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An Antimicrobial Peptide Gene Found in the Male Reproductive System of Rats

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Little is known about the innate defense mechanisms of the male reproductive tract. We cloned a 385–base pair complementary DNA and its genomic DNA named Bin1b that is exclusively expressed in the caput region of the rat epididymis and that is responsible for sperm maturation, storage, and protection. Bin1b exhibits structural characteristics and antimicrobial activity similar to that of cationic antimicrobial peptides, β-defensins. Bin1b is maximally expressed when the rats are sexually mature and can be up-regulated by inflammation. Bin1b appears to be a natural epididymis-specific antimicrobial peptide that plays a role in reproductive tract host defense and male fertility.

Reproductive tract infections remain a global public health problem. However, the mechanisms that protect genitourinary organs from ascending infection by sexually transmitted microorganisms, especially along the male reproductive tract, are poorly understood. The epididymis is a male accessory sex organ that consists of a caput (head), corpus (body), and cauda (tail) and is responsible for sperm maturation, storage, and protection (*1*). It may also act as a reservoir of sexually transmitted bacteria and viruses, including human immunodeficiency virus (HIV), as suggested by the fact that epididymitis occurs in 5 to 10% of the sexually active population and can result in 20 to 40% of infected males becoming infertile (*2*). The defensins are innate immune effectors comprising a family of cationic antimicrobial peptides divided into two subfamilies, α-defensins and β-defensins, differing in amino acid sequence, pairing of the six cysteines, and number of exons (*3*). Although defensins are expressed in a wide variety of tissues of different species of vertebrate and invertebrate animals and have been implicated in phagocytic and epithelial host defense (*3–6*), none has been found in the epididymis and their role in the male reproductive tract has not been elucidated.

Using differential display analysis of mRNAs (*7*), we cloned a cDNA fragment named Bin1b from the rat epididymis. With two 5′ rapid amplification of cDNA ends (5′-RACE) approaches (*8*), the cap site of Bin1b was identified and its full-length cDNA was 385 base pairs (bp) (excluding the polyadenylate tail), with an open reading frame of 204-bp nucleotides, encoding a 68–amino acid protein (including a 16–amino acid signal peptide) (*9*). This amino acid sequence has 20.3% (14/68) identity with several β-defensins such as bovine neutrophil beta-defensin-9, -3, and -7 found in the cattle (*9*). β-defensins generally have a 60– to 90–amino acid (including a signal peptide) protein precursor, which is digested by endogenous protease into mature functional peptides of 38 to 42 amino acids. Although sequence similarity between Bin1b and the β-defensins appears unimpressive, both the size and structure of Bin1b coincide with the consensus structural characteristics of the β-defensin family (*10*) such that the six cysteine residues are invariantly spaced to form three disulphide bonds and the mature peptide has a net positive charge of +7 (*9*). Furthermore, its cloned and sequenced genomic DNA also exhibited two exons separated by a 1.3-kb intron (*9*). This type of organization is similar to that of other β-defensins reported (*10*).

Unlike other members of β-defensin family, which have been shown to be expressed in a wide array of epithelial tissues, Bin-1b is unique in being epididymis-specific. Of 18 organ tissues examined by Northern blot analysis, Bin1b mRNA was only found in the epididymis (Fig. 1A) and was confined to epithelial cells in the middle part of the caput region (Fig. 1B), as shown by in situ hybridization (Fig. 1, C to E). Monitoring Bin1b expression throughout the life-span of rats indicated that it was developmentally regulated (Fig. 1F). Its expression started at 30 days of age, reached a maximum during the sexually mature period, and then decreased in old rats. Such a region- and development-specific expression pattern of Bin1b suggests its importance in epididymis function and fertility.

