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Mouse Heparin Binding Protein-44 (HBP-44) Associates with Brushin, a High-Molecular-Weight Glycoprotein Antigen Common to the Kidney and Teratocarcinomas¹

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Heparin binding protein-44 (HBP-44) is a heparin binding protein of 44 kDa, found by cDNA cloning using antibodies against teratocarcinoma glycoproteins [Furukawa, T. *et al.* (1990) *J. Biochem.* 108, 297-302]. The N-terminal sequence analysis reported in this publication establishes the structure of its mature form. Immunohistochemical staining revealed that HBP-44 was located in the tubular brush border of the kidney. HBP-44 formed a complex with brushin, a high molecular weight (450 kDa) glycoprotein antigen common to the kidney and teratocarcinoma, but not with OR8 antigen, another antigen (350 kDa) of the same category. Brushin was shown to be the mouse counterpart of rat Heymann nephritis antigen, called gp330. The association between HBP-44 and brushin was revealed not only by co-precipitation upon indirect immunoprecipitation, but also by ligand blotting with HBP-44-maltose binding protein fusion protein. Calcium ion stabilized the association. Disulfide bonds in brushin seemed to be necessary for the complex formation, since reductive cleavage of the bonds resulted in failure of the protein to associate with HBP-44 in a ligand blotting experiment. Association of HBP-44 with brushin occurred both in teratocarcinoma cells, in which these molecules are mainly located in extraembryonic endoderm cells, and in the kidney, suggesting that the complex has an unknown common function in the renal tubular brush border and the extraembryonic endoderm.

Differentiation antigens, which are selectively expressed in certain differentiated cells or in subpopulations of cells in the intermediate stage of differentiation, serve as useful markers in cell identification and separation. Furthermore, these antigens may reflect functional characteristics of the positive cells, particularly in the cell surface, giving clues to analyze specific cell functions in mature and differentiating cells. Among many differentiation antigens found in the mouse teratocarcinoma system, several are also expressed in the kidney (1-5).

Heparin binding protein-44 (HBP-44), one such antigen, is a 44 kDa heparin binding protein found by cDNA cloning using antibodies against glycoproteins from teratocarcinoma cells (5). HBP-44 mRNA is intensely expressed in the kidney and the extraembryonic endoderm cells of teratocarcinomas (5). A 39 kDa human protein subse-

quently identified (RAP, receptor-associated protein) has 77% amino acid identity with HBP-44 (6), and is probably the human homolog of HBP-44.

Brushin is defined by polyclonal antibodies raised against teratocarcinoma glycoproteins which bind to a lectin, *Dolichos biflorus* agglutinin (DBA) (3). OR8 antigen is defined by a monoclonal antibody raised against RCA (*Ricinus communis* agglutinin)-binding glycoproteins from murine teratocarcinomas (4). Brushin and OR8 antigen are expressed in the visceral endoderm and the renal tubular brush border, and are of high molecular weight (4). Furthermore, renal distribution of a high molecular weight glycoprotein of the rat, gp330 (7, 8), which is a causative antigen of an experimental autoimmune nephritis called Heymann nephritis (9), is similar to brushin and OR8 antigen. Although we observed no immunological cross reaction between HBP-44 and brushin, a partial protein sequence of rat gp330 reported by Pietromonaco *et al.* was surprisingly similar to that of HBP-44 (10).

Herein we report further studies on HBP-44. We were particularly interested in clarifying the relationship between HBP-44 and the high molecular weight renal antigens. Since RAP, which is probably the human HBP-44, associates with α_2 -macroglobulin receptor (identical to LDL-receptor-related protein, LRP) (6), HBP-44 may be associated with the high-molecular-weight antigens.

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Abbreviations: α_2 MR, α_2 -macroglobulin receptor; DBA, *Dolichos biflorus* agglutinin; EGTA, ethyleneglycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid; HBP-44, heparin binding protein-44; LRP, LDL receptor-related protein; MBP, maltose binding protein; PBS(-), Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} ; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RAP, receptor associated protein; RCA, *Ricinus communis* agglutinin.

MATERIALS AND METHODS

Cells and Tumors—PYS-2 cells and teratocarcinoma OTT6050 were cultured or maintained as described previously (5).

Primer Extension—Primer extension was performed as described previously (5).

