Defect in SHAP-hyaluronan complex causes severe female infertility:
A study by inactivation of the bikunin gene in mice*

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Running title: Physiological functions of the SHAP-HA complex and ITI family
Summary

Hyaluronan (HA) associates with proteins and proteoglycans to form the extracellular HA-rich matrices that significantly affect cellular behaviors. So far, only the heavy chains of the plasma inter-α-trypsin inhibitor (ITI) family, designated as SHAPs (serum derived hyaluronan-associated proteins), has been shown to bind covalently to HA. The physiological significance of such a unique covalent complex has been unknown but is of great interest because HA and the ITI family are abundant in tissues and in plasma, respectively, and the SHAP-HA complex is formed wherever HA meets plasma. We abolished the formation of the SHAP-HA complex in mice by targeting the gene of bikunin, the light chain of the ITI family members, which is essential for their biosynthesis. As a consequence, the cumulus oophorus, an investing structure unique to the oocyte of higher mammals, had a defect in forming the extracellular HA-rich matrix during expansion. The ovulated oocytes were completely devoid of matrix and unfertilized, leading to severe female infertility. Intraperitoneal administration of ITI, accompanied by the formation of the SHAP-HA complex, fully rescued the defects. We conclude that the SHAP-HA complex is a major component of the HA-rich matrix of the cumulus oophorus and is essential for fertilization in vivo.
Introduction

Not all, but most cells undergoing active proliferation, differentiation and locomotion *in vitro* and *in vivo* form extracellular hyaluronan (HA)-rich matrices, which have been shown to play significant roles in many biological processes, such as tissue organization, morphogenesis, inflammation and cancer metastasis (1-3). We previously identified the covalent SHAP (serum-derived hyaluronan-associated protein)-HA complex in the extracellular HA-rich matrix of cultured mouse dermal fibroblasts, and found that the SHAPs are the heavy chains of the inter-α-trypsin inhibitor (ITI) family in sera supplemented to culture media (4-6).

The ITI family members are synthesized and assembled in liver and secreted into blood at high concentrations (0.15-0.5 mg/ml plasma) (7). The members are composed of a common light chain, bikunin, and one or two of the three genetically different heavy chains (HC1, HC2 and HC3) (8). Free bikunin in circulation is excreted rapidly into the urine (9) where it is present as the urinary trypsin inhibitor (UTI) (10). The serine residue at position 10 of bikunin contains an O-glycosidically linked chondroitin-4-sulfate chain (11), to which the C-terminal of a heavy chain is covalently bound via a unique ester bond to aspartate(12). The SHAPs link to HA via an equivalent ester bond, suggesting that the formation of the SHAP-HA complex is a substitution reaction, namely HA replaces the chondroitin sulfate of bikunin to link to a heavy chain (Fig 1) (6). Plasma includes an enzyme activity that catalyzes the reaction (13). Therefore, we might expect that the SHAP-HA complex would be formed once plasma meets HA, thereby contributing to some physiological and pathological processes.

This paper attempts to clarify not only the physiological function of the SHAP-HA complex but also that of the ITI family by eliminating their formation in mice in which the bikunin gene has been targeted.
Experimental Procedures