The antimicrobial activity of Bin1b was tested with primary cultures of polarized caput and cauda epididymal epithelia by a previously established technique used for studying ion transport properties (*11*). A total of 100 colony-forming units (CFU) of *Escherichia coli* was added to the apical compartment of the epithelial cells after 3 days in culture (0.25×10^6 cells per well), and the medium was collected 16 hours later for CFU counting. No bacterial colony growth was detected in the medium collected from caput cultures, indicating the ability of Bin1b to suppress bacterial colonization, whereas substantial numbers of CFU were observed in the cauda cultures (Fig. 2A). This was consistent with our finding that Bin1b was exclusively expressed in the caput but not the cauda of the epididymis. To confirm that the antimicrobial activity was indeed contributed by Bin1b, we designed antisense oligos of Bin1b and added them to cultures 24 hours before the addition of bacteria. The antibacterial capability

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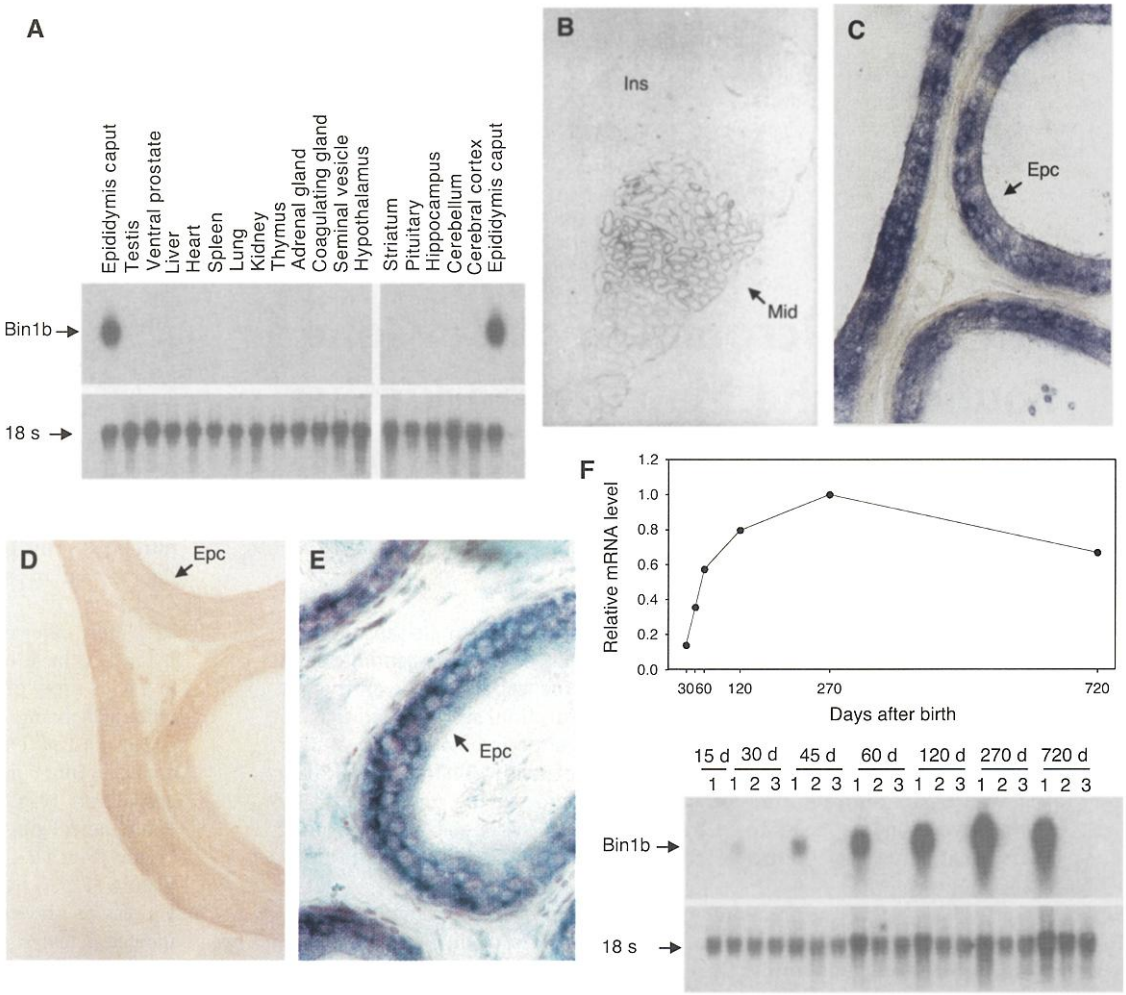

