Hensin, a New Collecting Duct Protein Involved in the In Vitro Plasticity of Intercalated Cell Polarity

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Abstract

Two forms of intercalated cells are present in kidney collecting tubules, the α cell has apical endocytosis, apical H^+-ATPase and basolateral band 3, while β cells have reversed polarity of these proteins and no apical endocytosis. When a β cell line was seeded at high density, it changed into the α form. We previously showed that a partially purified 230 kD extracellular matrix protein of high density cells was able to retarget band 3 from apical to basolateral domains and stimulated apical endocytosis in vitro (Van Adelsberg, J., J.C. Edwards, J. Takito, B. Kiss, and Q. Al-Awqati. 1994. *Cell.* 76:1053–1061). We now purify this protein, which was named hensin, to near homogeneity and find that it belongs to the macrophage scavenger receptor cysteine rich (SRCR) family. An antibody, generated against a fusion protein made from a partial cDNA recognized a 230-kD protein in rabbit kidney and in the intercalated cell line. In vitro, the hensin antibody inhibited expression of apical endocytosis. Hensin was secreted in a polarized manner and bound to the basolateral membrane and extracellular matrix. Immunohistochemistry of the kidney showed that it was expressed only in collecting tubules. Double immunofluorescence with hensin and peanut lectin, H^+-ATPase, or band 3 showed many patterns; most α-cells had hensin staining while 50% of β-cells did not. These results suggest that hensin may also be involved in the polarity reversal of intercalated cells in vivo. (*J. Clin. Invest.* 1996. 98:2324–2331.) Key words: rabbit kidney • pH homeostasis • extracellular matrix • band 3 • H^+-ATPase

Introduction

Transport of H^+ and HCO_3^- by the collecting tubules of the kidney is one of the major mechanisms by which the kidney regulates the acid–base balance of the body fluids. In the cortical and outer medullary segments of this tubule there are two cell types, principal and intercalated. Only the latter, accounting for about a third of the cells, is responsible for acid–base transport (1, 2). While many nephron segments acidify their lumens, the cortical collecting tubule of rabbits usually secretes

HCO_3^-. However, treatment of the animals with an acid diet reverses the secretion of alkali to that of acid (for review see reference 3).

Based on purely mophological criteria, it had been known that the intercalated cells were present in at least two forms (4). Using a combination of cell pH, endocytosis and lectin binding we initially found that the intercalated cells exist in two functional forms, one that secretes H^+ while the other secretes HCO_3^- into the lumen (5). In the acid-secreting type (here termed α) a proton translocating ATPase is packaged in endocytic vesicles and is inserted into the apical membrane by a vigorous process of apical endocytosis and exocytosis. Pumping of H^+ results in the accumulation of cellular HCO_3^- which is then transported across the basolateral membrane by a Cl^-:HCO_3^- exchanger that is an alternatively spliced form of the red cell anion exchanger, band 3 (6, 7). The alkali secreting cell (β type), has a basolateral proton translocating ATPase and an apical Cl^-:HCO_3^- exchanger, but no apical endocytosis. We had suggested that the mechanism by which the tubule converts its alkali secretion to that of acid was due to a reversal of the polarity of these proteins in the β intercalated cell; a process we termed plasticity in epithelial polarity (5).

Since that proposal, several investigators developed immunocytochemical reagents to examine the polarized distribution of the ATPase and band 3, and the results have suggested that there is a much greater heterogeneity of types of intercalated cells than previously recognized (for review see reference 3). While "canonical" α and β cells exist, many others had their H^+-ATPase or band 3 in vesicles that were distributed diffusely in the cytoplasm or were localized to one or another pole of the cell (8, 9). In the most comprehensive of such studies, Bastani et al. analyzed quantitatively the distribution of the H^+-ATPase in the rat kidney under various acid-base states (10). As many as seven patterns were recognized and the "population density" of each of these patterns was found to change with acidosis or alkalosis. Since the total number of intercalated cells did not change with acid–base changes, it follows that the changes in the pattern reflect some degree of conversion of one cell type to another. However, these studies were not interpreted within the framework of the plasticity of epithelial polarity.

To test the plasticity model rigorously, we generated an immortalized cell line of the β-intercalated cell which exhibited all of the known characterstics of this cell. These included transepithelial HCO_3^- secretion by an apical Cl^-:HCO_3^- exchanger, basolateral H^+-ATPase, apical peanut lectin binding, and no apical endocytosis (11, 12). When these clonal cells were seeded at subconfluent density and allowed to grow to confluence, they secreted HCO_3^- by an apical band 3 (13). However, when they were seeded at high density, they reversed their polarity to that of the α type, secreting H^+ into the apical medium by a H^+-ATPase that is actively endocytosed and exocytosed. In addition, band 3 reversed its polarity from the apical

to the basolateral membrane. We found that high density cells secreted a factor into the extracellular matrix which was capable of reversing the polarity of low density cells. The ECM[1] (extracellular matrix) fraction was enriched in a protein of 230 kD and another of 45 kD. Partially purified 230 kD protein stimulated endocytosis and reversed the polarity of band 3 in low density cells. Because retargeting of one protein (band 3) to the basolateral membrane was associated with induction of endocytosis on the opposite membrane, we suggested that extracellular cues were capable of reorganizing the targeting machinery of the cell (14).

In the present study, we purify this protein to near homogeneity and provide preliminary sequence of its purified peptides which show that it is a previously unidentified protein. Because of its role in the determination of intercalated cell type, we term this protein hensin, from the Japanese, for "change in body" or metamorphosis. It belongs to a newly identified family of proteins, the SRCR (scavenger receptor cysteine rich) family (15, 16). While the function of the SRCR domain is not entirely clear, it appears to mediate protein–protein interaction. In addition, we find that this protein is a secreted protein that is basolaterally targeted. In the kidney, the protein is restricted to the collecting tubule. Co-localization studies suggest that it has a heterogenous expression in intercalated cells; half of the β-intercalated cells do not express it, while most of the α-intercalated cells are enriched in it. These studies suggest that this protein which plays an important role in the plasticity of intercalated cell polarity in vitro might also have similar functions in vivo.

