 2B). Therefore, the molar ratios of *n*-octanoyl ghrelin/total ghrelin decreased in glyceryl trihexanoate-treated mice and increased in glyceryl tridecanoate-treated mice (Fig. 2C). Throughout the experimental period (0–2 wk), no significant differences in body weight or total food consumption could be observed between triacylglycerol-fed and control groups.

Molecular forms of ghrelin peptide after triacylglycerol ingestion

To clarify the molecular forms of ghrelin peptide present after triacylglycerol ingestion, we measured ghrelin immunoreactivity by C-RIA in fractions of stomach extracts isolated by HPLC to reveal the ghrelin molecular forms (Fig. 3) present in mice fed glyceryl trihexanoate, trioctanoate, and tridecanoate. Based on the observed retention times of synthetic des-acyl or acyl-modified ghrelin peptides, 11.2 min for des-acyl ghrelin, 13.8 min for *n*-butyryl ghrelin, 17.2 min for *n*-hexanoyl ghrelin, 20.2 min for *n*-octanoyl ghrelin, 22.6 min for *n*-decanoyl ghrelin, 24.2 min for *n*-decanoyl, 27.6 min for *n*-lauryl ghrelin, and 34.6 min for *n*-palmitoyl ghrelin, peaks a, d, h, and k corresponded to a des-acyl ghrelin lacking any fatty acid modification. Peaks b, f, i, and l corresponded to a *n*-octanoyl ghrelin, the form modified at Ser^3 by *n*-octanoic (C8:0) acid. Peaks c, g, j, and m corresponded to a *n*-decanoyl ghrelin form bearing an *n*-decanoic (C10:1) acid modification.

Ingestion of glyceryl trioctanoate stimulated the production of *n*-octanoyl ghrelin (peak i in Fig. 3). The molar ratio of *n*-octanoyl/total ghrelin reached greater than 60% in treated mice (Table 1). This high *n*-octanoyl ghrelin ratio was not observed in mice fed normal food and water (Table 1). Because the stomach content of *n*-octanoyl ghrelin also increased after *n*-octanoic acid ingestion, both glyceryl trioctanoate and *n*-octanoic acid can increase the stomach concentrations of *n*-octanoyl ghrelin.

n-hexanoyl ghrelin could be detected only at very low levels in stomach of mice fed normal chow. When fed glyceryl trihexanoate, however, the stomach concentrations of *n*-hexanoyl ghrelin, bearing the *n*-hexanoic (C6:0) acid modification, increased drastically (peak e). We also observed significant decreases in *n*-octanoyl ghrelin concentrations in these mice (peak f in Fig. 3 and Table 1) in comparison with levels observed in control mice (peak b in Fig. 3 and Table 1). The content of *n*-hexanoyl ghrelin also increased after ingestion of *n*-hexanoic acid (data not shown).

Moreover, after ingestion of glyceryl tridecanoate, the stomach concentration of the *n*-decanoyl ghrelin form modified by *n*-decanoic (C10:0) acid increased (peak n).

Ghrelin peaks eluting at the same retention times as synthetic *n*-butyryl (C4:0), *n*-lauryl (C12:0), and *n*-palmitoyl (C16:0) ghrelin could not be observed in the stomach extracts of mice given glyceryl tributyrate, trilaurate, or tripalmitate (data not shown), indicating that neither glyceryl tributyrate nor tripalmitate could be transferred to ghrelin in mice.

To examine the influence of a high-fat intake on the distribution of des-acyl and acyl-modified ghrelins in mouse stomach, we fed mice a HF diet with 48.4% of the total calories from animal fat containing a high proportion of LCTs. We compared the distribution of stomach ghrelin in mice ingesting the HF diet with control mice fed an AIN-76A control diet (deriving 12.4% of the total calories from fat). We observed a faint, but significant, decrease in both the amount and proportion of des-acyl ghrelin in the stomach after a 2-wk administration of the HF diet (Table 2). We also observed a significant increase in the proportion of total ghrelin that bore the *n*-octanoyl modification (C8:0) in the HF diet group in comparison with the control animals. Whereas the total amount of stomach *n*-decanoyl (C10:0) ghrelin also increased in the HF diet group, we observed a faint decrease in the proportion of total ghrelin that was *n*-decanoylated (C10:1) in the HF diet group. Whereas the total amount of stomach ghrelin decreased slightly in mice fed a HF diet, there was no significant difference between the HF diet and control groups. These changes in the distribution of stomach ghrelins after administering the HF diet were small in com-

TABLE 2. The effect of HF diet on the distribution of stomach ghrelins

Diet	AIN-76A diet	HF diet
Total ghrelin	1058 ± 108	992 ± 122
des-acyl Ghrelin	275.2 ± 39.6 (26.0 ± 2.4)	229.3 ± 46.1[a] (23.0 ± 2.8)[a]
n-Hexanoyl ghrelin	32.8 ± 5.2 (3.1 ± 0.3)	29.4 ± 4.9 (3.0 ± 0.4)
n-Octanoyl ghrelin	452.0 ± 45.2 (42.8 ± 2.0)	464.3 ± 43.7 (48.0 ± 2.4)[b]
n-Decenoyl ghrelin	187.2 ± 17.1 (17.8 ± 1.1)	138.2 ± 19.7[a] (13.9 ± 1.1)[c]
n-Decanoyl ghrelin	33.9 ± 5.1 (3.2 ± 0.3)	38.0 ± 6.7 (3.8 ± 0.6)[a]

The content of each ghrelin molecule was measured by ghrelin C-RIA after HPLC fractionation. Data represent mean ± SD of the amount (fmol/0.2 mg) of each ghrelin molecule (n – 8). Data in *parentheses* represent the proportion (percentage) of each ghrelin molecule for total ghrelin.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ *vs.* control (AIN-76A diet group).

parison with those observed after treatment with MCFAs or MCTs.

Time course of n-octanoyl ghrelin production after glyceryl trioctanoate ingestion

To examine time-dependent changes in *n*-octanoyl ghrelin production after ingestion of glyceryl trioctanoate, we fed mice glyceryl trioctanoate-containing chow (5%, wt/wt) after a 12-h fasting period. We then measured the stomach concentrations of *n*-octanoyl and total ghrelins at the indicated times. *n*-octanoyl ghrelin production (Fig. 4) increased significantly in the stomach 3 h after the ingestion of glyceryl trioctanoate. When continuously given glyceryl trioctanoate, the stomach concentrations of *n*-octanoyl ghrelin gradually increased, peaking 24 h after the beginning of administration. The stomach concentrations of *n*-octanoyl ghrelin measured 14 d after continuous feeding of the glyceryl trioctanoate-mixed chow remained significantly higher than those of mice fed normal chow (Fig. 4A). Under these conditions, however, no significant changes in the stomach content of total ghrelin, measured by C-RIA, could be observed (Fig. 4B).

Ghrelin mRNA expression after glyceryl trioctanoate ingestion

To examine whether the ingestion of MCTs affects ghrelin mRNA expression, we quantitated ghrelin RNA in mouse stomach by Northern blot analysis after 4 d of glyceryl trioctanoate ingestion (Fig. 5). The expression levels of gastric ghrelin mRNA did not change after the ingestion of glyceryl trioctanoate. Because the ingestion of glyceryl trioctanoate increased the stomach content of *n*-octanoyl ghrelin without changing the total ghrelin concentration, we propose that ingestion of glyceryl trioctanoate stimulates the octanoyl modification of ghrelin only.

Molecular forms of ghrelin peptides after glyceryl triheptanoate ingestion

To examine the direct use of ingested FFAs for acyl modification of ghrelin, mice were fed MCTs that are not present in food sources nor naturally synthesized in mammals. We selected synthetic glyceryl triheptanoate as an unnatural FFA source because *n*-heptanoic acid (C7:0), a hydrolyzed from of glyceryl triheptanoate, does not naturally occur in mammals. Moreover, *n*-heptanoyl ghrelin can be easily separated from natural ghrelin by HPLC. We examined ghrelin content in stomach extracts from mice fed glyceryl triheptanoate by examining ghrelin immunoreactivity by C-RIA in HPLC fractions. The retention times of the peaks a and c corresponded to des-acyl ghrelin and *n*-octanoyl ghrelin, respectively (Fig. 6). Peak b immunoreactivity was observed only in mice fed glyceryl triheptanoate. This peak was not observed in mice fed any of the other FFAs or triglycerides examined, including *n*-hexanoic acid, *n*-octanoic acid, *n*-lau-

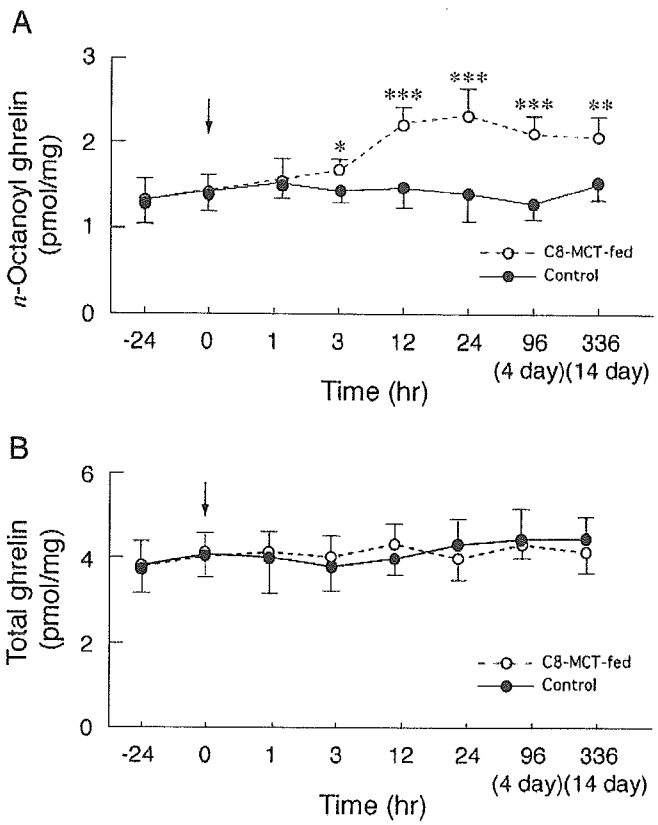

FIG. 4. Time-dependent changes in stomach concentrations of ghrelin in mice fed glycerol trioctanoate. A, *n*-Octanoyl ghrelin content was measured by ghrelin N-RIA. B, Total ghrelin content was measured by ghrelin C-RIA. After 12 h of fasting, mice were given glyceryl trioctanoate (5% wt/wt)-containing food beginning at the time (0 h) indicated by the *arrow*. Stomach samples were isolated from control mice fed standard laboratory chow (*closed circles*) and mice fed glyceryl trioctanoate (C8-MCT; *open circles*) at the indicated times. Each point represents mean ± SD (n = 8). Statistical significance is indicated by *asterisks*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

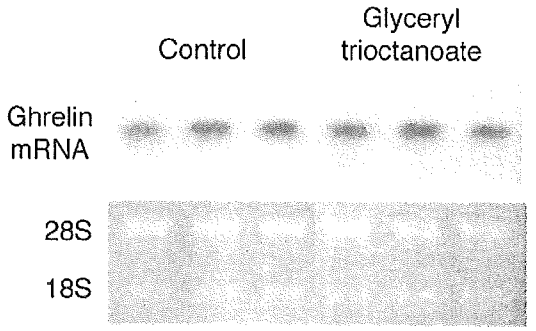

FIG. 5. Northern blot analysis examining stomach ghrelin mRNA expression after ingestion of glyceryl trioctanoate-containing food. Each lane contained 2 μg of total RNA. The *lower panel* indicates the intensity of 28S and 18S rRNAs internal controls.

ric acid, *n*-palmitic acid, and the corresponding triglyceride forms. The estimated retention time of peak b was between that of *n*-hexanoyl and *n*-octanoyl ghrelin, consistent with *n*-heptanoyl ghrelin.

