

# Kallikrein-binding protein is induced by growth hormone in the dwarf rat

HEATHER C. HATCHER,\* NANCY M. WRIGHT,<sup>†</sup> JULIE CHAO,<sup>‡</sup> LEE CHAO,<sup>‡</sup> AND JIAN-XING MA\*<sup>1</sup>

\*Department of Ophthalmology, <sup>†</sup>Department of Pediatrics, Division of Endocrinology, <sup>‡</sup>Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, USA

**ABSTRACT** Rat kallikrein-binding protein (KBP), a member of the serpin family, is a tissue kallikrein inhibitor. It has been shown to be a potential pathogenic factor of diabetic retinopathy and may play a role in animal development and growth. To determine whether reduced KBP expression is involved in retarded animal growth, we examined the *in vivo* effect of growth hormone (GH) deficiency on the expression of KBP in the Lewis dwarf (*dw/dw*). We found that serum levels of functionally active KBP were reduced in the dwarf rat ( $P < 0.05$ ) as determined by complex formation assay between serum KBP and <sup>125</sup>I-labeled rat tissue kallikrein. Enzyme-linked immunosorbent assay showed that KBP levels were significantly reduced in the serum of the dwarf rat compared to the Lewis rat (213.8 ng/ml vs. 413.8 ng/ml,  $n = 4$ ,  $P < 0.01$ ). The decreased KBP levels were confirmed by Western blot analysis. Moreover, treatment of the dwarf rat with recombinant human GH for 4 wk resulted in a significant increase in KBP activity ( $P < 0.01$ ) and serum KBP levels compared with the untreated dwarf rat (549.8 ng/ml,  $n = 5$ , vs. 213.8 ng/ml,  $n = 4$ ,  $P < 0.02$ ). Northern blot analysis and densitometry showed that liver KBP mRNA levels were reduced by fivefold in the dwarf rat compared to the Lewis rat and the decrease was reversed by the GH treatment. These results indicate that the KBP levels are regulated at the RNA level. Furthermore, *in vitro* studies using cultured rat hepatocytes showed that GH may have a direct regulatory effect on KBP expression since KBP levels increased in the conditioned media of cells treated with GH. These results demonstrated that KBP is reduced in the genetic dwarf rat and is restored to normal by GH; therefore, KBP is a GH-dependent protein and may be a new target for studying the mechanism of pathological animal growth.—Hatcher, H. C., Wright, N. M., Chao, J., Chao, L., Ma, J.-x. Kallikrein binding protein is induced by growth hormone in the dwarf rat. *FASEB J.* 13, 1839–1844 (1999)

*Key Words:* growth hormone deficiency • serpins • tissue kallikrein

THE MECHANISMS SURROUNDING the growth-promoting actions of growth hormone (GH)<sup>2</sup> are not well understood, although several lines of evidence suggest that GH and somatomedin/insulin-like growth factors coordinate to promote growth (1). It is unclear whether the effects of GH result from direct action on target cells or are mediated indirectly by stimulation of growth factors (1). Since the liver is a major site of serum protein synthesis, GH may have a significant effect on this organ. Investigation of those proteins responsive to GH thus may generate a greater understanding of the molecular mechanisms pertaining to growth hormone action. The exact mechanisms of growth hormone action in the liver and GH regulation of gene transcription are unknown. Liver proteins initially identified as being GH responsive include  $\alpha_{2u}$ -globulin (2–5), insulin-like growth factor 1 (IGF-1) and its binding proteins (6), and a protein called ‘growth hormone-responsive acidic protein’ (7–9), identified as kallikrein binding protein (KBP) (10, 11).