Methods

Materials. L-[^{35}S]methionine (1.2 mCi/mmol) and [α-^{32}P]dCTP were from DuPont-New England Nuclear (Cambridge, MA). Restriction endonucleases were from Boehringer Mannheim (Indianapolis, IN). All other analytical-grade biochemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Cell culture. Stock cultures of clone C of β-intercalated cells established from rabbit kidney cortex were maintained as described (11–14). The cells were trypsinized and seeded on Costar (Cambridge, MA) polycarbonate filters (pore size 0.4 μm) at a density of 4×10^4 cells/cm^2 (low density) or 2×10^6 cells/cm^2 (high density) and transferred to 40°C to inactivate the T antigen.

Purification and partial amino acid sequence determinations of hensin. Mono-Q column fractions capable of inducing apical endocytosis were obtained from 1.6 l of the ECM of clone C plated at high density as described previously (14). These were first equilibrated with 7 M urea, 0.2 M NaCl, 2 mM EDTA, 0.1% SDS, and 50 mM Tris-HCl (pH 7.5) and further purified using Superdex HR200 10/30 chromatography. The hensin-rich fractions were combined, electrophoresed on SDS-PAGE and transferred to a PVDF membrane. The 230-kD band was cut out from the membrane and processed for microsequencing. The amino acid sequences obtained were compared to those of GenBank and EMBL using the GCG software package (Genetics Computer Group, Madison, WI).

Generation of hensin antibodies. Using these peptide sequences we cloned a partial cDNA clone by PCR and expressed the recombinant protein in *E. coli.* Total RNA was prepared from clone C after plating at high density using the RNAzol B (TEL-TEST, Friendswood, TX). cDNA was reverse transcribed from the total RNA using random primers with the cDNA cycle kit (Invitrogen, La Jolla, CA). RT-PCR was performed with degenerate primers designed from the amino acid sequences obtained as follows: 5′-TCGAATTCTC(G/A)TC(A/G)CAIAC(A/G/T/C)GT(A/G/T/C)CCCCA-3′; 5′-TCGAATTCTT(T/C)GGICA(A/G)GGICC(A/G/T/C)GG(A/G/T/C)CA(A/G)AT-3′; 5 min at 94°C; 2 cycles of 1 min denaturation at 94°C, 2 min annealing at 37°C, 2 min extension at 72°C followed by 30 cycles of 1 min denaturation of 94°C, 2 min annealing at 45°C, 2 min extension at 72°C; and a final extension of 7 min at 72°C. Amplified bands were recovered from agarose gels and cut with EcoRI, cloned into Bluescript SK(−) vector (Stratagene, San Diego, CA) and sequenced. The vector introduced six histidines into the COOH-terminus of the recombinant hensin fusion protein (Qiagen, Chatsworth, CA). A 700-bp clone of hensin was excised from the Bluescript and ligated with pQE-50 and pQE-16 vectors. The recombinant fusion protein (containing the 6 × His tag) was expressed in *E. coli* and purified using a Ni-NTA resin. Amino acid analysis of the recombinant protein confirmed its deduced composition (data not shown). The recombinant protein was injected into guinea pigs or chickens to generate polyclonal antibodies.

Western blotting and Northern blotting. The ECM fraction was obtained from clone C plated at high density as described (14). Kidney cortex, medulla, and papilla were cut from frozen rabbit kidney with a razor and homogenized in 10 vol of PBS, 0.1% Triton X-100, 0.05% SDS, and 1 mM PMSF with Polytron. The homogenate was centrifuged at 1,000 *g* for 10 min and centrifuged again at 10,000 *g* for 10 min. The resulting supernatant was processed for immunoprecipitation. After electrophoresis, proteins were transferred to nitrocellulose membranes in 10% methanol, 24 mM Tris, 19.3 mM glycine at 0.25 amp for 5 h. Western blotting was performed as described by Harlow and Lane (17). For Northern blotting, total RNA was prepared from clone C plated on a plastic tissue-culture flask and the frozen rabbit kidney with RNAzol B. Poly A$^+$ RNA was purified from the total RNA with Oligotex mRNA kit (Qiagen). Northern blotting was carried out as described (18).

Immunocytochemistry and immunohistochemistry. For immunocytochemistry, clone C cells were seeded at high density on filters (Costar, diameter 6.5 mm, pore size 0.4 μm) and cultured for 2 d. The cells were fixed with 100% methanol for 2 min at room temperature, blocked with 10% FBS in PBS for 1 h. Then the filter was cut from the cup and incubated with anti-hensin IgG at a dilution of 1:50. IgG was prepared from guinea pig sera using protein A (17). For immunohistochemistry, 10-μm cryostat sections from fresh rabbit kidneys were embedded in Tissue-Tek OCT Compound (Miles Laboratories, Elkhart, IN). The sections were fixed with ice-cold 100% acetone in PBS for 5 min and blocked with 1% BSA in PBS for 1 h. For double staining, primary antibodies were mixed and incubated with the section in 1% BSA and PBS for 1 h at room temperature: hensin antibody dilution at 1:100, undiluted hybridoma medium of E11 (19), IVF 12 dilution at 1:200 (20). Fluorophore-conjugated secondary antibodies (Jackson Immuno Research Inc., West Grove, PA) were used at dilution 1:200–1:400. TRITC labeled peanut lectin (Sigma Co.) at dilution 1:20 was incubated with the specimens for 10 min after the secondary antibody incubation (21). Confocal images were collected on a Bio-Rad MRC 600 confocal laser scanning microscope.