Construction of targeting vector and generation of Bikunin-deficient mice—To delete the chondroitin sulfate attachment site encoded by exon 7 of the α-1-microglobulin/bikunin precursor gene (14), we created an Eco47III site by site-directed mutagenesis of the codon for the serine residue at position 7 in bikunin from AGT to CGCT (Quickchange™ kit, Stratagene). After the Eco47III digestion, the 5′-part of exon 7 was linked to a SV 40-derived sequence between a HpaI site (position 2474) and a BamHI site (position 2605) in pMAMneo-s vector (Clontech), which provided a new stop codon and polyadenylation signal sequences for the α-1-microglobulin gene (Fig. 2). A neomycin-resistant gene was linked to the SV 40 sequence. The 6 kb long sequence between ClaI site in exon 3 and the mutated site was used as the long arm of the targeting construct, and the 1.6 kb long sequence flanked by an EcoRI site in intron 8 and a downstream PvuII site as the short arm. The whole fragment was cloned into a vector containing the thymidine kinase gene. The linearized vector was electroporated into E14 embryonic stem (ES) cells. After positive and negative selection, about 2% of the resistant colonies were confirmed to have undergone homologous recombination by nested PCR analysis with the primers: GACATTGGGTGGAAACATTCCAGG and TGGGGTACTGTGTGGATGCAGTTAG for the first PCR, and CGAAGCTTGGCAGCTAAACTCC and ATTCTCCAGGGCATGGGCATAGGCC for the nested PCR (arrows in Fig. 2a), and then further by southern blot analysis. Four almost 100% chimera mice were obtained from an ES clone by aggregating ES cells with the blastocysts from C57BL/6 female mice. All of them gave F1 offspring carrying the mutation in a heterozygous state when mated to C57BL/6 females. Homozygous mutants were obtained by sibling intercross. Animals at generation F2 to F4 were used for analysis.

RT-PCR—Total RNA was purified from liver with TRIzol® reagent (Life Technologies). The primers used for RT-PCR were: a) CAAGAATTCCAGGGCAACCTG and TTCTCGAGCACAGCCTGGTCCCTCC for heavy chain 1 (HC1); b) ACGGATCCTCTT
CAGCTCAAGAAAT and CCCTCGAGTTTCCAAGATGA for HC2; c) CCAAGAGAGTAGGGGTCAGGGACT and GGGTCCAGATCATCTAAGCT for bikunin; d) GCTGATCATCGTCAAACACTG and CGTGATCATCTGGCAATTGA CGTGGGC for α-1-microglobulin; and e) CCTGGAATGTTTCCACCCAATGTCG and CCATGATATTGCAGCAAGC for the neomycin-resistant gene.

**Immunostaining analysis**—ITI family molecules were detected by rabbit antibody against human ITI (DAKO, Denmark) or rabbit antiserum against human bikunin (Yanaihara, Japan) followed by peroxidase-conjugated goat antibody against rabbit Ig (DAKO) or Alexa 594-labeled goat anti-rabbit IgG antibodies (highly cross-adsorbed, Molecular Probes, Inc., USA). The antigen, bikunin, was purified from Miralcl® (Mochida Pharmaceutical Co., Japan) by elution on a DEAE-Sepharose column (15). The α-1-microglobulin was detected with sheep antibody against human α-1-microglobulin (The Binding Site, England) followed by peroxidase-conjugated rabbit antibody against sheep Ig (DAKO). Hyaluronan was detected by biotinylated hyaluronan-binding protein (Seikagaku Co., Japan) followed by Alexa 488-labeled streptavidin (Molecular Probes). The peroxidase-conjugated second antibodies were visualized with Renaissance® chemiluminescence reagent plus (NEN, Boston) and exposed to Hyperfilm ECL film (Amersham Japan).

**Purification of mouse ITI**—Mouse ITI was purified from the pooled serum of wild type or Bik+/− mice by Q-Sepharose chromatography and ammonium sulfate precipitation according to the method by Yamamoto et al. (16).