KBP is a potent modulator of tissue kallikrein activity and bioavailability (12). KBP, which is produced primarily in the liver, is induced by multiple hormones including GH, triiodothyronine, glucocorticoid hormones, estrogen, and progesterone. Its expression levels correlate with animal growth rates, indicating it may play a role in animal development in addition to modulating kallikrein activity (8, 9, 11, 13). A growth hormone-response element in the 5′ flanking region of the KBP gene may mediate the induction of KBP by growth hormone (14, 15). The physiological function of KBP is currently unknown; however, several lines of evidence

<sup>1</sup> Correspondence: Medical University of South Carolina, Department of Ophthalmology, 171 Ashley Ave., Charleston, SC 29425, USA. E-mail majx@musc.edu

<sup>2</sup> Abbreviations: EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunoassay; GH, growth hormone; IGF, insulin-like growth factor; KBP, kallikrein binding protein; PBS, phosphate-buffered saline; rhGH, recombinant human growth hormone; s.c., subcutaneously; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STZ, streptozotocin.

indicate that it may participate in blood pressure regulation, inflammatory response, growth, and development (8, 12, 16–18).

KBP has been proposed to have a role in the development of hypertension (10, 16, 17) and diabetes (19, 20). Studies in both diabetic animal models and diabetic humans reveal potential involvement of the tissue kallikrein–kinin system in diabetes mellitus (21, 22). Our recent studies have suggested the potential function of KBP in ocular tissues as high levels of KBP were identified in human retinal neuronal and capillary endothelial cells (23). Vitreous KBP concentrations in diabetic humans were significantly lower than those in nondiabetic vitreous fluids (19). This finding was supported by our results showing that retinal and serum KBP levels were significantly reduced in the streptozotocin (STZ)-induced diabetic rat (20). Although the mechanism of the decreased KBP in diabetes is unknown, these studies suggest the potential involvement of KBP in the development of diabetic retinopathy.

Several studies have shown that GH levels are decreased in the STZ-induced diabetic rat and diabetic (*db/db*) mouse (24–27). Therefore, alterations in levels of GH in the STZ-diabetic rat may directly or indirectly regulate KBP expression. The aim of this study is to examine GH regulation of KBP in the genetic GH-deficient dwarf rat by measuring liver and serum levels of KBP and to determine whether KBP deficiency is involved in the retarded animal growth in the genetic dwarf rat. The genetic dwarf rat is known to be deficient in GH while having normal secretion of all other pituitary hormones (28). The amount of GH secreted from the pituitary by homozygous dwarf female rats is only 6% that of normal controls (28). Therefore, the effects of GH treatment on KBP can more readily be determined when using this animal model.

## MATERIALS AND METHODS

### Animals

Three age- and gender-matched animal groups were studied: the untreated and GH-treated dwarf rat and the Lewis rat. Homozygous dwarf (*dw*) rat breeding pairs (*dw-4-ola-hsd*) were obtained from Harlan Olac (Bicester, England) and bred as previously reported (29). Female offspring were used for this study. The mutation was originally discovered in an inbred colony of the Lewis rat in Oxford, U.K. Breeding difficulty required the mutation to be introduced onto the NIMR/AS rat strain, with subsequent back-crossing onto the Fischer rat strain (Harlan Olac). Timed pregnant Lewis rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) and female offspring were used as controls. Animals were housed in standard rat cages in controlled animal facilities (23°C, 14 h light) according to procedures outlined in the *NIH Guide for the Care and Use of Laboratory Animals*. All

animals were weaned at 3 wk of age, fed standard rat chow *ad libitum*, and allowed continuous access to water.

Recombinant human growth hormone (rhGH, Humatrope) was kindly provided by Eli Lilly and Co. (Indianapolis, Ind.). Animals in the treatment group received 200 µg/day subcutaneously (s.c.) of rhGH from weeks 2 to 6. Serum was obtained from the animals by cardiac puncture at the time of death.

### Tissue kallikrein-KBP complex formation

Serum (1 µl/animal) was incubated with [<sup>125</sup>I]-labeled rat tissue kallikrein (10,000 cpm per sample) in 10 mM sodium phosphate buffer, pH 7.0, at 37°C for 1 h as described previously (16). The binding was stopped by adding one-third volume of 3× sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris-Cl, pH 6.8, 30% glycerol, and 5% SDS) and boiling for 5 min. The mixture was then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions and stained with Coomassie blue. The gel was dried and exposed to Kodak X-Omat film. The band formed by the complex (92 kDa) was analyzed by densitometry using NIH Image V1.57 (NIH, Bethesda, Md.).