Liberation of hensin from membrane vesicles. Clone C was seeded on a filter at high density and cultured for 6 d. The cells were scraped, homogenized in 250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl (pH 8.0), and centrifuged at 1,000 *g* for 10 min. The resulting supernatant was centrifuged at 100,000 *g* for 1 h and the pellet was resuspended in 1 mM EDTA and 10 mM Tris–HCl (pH 8.0) and used as the membrane fraction. The membrane was diluted in 500 μl of 1 mM EDTA, 10 mM Tris–HCl (pH 8.0) with 1 M KCl, 1 M KSCN, or 0.1 M Na$_2$CO$_3$ (pH 11) and passed through 20 times with 26 gauge needle attached to a 1-ml syringe. The supernatant and the precipitate were separated by ultracentrifugation. The sample was analyzed by SDS-PAGE on 7.5% acrylamide gel, and processed for immunoblotting with anti-hensin serum diluted 1:500.

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; PNA, peanut lectin; SRCR, scavenger receptor cysteine rich.
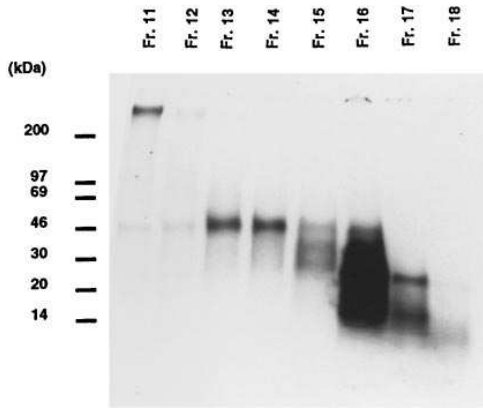
Figure 1. Separation of hensin on gel filtration chromatography. Hensin was extracted from the ECM of the clone C cell line, purified with gelatin affinity and Mono-Q columns (14). Hensin-enriched fractions obtained by Mono-Q chromatography were further applied on Superdex HR200 10/30 in 7 M urea, 0.2 M NaCl, 2 mM EDTA, 0.1% SDS and 50 mM Tris–HCl (pH 7.5). The eluate was precipitated with TCA, separated by reduced SDS-PAGE on a 4–20% acrylamide gradient gel, dried, and then visualized by autoradiography.

Results

Purification of hensin and sequence of peptides. We previously reported that the extracellular matrix of the intercalated cell line seeded at high density was capable of reversing the polarity of cells seeded at low density (14). Partial purification of the ECM fraction on gelatin sepharose followed by Mono-Q ion exchange chromatography yielded a protein fraction that, by itself reversed the polarity of band 3 in this clone of intercalated cells. This fraction contained a 230-kD protein (hensin) and another 45-kD protein. Sequence of the 45-kD protein showed that it was an intermediate filament of the cytokeratin (Type I.18) (22).

The assay used to follow polarity reversal during chromatographic separation was induction of apical endocytosis, a feature of α- but not β-intercalated cells. To purify these two proteins further we used several chromatographic resins with no success. Eventually, when these fractions were denatured with
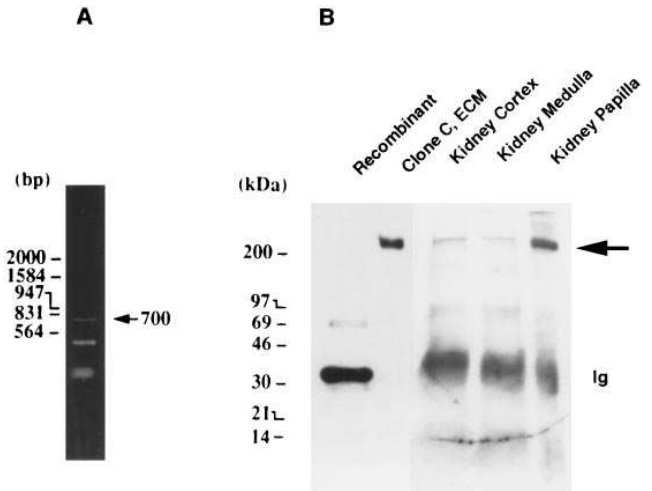
Figure 2. Generation of hensin antibody from a recombinant fusion protein with a 700 bp DNA cloned by RT-PCR. (*A*) A 700- and 400-bp DNA were cloned by RT-PCR with degenerate primers designed from the amino acid sequences obtained from the purified protein. The PCR products were analyzed on 1.2% agarose gel, and visualized with ethidium bromide staining. The arrow indicates the position of a 700-bp DNA. (*B*) Western blotting. Purified recombinant protein (0.1 μg) and ECM of clone C plated at high density were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antihensin guinea pig serum. Proteins (1 mg) from 10,000 *g* supernatant of the cortex, medulla, and papilla were immunoprecipitated with anti guinea pig hensin antibody (dilution 1:500) and processed for immunoblotting with anti-hensin chicken IgY. Signals were detected by ECL system (Amersham International, Buckinghamshire, England). The arrow indicates the position of hensin. The broad bands in kidney cortex, medulla and papilla are Ig cross-reacted with anti-chicken IgY secondary antibody.

7 M urea, and 0.1% SDS, we obtained a nearly homogenous band of hensin after Superdex 200HR chromatography (Fig. 1). Purified hensin did not induce apical endocytosis. However combinatorial experiments with different fractions separated by the Superdex column also failed to induce endocytosis suggesting that the activity loss was due to irreversible denaturation by the harsh conditions in the final step.