Purification of n-heptanoyl ghrelin

To confirm the use of the ingested glyceryl triheptanoate for *n*-heptanoyl ghrelin modification, we purified acyl-modified ghrelins from the stomachs of mice fed glyceryl triheptanoate-containing food for 4 d. This purification of ghrelin peptides from the stomachs of treated mice identified peak

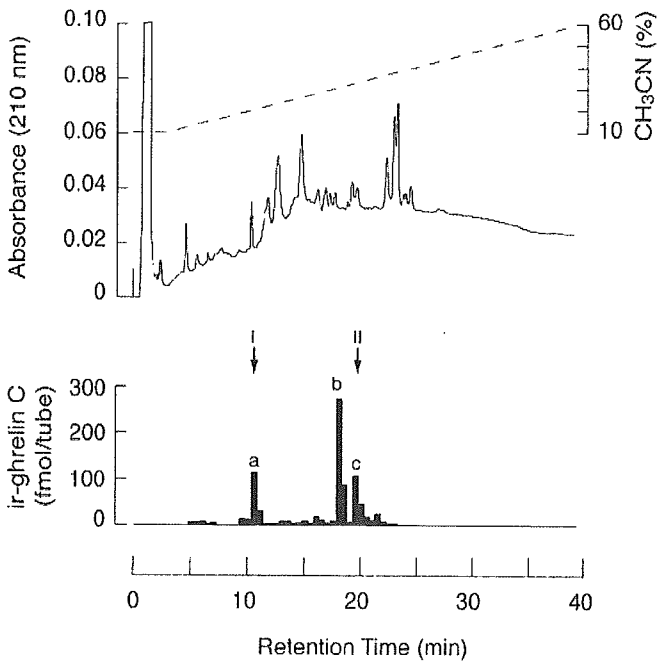

FIG. 6. The HPLC profile of peptides extracted from the stomachs of mice fed glyceryl triheptanoate. Stomach extracts of glyceryl triheptanoate-treated mice were fractionated by HPLC (*upper panel*). The concentration of ghrelin in each fraction (equivalent to 0.2 mg stomach tissue) was monitored by C-RIA (*lower panel*). Ghrelin immunoreactivity (ir-ghrelin),represented by *solid bars*, was separated into three major peaks (peaks a, b, and c) by C-RIA. Peak b was observed only after the ingestion of glyceryl triheptanoate. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl (II) ghrelin.

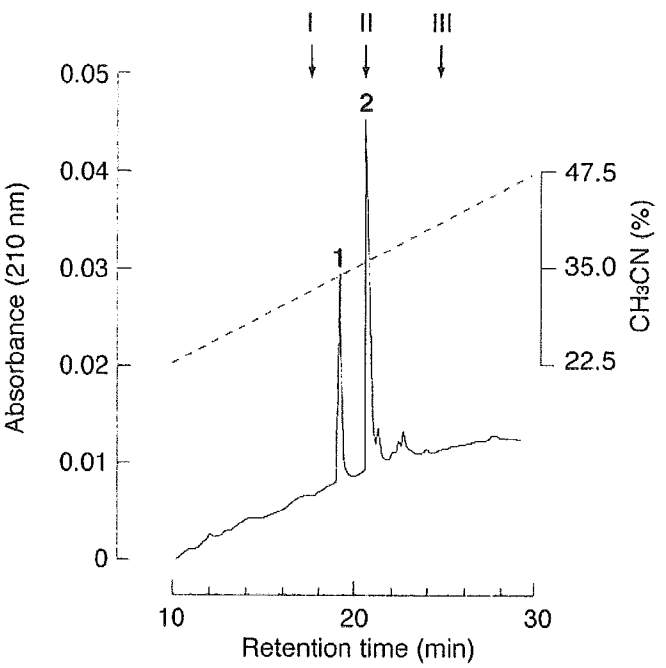

FIG. 7. Purification of *n*-heptanoyl ghrelin. Ghrelin peptides were purified from the stomachs of mice fed glyceryl triheptanoate. Samples eluted from an antirat ghrelin immunoaffinity column were subjected to HPLC. Peak 1 was observed only in samples from glyceryl triheptanoate-treated mice. Based on the retention times of control samples in HPLC and MALDI-TOF-MS analysis, peak 2 corresponded to *n*-octanoyl ghrelin. *Arrows* indicated the elution positions of *n*-hexanoyl (I), *n*-octanoyl (II), and *n*-decanoyl (III) ghrelin.

2 (Fig. 7) as *n*-octanoyl ghrelin from its HPLC retention time. The extra peak eluting at a retention time of 18.4 min (peak 1 in Fig. 7), observed only after ingestion of glyceryl triheptanoate, eluted at a retention time between that of *n*-hexanoyl- and *n*-octanoyl ghrelin. We purified this peak 1 peptide and subjected it to amino acid sequence analysis and mass spectrometry.

The purified HPLC peak 1 peptide (Fig. 7) contained 28 amino acids with an identical amino acid sequence to that of mouse ghrelin. The average m/z value of the peak 1 peptide measured by MALDI-TOF-MS was 3301.9 (Fig. 8A). The estimated molecular weight of this peptide, calculated from this MALDI-TOF-MS m/z value, was 3300.9. Modification of ghrelin at Ser^3 with an *n*-heptanoyl group would produce a theoretical molecular weight of approximately 3300.86 (Fig. 8B), an almost identical molecular weight as that measured by MALDI-TOF-MS. Thus, we concluded that the purified peak 1 peptide was *n*-heptanoyl ghrelin. No additional peaks were observed in the final purification, indicating that, after hydrolysis from the ingested glyceryl triheptanoate, the *n*-heptanoyl group could be directly transferred to Ser^3 of ghrelin.

Molecular forms of circulating ghrelin peptides after glyceryl triheptanoate ingestion

To examine whether *n*-heptanoyl ghrelin synthesized after glyceryl triheptanoate ingestion is secreted into the circulation, we determined the molecular forms of acyl-modified

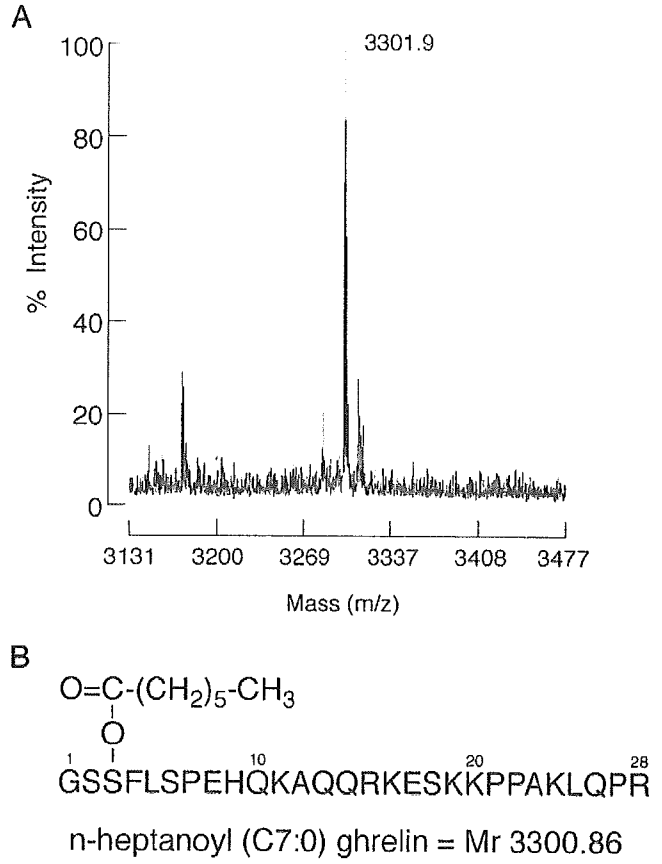

FIG. 8. A, MALD-TOF-MS of the purified ghrelin-like peptide in Fig. 7, peak a. The mass ranges from 3131.0 to 3477.0 (m/z). From the average of 100 mass spectra acquired in positive ion mode (average $[M+H]^+$: 3301.9), the molecular weight of the peak a peptide was calculated to be 3300.9. B, The structure of *n*-heptanoyl (C7:0) ghrelin provides a calculated molecular weight for *n*-heptanoyl ghrelin of 3300.86.

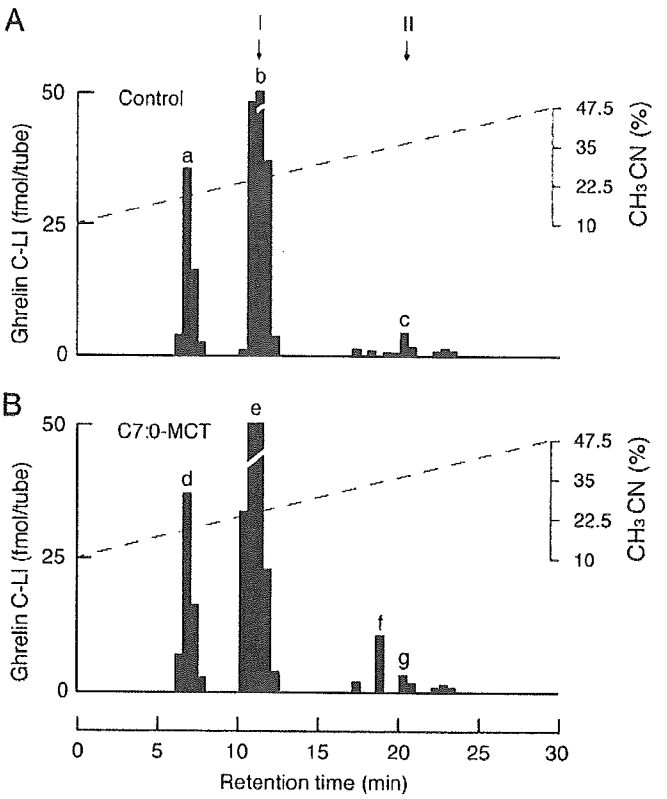

FIG. 9. The molecular forms of plasma ghrelin peptides isolated from mice fed glyceryl triheptanoate-containing chow. Plasma samples from mice fed standard chow (A) or glyceryl triheptanoate-containing food (B) were fractionated by HPLC. Ghrelin immunoreactivity was then measured by C-RIA. *Arrows* indicate the elution positions of des-acyl ghrelin (I) and *n*-octanoyl ghrelin (II). Plasma ghrelin immunoreactivity is represented by *solid bars*. Based on the retention times of each peak, peaks b and e correspond to des-octanoyl ghrelin, whereas peaks c and g correspond to *n*-octanoyl ghrelin. Peak f exhibited a similar elution profile as that of *n*-heptanoyl ghrelin isolated from the stomachs of mice given glyceryl triheptanoate.

ghrelin found in the plasma of mice fed glyceryl triheptanoate-containing food for 4 d (Fig. 9, A and B). Plasma samples, fractionated by RP-HPLC, were assessed for ghrelin immunoreactivity by C-RIA. Plasma ghrelin immunoreactivity in control mice was separated into two major peaks (peaks a and b in Fig. 9A) and a minor peak (peak c in Fig. 9A). Plasma ghrelin immunoreactivity in glyceryl triheptanoate-treated mice was separated into two major peaks (peaks d and e in Fig. 9B) and two minor peaks (peaks f and g in Fig. 9B). Based on the elution profiles, peaks b and e corresponded to *des*-acyl ghrelin, whereas peaks c and g corresponded to *n*-octanoyl ghrelin. Although peaks a and d are thought to be a C-terminal fragment of the ghrelin peptide resulting from protease digestion, the exact molecular form of this peptide has not yet been determined.