**Test-tube reaction for the formation of SHAP-HA complex**—The reaction mixture (2.75 ml) including 0.5 ml of serum, 0.1 ml of HA (5 mg/ml, Seikagaku, Japan) and 5 mM of MgCl₂ in Hank’s medium was incubated overnight at 37°C; 0.2 mg of mouse ITI was added if necessary. After incubation, a 4 M guanidine HCl solution was prepared and adjusted to a density of 1.37 g/ml with solid CsCl. After centrifugation at 40,000 rpm for 48 h, the SHAP-HA complex in the bottom fraction (ρ≥1.45 g/ml) was precipitated with ethanol, digested with *Steptomyces* hyaluronidase (Seikagaku Corp., Tokyo, Japan) at 60°C for 2 h and subjected to immunoblot analysis (4-6).
Superovulation—Three- to four-weeks-old female mice were injected intraperitoneally with 5 IU of pregnant mare's serum (Sigma) at noon and 5 IU of human chorionic gonadotropin (hCG) (Sigma) 48 hours later. If necessary, purified mouse ITI (0.35 mg/mouse) or human bikunin (67 µg/mouse, prepared as above) was mixed with hCG and injected at the same way. Then the Bik^{-/-} females were caged overnight with males, and their oocytes were examined at 0.5 dpc and 1.5 dpc.
Results

Gene targeting of bikunin abolished the formation of the ITI family and the SHAP-HA complex—Bikunin is synthesized as a fusion protein with another plasma protein, α-1-microglobulin, and is separated from α-1-microglobulin by post-translational proteolysis (17,18). We successfully truncated the α-1-microglobulin/bikunin precursor gene (AMBp) to abolish bikunin gene expression while retaining normal α-1-microglobulin gene expression (Fig. 2A-E). The Bik⁻/⁻ mice have a normal plasma level of α-1-microglobulin protein, which forms complexes with other plasma proteins normally (Fig. 2F) (19). On the other hand, as predicted, ITI (230 kDa) and PαI (130 kDa) were absent in Bik⁻/⁻ mice (Fig. 2F). The Bik⁺/⁻ mice, with less bikunin transcripts (Fig. 2E), showed normal plasma levels of ITI and PαI (Fig. 2F).

A new heavy chain-related protein appeared in the serum of Bik⁻/⁻ mice (Fig. 2F). During the assembly of members of the ITI family, the propeptides of heavy chains have their C-terminal extensions (240-280 amino acid residues) proteolytically removed while linking to bikunin (20). The molecular weight (110 kDa) of the new protein is consistent with that expected for the unprocessed heavy chain. The new protein was not altered by chondroitinase ABC digestion (data not shown), which indicates that the heavy chains do not link to other chondroitin sulfate proteoglycans in the absence of bikunin.

Incubation of the serum of Bik⁻/⁻ mice with exogenous HA resulted in no formation of the SHAP-HA complex, indicating that the unprocessed heavy chains have no ability to form the SHAP-HA complex with HA (Fig. 2G). Therefore, the ester bond between a heavy chain and the chondroitin sulfate chain of bikunin is essential for the substitution reaction to form the SHAP-HA complex. Thus, we have succeeded in developing an in vivo system where there is no biosynthesis of the ITI family and consequently no formation of the SHAP-HA complex.

Bik⁻/⁻ female mice are infertile—Bikunin deficiency did not significantly affect ontogenesis. Segregation of bikunin alleles followed Mendel's law (data not shown). Gross inspection failed to
discriminate between the mutant and wild type animals. Adult Bik\(^{-/-}\) mice showed normal copulating behaviors. However, in contrast to the full fertility of males, the females showed severe infertility (Table 1). Although the vaginal plug was documented many times, more than half of the Bik\(^{-/-}\) females were not pregnant. The others had only small litters (1.6 in average) and were indifferent to their pups. The neonatal pups usually died within 2 days, but the ones that were fostered by Bik\(^{+/+}\) mothers survived.

We examined the gestation processes in Bik\(^{-/-}\) females. The ovaries appeared normal because those of 4-week-old females responded normally to gonadotropin treatment, and those of the adults included follicles at all maturation stages as well as the well defined corpus luteum (data not shown). Vaginal cytological examination revealed normal menstrual cycles (data not shown). In contrast, when examining the uteri at 5.5-7.5 days post coitus (dpc), only one implanted embryo was found in seven uteri (Fig. 3A). These results, together with the rare, but complete success in gestation, indicated normal uterine and ovarian functions and suggested impairment at fertilization or implantation in Bik\(^{-/-}\) females.