Table I. Hensin Belongs To the SRCR Family of Proteins

SRCR concensus	R LV - - - G - - - CEG - **VE** - - - - - - **WGTV** - - **CD** - - **W** - - - - - - - - - **VVC**	
Hensin-1	**VE**IYHGGR	
Hensin–2	<u>GS**WGTV** **CD**DSW</u>DINDAS**VVC**	
SRCR consensus	R - **LGCG** - G - - - - - - - - - - - - - - **F** - - - - - - - - - - - - - - - **V** - **C** - **G** - **E** - S**L**	
Hensin–3	-Q**LGCG**MAVSAPGSAR	
Hensin–4	-Q**LGCG**WAVSAPGSAR	
Hensin–5	-Q**LGCG**T A VSAPGSAR	
Hensin–6	**F**GQGPGQIVLDD**V**S**CSG**QEL	
Hensin–7	<u>**F**GQGPGQI</u>VLDD**V**S**CSG**QEPY**L**	
Unassigned sequences		
Hensin–8	GSFTSSNFLR	
Hensin–9	FVSCDSI	

Seven out of the nine amino acid sequences were aligned to the SRCR consensus (16). Two sequences (hensin–8 and –9) were unique. The sequences which were used to design the degenerate oligonucleotide primers are underlined.

Table II. Deduced Amino Acid Sequences of Two cDNA Fragments of Hensin

Hensin–1

1	SGQGPGQIVL	DDVSCSGQEP	YLWSCHHRGW	LSHNCGHQED	AGVICSGAMD
51	TTTPTLPDTWP	TTVIYESTPV	HISGLQLRLV	NGSDRCEGRV	EVLYQGSWGT
101	VCDDSWDLND	ASVVCRQLGC	GTALSAPASA	QFGQSSGSIV	LDDVSCSGSE
151	PNLWCSCHRG	WLSHNCGHHE	DAGVVCSGPD	SRLAVRLVNG	STRCQGRVEV
201	LYRGSWGTVC	DDSWDINDAS	VVCRQLGCGW	AVSAPGSARF	GQGSGSIFLD
251	EVSCSGQEPY	LWNCSHRGWL	SHNCGHYEDA	GVICSDGWTT	VTPPAPTTDW
301	WEPTVTTTVG	PSS			

Hensin–II

1	FPRDASLRLV	SGNSSYGACA	GRVEIYHGGR	WGTVCDDSWD	TQDAQVVCRQ
51	LQCGDAVSAP	GGAYFGSGSG	PITLDDVNCS	GTEATLWQCR	SQSWFSHNCG
101	HHEDASVICT	GNYGTTTASV	PNISTSNAS		

The SRCR domains are shown in bold, sequence I contains two and a half domains while sequence II contains only one SRCR domain. Underlined sequences represent sequences identified by peptide sequence of proteolytic fragments. The fused recombinant protein was generated to amino acid 1-219. The nucleotide sequences of the two clones have been deposited in the GenBank database and have an accession number of U69143 for hensin–I and U69144 for hensin–II.

Several peptides were obtained from a tryptic digest of the purified hensin and their partial amino acid sequence determinations revealed that this protein was a new member of the SRCR family of proteins (15, 16). Seven of nine peptide sequences were similar to the SRCR consensus motif (Table I). Homology search of the databases showed that these hensin peptides had 50–70% homology to WC 1.1., a differentiation marker of $CD4^-$, $CD8^-$ bovine T cells (23). Two other peptides had unique sequences without significant homology ($< 30\%$) to other reported proteins.

Cloning of a partial cDNA of hensin. RT-PCR allowed us to clone 700-bp and 400-bp DNA fragments of hensin using a pair of degenerate primers designed from the peptide sequences described above (Fig. 2 *A*). Using the sequence of this fragment we cloned two additional cDNAs. The deduced amino acid sequence of two of the cDNA clones is shown in Table II. Sequence of hensin-I includes two and a half SRCR domains each about 100 amino acids long (shown as bold letters). This sequence includes all of the 700 nucleotides of the PCR fragment. The SRCR domains in this fragment are 83% identical to each other. Hensin-II contains one complete SRCR motif which is 65% identical to those in hensin-I. These domains are separated by short unique intervening sequences. Importantly, the two sequences deduced from DNA include three peptides previously identified by sequence of the purified protein demonstrating that these sequences are authentic hensin sequences and not another homologous SRCR protein.

The 31-kD recombinant fusion protein (from the 700-bp DNA, representing aa 1-219 in hensin-I) was purified using its inserted histidine tag from an *E. coli* expression system and used to generate polyclonal antibodies. These antibodies recognized the 31-kD fusion protein. They also stained a 230-kD band, the same molecular weight of purified hensin, in the extracellular matrix of clone C plated at high density (Fig. 2 *B*). A combination of immunoprecipitation followed by Western blotting of rabbit kidney showed a 230-kD band in the cortex, medulla, and papilla with the papilla showing the highest expression (Fig. 2 *B*). Direct Western blotting without prior immunoprecipitation detected the protein in rabbit kidney medulla and papilla, but not cortex, probably reflecting the low abundance in the cortex (data not shown). As will be demon-

strated below, the only structures that stain with these antibodies were the collecting tubules. Northern blotting, performed using the mixture of 400 and 700 bp DNA cloned by RT-PCR, detected a single species in poly A$^+$ fractions of clone C, rabbit kidney cortex, medulla, and papilla (Fig. 3). The size of the single message of 6-kb is compatible with its coding for a 230-kD protein. These results suggest that the sequences obtained from the purified proteins, antibodies, and DNA probes will be useful reagents in examining the role of hensin in the biology of intercalated cells.

Expression of hensin in different tissues. Using Northern analysis, we extracted RNA from a variety of epithelial and nonepithelial rabbit tissues. Cardiac and skeletal muscle did not express hensin. Several epithelia expressed hensin, with the intestine showing the highest expression (Fig. 4). The stomach, liver, and kidney cortex had a moderate level of expression but the lung had the lowest.