Peak f, which eluted at 18.0–18.5 min, was observed only in samples from glyceryl triheptanoate-treated mice. This analysis revealed the existence of a plasma ghrelin molecule with the same retention time as that of *n*-heptanoyl ghrelin purified from the stomachs of glyceryl triheptanoate-fed mice (peak f in Fig. 9B). These results indicate that despite the fact that *n*-heptanoyl ghrelin is an unnatural form of ghrelin synthesized *in vivo* after glyceryl triheptanoate ingestion, it can be released into the circulation.

Activity of n-heptanoyl ghrelin

Using the ghrelin calcium-mobilization assay, we examined GHS-R-stimulating activity of *n*-heptanoyl ghrelin purified from glyceryl triheptanoate-ingested mouse stomach. *n*-heptanoyl ghrelin induced intracellular-free calcium concentration $[Ca^{2+}]_i$ increases in GHS-R-expressing cells. The time course of these $[Ca^{2+}]_i$ changes was similar to those induced by *n*-octanoyl ghrelin (Fig. 10). Whereas the agonistic activity of *n*-heptanoyl ghrelin for GHS-R, calculated from the area under the curve (AUC) of the response curve, was approximately 60% that of *n*-octanoyl ghrelin, it was 3 times higher than that of *n*-hexanoyl ghrelin (Fig. 10). Thus, *n*-heptanoyl ghrelin possesses GHS-R-stimulating activity.

Discussion

We demonstrate here that ingested MCFAs and MCTs increase the stomach concentrations of acylated ghrelin without increasing either total peptide (measured by ghrelin C-

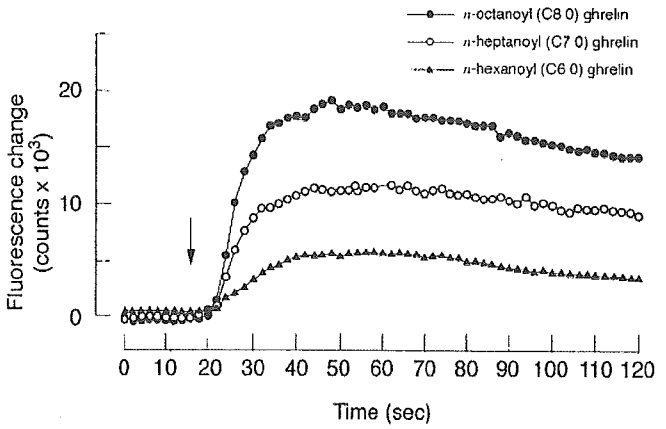

FIG. 10. Time course of fluorescence changes as a measure of $[Ca^{2+}]_i$ changes induced by *n*-octanoyl ghrelin (*closed circle*), *n*-heptanoyl ghrelin (*open circle*), and *n*-hexanoyl ghrelin (*closed triangle*) in GHS-R-expressing cells. Peptides (1×10^{-8} M) were added at the time indicated by the *arrow*.

RIA) or mRNA expression of ghrelin. These exogenous MCFAs and MCTs are directly used for ghrelin acyl modification. Ingestion of synthetic glyceryltriheptanoate or *n*-heptanoic acid produces an *n*-heptanoyl ghrelin that does not occur naturally, supporting the hypothesis of the direct use of MCFAs and MCTs as a fatty acid source for ghrelin acyl modification.

A putative ghrelin-specific acyl-modifying enzyme, ghrelin ser *O*-acyltransferase, may catalyze the acyl modification of *n*-hexanoyl, *n*-heptanoyl, *n*-octanoyl, and *n*-decanoyl ghrelins. Because we could not detect *n*-butyryl or *n*-palmitoyl ghrelin after ingestion of glyceryl tripalmitate, LCTs, or the short-chain triacylglyceride glyceryl tributyrate, the putative acyl-modifying enzyme may prefer MCTs (composed of C6:0-C10:0 FFAs) over either short-chain triacylglycerides or LCTs. Detailed *in vitro* studies will be required to examine the substrate specificity of this putative enzyme.

Ingested triacylglycerides are not the only source of FFAs used in mammals. In a dynamic triglyceride/fatty acid cycle (28), after storage in cells, triacylglycerides can be hydrolyzed, released into the circulation, and transferred to target tissues. Circulating protein-conjugated triglycerides can also be hydrolyzed to FFAs and again transferred to target cells. After conversion to the respective acyl-CoAs by acyl-CoA synthetase, reabsorbed FFAs within target tissues are used to produce ATP or are converted back into triglycerides (29, 30). *n*-octanoyl-CoA is a substrate for carnitine octanoyltransferase, a ubiquitously expressed enzyme abundant in gastrointestinal tissues, such as the stomach (31–34). Thus, *n*-octanoic acid and its derivatives are likely synthesized and stored in cells of this lineage. Thus, even in normal feeding conditions, *n*-octanoyl derivatives produced in normal lipid metabolism may serve as substrates for acyl modification of ghrelin.

Lee *et al.* (24) previously demonstrated that HF diets significantly lowered the rate of stomach ghrelin synthesis, as measured by ghrelin mRNA expression, and secretion, as determined by total ghrelin plasma levels. In contrast, a low-protein, high-carbohydrate diet increased the rate of stomach ghrelin synthesis and secretion (24). Although there were no significant changes in the amount of stomach total ghrelin in each of these feeding conditions, changes in the rate of ghrelin production and secretion may exert some influence on the proportion of acyl-modified ghrelin in the mouse stomach. In our HF diet experiment, we observed a faint, but significant, increase in the proportion of stomach *n*-octanoyl ghrelin in conjunction with a decrease in the levels of stomach *des*-acyl ghrelin. The effect of glyceryl trioctanoate (C8:0-MCT) on the amount and proportions of stomach *n*-octanoyl ghrelin, however, was far greater than that of the HF diet. These findings suggest that ghrelin acyl-modification after ingestion of MCT uses a slightly different mechanistic pathway than that used after administration of a HF diet.

In addition to new insights into the mechanism governing acyl modification of ghrelin, our experiments have also shed light on the role of MCTs in ghrelin synthesis. It is interesting to reexamine the physiological effects of MCT, a naturally occurring component of coconut oil, butter, and other palm kernel oils (27, 35) that is also present in milk from rodents (36) and humans (37, 38), on ghrelin synthesis, modification, and activity. Through the acyl modification of Ser^3, these orally ingested MCTs may modify the ghrelin biological activity.

Whereas both further *in vivo* and *in vitro* studies will be required to elucidate the mechanism of ghrelin acyl modification, our study provides a number of important clues enhancing our understanding of this process. In addition, modification of ghrelin activity through administration of exogenous FFAs may be a potential therapeutic modality for the clinical manipulation of energy metabolism.

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Modulation of Androgen Receptor Transactivation by FoxH1

*A NEWLY IDENTIFIED ANDROGEN RECEPTOR COREPRESSOR**

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Androgen signaling plays key roles in the development and progression of prostate cancer, and numerous ongoing studies focus on the regulation of androgen receptor (AR) transactivity to develop novel therapies for the treatment of androgen-independent prostate cancer. FoxH1, a member of the Forkhead-box (FOX) gene family of transcription factors, takes part in mediating transforming growth factor-β/activin signaling through its interaction with the Smad2·Smad4 complex. Using a series of experiments, we found that FoxH1 repressed both ligand-dependent and -independent transactivation of the AR on androgen-induced promoters. This action of FoxH1 was independent of its transactivation capacity and activin A but relieved by Smad2·Smad4. In addition, the repression of the AR by FoxH1 did not require deacetylase activity. A protein-protein interaction was identified between the AR and FoxH1 independently of dihydrotestosterone. Furthermore, a confocal microscopic analysis of LNCaP cells revealed that the interaction between the AR and FoxH1 occurred in the nucleus and that FoxH1 specifically blocked the foci formation of dihydrotestosterone-activated AR, which has been shown to be correlated with the AR transactivation potential. Taken together, our results indicate that FoxH1 functions as a new corepressor of the AR. Our observations not only strengthen the role of FoxH1 in AR-mediated transactivation but also suggest that therapeutic interventions based on AR-coregulator interactions could be designed to block both androgen-dependent and -independent growth of prostate cancer.

Prostate cancer is a significant cause of morbidity and mortality worldwide. Androgens play major roles in promoting the development and progression of prostate cancer (1–3), and therefore, androgen ablation and blockade of androgen actions through the androgen receptor (AR)[3] have been the cornerstones of treatments for advanced prostate cancer. Despite these regimens, prostate cancer invariably progresses to a fatal, androgen-refractory state (4, 5). However, although such relapsed tumors are androgen-independent, they are still dependent on the AR for their growth and survival (4, 6–8). Therefore, identification of the precise mechanisms underlying the regulation of AR function is of critical importance for the design and development of novel therapies and pharmaceutical targets for treating prostate cancers.

The AR shares a characteristic structure with other members of the steroid hormone receptor family (comprised of receptors for estrogens (ERs), progesterone (PR), glucocorticoids (GR), and mineralocorticoids), namely a variable NH_2-terminal transactivation domain (NTD) possessing an activation function 1 (AF-1) domain, a highly conserved zinc finger-type DNA binding domain (DBD), and a ligand binding domain (LBD) that usually contains a second activation domain (AF-2) (9, 10). AF-1 functions in a ligand-independent manner, whereas the activity of AF-2 requires cognate ligand binding (9, 11, 12). Upon activation by ligands, the AR translocates to the nucleus, where it binds to androgen response elements and regulates the transcription of target genes. Moreover, it has become clear that the transactivity of nuclear receptors, including the AR, is regulated by coregulator proteins that enhance (coactivators) or reduce (corepressors) the target gene transcription by various mechanisms (10, 13, 14). Although most of the AR coregulators identified to date have been coactivators, it is conceivable that AR corepressors are also required for precise and efficient regulation of the AR activity in cells (13, 15). Therefore, further characterization of AR corepressors may provide insights into the signaling events that occur within prostate cancer and pave the way to the development of individualized therapies.