### Enzyme-linked immunosorbent assay (ELISA)

The ELISA was modified from a previously described method (30). A 96-well microtiter plate was coated with unlabeled anti-KBP IgG [1 µg/ml in phosphate-buffered saline (PBS), 100 µl/well] overnight at 4[de]C. The plate was then blocked with 200 µl/well of PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) containing 1% BSA at 37°C for 1 h and washed three times with PBS containing 0.1% Tween-20 (washing solution). Dilutions of purified KBP (0.4 to 25 ng/ml) and rat sera, which were diluted in 100 ml of freshly made dilution buffer (PBS containing 0.05% Tween-20 and 0.5% gelatin), were added to individual wells. The plate was incubated at 37°C for 90 min and washed three times with the washing solution. Biotin-labeled anti-KBP antibody (31) was added into each well at a concentration of 1 µg/ml in a total volume of 100 µl. The plate was incubated at 37°C for 1 h and washed three times with the washing solution and once with PBS. Freshly prepared substrate solution [10 µl of 0.1 M citrate buffer, pH 4.3, 3 mg of 2,2'-azino-bis(3-ethylbenzthiazoline-sulfonic acid), and 10 µl of 3% H<sub>2</sub>O<sub>2</sub>] were added into each well (100 µl/well). After 30 min the plate was measured with an ELISA reader at 414 nm for absorbance.

### Western blot analysis

Rat sera or purified KBP was resolved on SDS/PAGE and electrotransferred onto nitrocellulose membranes. The antigen overlay method for immunoblotting has been described previously (32). Briefly, the membranes were blocked with BLOTTO solution [5% (w/v) nonfat dry milk in TBST (20 mM Tris base, 137 mM sodium chloride, pH 7.6, and 0.05% (w/v) polyoxyethylene sorbitan monolaurate (Tween 20))] overnight at 4°C. The membranes were then incubated with rabbit anti-KBP antiserum (1:250 in BLOTTO solution) for 3 h with gentle shaking. The membranes were washed three times with BLOTTO, followed by an incubation with [<sup>125</sup>I]-labeled KBP for 1.5 h. The membranes were washed three times with BLOTTO and once with TBST, air-dried, and exposed to Kodak X-Omat film. All procedures were done at room temperature. Densitometry of the specific KBP band was done using NIH Image V1.57.

## RNA extraction and Northern blot analysis

RNA was isolated from rat livers as previously (20). RNA was electrophoresed through a 1.2% agarose gel containing formaldehyde. The samples were prepared by mixing the following in a sterile Microfuge tube: 4.5  $\mu$ l (20  $\mu$ g) RNA, 2.0  $\mu$ l 5 $\times$  MOPS running buffer [1 $\times$  MOPS running buffer = 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0, 8 mM sodium citrate, 1 mM ethylenediaminetetraacetic acid (EDTA)], 3.5  $\mu$ l formaldehyde, and 10.0  $\mu$ l formamide. The samples were heated for 15 min at 65°C, then chilled on ice. After a brief centrifugation, 2  $\mu$ l of formaldehyde gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was added to each tube. The gel was run submerged in 1 $\times$  MOPS buffer at 3–4 V/cm for ~3 h. At the end of the run, the molecular weight marker was cut from the gel, stained with ethidium bromide for 30 min, and photographed under UV light. The RNA from the gel was transferred to a nylon membrane by capillary transfer using 20 $\times$  SSC (1 $\times$  SSC = 0.15 M sodium chloride, 15 mM sodium citrate, pH 7.0). The RNA was UV-cross-linked to the nylon membrane. Full-length KBP and  $\beta$ -actin cDNAs were nick-translated with [ $\alpha$ - $^{32}$ P]dCTP and hybridized with the membrane in a hybridization solution containing 5 $\times$  SSPE (1 $\times$  SSPE = 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.4), 5 $\times$  Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, and 100  $\mu$ g/ml denatured herring sperm DNA at 42°C overnight (11). The membrane was washed in a final solution of 6 $\times$  SSPE, 0.1% SDS twice at 42°C, and exposed to Kodak X-Omat film at -70°C. To account for loading efficiency of the gel, the membrane was stripped, boiled, and reprobed with  $\beta$ -actin cDNA. Densitometry of the KBP band was measured using NIH Image V1.57. KBP mRNA levels in the three groups were normalized by the  $\beta$ -actin mRNA level.