A role for hensin in the in vitro reversal of polarity. To document the role of hensin in the conversion of epithelial polarity of intercalated cells, clone C was seeded on filters at high den-
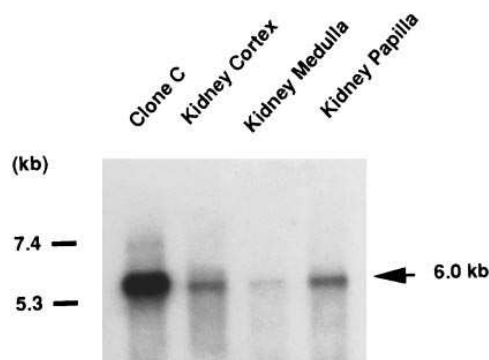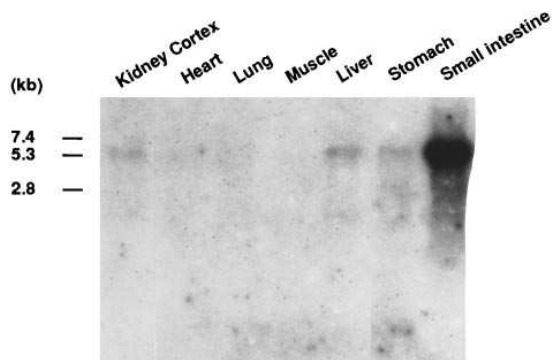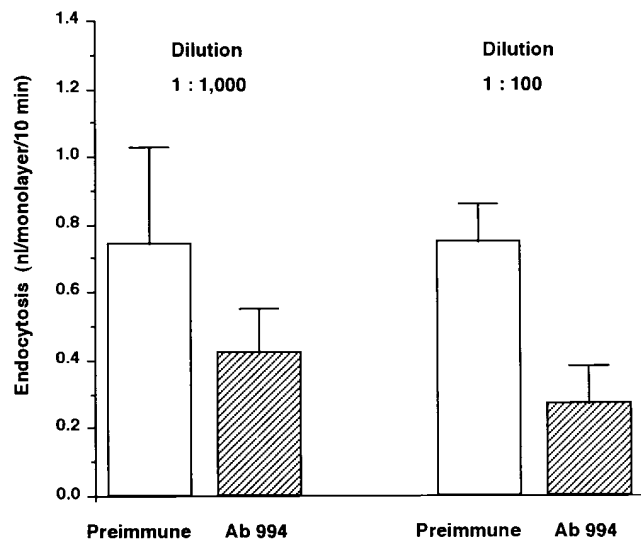

Figure 3. Expression of hensin transcript in rabbit kidney cortex, medulla, and papilla. Poly A$^+$ RNA (1 μg) was purified from clone C and the frozen rabbit kidney and processed for northern blotting for hybridization under high stringency to a mixture of 700-bp and 400-bp cDNA probe cloned by RT-PCR. Signals were detected by autoradiography.
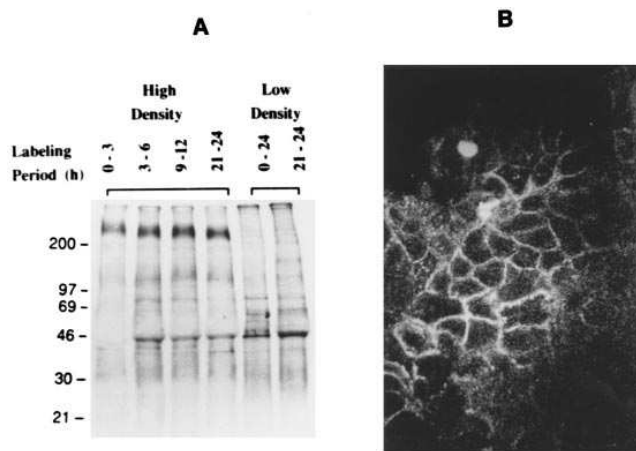
Figure 4. Expression of hensin transcripts in various tissues. Poly A[+] RNA (0.5 μg) was purified from rabbit tissues and processed for Northern hybridization under high stringency using a 400-bp cDNA probe cloned by RT-PCR. Signals were detected by autoradiography.

sity and cultured for 8 d. The media contained preimmune or immune anti-hensin sera at a dilution of 1:1000 or 1:100, which were replaced with fresh media on days 2 and 5. Apical endocytosis of horseradish peroxidase was then measured as the difference between uptakes at 37°C and 4°C as described previously (14). The results are shown in Fig. 5, and demonstrate that the antibodies inhibited the apical endocytosis even at high dilution. These results suggest that hensin is an important component of the protein(s) needed for the density dependent conversion of the polarity of intercalated cells in vitro.

Hensin is a secreted protein. Clone C cells were plated at high density on a filter, labeled with [^{35}S]methionine, and the

Figure 6. Cell density dependent appearance of hensin in the ECM of clone C (*A*) and indirect immunofluorescence of hensin in clone C (*B*). (*A*), Clone C was seeded on a filter at high or low density and labeled with 100 μCi/ml [^{35}S]methionine for indicated time points. The ECM fraction was prepared, dialyzed against PBS overnight, immunoprecipitatead with hensin antiserum (dilution 1:50), recovered with protein A beads. The proteins were separated by SDS-PAGE, dried and visualized by autoradiography. (*B*) Clone C was seeded on a filter at high density and cultured for 2 d. The cells were fixed with 100% methanol, and incubated with anti hensin IgG (dilution 1:20). Signal was detected with FITC-labeled anti-guinea pig IgG. The image was photographed with a confocal laser scanning microscope. No staining was observed with preimmune serum. ×400.