Activins, which are members of the TGF-β superfamily, are composed of two β subunits, βA and βB, which form activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (16). In addition to their stimulatory effects on pituitary follicle-stimulating hormone synthesis, activins have also been implicated in the control of many other cellular processes, including growth and tumorigenesis (17, 18). The presence of activin A and its receptors in the prostate (19–22) and the ability of activin A to inhibit prostate cancer cells grown in culture (23–26) suggest an important role for activin A in the regulation of prostatic growth. Moreover, activin A has been shown to induce the expression of prostate-specific antigen (PSA), prostatic acid phosphatase, and the AR (26), genes that are also induced by androgen. Although the molecular mechanism through which activin A regulates gene expression and growth in the prostate has not yet been fully elucidated, cross-talk between activin A and androgen-mediated signaling pathways may play critical roles in these processes (27).

Smads are a family of proteins that function as effectors of the TGF-β/activin-signaling pathway. Ligand addition induces the phosphorylation of specific receptor-regulated Smads (R-Smads), which then oligomerize with the common mediator Smad4 and move into the nucleus. Once there, the R-Smad·Smad4 complex interacts with a vari-

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[3] The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; NTD, NH_2-terminal transactivation domain; AF-1, activation function 1; DBD, DNA binding domain; LBD, ligand binding domain; AF-2, activation domain 2; PSA, prostate-specific antigen; TGIF, 5′TG3′ interacting factor; GFP, green fluorescent protein; MMTV, mouse mammary tumor virus; LUC, luciferase; DHT, dihydrotestosterone; TSA, trichostatin A; TGF, transforming growth factor; CBP, cAMP-response element-binding protein (CREB)-binding protein; RT, reverse transcription.

ety of transcription factors and coregulators and becomes targeted to a diverse array of gene promoters (28, 29). Interestingly, some coregulators of Smads, such as AP-1 (30), CBP/P300 (31, 32), and TGIF (33), can also regulate AR-mediated transactivation (34–37). FoxH1 (also known as FAST-1), a member of the Forkhead-box (FOX) gene family of transcription factors, plays an important role in mediating TGF-β/activin signals through its interaction with the Smad2·Smad4 complex (38–40). We hypothesized that FoxH1 may also function as a coregulator to regulate the AR transactivation potential and, therefore, be involved in the cross-talk between activin A and androgen-mediated signaling. In the present study we found that FoxH1 could repress ligand-dependent and -independent transactivation of the AR on androgen-induced promoters. However, the repression of the AR by FoxH1 was not alleviated by activin A, indicating that FoxH1 was dispensable for the stimulatory effect of activin A on PSA expression (26, 27, 41). Nonetheless, our results demonstrate that FoxH1 is a new corepressor of the AR.

EXPERIMENTAL PROCEDURES

Plasmids—pCMVhAR, pCMXhGR, pcDNA3-ANT-1, pcDNA3-Smad2, pcDNA3-Smad4, pAR-GFP, and pCBP-GFP as well as the reporter plasmid pMMTV-LUC (containing the luciferase gene driven by the mouse mammary tumor virus (MMTV) long terminal repeat harboring hormone response elements for AR, GR, and PR) were described previously (42–47). The human FoxH1 expression vectors pCMVFoxH1 and pCMVFoxH1$_{H83R}$ were kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center, Baltimore, MD) (39). The human ER expression vectors pSG5-ERα and pSG5-ERβ as well as a reporter plasmid for ER (pERE2-tk109-LUC) were kind gifts from Dr. Shigeaki Kato (University of Tokyo, Tokyo, Japan). The reporter plasmid pPSA-LUC, containing the luciferase gene under the control of a 6.1-kilobase promoter fragment of the human PSA gene, was kindly provided by Dr. Jer-Tsong Hsieh (University of Texas Southwestern Medical Center, Dallas, TX). The human PR expression vectors pSG5-PRA and pSG5-PRB were kind gifts from Dr. Pierre Chambon (INSERM, Illkirch, France). pRL-SV40, pG5-LUC, pBIND, and pACT expression vectors were obtained from Promega (Madison, WI).

AR and FoxH1 expression vectors for mammalian two-hybrid assays were subcloned into the pBIND and pACT expression vectors, respectively. pFoxH1-Myc and pFoxH1$_{H83R}$-Myc were constructed by subcloning the FoxH1 and FoxH1$_{H83R}$ cDNAs into pcDNA3.1/Myc-His, respectively. pFoxH1-GFP was constructed by inserting the FoxH1 cDNA into pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). The validity of the structure of each construct was confirmed by DNA sequencing and Western blot analysis of transfected COS-7 cells.

RNA Preparation and RT-PCR—Total RNA was extracted using ISOGENE (Wako, Osaka, Japan) according to the manufacturer's instructions. The concentration and purity of the RNA were determined spectrophotometrically. Next, 5 μg of total RNA was reverse-transcribed into first-strand cDNA using a SuperScript III kit (Invitrogen) in a final volume of 20 μl. To analyze the expression of FoxH1 in prostate cancer cell lines, a sensitive RT-PCR was performed using previously described primers (39). The PCR was carried out in a 50-μl reaction mixture containing 2.5 mM $MgCl_2$, 0.3 mM dNTP, and 2.5 units of *Taq* DNA polymerase (Promega) under the following conditions: 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. Aliquots of the PCR products were electrophoresed in 2% agarose gels containing 0.5 mg/ml ethidium bromide and then photographed under UV light using a positive/negative instant film (Polaroid 665; Nippon-Polaroid, Tokyo, Japan). The authenticity of each PCR product was confirmed by sequencing.

Transactivation Assays—The human prostate cancer cell lines LNCaP and PC-3 were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sera Laboratories Ltd., Sussex, UK). COS-7 monkey kidney cells and ALVA41 human prostate cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were cotransfected with the indicated expression vectors in 24-well plates using an Effectene transfection kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's protocol. The cells were then incubated in RPMI 1640 or Dulbecco's modified Eagle's medium containing 0.5% dextran-coated charcoal-stripped fetal bovine serum and vehicle (0.1% ethanol) or ligands as indicated. After 24 h, the firefly and *Renilla* luciferase activities were assayed using the Dual-Luciferase® Reporter assay system (Promega) according to the manufacturer's protocol in a Minilumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). The results were normalized for the internal *Renilla* control and presented as the relative luciferase activity. All transfection experiments were carried out in triplicate wells and repeated at least three times using two sets of plasmids prepared separately. Data were calculated as the mean ± S.D.

Stable Transfection of LNCaP Cells with pFoxH1-Myc, Semiquantitative RT-PCR, and Western Blotting—LNCaP cells were cultured in six-well plates and then transfected with pFoxH1-Myc using an Effectene transfection kit. After 4 weeks of culturing and selection with 400 μg/ml of Geneticin (Invitrogen), 6 colonies were harvested. After limited dilution, 2 independent FoxH1-Myc-expressing clones (designated LNCaP/FoxH1-1 and LNCaP/FoxH1-2) were identified by Western blotting with an anti-Myc antibody (1:1000; sc-40; Santa Cruz Biotechnology, Inc.) and further maintained as stable cell lines in RPMI 1640 supplemented with 200 μg/ml Geneticin. Cells stably transfected with the empty vector (LNCaP/Vector) served as a control.

The effect of stably expressing FoxH1 on the endogenous PSA level in the absence or presence of 100 nM dihydrotestosterone (DHT) was investigated by semiquantitative RT-PCR and Western blotting, respectively. For semiquantitative RT-PCR, total RNA was extracted and reverse-transcribed as described above. Preliminary experiments were conducted to ensure linearity for the semiquantitative procedures. Hot-start PCR was performed by heat-activating AmpliTaq Gold DNA polymerase (PerkinElmer Life Sciences) at 95 °C for 10 min. Optimized cycling condition was 30 cycles (for PSA) or 26 cycles (for glyceraldehyde-3-phosphate dehydrogenase) of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. The primer sequences specific for PSA and glyceraldehyde-3-phosphate dehydrogenase were described previously (48, 49). For Western blotting, the cells were harvested, and the protein concentration of each sample was measured using a BCA protein assay kit (Pierce). Aliquots containing 20 μg of protein were separated in 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the anti-human PSA antibody A67-B/E3 (1:200; sc-7316; Santa Cruz Biotechnology Inc.) or an anti-β-actin antibody (1:500; AC-74; Sigma). Bands were visualized using an alkaline phosphatase system.

Microscopy and Imaging Analyses—Microscopy and imaging analyses were performed essentially as described previously (44–46). The cells were imaged for green fluorescence by excitation with the 488-nm line from an argon laser, and the emission was viewed through a 496–505-nm band pass filter.

Immunoprecipitation and Western Blotting—COS-7 cells were cotransfected with 2 μg of pFoxH1-Myc or pFoxH1$_{H83R}$-Myc together