Fusion Proteins—Protein A-HBP-44 fusion protein was prepared as follows. The plasmid clone pO20 (5) was digested with *EcoRI*, blunt-ended with Klenow fragment, and then digested with *PstI*. The fragment was subcloned into the *SmaI*-*PstI* site of a protein A fusion protein vector pRIT2T (Pharmacia), and the recombinant plasmid was introduced into *Escherichia coli* N4830-1 cells. The bacterial culture and the purification of the fusion protein were performed as described previously (11).

For producing maltose binding protein (MBP)-HBP-44 fusion protein, *EcoRI*-*PstI* fragment of pO20 was subcloned into an MBP fusion protein vector, pMAL-cRI (Bio-Labs). *E. coli* TB1 cells were transformed with the recombinant plasmid, which encodes the fusion protein. The cells were cultured, induced for fusion protein production, and collected according to the protocol of the producer (Bio-Labs). The collected cell pellet was suspended in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(-)] and disrupted by sonication. After centrifugation of the sonicate at $20,000 \times g$ for 30 min, the supernatant was applied to a column of amylose-resin (Bio-Labs) (column volume = 1 ml), and the column was washed with 5-10 column volumes of PBS(-) containing 0.1% Tween 20 and with PBS(-) successively. The fusion protein was eluted with 10 mM maltose in PBS(-). About 10 mg of fusion protein was obtained from 1 liter of bacteria culture solution.

Antibodies—Rabbit anti-brushin antibodies and rat OR8 monoclonal antibodies were prepared as described previously (3, 4).

Isolation of rat gp330 and preparation of anti-gp330 antibodies were performed as follows. Renal tubular membrane fraction was prepared as described previously (12), and subjected to preparative SDS-PAGE (5% gel, under non-reducing conditions). The separation of gp330 from the other proteins including maltase was confirmed by Coomassie Blue staining. Proteins in the gel were then transferred electrophoretically to a nitrocellulose membrane and the position of gp330 was identified by Western blotting using sera of Heymann nephritis rats, which contain anti-gp330 autoantibodies. Most of the gp330 in the gel was not transferred to nitrocellulose membrane because of its size. A piece of the gel containing gp330 was collected and this isolation of gp330 was repeated. The gel (4 ml) containing gp330 was then homogenized in 4 ml of PBS and mixed with 8 ml of Freund's complete adjuvant. Rabbits were immunized with the mixture and boosted twice, 4 and 9 weeks later, with the antigen in Freund's incomplete adjuvant. The animals were bled several times 1 to 3 weeks after the last injection and the presence of antibodies was determined by enzyme immunoassay. Upon Western blotting, the antibody prepared by this method reacted to only one band with a molecular weight of 440-450 kDa, which showed identical mobility to that of the antigen reacting to anti-gp330 monoclonal antibody (13).

Anti-HBP-44 antibodies were prepared as follows. New Zealand White rabbits were immunized with 100 μg of the protein A-HBP-44 fusion protein which had been mixed with an equal volume of Freund's complete adjuvant and given 3 booster injections at 2-week intervals. Ten days after the last injection, the rabbits were bled and antisera were collected. For affinity purification of antibodies, 1 mg of MBP-HBP-44 fusion protein was coupled to 1 ml of CNBr activated Sepharose (14). The antisera were applied to the MBP-HBP-44 column. After washing of the column with 10-20 column volumes of PBS(-), specific antibodies bound to the column were eluted with 0.1 M glycine-HCl buffer, pH 2.5, and immediately neutralized with 1.5 M Tris-HCl buffer, pH 8.8. The antibody concentration of the eluate was 0.4 mg/ml.

Immunohistochemistry—For cryostat sections, the specimen was frozen in dry ice-acetone, cut into 4 μm slices with a cryostat, and fixed with acetone. Endogenous peroxidase activity was blocked by treatment of the sections with methanol containing 0.3% H_2O_2 . The section were treated first with anti-HBP-44 antibodies [0.4 $\mu\text{g}/\text{ml}$ in PBS(-)] at room temperature overnight and then with peroxidase-conjugated goat anti-rabbit IgG (Jackson) diluted 100-fold with PBS(-) at room temperature for 1 h. The sections were washed in PBS(-) between incubations. The reaction products were visualized by reaction with a solution of diaminobenzidine (15).