Figure 5. Antihensin antibody blocks the induction of apical endocytosis of clone C. The culture medium was diluted with preimmune or antihensin guinea pig sera (Ab 994) at 1:100 or 1:1,000 and sterilized. Clone C was suspended in the medium, seeded on a filter at high density, and cultured for 8 d to allow the formation of tight monolayers. The medium was replaced with fresh media on day 2 and 5. Apical endocytosis of horseradish peroxidase was defined as the difference between uptakes at 37°C and 4°C. The activity of horseradish peroxidase was determined by measuring the initial rate of hydrolysis of *o*-dianisidine photometrically, and converted into the volume of endocytosis. The activity at 4°C was 0.15±0.04 nl/monolayer/10 min. For each column, the error bar represent standard deviation, *n* = 3.

ECM was extracted and subjected to immunoprecipitation. As early as 3 h after plating, hensin was found in the ECM and the amount gradually increased up to 24 h (Fig. 6 *A*). In contrast, hensin was not observed in the low density ECM even after 24 h after plating. These results confirm our previous observation that the 230-kD protein appeared only in the ECM of high density cells (14). Immunocytochemistry of clone C was performed to determine the subcellular localization of hensin. The confocal image of hensin staining in clone C plated at high density showed basolateral localization with small vesicles in the cytoplasm (Fig. 6 *B*).

The basolateral localization rather than a purely basal ECM type of staining raised the question of whether hensin might be a membrane protein. We prepared a postnuclear membrane fraction from clone C 6 d after seeding at high density by centrifugation at 100,000 *g*. By immunblots, more than 60% of hensin in this membrane fraction was released into the supernatant by shearing force alone, a process accelerated by the use of 100 mM Na$_2$CO$_3$, but not by 1 M KCl or 1 M KSCN. That alkaline solutions extracted more than 90% of hensin from the membrane eliminated the possibility that hensin was an integral membrane protein (data not shown).
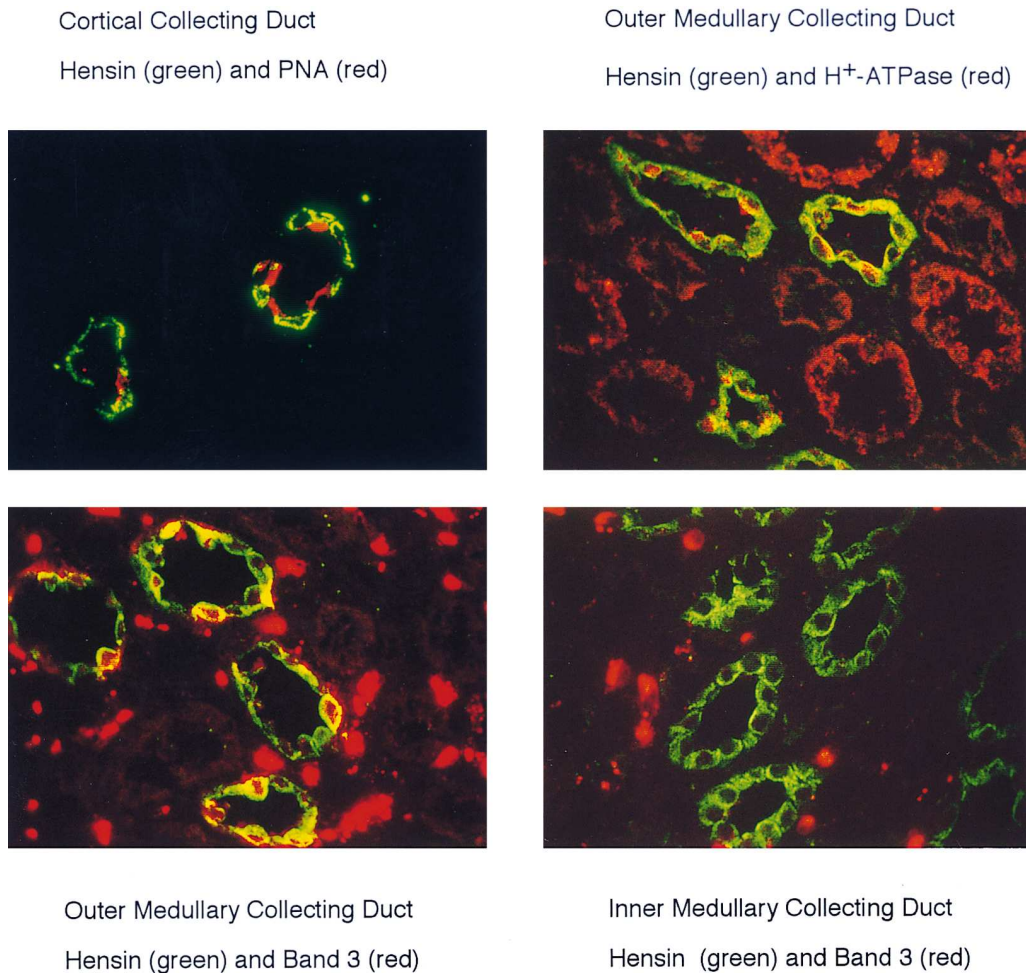
To test whether hensin is a secreted protein, clone C was seeded at high density for 6 d by which time they formed tight monolayers. Cells were radiolabeled with [^{35}S]methionine, chased for 3 h, and the whole cell extracts, the apical and basolateral media were immunoprecipitated (Fig. 7). The results showed that clone C plated at high density secreted hensin basolaterally, suggesting that hensin was secreted and bound to the basolateral membrane or the ECM.
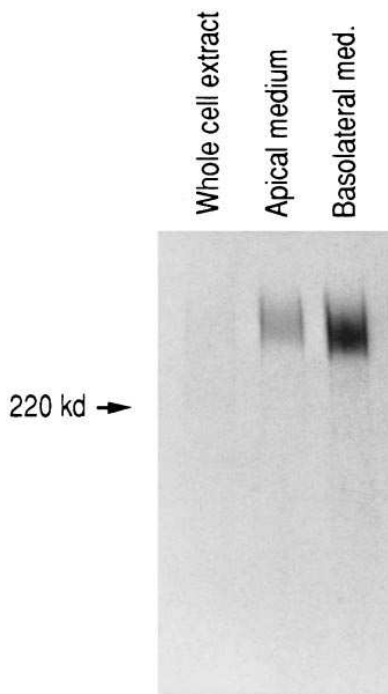
Figure 7. Polarized secretion of hensin into the basolateral medium in culture. Clone C was seeded on a filter at high density and cultured for 6 d, labeled with 50 μCi/ml [^{35}S]methionine for 3 h, then chased for 3 h with a normal medium plus 10 mM cold methionine. After the apical and basolateral medium were taken off, cells were scraped and extracted with 1% SDS, 1% Triton X-100, 1 mM EDTA and 10 mM Tris–HCl (pH 8.0). The extract was diluted 10-fold with 1 mM EDTA and 10 mM Tris–HCl (pH 8.0). The whole cell extract, apical and basolateral medium were processed for immunoprecipitation with hensin antiserum (dilution 1:500). The immunoprecipitates were separated on SDS-PAGE and dried. Signal was visualized by autoradiography.