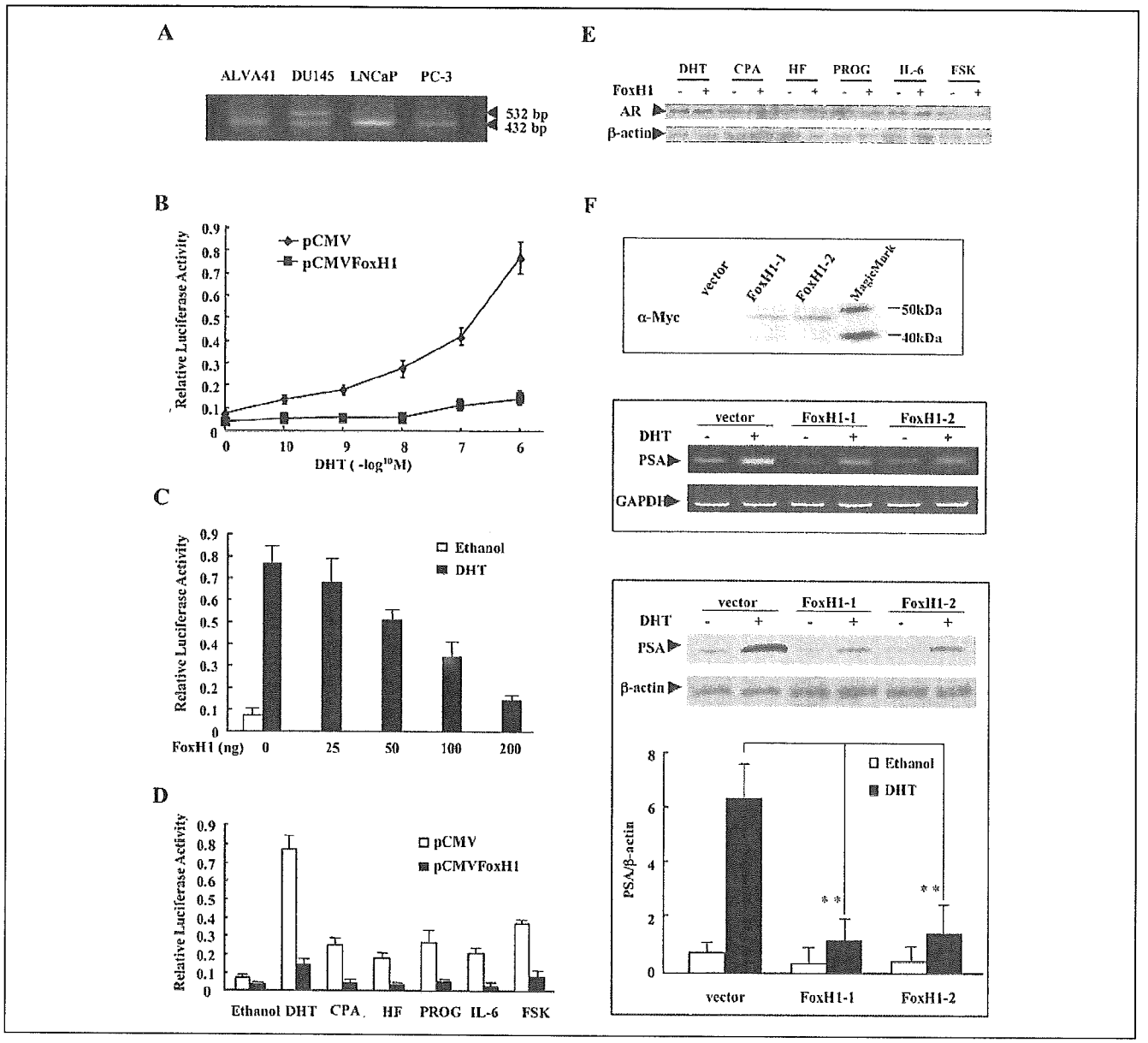

FIGURE 1. **Inhibitory effect of FoxH1 on AR transactivation in prostate cancer cells.** *A*, endogenous expression of FoxH1 in 4 prostate cancer cell lines. Total RNA was prepared from each prostate cancer cell line and subjected to RT-PCR as described under "Experimental Procedures." The cell lines examined are listed *above* the *panel*. *B–F*, inhibitory effects of FoxH1 on both ligand-dependent and -independent transactivation of the AR in LNCaP cells. *B–E*, LNCaP cells were transiently cotransfected with a DNA mixture containing 50 ng of pPSA-LUC, 1.5 ng of pRL-SV40, and increasing amounts (0–200 ng/well) (*C*) or 200 ng (*B* and *D*) of pCMVFoxH1 adjusted with the empty pCMV vector to produce equimolar amounts of the pCMV vector. The total amount of DNA in each well was brought to 250 ng with pBSK+ DNA. *B*, cells were treated with increasing concentrations of DHT as indicated. *C*, cells were exposed to 1 μM DHT. *D*, cells were exposed to the following concentrations of ligands: 1 μM DHT, 1 μM cyproterone acetate (*CPA*), 1 μM hydroxyflutamide (*HF*), 1 μM progesterone (*PROG*), 25 ng/ml interleukin-6 (*IL-6*), or 10 μM forskolin (*FSK*). *E*, AR expression in extracts (20 μg of protein) of LNCaP cells, corresponding to the same samples shown in *D*, were assessed by immunoblotting using the anti-AR antibody N-20 (1:500). The names of the samples are listed *above* the *panels*. *F*, pFoxH1-Myc was stably transfected into LNCaP cells, and the expression of FoxH1-Myc was investigated by immunoblotting (*top panel*). Endogenous PSA expression was also investigated by semiquantitative RT-PCR (*middle panel*) and immunoblotting (*bottom panel*) as described under "Experimental Procedures." Bands were measured by densitometry. Data are presented as the mean (±S.E.) ratio of PSA:β-actin proteins from three independent experiments (*bottom panel*). The blot shown in the figure shows the results of a typical experiment. **, $p < 0.01$. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

with 2 μg of the pCMVhAR or pCMV parent vector in 6-well plates using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected cells were cultured in the presence or absence of 10 nM DHT for 24 h and then harvested in cellLytic™-M (Sigma) containing 1× Complete Mini-EDTA-free protease inhibitor mixture (Roche Applied Science). The protein concentration of each sample was measured and adjusted to 1 mg/ml. Next, an aliquot (160 μg) of each lysate was incubated with 3 μg of the anti-AR antibody C-19 (sc-815; Santa Cruz Biotechnology Inc.) or normal rabbit IgG as a control in TNE buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl) containing 1× Complete Mini-EDTA-free protease inhibitor mixture and then further incubated with 25 μl of protein G magnetic beads (New England Biolabs Inc., Beverly, MA) at 4 °C for 2 h on a rotating platform. The beads were collected using a magnet, and the bound proteins were eluted in 1× SDS-PAGE sample buffer and subjected to 10% SDS-PAGE. A Western blot analysis was performed using the anti-Myc antibody (1:500) as described above, and the positive bands were detected using an enhanced chemiluminescence detection

system (Amersham Biosciences) and a VersaDoc™ imaging system (Bio-Rad).

Mammalian Two-hybrid Assays—Mammalian two-hybrid assays (Promega) were mainly performed according the manufacturer's protocol, with some modifications. NIH3T3 cells were transiently cotransfected with the indicated vectors in 24-well plates using the Superfect transfection reagent (Qiagen K. K.) in the presence or absence of 10 nM DHT. Luciferase activities were assayed as described above.

Statistical Analysis—Statistical significance was determined by one-factor analysis of variance followed by a post hoc test (Fisher's protected least significant difference test). $p < 0.05$ was considered statistically significant.

RESULTS

Expression of FoxH1 in Prostate Cancer Cell Lines—A previous study (39) reported that human FoxH1 gene expression was ubiquitous and could be detected in all normal human tissues tested as well as in several cancer cell lines. However, whether FoxH1 is expressed in LNCaP cells as well as in other prostate cancer lines has yet not been elucidated. Therefore, to investigate the role of FoxH1 in the cross-talk between activin A- and androgen-mediated signaling in prostate cancer, we initially used the primers described in the above-mentioned study (39) to investigate FoxH1 expression in the prostate cancer lines ALVA41, DU145, LNCaP, and PC-3 by RT-PCR. These primers spanned a 100-bp intron and discriminated between the spliced (423 bp) and unspliced (523 bp) products. As shown in Fig. 1*A*, a 423-bp band was detected in all 4 prostate cancer cell lines. Moreover, three of the cell lines also contained the unspliced product, which may arise from either genomic DNA or unprocessed transcripts.

FoxH1 Represses Both Ligand-dependent and -independent Transactivation of the AR in LNCaP Cells—Next, we examined the possible roles of FoxH1 expression in ligand-dependent and -independent transcription of the PSA promoter induced by endogenous AR by cotransfecting LNCaP cells, the most commonly used androgen-sensitive prostate cancer cell line, with a FoxH1 expression plasmid and a PSA-luciferase reporter gene. As shown in Fig. 1*B*, DHT activated the AR in a concentration-dependent manner, and an ~10-fold higher induction was observed in the presence of 1 μM DHT compared with vehicle treatment. Cotransfection of the FoxH1 expression plasmid brought about a marked repression of the DHT-induced AR activation at all concentrations of DHT tested (Fig. 1*B*), and the repression was dose-dependent (Fig. 1*C*). Furthermore, FoxH1 completely blocked the stimulatory effects of progesterone and the anti-androgens cyproterone acetate and hydroxyflutamide on the AR in LNCaP cells, which contains a mutation (T877A) that results in alterations of the specificity and sensitivity of the receptor to these molecules (50, 51) as well as the ligand-independent transactivation of the AR by interleukin-6 (52–54) and forskolin (55, 56) (Fig. 1*D*). In these and subsequent experiments, the inhibitory effect of FoxH1 on the AR transactivity was not due to a reduced AR expression level, since immunoblot analyses of extracts from the transfected cells revealed comparable amounts of immunoreactive protein (Fig. 1*E*). Together, these results demonstrate that FoxH1 represses both ligand-dependent and -independent transactivation of endogenous AR in LNCaP cells.

To further explore the relevance of FoxH1 in AR-mediated transactivation, we examined the relationship between FoxH1 expression and endogenous PSA expression in LNCaP cells. pFoxH1-Myc was stably transfected into LNCaP cells, and 2 isolated clones expressing similar protein levels of FoxH1-Myc, LNCaP/FoxH1-1, and LNCaP/FoxH1-2 were established (Fig. 1*F*, *top panel*) to investigate the effect of FoxH1 on

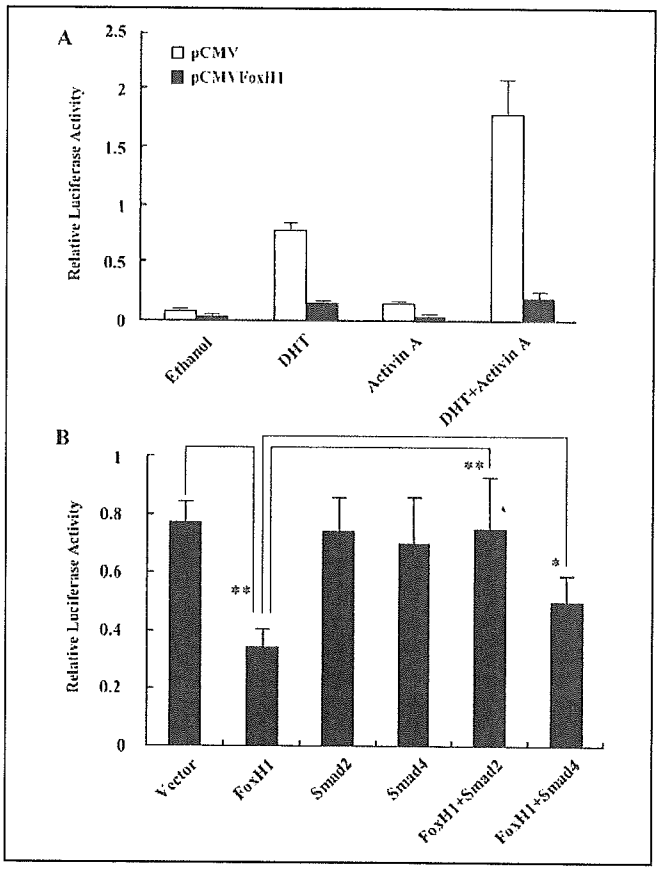

FIGURE 2. **Repression by FoxH1 is not rescued by activin A but is relieved by Smad2/4.** *A*, activin A has no effect on the FoxH1-mediated repression of PSA expression. LNCaP cells were cotransfected as described in the legend for Fig. 1, *C* and *D*, and then treated with 0.1% ethanol, 1 μM DHT, 25 ng/ml activin A, or DHT + activin A. *B*, LNCaP cells were cotransfected with 50 ng of pPSA-LUC and 1.5 ng of pRL-SV40 as well as 100 ng of pCMVFoxH1, 100 ng of a Smad expression vector alone, or 100 ng each of pCMVFoxH1 and a pcDNA3-Smad vector. The parent expression vectors were used to maintain equimolar concentrations across all cultures. After transfection, the cells were treated with 1 μM DHT. *, $p < 0.05$; **, $p < 0.01$.

PSA expression. As expected, stable expression of FoxH1 resulted in a parallel reduction of PSA expression in either mRNA or protein levels in both the absence and presence of DHT but had little effect on the levels of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase and β-actin (Fig. 1*F*, *middle* and *bottom panels*), indicating that FoxH1 could regulate the expression of endogenous androgen-responsive genes.