Immunoprecipitation—Up to 1 g of murine organs or teratocarcinoma OTT6050 was homogenized in 4 ml of PBS(-) containing 2 mM CaCl_2 with a Polytron, and the homogenate was centrifuged at $60,000 \times g$ for 1 h. The pellet was re-homogenized in 2% Triton X-100, 2 mM CaCl_2 in PBS(-), and centrifuged at $60,000 \times g$ for 30 min. The supernatant (0.5 ml) was incubated with 200 μl of 20% protein A Sepharose CL-4B (Pharmacia), which had been washed with the washing buffer [0.1% Triton X-100, 2 mM CaCl_2 , 1 mg/ml of ovalbumin in PBS(-)] for 15 min. After centrifugation, the supernatant was incubated with 10 μg of antibodies for 1 h, and immune complexes were collected with 50 μl of 20% protein A-Sepharose CL-4B. In the case of OR8 antibodies, rabbit anti-rat IgG (Jackson) was used as the second antibodies before incubation with protein A-Sepharose. The resin was washed with the washing buffer 3 times, then suspended in 50 μl of SDS-PAGE sample buffer (16) and boiled for 5 min. After centrifugation, the supernatant was subjected to SDS-PAGE as described by Laemmli (16) on a 4% gel, except that 2-mercaptoethanol was omitted from the sample buffer, since anti-gp330 antibodies and OR8 reacted only under non-reducing conditions.

Western Blot Analysis and the Analysis of the Association of HBP-44 with Immobilized Brushin—Western blot analysis was carried out as described previously (5): antigens reacted with rabbit or rat antibodies were visualized by reaction with horseradish peroxidase-conjugated goat Fab anti-rabbit (or rat) IgG followed by 4-chloro-1-naphthol and H_2O_2 treatment.

To test the association of HBP-44 with immobilized brushin, immunoprecipitate of the kidney with anti-gp330 antibodies was prepared as described under "Immunoprecipitation," subjected to SDS-PAGE, and transferred to a nitrocellulose filter. The filter was incubated with MBP-HBP-44 fusion protein or MBP (10 $\mu\text{g}/\text{ml}$ each) in PBS(-)

containing 2 mM CaCl_2 and 4% skim milk for 2 h at room temperature, followed by incubation with rabbit anti-MBP antibodies (10 $\mu\text{g}/\text{ml}$) in the same buffer for 1 h at room temperature. The bound antibodies were detected as described previously (5), using peroxidase-conjugated goat Fab anti-rabbit IgG.

Ca^{2+} Dependency of the Association of HBP-44 with Brushin—Immunoprecipitation using anti-GP330 antibodies was performed as mentioned under "Immunoprecipitation" except that Tris-HCl buffer containing 5 mM CaCl_2 or 5 mM EGTA was used instead of PBS(−) containing 2 mM CaCl_2 .

The effect of Ca^{2+} on the association of HBP-44 with immobilized brushin was tested as follows. Immunoprecipitates of the kidney with anti-gp330 antibodies were prepared as described above using Tris-HCl buffer (pH 7.4) containing 5 mM CaCl_2 . After SDS-PAGE followed by transfer to a nitrocellulose filter, the incubation of the filter with MBP-HBP-44 or MBP, and antibodies was carried out as mentioned above except that Tris-HCl buffer (pH 7.4) containing 5 mM CaCl_2 or 5 mM EGTA was used instead of PBS(−) containing 2 mM CaCl_2 .

NH_2 -Terminal Sequence Analysis—HBP-44 was isolated from RCA-agarose binding glycoproteins of teratocarcinoma OTT6050 (4) by immunoaffinity chromatography using anti-HBP-44, performed essentially as described before (3). After SDS-PAGE and transfer of the 44 kDa band to polyvinylidene difluoride membrane, the amino acid sequence of HBP-44 was determined as described (17) except that 8% gel was used and that transfer to the membrane was performed in 25 mM Tris-HCl, 10 mM glycine solution.

RESULTS

Further Characterization of HBP-44—We performed primer extension to obtain a cDNA clone of HBP-44 with the full coding sequence; the cDNA clones we previously analyzed had lacked initiator methionine (5). By screening a library constructed by primer extension, we isolated a desired clone. The initiator methionine was found just one amino acid residue on the N-terminal side from the previously identified HBP-44 sequence (Fig. 1), and the position corresponded to that of human RAP (6). The result is consistent with our previous conclusion that the cDNA clone, which we analyzed, specifies a 44 kDa glycoprotein (5), not a 330 kDa glycoprotein as proposed by Pietromonaco *et al.* (10).