## Cell culture

Rat hepatocytes (HF1B) obtained from frozen stock were grown on 75 cm<sup>2</sup> tissue culture flasks and incubated under constant humidity in an atmosphere of 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotic/antimycotic mixture (200 U/ml penicillin, 200  $\mu$ g/ml streptomycin, and 0.5  $\mu$ g/ml amphotericin B). The cells were subcultured by enzymatic dissociation using 0.05% trypsin/0.53 mmol/l EDTA solution. All cell culture reagents were obtained from Gibco/BRL Life Technologies (Gaithersburg, Md.). To study the effects of growth hormone, cells were cultured in OptiMEM serum-free medium for 48 h with the addition of 500 ng/ml rhGH or albumin. After 48 h, the conditioned media was collected and an ELISA was performed as described previously.

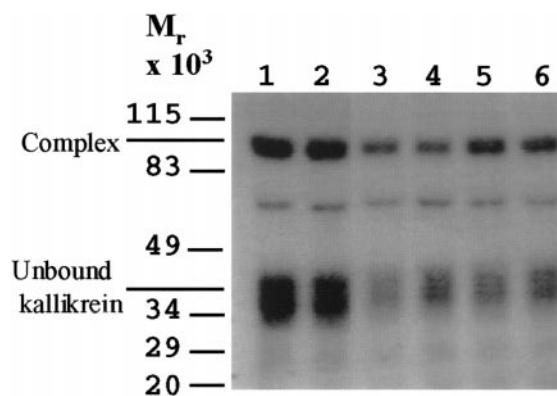
## Statistical analysis

Serum KBP levels were averaged within a group and presented as the mean  $\pm$  SE. The significance of the difference between two groups was determined by Student's *t* test. Differences were considered significant at a value of  $P < 0.05$ .

## RESULTS

### Kallikrein binding activity in rat sera

The activity of KBP was assayed by the ability to bind to [ $^{125}$ I]-labeled rat tissue kallikrein. **Figure 1**

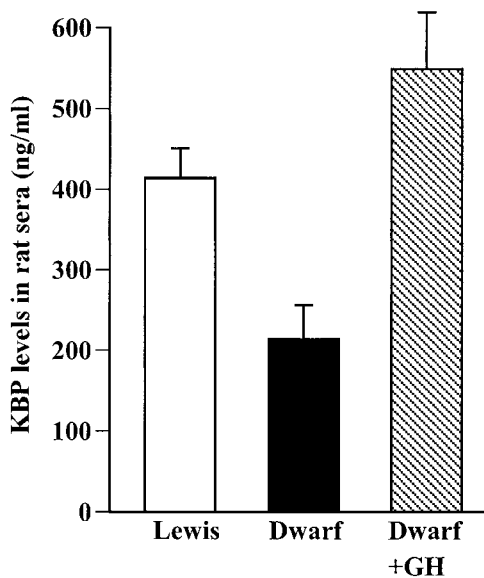
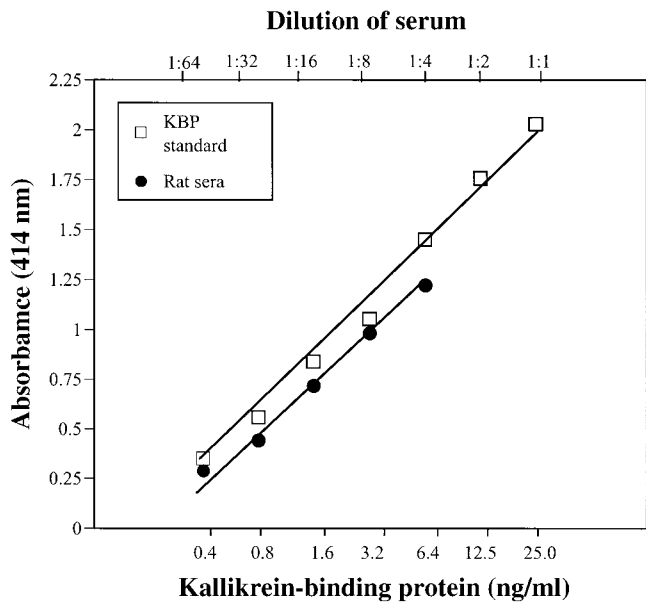