Repression of FoxH1 Is Not Rescued by Activin A but Is Relieved by Smad2/4 Proteins—Because FoxH1 mediates transcriptional responses to TGF-β/activin in a ligand-, receptor-, and Smad-dependent fashion (39)and activin A has been shown to induce PSA expression in LNCaP cells (26, 27, 41), we next investigated whether activin A and its effectors Smad2·Smad4 could alleviate the repression of the AR by FoxH1 in LNCaP cells. Consistent with previous reports, activin A induced transcription from the PSA promoter, and the PSA promoter induction after treatment with both DHT and activin A was additive compared with the values observed with either reagent alone (Fig. 2*A*). Unexpectedly, FoxH1 could still repress the AR-mediated transactivation of the PSA promoter in the presence of activin A, indicating that FoxH1-mediated inhibition was independent of activin A in LNCaP cells and that FoxH1 was dispensable for the stimulatory effect of activin A on PSA expression (26, 27, 41). On the other hand, in agreement with a previous report (57) neither Smad2 nor Smad4 alone had a signifi-

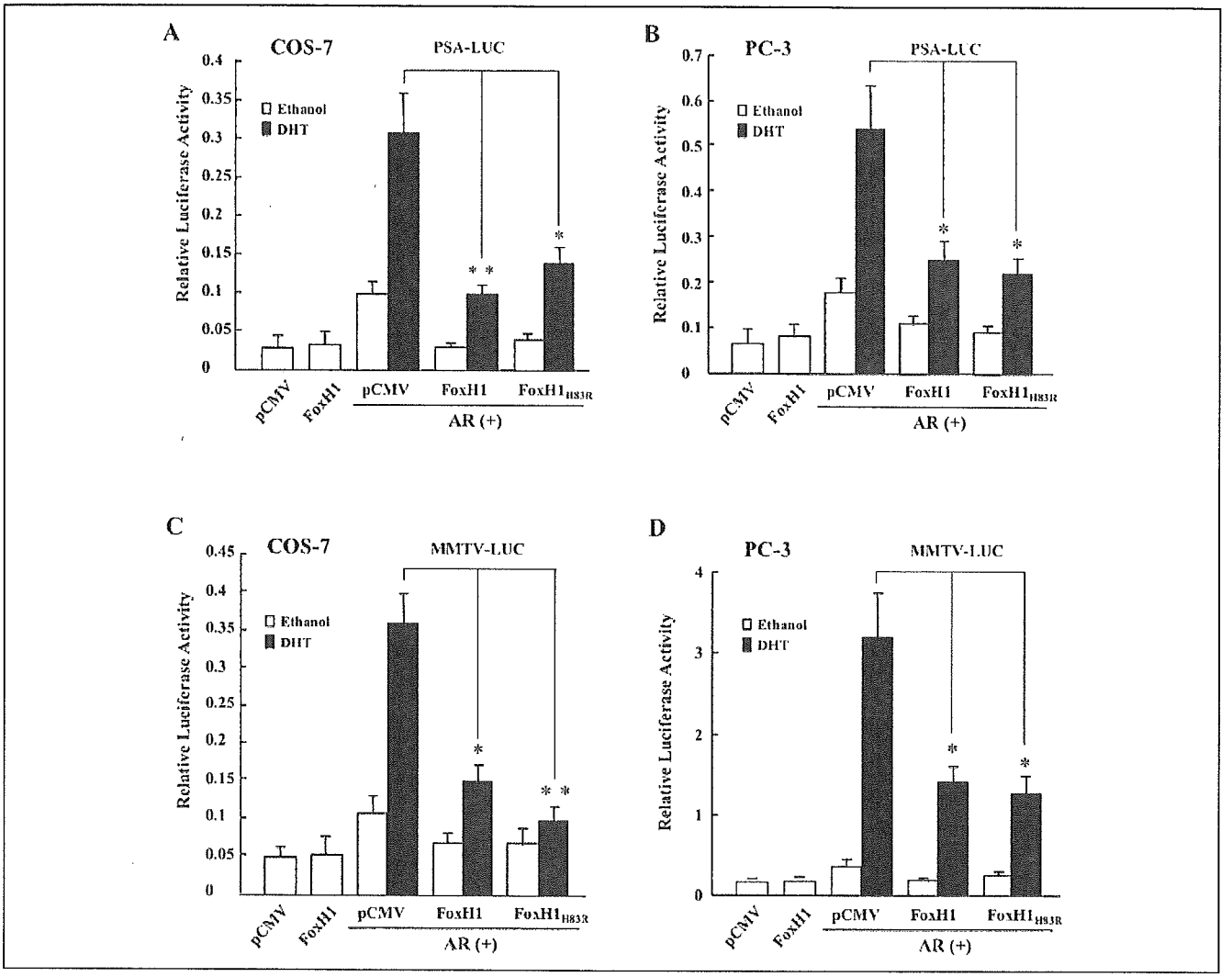

FIGURE 3. **Repression by FoxH1 is neither cell type- nor promoter context-dependent.** *A* and *B*, PSA-LUC reporter assays performed in COS-7 cells (*A*) or PC-3 cells (*B*). *C* and *D*, MMTV-LUC reporter assays performed in COS-7 cells (*C*) or PC-3 cells (*D*). The cells were cotransfected with 50 ng of pPSA-LUC or pMMTV-LUC, 1.5 ng of pRL-SV40, and 150 ng of pCMVFoxH1 or pCMVFoxH1$_{H83R}$ or an equimolar amount of the empty pCMV vector with or without 50 ng of a wild-type AR expression vector as indicated. After transfection, the cells were treated with 0.1% ethanol or 10 nM DHT. *, $p < 0.05$; **, $p < 0.01$.

cant effect on the AR-dependent transcription, whereas coexpression of Smad2 or Smad4 resulted in complete or partial relief of the repression by FoxH1, respectively (Fig. 2*B*). These results suggest that Smad2·Smad4 may be negative regulatory factors for the repression of the AR by FoxH1 through competition with the AR for binding to the limiting cellular FoxH1.

Repression of FoxH1 Is Neither Cell-type- Nor Promoter Context-dependent—To further explore the biological significance of the FoxH1-mediated repression, we examined the repression effect of FoxH1 on the wild-type AR in COS-7 and PC-3 cells using both pPSA-LUC and pMMTV-LUC as reporter genes. As shown in Fig. 3, FoxH1 as well as its mutant FoxH1$_{H83R}$, which has a mutation in the DNA binding Forkhead domain and completely lacks transcriptional activity (39), still significantly repressed AR-regulated transcription from either the PSA or MMTV promoter in both COS-7 and PC-3 cells. Notably, the basal transcription from the reporter genes was also reduced in the presence of FoxH1 but only in the presence of the AR (Fig. 3). In addition, the internal control *Renilla* luciferase activity was not influenced by FoxH1. Therefore, the repression by FoxH1 was specific for the AR and not a general transcriptional inhibition. These observations suggest that the inhibitory effect of FoxH1 on the AR is neither cell type- nor promoter context-dependent and is independent of its transactivity.

Selective Repression by FoxH1—Several of the corepressors identified to date, such as N-CoR and SMRT, can repress the transcriptional activity of other steroid hormone receptors as well as that of the AR (58–61). Therefore, it is important to investigate whether the repression by FoxH1 is specific for the AR or a more general phenomenon. To address this question, we tested GR, ER-α, ER-β, and PR isoforms in PC-3 cells under similar experimental conditions to those used for the AR. As shown in Fig. 4, these receptors showed ligand-dependent transactivation in the presence of their appropriate ligands, and cotransfection of FoxH1 resulted in obvious repressions of ER-α and ER-β as well as of the AR. In contrast, FoxH1 showed no significant repression of the transactivation of the GR and PR isoforms, demonstrating that the repression of the AR by FoxH1 did not result from competition with the AR for binding to the hormone response elements. In these experiments, the internal control *Renilla* luciferase activity was stable. Collectively, these results suggest that FoxH1 may be involved in multiple regulatory processes mediated by steroid hormone receptors.

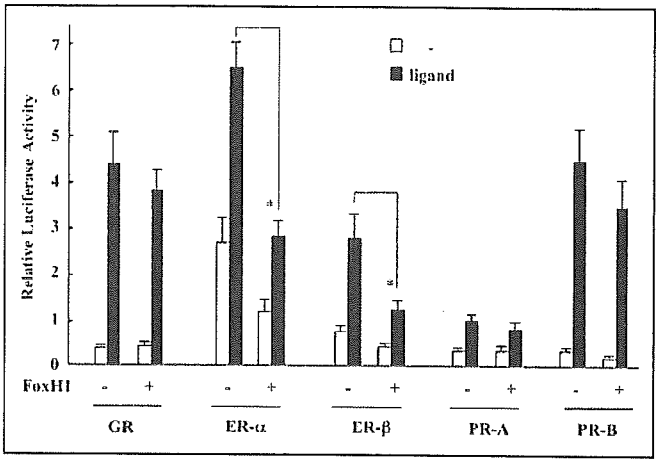

FIGURE 4. **Repression by FoxH1 is selective.** For each sample 50 ng of pMMTV-LUC or pERE2-tk109-LUC, 50 ng of the indicated corresponding steroid receptor expression constructs, and 1.5 ng of pRL-SV40 together with 150 ng of pCMVFoxH1 or an equimolar amount of the empty pCMV vector were cotransfected into PC-3 cells. After transfection the cells were treated with 10 nM concentrations of the specific ligand for each receptor. The ligands used were dexamethasone, 17β-estradiol, and progesterone. *, $p < 0.05$.

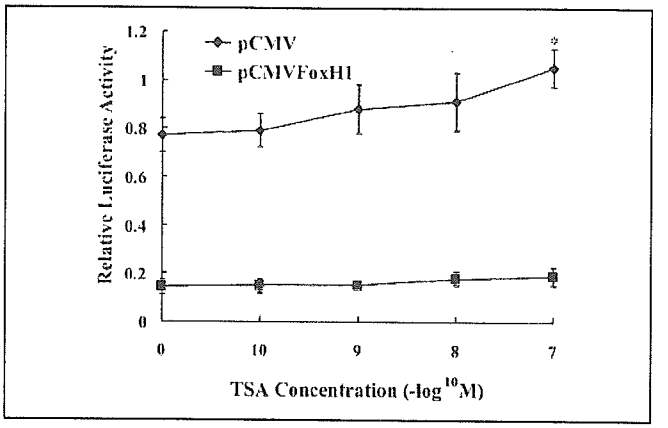

FIGURE 5. **Repression of the AR by FoxH1 does not require deacetylase activity.** LNCaP cells were cotransfected with 50 ng of pPSA-LUC, 1.5 ng of pRL-SV40, and 200 ng of pCMVFoxH1 or an equimolar amount of the empty pCMV vector. After transfection the cells were treated with 1 μM DHT alone or in combination with different concentrations of trichostatin A (*TSA*) as indicated. *, $p < 0.05$ *versus* the control.