N-Terminal amino acid sequence analysis (Table I) revealed that HBP-44 had a signal peptide and that the position of cleavage was between Lys³⁷ and Tyr³⁸, the same as that of human RAP (6). The mature protein has a molecular weight of 38,090, and considering the contribution of carbohydrate chains, this value is in agreement with the estimate of 44 kDa based on SDS-PAGE.

We then studied the distribution of HBP-44 in the kidney by immunohistochemical analysis. As shown in Fig. 2, HBP-44 was present on the brush border of the proximal tubules and the inner surface of Bowman's capsules. This distribution is similar to those of brushin (3) and OR8 antigen (4).

Correlation between Brushin, OR8 Antigen and gp330—Brushin was isolated by indirect immunoprecipitation from

the kidney and teratocarcinoma OTT6050. After SDS-PAGE and blotting with anti-brushin antibodies, two bands (450 and 350 kDa) were detected in the preparation from the teratocarcinoma (Fig. 3, lane 1). In the case of the kidney, these two bands and a minor band just below the 450 kDa one were detected (lane 2). Anti-rat gp330 antibody reacted with the larger, predominant band both in the kidney and teratocarcinoma (lanes 3 and 4). The minor band just below the 450 kDa one was also reactive with anti-gp330 and was considered to be a modified form of the

	GT	2
CCTCTCAGTCTGCTAGCGCTTCAGAGGAAGATGGCGCTCGAAGAGAGAGGGTCTCTACG		62
<u>M A P R R E R V S T</u>		10
CTGCCCGGGCTCCAACTGCTAGTGTGTGTGTCTGCCGCTGATGCTGTGTGCCCGAGCCC		122
<u>L P R L Q L L V L L L L L P L M L V P Q P</u>		30
ATAGCAGGCCATGGCGGCAAGTACTCGGAGAGAAGAACGAGCCGAGATGGCCGCCAAG		182
<u>I A G H G G K Y S R E K N E P E M A A K</u>		50
CGCGAGTCCGSGGAGGAGTTCCGCATGGAGAAGCTGAACAGCTATGGGAGAGGCCAAG		242
<u>R E S G E E F R M E K L N Q L W E K A K</u>		70
CGGCTTCATCTGTCTCTCTGTGAGGCTGGCGAGTGCATCTGACCTGAAGATACAAGAG		302
<u>R L H L S P V R L A E L H S D L K I Q E</u>		90
AGGGATGAATCAACTGGAAAAAGCTGAAGGTGGAAGGCTTGGATAAGGATGGGGAGAAA		362
<u>R D E L N W K K L K V E G L D K D G E K</u>		110
GAAGCAAACTGATCCACAACCTCAACGTATCTGGCCAGATACGAGCTGGATGGGAGG		422
<u>E A K L I H N L N V I L A R Y G L D G R</u>		130
AAGGACCCGAGATGGTGCACAGCAACGCCCTCAATGAAGACACCCAGGATGAGCTGGG		482
<u>K D A Q M V H S N A L N E D T Q D E L G</u>		150
GACCCAGGCTGGAAGCTGTGGCACAAGGCAAGACATCAGGGAATTCCTCAGTGAA		542
<u>D P R L E K L W H K A K T S G K F S S E</u>		170
GAGCTGGACAAGCTGTGGAGAGAGTTTCTGCATTACAAAGAGAAGATCCAGGAGTACAA		602
<u>E L D K L W R E F L H Y K E K I Q E Y N</u>		190
GTGCTGTAGACACACTGAGCAGAGCTGAAGAAGTTATGAGAACCTTCTCAGTCCCTCG		662
<u>V L L D T L S R A E E G Y E N L L S P S</u>		210
GACATGGCCACATCAAGAGCGACACCTGATCAGCAAGCAGTGAAGTGAAGGACAGA		722
<u>D M A H I K S D T L I S K H S E L K D R</u>		230
CTGCGCAGTATCAACAGGGCTTGGACCGCTCGGGAAGGTGAGCCACAGGGCTATGGC		782
<u>L R S I N Q G L D R L R K V S H Q G Y G</u>		250
TCCACCACTGAGTTTGAAGAGCCCGGGTGTAGATCTGTGGACCTGGCTCAGTCTGCC		842
<u>S T T E F E E P R V I D L W D L A Q S A</u>		270
AACTTCACTGAGAAGGAAGTGGAGTCACTCAGGAGGAGCTCAAGCACTTTGAGGCCAAA		902
<u>N F T E K E L E S F R E E L K H F E A K</u>		290
ATTGAAAGCACAACTACCAAGAGCAGCTGGAGATTTCCACCAAGAGCTGAAGCAC		960
<u>I E K H N H Y Q K Q L E I S H Q K L K H</u>		310
GTGGAGAGCATCGCGACCCGAGCACATCAGCCGCAACAGGAGAAATACGTGTGTCTG		1022
<u>V E S I G D P E H I S R N K E K Y V L L</u>		330
GAGGAGAAGACCAAGGAGCTGGGCTCAAGGTGAAGAAGCATCTACAGGACCTGTCTAGC		1082
<u>E E K T K E L G Y K V K K H L Q D L S S</u>		350
AGGGTCTCAAGGGCTCGGCACAATGAGCTCTGAGGACCAAGGCCACCCAGCAGCAGCCTA		1142
<u>R V S R A R H N E L</u>		360
GAGAGAACACTTGAAGACACCGGAGCTGTGAGCATGTCTCGGCTTGCATAGACCTGAG		1202
<u>GTGACTGGTGTGGCTGACCAACCGTGGCAAGGAGGATCCCTTGAAATACCAAGCTGATCCT</u>		1262
ACAGTGGCTGGCAAGGACTTATTTCTCTCAAGCAAGTGTAGTTGTACCAACCTGGACG		1322
<u>AGGGCTTGGGTACCGCTACCACTGAGATAGGACTGGACTCCGAGCTGCAGCACACACT</u>		1382
TTATATTGAAATCACATAAACCTGGCTGCCACTGGAAACATTCTGTACACATCCTTAAT		1442
<u>ACATGGCAGAGTTTGAAGAAAAA</u>		1478