Role of ambient pH in hensin production. The idea for reversal of polarity originated from the finding that feeding an acid diet caused this phenomenon. However, using the clonal intercalated cell line, we found that reducing the ambient pH (at least for short periods of time) had no effect on the reversal of polarity of band 3 or the induction of apical endocytosis (14). However, these cells were quite sensitive to reduction in pH and developed gross morphological changes when the pH was lowered for 3 h or more. To test for an effect of lowering pH on hensin biosynthesis, we seeded cells at high or low density for 24 h, and then labeled the cells with [^{35}S]methionine for 2 h in the presence of low ambient pH (pH 6.7). Media, cell extracts, and ECM were subjected to immunoprecipitation followed by SDS-PAGE and autoradiography. We found that this short period of exposure to low pH had no effect on the biosynthesis or secretion of hensin in low density or high density cells. Whether longer periods are required or that hensin induced polarity reversal is independent of the pH regulated mechanism remains to be determined.

Immunocytochemistry of hensin. We determined the localization of the protein in rabbit kidney by indirect immunofluorescence using confocal microscopy (Fig. 8). Hensin staining was restricted to the collecting tubule of kidney cortex, medulla, and papilla where it stained plasma membranes and cytoplasmic vesicles of most cells. The percentage of hensin-positive cells in the cortical collecting ducts was 82% (257 cells of 314 cells counted). Since intercalated cells only form a third of

Cortical Collecting Duct

Hensin (green) and PNA (red)

Outer Medullary Collecting Duct

Hensin (green) and H$^+$-ATPase (red)

Outer Medullary Collecting Duct

Hensin (green) and Band 3 (red)

Inner Medullary Collecting Duct

Hensin (green) and Band 3 (red)

Figure 8. Subcellular localization of hensin in rabbit kidney by double immunostaining. Sections of fresh rabbit kidney were doubly stained with hensin IgG and TRITC-conjugated PNA, H$^+$-ATPase antibody, or band 3 antibody. Signals were detected by FITC-conjugated secondary antibody (hensin, *green*) or TRITC-conjugated secondary antibody (H$^+$-ATPase and band 3, *red*) and images were photographed on a laser confocal microscope. The bright red staining outside of the collecting tubules were derived from band 3 in erythrocytes. ×400.
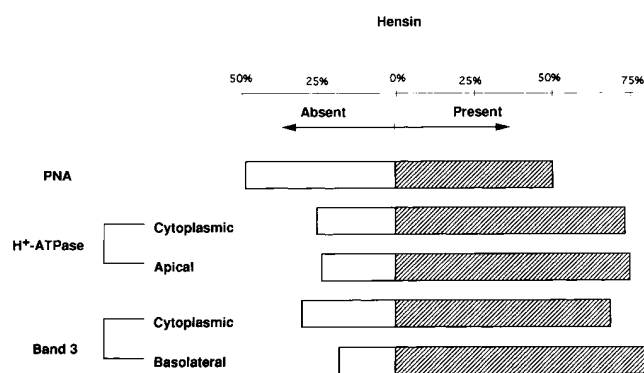
Figure 9. Subcellular distribution of hensin in the rabbit kidney intercalated cells. Rabbit kidney was doubly-stained with hensin IgG and PNA, H$^+$-ATPase or band 3 antibody. The staining pattern of α-intercalated cells was divided into two:diffuse cytoplasmic staining or plasma membrane staining. β-intercalated cells were identified by PNA binding. Total number of H$^+$-ATPase-, band 3-, and PNA-positive cells were 82, 148, and 123, respectively. Over 20 collecting tubules were randomly selected for the cell counting.

the collecting tubule cells, these results suggest that hensin also binds to the principal cells.

Kidney sections were doubly stained with hensin antibody and peanut lectin (PNA), a monoclonal H$^+$-ATPase antibody (E11), or a monoclonal antibody for band 3 (IVF12). PNA specifically binds to the apical membrane of the β-intercalated cells of rabbit kidney cortex (21). H$^+$-ATPase antibody (19) and band 3 antibody (20) were used for the identification of α-intercalated cells.

52% of PNA-positive cells (largely β-intercalated cells) were devoid of hensin staining (Fig. 9). In the subgroup of cells which expressed it, hensin was present in a basal and/or lateral location. It is noteworthy that there were some PNA positive cells that had strong lateral staining without basal staining. The remainder (13%) of these cells showed diffuse cytoplasmic staining without clear membrane staining.