Repression of the AR by FoxH1 Does Not Require Deacetylase Activity—Recent studies have shown that the transcriptional repression of the AR by some corepressors, such as TGIF (37) and ARR19 (62), is mediated through histone deacetylase pathways. In this regard, we examined whether trichostatin A (TSA), a specific inhibitor of histone deacetylase activity (63), had any influence on the repression of the AR by FoxH1 in LNCaP cells. Consistent with a previous report (64), treatment with 10^{-7} M TSA slightly increased the DHT-induced AR transactivity by about 30% (Fig. 5). However, TSA had no significant effect on the FoxH1-mediated inhibition at any of the doses tested, indicating that the repression of the AR by FoxH1 does not require deacetylase activity and that FoxH1 may repress AR-mediated transactivation through other mechanisms.

FoxH1 Abrogates Nuclear Foci Formation by the AR—We previously reported that DHT-bound AR formed foci in the nucleus, which were correlated with AR-mediated transactivations (44, 45). More recently, it was reported that the Tob-mediated suppression of AR activity may result from inhibition of the AR foci formation (65). Therefore, the prevention of AR foci formation may be responsible for the repression by FoxH1. To verify this hypothesis, we first examined the intracellular localization of the FoxH1 protein. As expected, the majority of FoxH1-GFP was homogeneously distributed in the nucleus in all the cell lines examined (Fig. 6, *A–C*). No significant changes were observed in the subcellular distribution of FoxH1-GFP before and after the addition of DHT or activin A. Consistent with our previous report (44), after cotransfection of LNCaP cells with pAR-GFP and the empty pCMV vector, the majority of AR-GFP was located homogeneously in the cytoplasm in untreated control cells (Fig. 6*D*), whereas treatment with 10 nM DHT resulted in nuclear translocation and fine foci formation by the AR in the nucleus (Fig. 6*E*). However, the addition of activin A (25 ng/ml) had little effect on the distribution of either unliganded or liganded AR (Fig. 6, *F* and *G*). In contrast, cotransfection with FoxH1 resulted in disruption of the DHT-induced foci formation, and AR-GFP was distributed homogeneously in the nucleus (Fig. 6*H*). In agreement with the transactivation assays, treatment with activin A did not recover the disruption of the DHT-induced foci formation by FoxH1 (Fig. 6*I*). Furthermore, replacement of pCMVFoxH1 by pCMVFoxH1$_{H83R}$ produced similar results (Fig. 6, *J* and *K*). Notably, and consistent with our previous report (45), CBP, a general integrator for nuclear receptors, was distributed in a mixed pattern with fine foci formation in a diffuse background in the nucleus (Fig. 6*L*). Cotransfection of FoxH1 had no obvious effect on the nuclear distribution of CBP (Fig. 6*M*), demonstrating that the disruption of foci formation by FoxH1 was specific for the AR rather than a general phenomenon.

FoxH1 Interacts Physically with AR—As a result of the above observations, we examined whether there was a physical interaction between the AR and FoxH1 using a coimmunoprecipitation analysis. pFoxH1-Myc was cotransfected into COS-7 cells with the empty pCMV vector or pCMVhAR. Cell extracts were immunoprecipitated with normal rabbit IgG (negative control) or an anti-AR antibody (C-19) followed by Western blotting with an anti-Myc monoclonal antibody. As shown in Fig. 7*A*, *upper panel*, FoxH1 protein was detected in anti-AR immunoprecipitates from cells cotransfected with the AR and FoxH1 in the absence or presence of 10 nM DHT but not in those from cells transfected with the AR or FoxH1 alone or when normal rabbit IgG was used. A similar result was obtained when pFoxH1-Myc was replaced by pFoxH1$_{H83R}$-Myc (Fig. 7*A*, *lower panel*). These data indicate that either FoxH1 or FoxH1$_{H83R}$ can form a specific complex with the AR.

Involvement of AF-1 in the Interaction of the AR with FoxH1—To further define the individual domains of the AR involved in binding to FoxH1, mammalian two-hybrid assays were carried out. The AR constructs consisted of amino acids 1–919 (pBIND-AR), amino acids 1–660 (pBIND-AR-NTD/DBD), and amino acids 615–919 (pBIND-AR-LBD) fused to the DBD of GAL4, whereas full-length FoxH1 was fused to the VP16 activation domain (pACT-FoxH1). As shown in Fig. 7*B*, the pG5-LUC reporter was induced after cotransfection of pBIND-AR-NTD/DBD and the empty pACT vector but not after cotransfection of the pBIND-AR-LBD construct, consistent with previous evidence that AF-1 in the NTD is responsible for most of the AR transactivation (66). Further induction was observed when pACT-FoxH1 was cotransfected with either pBIND-AR or pBIND-AR-NTD/DBD but not pBIND-AR-LBD. The addition of DHT (10 nM) further enhanced the transcription by about 2-fold in the case of pBIND-AR, whereas no significant induction was observed in the case of pBIND-AR-LBD. Moreover, pBIND-AR-NTD/DBD was found to have even stronger induction ability with FoxH1 than the full-length AR. Interestingly, when ANT-1, which specially enhances AR transactivation through a direct interaction with AR-AF-1 (46), was coexpressed, the induction of pG5-LUC by pACT-FoxH1 and pBIND-AR-NTD/DBD was completely blocked, suggesting that ANT-1 competed with FoxH1 for bind-

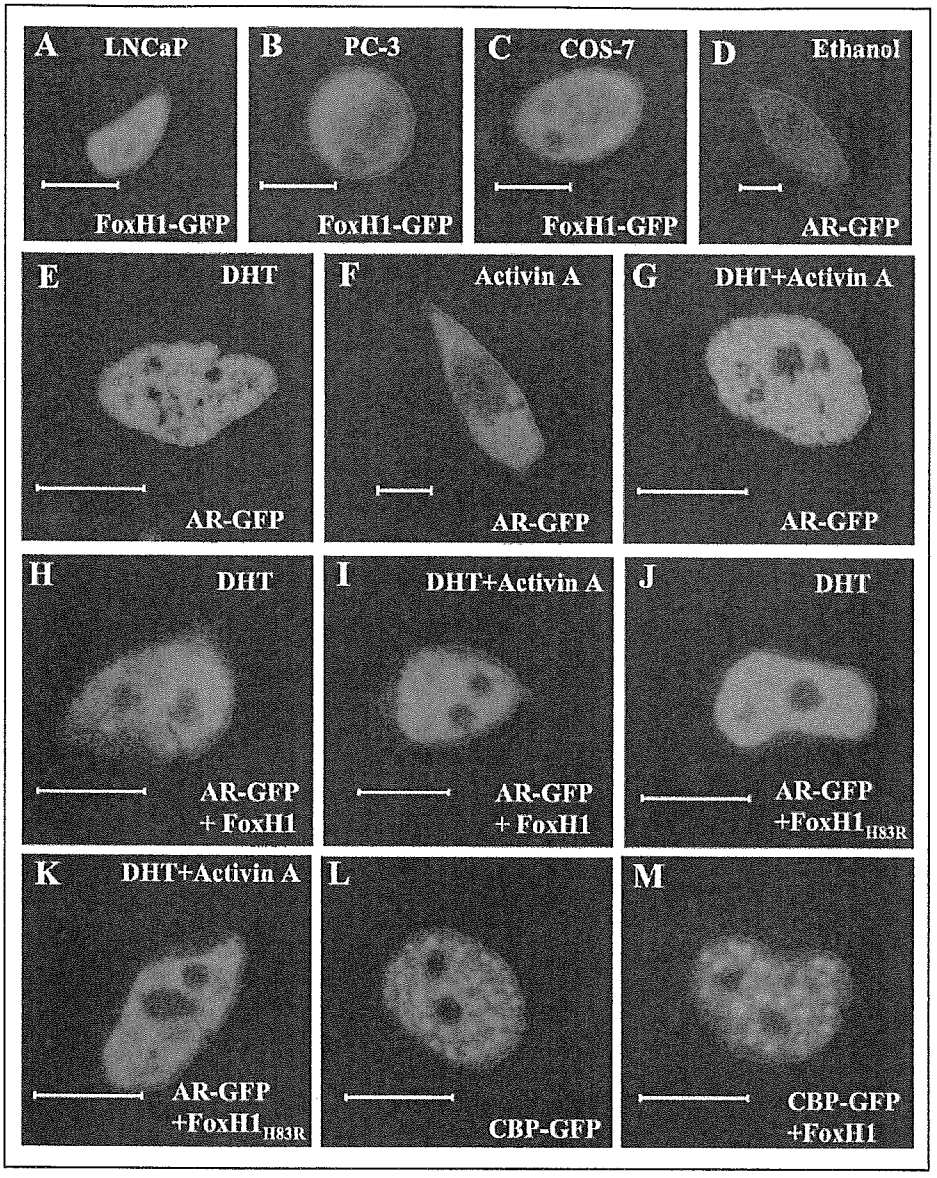

FIGURE 6. **Confocal laser microscopy images of FoxH1-GFP, AR-GFP, and CBP-GFP.** *A–C*, intracellular localizations of FoxH1 protein. pFoxH1-GFP was transiently transfected into LNCaP cells (*A*), PC-3 cells (*B*), and COS-7 cells (*C*), and the fluorescent signals in the cells were collected by confocal laser scanning microscopy. *D–M*, specific disruption of the AR nuclear foci formation by FoxH1. pAR-GFP or pCBP-GFP was cotransfected into LNCaP cells with the pCMV parent vector (*D–G* and *L*), pCMVFoxH1 (*H*, *I*, and *M*), or pCMVFoxH1$_{H83R}$ (*J* and *K*). After transfection the cells were treated with 0.1% ethanol (*D*), 10 nM DHT (*E*, *H*, and *J*), 25 ng/ml activin A (*F*), or DHT and activin A (*G*, *I*, and *K*) as indicated, and the fluorescent signals from AR-GFP or CBP-GFP were collected after 2 h. *Bars*, 10 μm.

ing to AR-AF-1. These data suggest that AF-1 may be involved in the interaction of the AR with FoxH1 in both the presence and absence of androgens.

FoxH1 Represses AR-AF-1 Function—Because our data implicated AF-1 as the site of FoxH1 corepressor activity, we investigated whether AF-1-dependent transactivation was sensitive to FoxH1 repression. As shown in Fig. 7*C*, when pBIND-AR-NTD (amino acids 1–532) was cotransfected together with pG5-LUC, there was a marked increase in the luciferase activity which was dramatically reduced when FoxH1 was coexpressed. These results provide additional evidence that AF-1 may be involved in the interaction between the AR and FoxH1 in cells.

DISCUSSION

To date many coregulators of the AR have been identified and characterized (13, 15). Compared with the coactivators, the AR corepressors identified are relatively fewer and less well characterized. The data obtained in the present study demonstrate a new function for FoxH1, which was expressed in LNCaP cells and the other prostate cancer cell lines tested, as a corepressor that attenuates the transactivation potential of the AR. This effect occurred in both the presence or absence of activin A, indicating that activin A was not involved in the repression of the AR by FoxH1 and, conversely, that FoxH1 was dispensable for the stimulatory effect of activin A on PSA expression (26, 27, 41). However, the possible roles of the FoxH1-mediated repression of the AR in activin signaling in prostate cancer require further investigation since Smad2/4 can rescue the repression of the AR by FoxH1, and a mutually antagonistic interaction between activin and androgen signaling has been shown to be involved in modulating the expressions of cell cycle regulatory proteins such as Rb, E2F-1, and p27 (27), thereby playing an important role in the regulation of prostate cancer cell growth. Hence, further clarification of the effect of the FoxH1-mediated repression of the AR on the expressions of these cell cycle regulatory proteins would help to elucidate the molecular mechanism through which activin A regulates gene expression and growth in the prostate.