Fig. 1. Sequence of cDNA encoding HBP-44 and the deduced amino acid sequence. The signal sequence is underlined, and a potential N-glycosylation site is shown by an asterisk.

TABLE I. N-terminal sequence analysis of HBP-44. Undefined residues are represented as X.

Cycle No.	1	2	3	4	5	6	7	8	9	10	11	12
Amino acids	Tyr	Ser	X	Glu	Lys	Asn	X	Pro	Glu	Met	Ala	Ala

Fig. 2. The distribution of HBP-44 in the kidney. A frozen section of the kidney was stained as described in "MATERIALS AND METHODS." Bar: 50 μ m.

Fig. 3. Correlation between brushin, OR8 antigen, and gp330. Triton X-100 extracts from the kidney or teratocarcinoma OTT6050 were immunoprecipitated with rabbit anti-brushin antibodies, separated by SDS-PAGE under non-reducing conditions using 4% gel and subjected to Western blot analysis with rabbit anti-brushin antibodies (lanes 1 and 2), rabbit anti-gp330 antibodies (lanes 3 and 4), or OR8 monoclonal rat antibody (lanes 5 and 6). Lanes 1, 3, 5: teratocarcinoma OTT6050; lanes 2, 4, 6: the kidney. Since final detection depended on reaction with the first antibody, rabbit immunoglobulins (around 150 kDa) in the immunoprecipitates were detected in lanes 1-4. Therefore the region above molecular weight 200 kDa is shown in the figure.

450 kDa component (lane 4). Thus, brushin which we had previously identified was mainly composed of two components, the 450 and 350 kDa ones. The 450 kDa one is the mouse counterpart of rat gp330. OR8 antibody did not react with the 450 kDa band but reacted with the 350 kDa band (lanes 5 and 6). Thereafter we will use the term brushin for the major 450 kDa component, and the 350 kDa one will be

Fig. 4. Immunoprecipitation of the kidney, the liver, and OTT6050 with anti-HBP-44 antibodies. The murine kidney (lane 1), liver (lane 2), or teratocarcinoma OTT6050 (lane 3) was solubilized with 2% Triton X-100, immunoprecipitated with anti-HBP-44 antibodies, and separated on 4% polyacrylamide gel. The gel was stained with silver nitrate.