α-Intercalated cells are characterized by apical H$^+$-ATPase and basolateral band 3 staining, but we observed diffuse staining as well. Hence, these cells were subdivided into two classes each and analyzed separately for hensin staining. Most of the cells with apical H$^+$-ATPase or basolateral band 3 staining were hensin positive; (75% of the former and 80% of the latter). Similarly, those with diffuse ATPase and band 3 staining also had hensin staining (73% and 69% respectively). Thus the vast majority of α type intercalated cells expressed hensin while half of the typical β cells did not. The medullary and cortical intercalated cells were quite similar in the distribution of hensin negative and positive cells, hence the data were combined and presented in Fig. 9.

In the outer medulla, almost all of the intercalated cells have some form of α staining pattern and there were no recognizable β- intercalated cells. The staining pattern of hensin in medullary intercalated cells differed from that in the cortex by having a brighter intensity. In addition, there were some α-intercalated cells with the hensin staining in the apical pole (Fig. 8). This was confirmed by the overlap of hensin staining with the apical H$^+$-ATPase staining. A small percentage of α-cells did not stain with hensin antibodies.

In inner medullary collecting ducts, hensin antibody stained many cells in their apical pole (Fig. 8). The staining pattern appears to be rather uniform. There was no H$^+$-ATPase-positive nor band 3-positive cells, confirming the results of others (3).

Discussion

The SRCR motif, originally found in the macrophage scavenger receptor is a 110 amino acid stretch that contains 6 or 8 cysteine residues predicted to form β sheets by intramolecular disulfide bonds (15, 16). Proteins that contain these domains include both secreted (24) and membrane spanning proteins (25, 26). Many are involved in cell:cell recognition (e.g., CD5, CD6, WC1.1,) or immune functions (complement factor I). Hensin is most similar to WC1.1 a bovine T cell membrane protein with a similar molecular mass though with a smaller message size (4.5 vs. 6.0 kb for hensin). While the function of the SRCR domain is unknown, recent studies have identified the "receptor" for one of the CD6 SRCR domains as the activated leukocyte cell adhesion molecule (27). Hence, these domains may mediate protein:protein interaction.

Our biochemical studies have shown that while hensin is bound to membranes, it is easily released by alkali treatment. Further, pulse chase analysis demonstrated that it is secreted to the basolateral medium. While this is compatible with its being a secreted extracellular matrix protein, its immunolocalization differs from typical ECM proteins like collagen and laminin. ECM proteins are strictly basal in distribution while hensin is present in the lateral membrane as well. The images of Figs. 6 *B* and 8 resemble more those of cell adhesion molecules such as E-cadherin (28). Hence, the function of hensin is more likely to mediate cell to cell interaction rather than purely cell substrate interactions, however the two are not mutually exclusive. It is interesting in this regard, that the protein's function was manifest when the cells were plated at high cell density, an experimental paradigm of increased cell to cell interaction. That the cloned fragment contained two SRCR domains and the polyclonal antibody blocked its function suggests that the SRCR domains might be involved in these interactions. However, more direct experiments will need to be performed.

Our previous studies demonstrated that hensin is directly involved in the conversion of the polarity of the clonal intercalated cell line. Its localization in the ECM fraction of high density cells (α-like) was the determining factor in that cell's phenotype. In low density cells (β-like), no hensin was seen in the ECM, but plating these cells on hensin-conditioned filters reversed their polarity. Although it is premature to conclude whether it has a similar function in the acidosis-induced plasticity of these cells in vivo, some findings are intriguing enough to warrant a few speculations. It is now well-established that intercalated cells have heterogenous patterns of staining with H$^+$-ATPase, band 3 antibodies and in PNA binding. Hensin staining is also heterogenous. As many as half of the PNA binding cells (β type) do not have hensin in their intercellular space or ECM, reminiscent of the low density cells. This is in contrast to those with apical H$^+$-ATPase or basolateral band 3 (α type), where most of the cells, like the high density cells express hensin in their ECM (Fig. 8). A more refined analysis will need to be performed where the pattern of subcellular expression of hensin will have to be correlated with that of the ATPase and the anion exchanger, especially of how this pattern changes in response to feeding an acid diet. We had re-

cently suggested that the multiplicity of patterns shown for H$^+$-ATPase or band 3 localization (29) indicates that a large number of intermediate forms exist in a spectrum of types from α to β. Examination of the pattern of expression of these four proteins (hensin, band 3, the ATPase, and PNA binding protein) might help to provide a more solid foundation for the acidosis-induced plasticity hypothesis.

In our preliminary studies on hensin expression we noted that hensin is synthesized and deposited in the ECM of high density cells immediately after plating (14). The half life of hensin was very long in high density ECM. However, it appeared that it was no longer synthesized after the cells were plated for one week or more. Two conclusions can be drawn from this; one that hensin has the property of a molecular switch, i.e., it is "turned on" and remains "on," with no necessity of further synthesis. Another, is that if it was going to be "turned off," it would have to be degraded. Hence, proteases and protease inhibitors are likely to be important regulators of its function. It is also intriguing that some cells (both β and α types) do not have hensin in their ECM. Was it produced and then degraded? Clearly more work needs to be done in this area.

Hensin was expressed in most collecting tubule cells suggesting that the principal cells also express it. Its function in these cells is unknown at present. Nor does this expression shed any light on an intrgiuing hypothesis that Fejes-Toth and Naray-Fejes-Toth (30) had proposed, that the β-intercalated cells can give rise to principal cells and α cells. Based on the developmental expression of a series of markers, Minuth and his co-workers (31) have suggested that all three cell types originate from one precursor ureteric bud cell type. This is the most likely hypothesis, but it would be important to test the other hypothesis directly by using either clonal cell lines or by marking a single ureteric bud cell and examining its progeny.

Acknowledgments

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