Fig. 1. Localization and developmental regulation of Bin1b. (**A**) Tissue distribution of Bin1b by Northern analysis. (**B**) Regional distribution of Bin1b in the epididymis by in situ hybridization (*30*). Bin1b is located in the middle (Mid) of the rat epididymis caput region. Ins, initial segment. (**C**) Cellular localization of Bin1b with antisense probe. Bin1b is located in the epithelial cells (Epc) of the epididymis. (**D**) Sense probe. (**E**) 18s probe as positive control. (**F**) The expression profile of Bin-1b mRNA in the rat epididymis during the whole life-span (15 to 720 days). 1, 2, and 3 represent the mRNA from the caput, corpus, and cauda regions, respectively.

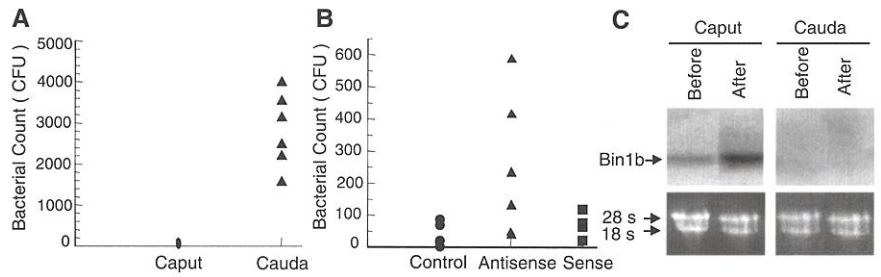

Fig. 2. Antimicrobial activity of Bin1b and its up-regulation of expression in response to inflammation. (**A**) Comparison of antimicrobial activity in caput and cauda cultures. One hundred CFU of *Escherichia coli* was added to cultures 16 hours before examination. (**B**) Effect of antisense of Bin1b on antimicrobial activity of the caput cultures. The cultures were transfected with antisense or sense oligos (5 μg/μl) for 20 hours. (**C**) Enhanced Bin1b mRNA in the caput but not the cauda region of the rat epididymis inflamed by 2-week ligation of the vas deferens.

of the caput culture was greatly attenuated by the treatment with antisense but not with sense oligos (Fig. 2B). Treatment of the cauda culture with Bin1b antisense did not affect CFU counts (*12*), which was expected, as Bin1b was not expressed in the cauda epididymis. These results indicate that Bin1b does have antimicrobial activity, just like members of β-defensin family, and thus is likely to be involved in the host defense of the epididymis. This notion was further supported by a threefold increase in Bin-1b mRNA expression in the caput but not cauda epididymis when the vas deferens was ligated (Fig. 2C); a surgical procedure induces inflammatory changes in the epididymis (*13*). This result is also consistent with the observation of augmented production of some epithelial defensins by infection or injury (*14*, *15*). Our findings may also provide an explanation for previously reported bacterial counts and lesions frequently observed in the cauda but not in the caput epididymis in experimental epididymitis caused by *Chlamydia trachomatis* in rats (*16*).