The AR shares hormone response element sequences in the DNA with GR and PR (67). In this regard, the NH_2-terminal region, which varies among these receptors, is considered to be responsible for the cell- and ligand-specific regulation of their target genes (66, 68, 69). The

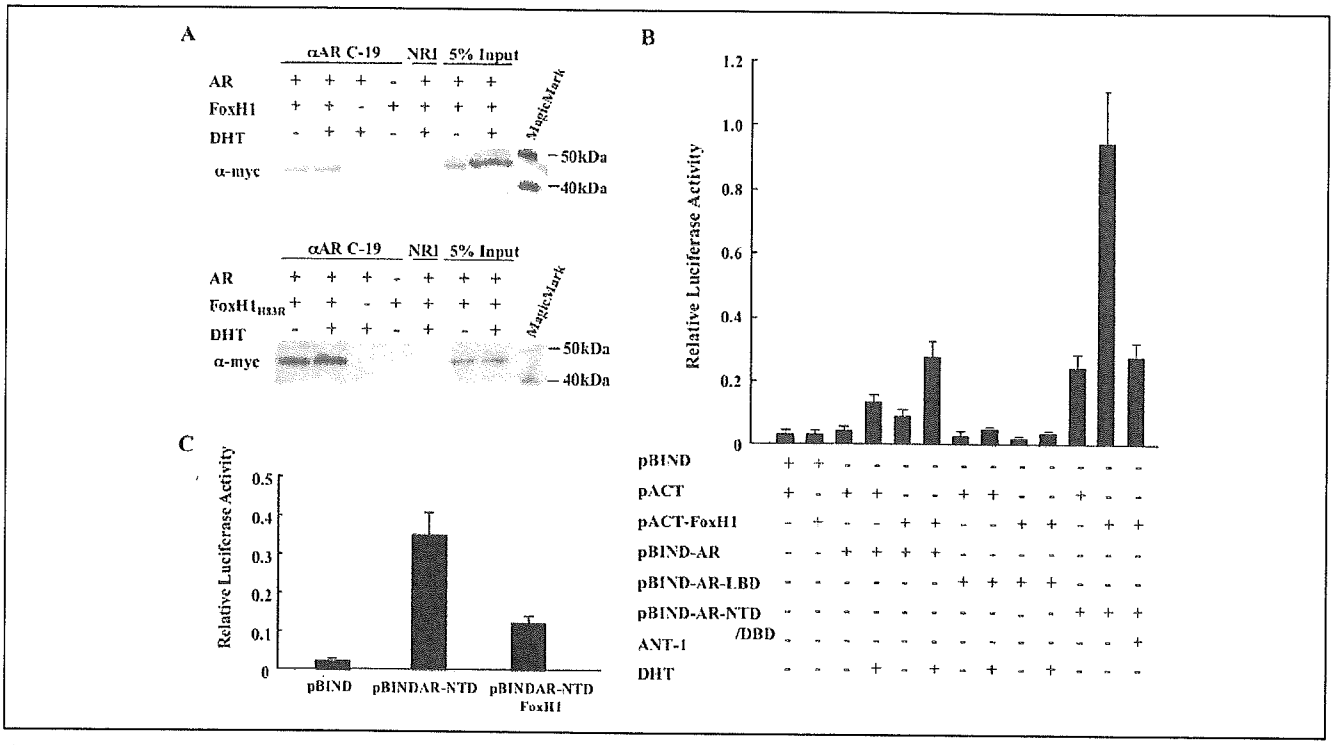

FIGURE 7. **Physical interaction between FoxH1 and the AR.** *A*, coimmunoprecipitation of the AR with FoxH1 or FoxH1H83R was performed as described under "Experimental Procedures." Whole cell extracts were immunoprecipitated with an anti-AR antibody (*C-19*) or normal rabbit IgG (*NRI*), and the immunoprecipitated fractions were analyzed by immunoblotting with an anti-Myc antibody. *B*, involvement of AF-1 in the interaction of the AR with FoxH1. Mammalian two-hybrid assays were carried out to test for an *in vivo* interaction between the AR and FoxH1. NIH3T3 cells were cotransfected with a DNA mixture containing 100 ng of pG5-LUC, 1.5 ng of pRL-SV40, 75 ng of pACT-FoxH1, or an equimolar amount of the pACT parent vector, 25 ng of pBIND-AR or equimolar amounts of pBIND-AR-NTD/DBD, pBIND-AR-LBD or the pBIND parent vector, and 225 ng of pcDNA3-ANT-1 or an equimolar amount of the pcDNA3 parent vector. The total DNA was adjusted to 600 ng/well with pBSK+ DNA. After transfection the cells were treated with 0.1% ethanol or 10 nM DHT. *C*, FoxH1 represses AR-AF-1 function. NIH3T3 cells were cotransfected with 100 ng of pG5-LUC, 1.5 ng of pRL-SV40, 50 ng of pCMV-FoxH1, or an equimolar amount of pCMV parent vector and 10 ng of pBIND-AR-NTD or an equimolar amount of the pBIND parent vector. The total DNA was adjusted to 600 ng/well with pBSK+ DNA. Approximately 24 h after transfection the cells were harvested, and the luciferase activity was measured.

AR is thought to be quite unique among the nuclear receptor superfamily members, since most if not all of its activities are mediated via the ligand-independent constitutive activity of the AF-1 (66). The fundamental role of the AR-AF-1 was further supported by our recent finding (43) that the absence of an AR-AF-1-specific coactivator resulted in androgen insensitivity syndrome. Our current observations show that AF-1 is involved in the interaction of the AR with FoxH1, which may explain why the inhibitory effect of FoxH1 on steroid receptors is selective. Because FoxH1 is expressed in mammary gland tissue (39), it is of interest to further elucidate whether FoxH1 is also able to interact with ER in breast cancer cell lines, such as MCF-7, and whether it is involved in the regulation of TGF-β/activin signaling during the growth of breast cancer.

In addition to SRC-1 (70) and CBP/P300 (71), which interact with both AF-1 and AF-2 of the AR, many coregulators, such as Hey1 (72), SMRT (60), SMAD3 (57), AES (73), cyclin D1 (74), ANT-1 (46), ARA24 (75), and ARA160 (76), have also been shown to interact preferentially with the AF-1. Interestingly, STAT3, a critical signaling molecule required for ligand-independent transactivation of the AR by interleukin-6, also associates with the AR via AF-1 (53, 77, 78). Because androgen levels do not fluctuate dramatically in adult males, the relative levels of coactivators *versus* corepressors binding to the AR may play a critical role in modulating AR function (15, 79). Indeed, Hey1 (72) and SMRT (60) have been shown to attenuate AR transcriptional activity through interrupting the interaction between the AR and SRC-1. Similarly, we demonstrated that ANT-1 competed with FoxH1 for binding to the AR. Therefore, interrupting the interactions between the AR and its coactivators may be responsible for the inhibitory effects of FoxH1 on the ligand-dependent and -independent transactivation of the AR. In addition, FoxH1, as well as Hey1, could attenuate the AR transactivation potential in both the presence and absence of androgens, suggesting that therapeutic interventions based on AR-coregulator interactions could be designed to block both androgen-dependent and -independent growth of tumor cells.

We previously reported a sensitive confocal laser microscopy approach that can clearly distinguish the transcriptionally active and inactive forms of the AR *in vivo* (44). More recently, we reported that transfer to common compartments (foci) of the nucleus and complex formation with coactivators, such as SRC-1, TIF2, and CBP, may be essential processes for eliciting the transactivation function of the AR (45). In the present study we observed that FoxH1 had no effect on the nuclear translocation of the AR but specifically blocked the DHT-induced foci formation by the AR in LNCaP cells. Therefore, the simplest explanation for our current finding that FoxH1 represses the AR-mediated transactivation is that FoxH1 maybe compete with AR coactivators, such as SRC-1 and ANT-1, for binding to the AR, thereby inhibiting the formation of the transcriptionally active complex. However, further studies are required to elucidate the precise mechanism of the FoxH1-induced repression of AR-mediated transactivation. Moreover, it will be interesting to investigate whether disruption of the foci formation is also involved in the repression of AR-mediated transactivation by other corepressors, such as Hey1 and SMRT.

In conclusion, this is the first report to demonstrate that association of FoxH1 with the AR can inhibit the transactivation potential of the AR.

Further studies of the expression of FoxH1 in prostate cancer at different stages and its role in the mutually antagonistic effects of androgen and activin A will provide fresh insights into the biology of prostate cancer and may lead to the development of new treatments.

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シンポジウム

4. 男性更年期外来の治療と症状スコア

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要旨：PADAM 症状調査票について aging male symptom score（AMS）と熊本の症状調査票を比較検討した。因子分析を行ってみると AMS については，各領域の独立性が低く，トータルのスコアでの症状評価には適切であっても領域別の解析には不適当であると思われた。一方熊本調査票は領域別の独立性が保たれていることから，領域別の解析に優れていることがわかった。

key words　症状スコア，PADAM，因子分析

「男性更年期外来」を受診する患者は 40 ～ 60 歳代で疲労，健康感の減少，抑うつ，いらつき，不眠，記憶力の低下，認知機能低下，性欲低下，性機能低下，筋肉痛，筋力低下，骨密度の低下，ほてり，発汗過多などの多彩な愁訴を訴えることを特徴とする。これらの症状の多くは女性の更年期障害と類似しているが，またうつ病とも共通する。症状の重症度評価は症状調査票を用いることが多い。精神・心理，身体，性機能についての 17 項目についての self-assessment 型の症状スコアである aging male symptom score が世界的に汎用されている。また DSM-Ⅳを用いうつ病についてのスクリーニングを行うことにより，精神科，心療内科とも連携を行っている。われわれの男性更年期外来で用いている症状調査票を表 1 に示した。aging male symptom score[1] は精神・心理，身体，性機能についての 17 項目について，5 段階評価の self-assessment 型の症状スコアである（表 2）。合計が 26 以下は正常，27 ～ 36 は軽度の異常，37 ～ 49 は中等度の異常，50 以上は重症とされている。われわれの検討では，男性更年期外来を受診した患者で AMS の合計スコアと血中フリーテストステロン値には相関が無かったものの，テストステロン補充後は AMS は有意に改善され，治療経過を見る上でよい surrogate marker であると思われた（図 1）。一方熊本は以前より健常者を対象に「健康調査票」での大規模な QOL 研究を行ってきたが，そこから得られた結果を元に PADAM についての症状調査票を作成した（表 3）。こちらも精神・心理，身体，性機能についての質問から構成されている。われわれはこの 2 つの質問紙を比較検討するために，2003 年 11 月から 2004 年 9 月まで帝京大学およ

表 1　質問紙を用いた症状評価

- □ Morley：スクリーニング
- □ Aging male symptoms score：Heinemann
- □症状調査票：熊本
- □うつの診断 DSM-IV
- □うつの症状スコア
  - ■ BDI
  - ■ SDS

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key words：AMS, Symptom score, PADAM

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