Fig. 5. The association of HBP-44 with brushin. A) Western blot analysis of proteins co-precipitated with HBP-44 in the kidney. The immunoprecipitate with anti-HBP-44 antibodies in the kidney was analyzed by Western blotting using anti-gp330 antibodies (lane 1) or an OR8 monoclonal antibody (lane 2). B) Evidence that HBP-44 is directly associated with brushin. Brushin was immunoprecipitated with anti-gp330 antibodies (lanes 1 and 3) or anti-HBP-44 antibodies (lanes 2 and 4), separated by SDS-PAGE (4% gel), and transferred to a nitrocellulose filter. The filter was incubated with MBP-HBP-44 fusion protein (lanes 1 and 2) or MBP (lanes 3 and 4). The association was detected as described in "MATERIALS AND METHODS."

Fig. 6. Requirements for association of brushin with HBP-44. A, B) Ca^{2+} dependency. A: Immunoprecipitation of Triton X-100 extract in the presence of 5 mM CaCl_2 (lane 1) or 5 mM EGTA (lane 2). The complex of brushin and HBP-44 was collected by using anti-HBP-44 antibodies from the kidney. The complex was separated by SDS-PAGE and subjected to Western blot analysis with anti-gp330 antibodies. B: Immobilized brushin was incubated with MBP-HBP-44 fusion protein (lanes 1 and 2) or MBP (lanes 3 and 4) in the presence of 5 mM CaCl_2 (lanes 1 and 3) or 5 mM EGTA (lanes 2 and 4). C) The effect of a reducing agent. Immunoprecipitated brushin was subjected to SDS-PAGE in the absence (lane 1) or presence (lane 2) of 2-mercaptoethanol. After transfer to a filter, the filter was incubated with MBP-HBP-44 fusion protein as in Fig. 5B.

called OR8 antigen.

HBP-44 Associates with Brushin Both in the Kidney and the Teratocarcinoma—Although HBP-44/RAP associates with $\alpha_2\text{MR/LRP}$, their patterns of tissue distribution do not correlate with one another. $\alpha_2\text{MR/LRP}$ is most highly expressed in the liver, the lung and the brain (18). On the other hand, HBP-44 is intensely expressed in the kidney followed by the brain and the testis, but only weakly in the liver (6). These data raise the possibility that in the kidney HBP-44/RAP associates with a molecule different from $\alpha_2\text{MR/LRP}$. To elucidate whether HBP-44 is associated with a molecule different from $\alpha_2\text{MR/LRP}$ in the kidney, immunoprecipitation with anti-HBP-44 antibodies was performed using Triton X-100 extracts from the kidney, the liver or OTT6050. A protein with a molecular weight of about 450 kDa was co-precipitated with HBP-44 in the kidney (Fig. 4, lane 1) and OTT6050 (lane 3), although a smaller protein (about 380 kDa), was co-precipitated in the liver (lane 2). The existence of HBP-44 in the immunoprecipitates was confirmed by Western blot analysis using a higher concentration gel (data not shown). The co-precipitated protein in the liver was probably $\alpha_2\text{MR/LRP}$, since it has been reported that human $\alpha_2\text{MR/LRP}$ was co-precipitated with RAP under similar conditions (6).

When the 450 kDa protein co-precipitated with HBP-44 in the kidney was subjected to Western blot analysis with rat anti-gp330 antibodies or OR8 antibodies, only anti-gp330 antibodies reacted with that protein (Fig. 5A). Thus, the molecule associated with HBP-44 in the kidney was brushin and not OR8 antigen. The same results were obtained with teratocarcinoma OTT6050 (data not shown).

To exclude the possibility that brushin was co-precipitated with HBP-44 due to cross-reactivity to anti-HBP-44 antibodies, we tested whether HBP-44 associated with immobilized brushin on the nitrocellulose membrane. A ligand blotting experiment (Fig. 5B) shows that MBP-HBP-44 fusion protein can associate with brushin on the nitrocellulose membrane, although control MBP cannot. This indicates that brushin was co-precipitated with HBP-44 due to their association and not to cross-reactivity.

Effects of Ca^{2+} and the Reducing Agent on the Association of HBP-44 with Brushin—Since the binding of human RAP to $\alpha_2\text{MR/LRP}$ requires Ca^{2+} (19), we tested the Ca^{2+} dependency of the association of HBP-44 with brushin.