Thus, Bin1b appears to be a β-defensin–like molecule in the rat epididymis. Bin1b also exhibits structural similarity to several isoforms of epididymis-specific genes recently found in humans and chimpanzees. HE2β1, one of the isoform mRNAs of HE2 gene in the human epididymis (*17*), has 29.3% (39/133) amino acid sequence identity with Bin1b (*9*). Frohlich *et al.* (*18*) have reported five variants of chimpanzee epididymal cDNA, of which the main transcript EP2A is the chimpanzee ortholog of human HE2. They noticed that the two less abundant transcripts, EP2E and EP2D, had certain amino acid sequence similarity with the β-defensin family, but they did not consider either as antimicrobial peptides because of the lack of positive charge at their COOH-terminals. However, we find that the amino acid sequence of EP2E has a greater identity, 60% (48/80), with Bin1b

(*9*). We aligned the amino acid sequence from all the β-defensins reported so far (including EP2E in chimpanzee), for sequence comparison and processed these data using Clustal. We found that Bin1b and EP2E located at the root of the tree. It seems that rat Bin1b might be the ancestor of these reported β-defensins but has gradually degenerated into a vestigial form similar to EP2E in the chimpanzee. Thus, this explains why Bin1b is abundant in the rat epididymis and why EP2E appeared as a minor transcript for the EP2 gene in chimpanzee epididymis.

The caput epididymis-specific localization of Bin1b presents an intriguing puzzle with regard to its function. The first β-defensin isolated from humans (hBD-1) and its cloned mouse homolog (mBD-1) are both expressed in salivary glands, airways, and the urogenital tract (*19*, *20*), suggesting that they contribute to host mucosal defense. But then, why should the epididymis also need a distinct and specific defensin? The expression pattern of Bin1b suggests that it may also be involved with sperm maturation in the epididymis, as well as sperm protection and preventing the ascent of microorganisms into the adjacent testes where sperm are produced. We have previously shown that the secretory activities of the epididymis in rats are highly regulated and fine-tuned in a region-dependent manner (*21*) and that epididymis-derived factors regulate sperm ion channels and receptors (*22*), supporting the notion that a unique and changing lumen environment, responsible for converting immature sperm into competent functional cells, is created by different regions of the epididymis. Changes in sperm motility patterns have also been observed along the epididymal tract, e.g., the microenviroment of the caput region was essential for sperm to acquire its forward motility (*23*). Bin1b may also influence the microenvironment of the caput region, in a similar way to the defensins that have secretagogue properties, which are produced and released from Paneth cells and known to influence the microenvironment of the intestinal crypt lumen (*24*).

The action of cationic antimicrobial peptides is not limited to direct killing of microorganisms (*3*) but also includes nonspecific cytotoxicity, membrane permeability, and opsoninlike abilities. Defensins also appear to have an important role in reproductive function as sperm-immobilizing activities (*25*), and cytotoxicity for mouse oocytes and preimplantation embryos have also been observed for several human defensins (*26*). Taken together, Bin1b appears to have different roles in epididymis function and may not only offer an interesting lead for work in contraception (*27*) but may also have therapeutic implications for sexually transmitted diseases (*28*, *29*).