**Figure 1.** KBP–kallikrein complex formation in rat serum. Serum (1  $\mu$ l/animal) was incubated with  $^{125}$ I-labeled rat tissue kallikrein and resolved by SDS-PAGE. The unbound kallikrein and the high molecular mass complex (92 kDa) formed between kallikrein and KBP, indicated at the left, were visualized by autoradiography. Lanes 1, 2: Lewis rat; lanes 3, 4: dwarf rat; lanes 5, 6: GH-treated dwarf rat. Functionally active KBP decreased twofold in the dwarf rat compared to the Lewis rat ( $P < 0.05$ ) and increased nearly twofold in the GH-treated dwarf compared to the untreated dwarf rat ( $P < 0.01$ ).

shows the formation of a tissue kallikrein–KBP complex on SDS-PAGE as visualized by autoradiography. The molecular mass of the complex (92 kDa) is identical to that of the complex formed from purified KBP and tissue kallikrein (10), indicating the existence of a functional KBP. Functionally active KBP in the serum of the dwarf rat (Fig. 1, lanes 3, 4) showed a twofold decrease compared to the Lewis rat (Fig. 1, lanes 1, 2) when the same amount of protein was used for the complex formation assay (Fig. 1). After GH treatment of the dwarf rat for 4 wk, the binding activity of KBP increased nearly twofold (Fig. 1, lanes 5, 6) compared to the untreated dwarf rat.

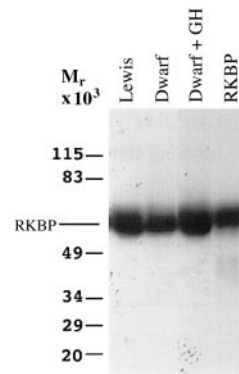
### Immunoreactive KBP levels in rat sera

An ELISA using a polyclonal antibody specific for KBP was performed to quantify KBP expression in the rat sera. As shown in **Fig. 2A**, the serial dilution curve of the rat sera is parallel to that of the KBP standard, suggesting its immunological identity. KBP levels in the dwarf rat were decreased compared to the Lewis rat (213.8 ng/ml of protein vs. 413.8 ng/ml of protein) (Fig. 2B). GH treatment of the dwarf rat for 4 wk restored serum KBP levels (549.8 ng/ml protein) (Fig. 2B). Immunoreactive KBP levels in sera were analyzed by Western blot using an antibody specific for KBP and the antigen overlay method. As shown in **Fig. 3**, the immunoreactive protein has a molecular mass of 60 kDa, which is identical to purified KBP (10). The semi-quantitative immunoblot showed that serum KBP expression was decreased in the dwarf rat and



**Figure 2.** A) Enzyme-linked immunosorbent assay of KBP in rat sera. The standard curve of KBP, which ranges from 0.4 to 25.0 ng/ml, is represented by solid squares. Serial dilution curve of rat sera (solid circles) are parallel to the KBP standard curve. B) Immunoreactive KBP levels in rat sera. Sera from Lewis, dwarf, and GH-treated dwarf rats were subjected to an ELISA. KBP levels were expressed as the mean  $\pm$  SE. The white bar represents the control Lewis rat,  $n=4$ ; the black bar represents the dwarf rat,  $n=4$ ; the striped box represents the GH-treated dwarf rat,  $n=5$ . KBP levels were significantly reduced in the untreated dwarf rat compared to the control Lewis rat ( $P<0.01$ ) and the GH-treated dwarf rat ( $P<0.05$ ).