When the immunoprecipitation was performed in the presence of EGTA, the amount of co-precipitated brushin significantly decreased, although the association was not completely abolished (Fig. 6A). Similar results were obtained when MBP-HBP-44 fusion protein was incubated with immobilized brushin. In the ligand blotting experiment the binding of the fusion protein to brushin on the membrane was drastically inhibited by EGTA (Fig. 6B).

When brushin was subjected to SDS-PAGE under reducing conditions and immobilized, HBP-44 could not associate with the molecule (Fig. 6C). The result indicates that a rigid structure of brushin preserved by disulfide bonds is required for binding to HBP-44.

DISCUSSION

We have presented evidence indicating that HBP-44 associates with brushin. A ligand blot experiment using MBP-HBP-44 fusion protein established that HBP-44 directly associated with brushin. Furthermore, the method enabled us to show that Ca^{2+} is involved in the association and that disulfide linkage in brushin is required for the association. Strictly speaking, immunoelectron microscopy is required to show that the molecular complex is not formed after detergent solubilization and that the two molecules form a complex on the membrane plane. However, because of the similar tissue distributions of HBP-44 and brushin (Fig. 2 and Ref. 3), we believe that the molecular complex is indeed present on the cell surface.

The relationship between brushin, gp330 and OR8 antigen was also clarified in the present investigation. The brushin preparation previously analyzed contained two polypeptides, a 450 kDa one and a 350 kDa one, of which the former was the major antigenic component. The 450 kDa one was identical to gp330. Since the molecular weight of this component is greater than 330 kDa and even higher than that of laminin heavy chain (400 kDa under reducing conditions) both in mouse and rat, we think the name gp330 may be inappropriate and in this publication we retain the name of brushin for the 450 kDa component. The 350 kDa component is called OR8 antigen, because monoclonal antibody OR8 specifically reacts with it.

Pietromonaco *et al.* had reported a cDNA sequence for a pathogenic domain of rat gp330 (10). However, since this

sequence has a strong homology with murine HBP-44 and human RAP, it is currently thought that the cloned cDNA encodes the rat homologue of HBP-44/RAP. During preparation of our manuscript, they reported that rat gp330 co-precipitates with a 44 kDa protein and that the 44 kDa protein is immunologically identical with human RAP (20). However, direct evidence that gp330 interacts with HBP-44 was not presented. It is not entirely clear why their cDNA clone produced a protein antigenically related to gp330; they mentioned the possibility that gp330 and the 44 kDa protein have a common antigenic domain (20). When we performed Western blot analysis of Triton X-100 extract from the kidney with affinity-purified antibodies raised against β -galactosidase-HBP-44 fusion protein or protein A-HBP-44 fusion protein, none of the antibodies reacted with brushin (data not shown). It seems unlikely that a common pathogenic domain exists, and it is certain that the cDNA cloned by them (20) specifies rat HBP-44/RAP, and not gp330.

On the other hand, Raychowdhury *et al.* isolated and sequenced partial cDNAs for rat gp330. The deduced amino acid sequence contained regions showing homology with human LDL-receptor (21). Thus, LDL-receptor, α_2 MR/LRP, and brushin/gp330 may be members of the same receptor family, and HBP-44/RAP associates with two of them, α_2 MR/LRP and brushin/gp330. The HBP-44/RAP binding sites have not been identified in α_2 MR/LRP or brushin/gp330. However, since both α_2 MR/LRP and brushin/gp330 have similar cysteine-rich repeats (18, 21), and since we reported here that reducing agents abolished the association of HBP-44 with brushin, some of these repeats might act as HBP-44/RAP binding sites. Furthermore, association of HBP-44 with brushin and α_2 MR/LRP requires Ca^{2+} for stability.

The molecular complex formed from brushin and HBP-44 is expected to be present on the tubular brush border in the kidney, since both molecules are co-localized there by immunohistochemistry. Brushin and HBP-44 also exist and form a complex in teratocarcinoma OTT6050. When PYS-2 parietal endoderm cells were stained with anti-HBP-44 antibodies (5), or with anti-gp330 antibodies (data not shown) both molecules showed similar distribution patterns. The same complex seems to exist in extraembryonic endoderm cells of teratocarcinoma and most probably those of embryos. Although the physiological functions of the complex and a distinct molecule, OR8 antigen, are un-

known, they may be involved in functions which are common to the renal brush border and extraembryonic endoderm cells, such as absorption of nutrients.

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