References and Notes

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7. The procedures for differential display of mRNA analysis [DD reverse transcriptase polymerase chain reaction (RT-PCR)] were as described by P. Liang and A. B. Pardee [*Science* **257**, 967 (1992)]. Two micrograms each of DNA-free total RNAs isolated from the caput, corpus, and cauda regions of the adult Sprague-Dawley rat epididymis were reverse transcribed with 2.5 μM lower primer T_{11}CA with 400 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). One-twentieth of the reverse transcription products was used as template to perform the amplification reaction with 2.5 μM upper primer 502 (5′-TGGATTGGTC-3′) and 0.5 μM lower primer T_{11}CA in a 20-μl volume containing 10 mM tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 4 μM each deoxynucleotide triphosphate (dNTP), 1 μCi ^{32}P-deoxyadenosine triphosphate, and 3 units of Taq polymerase. The PCR conditions were as follows: 94°C for 5 min, then 40 cycles of 94°C for 30 s, 40°C for 60 s, and 72°C for 50 s, with a final elongation at 72°C for 10 min. PCR products were precipitated and then fractionated on the 0.2-mm-thick 6% sequencing gel. The band (~340 bp) named Bin1 that is differentially displayed only in the RNA of caput region was cut out and left in boiling distilled water for 15 min. The eluted DNAs were ethanol precipitated and reamplified in the same conditions as described above but with 40 μM instead of 4 μM each dNTP and no isotope was involved. This 340-bp DNA fragments mixture was cloned into pBluescript SK$^-$. Fifty-two clones were screened by reverse Northern blot as described by L. Mou, H. Miller, J. Li, E. Wang, and L. Chalifour [*Biochem. Biophys. Res. Commun.* **199**, 564 (1994)] with ^{32}P-labeled total RNA derived from each region. The clone hybridized with a 0.4-kb mRNA that specifically appeared in the caput region on the Northern blot was named Bin-1b.
8. Two 5′-RACE approaches were used: one conventional method with DNA oligo–first strand cDNA ligation modified from A. N. Apte and P. D. Siebert [in *Reverse Transcriptase PCR*, J. W. Larrick, P. D. Siebert, Eds. (Horwood, London, 1995), pp. 232–244] and the other one with DNA oligo-RNA ligation [modified from K. Maruyama, S. Sugano, *Gene* **138**, 171 (1994)]. Total RNA (50 μg) from rat epididymis caput was digested with 400 unit of bacterial alkaline phosphatase (BAP) at 37°C for 30 min and additional incubation at 65°C for 30 min. The BAP was then digested with Proteinase K (50 ng/μl) at 37°C for 30 min. After purification, 10 μg of the RNA was further treated with 2 units of tobacco acid pyrophosphatase (TAP) at 37°C for 2 hours and then purified. The untreated RNA (0.75 μg, about 3 pmol), BAP-treated RNA (0.75 μg, about 3 pmol), and TAP-treated RNA (0.75 μg, about 3 pmol) were mixed with 1.25 pmol of the DNA oligonucleotide 7209 (5′-AATGGTACCGTGACGTGGTCC-3′) and ligated with T4 RNA ligase (1.2 unit/μl) in 10 μl of 50 mM tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM hexamine cobalt chloride, 25% PEG8000, and 1 mM adenosine triphosphate at 17°C for 18 hours. SuperScript II One-Step RT-PCR System (Gibco, BRL) was used to perform the RT-PCR reaction with 0.2 μl of the ligation products in 20 μl containing 200 nM Bin1b gene-specific primer (GSP) (5′-TGGCCCCGCTGCATGAAGCAC-3′) and oligo 7209 (5′-AATGGTACCGTGACGTGGTCC-3′). The RT-PCR reactions were run with PTC-200 (Peltier Thermalcycler Controller; MJ Research, Waltman, MA) as follows: 50°C for 30 min, 94°C for 2 min, then 35 cycles of 94°C for 5 s, 60°C for 15 s, and 72°C for 45 s, with a final elongation 72°C for 5 min. The second PCR was performed with 0.2 μl of the RT-PCR products in 10 μl containing 50 mM tris-HCl (pH 8.3), 1 to 3 mM $MgCl_2$, bovine serum albumin (250 μg/ml), 0.5% Ficoll 400, 1 mM tartrazine, 200 μM dNTP, 500 nM Bin1b GSP and oligo 7209, and 0.4 unit of Taq polymerase. PCR reactions were run in thin capillary tubes with 1605 Air-Thermo-Cycler (Idaho Technology, Idaho Falls, ID) as follows: 94°C for 1 min, then 60 cycles of 94°C for 0 s, 60°C for 0 s, and 77°C for 15 s, with a final elongation at 77°C for 5 min. The PCR product, about 200 bp, was cloned in pBluescript SK$^+$ T-Vector and then sequenced.
9. Sequence and structure characteristics of Bin1b, sequence similarity of Bin1b with β-defensins and its alignment with primate homologs are given in supplementary materials, available on *Science* online at www.sciencemag.org/cgi/content/full/291/5509/1783/DC1.
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