increased in the GH-treated dwarf rat (Fig. 3). As shown by Wright and colleagues (29), body weight, length, and serum IGF-I levels were decreased in the dwarf rat compared to the Lewis and the GH-treated dwarf rat. Our data show that KBP levels are correlated with body weight and length and the serum IGF-I levels of the animals. These

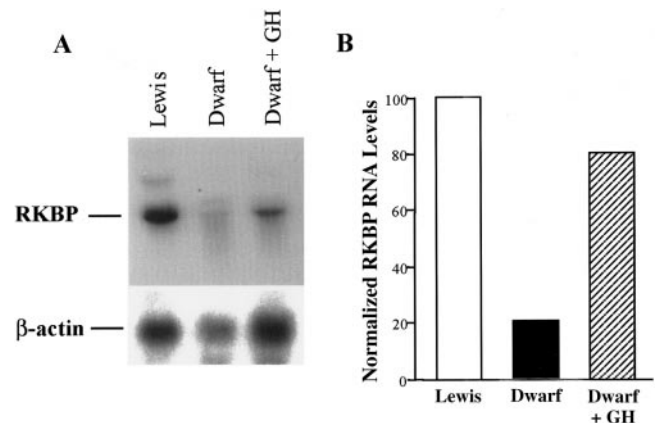


**Figure 3.** Western blot analysis of KBP in rat sera. Sera (2  $\mu$ l/animal) and purified KBP (6.6  $\mu$ g) were resolved on SDS-polyacrylamide gel electrophoresis and electrotransferred onto an Immobilon-P filter, which was blotted by an antigen overlay method. The membrane was blotted with rabbit anti-KBP antiserum, incubated with  $^{125}$ I-labeled KBP, and measured by densitometry as described in Materials and Methods.

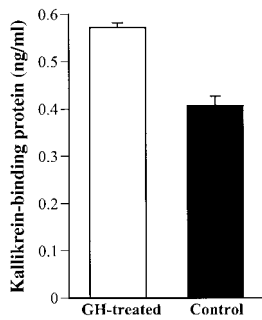
results suggest that KBP expression is correlated with animal growth rates.

### Northern blot analysis of KBP expression in rat liver

To determine whether the changes in KBP levels occurred at the translation or transcription level, the KBP mRNA from rat liver was measured, as the liver is the major source for KBP. Total RNA (15  $\mu$ g) from each group was subjected to Northern blot analysis using a full-length KBP cDNA probe. The Northern blot was stripped and reprobed with  $\beta$ -actin, a house-keeping gene, to account for loading accuracy. KBP mRNA levels were normalized by the mRNA levels of  $\beta$ -actin (Fig. 4A), using densitometry. The normalized levels are shown in Fig. 4B. A fivefold decrease was observed in the KBP mRNA level in the liver of the dwarf rat compared to the Lewis rat (Fig. 4A, B). GH treatment of the dwarf rat showed a nearly fourfold increase in KBP mRNA expression compared to the untreated dwarf rat (Fig. 4A, B). There-



**Figure 4.** Northern blot analysis of KBP mRNA level in rat liver. A) Total RNA (15  $\mu$ g) from the liver of Lewis, dwarf, and GH-treated dwarf rats were hybridized with a  $^{32}$ P-labeled RKBP and  $\beta$ -actin cDNA probes. B) KBP mRNA levels were normalized by the mRNA levels of  $\beta$ -actin. The white bar represents the control Lewis rat, the black bar represents the dwarf rat, and the striped box represents the GH-treated dwarf rat.



**Figure 5.** Immunoreactive KBP levels in conditioned media. Media from rat hepatocytes cultured for 48 h with 500 ng/ml rhGH or albumin were subjected to an ELISA. KBP levels were expressed as the mean  $\pm$  SE. The white bar represents the media from the cells cultured in the albumin control,  $n=4$ ; the black bar represents the media from the cells cultured in GH,  $n=4$ .

fore, the decrease of hepatic KBP in the dwarf rat occurred at the transcription level.

### Measurement of KBP in cultured rat hepatocytes

To examine whether GH has a direct effect on KBP expression, we assayed the conditioned serum-free media from rat hepatocytes that were cultured for 48 h with rhGH. The result of the 2 day treatment of rat hepatocytes with rhGH is presented in **Fig. 5**. KBP levels in the conditioned media from GH-treated rat hepatocytes increased significantly compared to that of the control albumin-treated cells (0.572 ng/ml vs. 0.407 ng/ml,  $n=4$ ,  $P < 0.002$ ) (Fig. 5). These results are consistent with the *in vivo* studies, indicating that GH directly regulates KBP expression.

## DISCUSSION

It has been suggested that the tissue kallikrein–kinin system may participate in several pathophysiological processes including hypertension, diabetes, and allergic responses (21, 33). KBP binds covalently with tissue kallikrein and inhibits its activities *in vitro* (10, 34). In addition, KBP has a profound effect on the clearance rate of tissue kallikrein *in vivo* as KBP prolongs the half-life of kallikrein (35). Therefore, KBP is a potent modulator of tissue kallikrein activity and bioavailability (12). The tissue kallikrein–kinin system participates in the regulation of vascular function and has been implicated in the diabetic complications of animal models and patients (36–38). Our previous studies showed that an endogenous kallikrein–kinin system exists in ocular tissues (23). KBP is decreased in the retina and serum of the STZ-induced diabetic rat (20); kallistatin, the human counterpart to KBP, is decreased in the vitreous of diabetic patients (19). The decreased level of retinal KBP in diabetes may allow overproduction of kinin due to increased kininogenase activity of tissue kallikrein, and thus may contribute to retinal vasodilation. Moreover, as KBP is an independent vascular regulator (18), the decreased KBP production may be involved in vascular abnormalities in diabetic retina. The direct cause of the KBP decrease in

STZ-diabetic rats is unknown. The present studies suggest that the reduced GH may be one of the mechanisms leading to the KBP reduction in STZ-diabetic rats.

The biological effect of GH is mediated by GH-dependent growth factors, such as IGF-1. In GH deficiency, these growth factors have been shown to be decreased (39, 40). The present studies present a novel observation to show that KBP is decreased in the genetic dwarf rat and KBP levels are restored by GH treatment. Furthermore, KBP levels paralleled the animal body weight and IGF-1 levels in the dwarf rat in response to GH treatment (29). Wright and colleagues found that IGF-1 levels and body weight were reduced in the dwarf rat, but restored to the level of the control rat after GH treatment (29). These findings support the notion that KBP may function as a GH-dependent growth factor in addition to acting as a kallikrein inhibitor. KBP is a major protein in the serum and is endogenously expressed in the neural retina, suggesting its physiological significance (12, 19). However, the physiological function of KBP is uncertain. Previous studies showed that KBP might have other activities independent of interactions with the kallikrein–kinin system (18). The present results support the role of KBP in retarded animal development resulting from GH deficiency, which may provide a clue to understand its physiological significance.

To explore the mechanism for the reduction of KBP in the dwarf rat, we treated rat hepatocytes, which endogenously express KBP, with GH. Our results showed that GH increased KBP expression in rat hepatocytes. This result indicates that decreased KBP may be a direct effect of reduced growth hormone or a growth-hormone dependent growth factor produced in the hepatocytes rather than a nonspecific secondary effect in dwarf rat. Further investigation of the mechanisms underlying the reduced KBP expression may reveal its significance in GH deficiency and diabetes. This may lead to beneficial clinical interventions to reverse this decrease of KBP in these disease processes. **[F]**

The authors would like to thank the laboratory of Dr. Rosalie K. Crouch in the Department of Ophthalmology (MUSC) for technical support and Zhirong Yang in the Department of Biochemistry (MUSC) for performing ELISA. This work was supported by a grant from Research to Prevent Blindness and an NIH grant EY12600.

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Received for publication November 30, 1998.

Revised for publication May 11, 1999.