We then collected the naturally ovulated oocytes from oviducts at 0.5 dpc. The oocytes of Bik\(^{-/-}\) females (3.5±1.8, \(n=13\)) were significantly less than the number observed in Bik\(^{+/+}\) females (8.1±2.3, \(n=11\)). More strikingly, all oocytes of Bik\(^{-/-}\) females were completely devoid of a cumulus oophorus, in sharp contrast to the cumulus-oocyte complexes collected from Bik\(^{+/+}\) females (Fig. 3B). The naked oocytes had intact zona pellucida. They remained at the single-cell stage at 1.5 dpc, when those of Bik\(^{+/+}\) females had already cleaved into two-cell oocytes (Fig. 3B). Therefore, the infertility of Bik\(^{-/-}\) females was due to the impaired fertilization of the cumulus oophorus-free oocytes.

Absence of the SHAP-HA complex caused the defect of the HA-rich matrix of the cumulus oophorus—The cumulus oophorus dissociates eventually after fertilization. However, the absence of cumulus oophorus in Bik\(^{-/-}\) females was independent of copulation as manifested by the equally naked oocytes induced by a gonadotropin priming. Ovarian histology revealed the presence of
normal cumulus oophori in the graffian follicles of Bik−/− females (Fig. 3C). However, after a gonadotropin surge in Bik−/− females, the matrix fails to form, and the cumulus cells were dispersed in the antral cavity (Fig. 3C).

During cumulus expansion, the blood-follicle barrier opens and allows the influx of members of the ITI family as well as the enzymatic factor required to form the SHAP-HA complex at the time when the cumulus cells initiate extensive HA synthesis (21,22). A granulosa cell-derived factor with similar enzyme activity has also been reported (23). Such a follicle environment would be very suitable for the formation of the SHAP-HA complexes. Thus, the defect of the SHAP-HA complex formation in Bik−/− females most likely impairs the construction of the cumulus HA-rich matrix with subsequent detachment of the cumulus cells from the oocyte. To verify this, we immunolocalized HA and SHAP in the cumulus-oocyte complex and found that the SHAP co-localized with HA perfectly throughout the matrix network (Fig. 4A). This finding indicates that the SHAP-HA complex is a major component of the cumulus matrix. Bikunin was not detectable in the cumulus-oocyte complex (Fig. 4A). An immunoblot result also showed that all SHAP-related immunoreactivities in the cumulus-oocyte complex were from the SHAP-HA complex, and no intact ITI family molecules were present in the cumulus matrix (Fig. 4B). Therefore, the infertility of the bikunin-deficient female mice was due to the absence of the cumulus SHAP-HA complex but not from the absence of bikunin per se.

ITI administration fully rescued the defect of cumulus expansion and oocyte fertilization—We further explored the possibility of rescuing the infertility of Bik−/− females by ITI administration. When injected intraperitoneally, the purified mouse ITI appeared in blood within 1 hour and remained detectable for more than 10 hours (Fig. 4C). This ensured the availability of plasma ITI during the process of induced ovulation (24). Oocyte examination revealed that ITI administration resulted in a full recovery of cumulus expansion and oocyte fertilization. The oocytes in oviducts regained the cumulus oophori at 0.5 dpc (3 mice) and were successfully fertilized and cleaved into 2-cell or 4-cell oocytes at 1.5 dpc (3 mice) (Fig. 4C). In contrast, administration of human UTI
resulted in no improvement of the naked oocytes (5 mice), although the rapid clearance of UTI from blood should not be overlooked (9). The recovery could be explained by the formation of the SHAP-HA complex between the exogenous ITI and the endogenous cumulus HA in accordance with the test-tube assay (Fig. 2G).
Discussion

The present results assign a definite physiological function to the SHAP-HA complex as well as the ITI family in the process of ovulation and fertilization. It is now clear that the blood has roles in fertilization not only indirectly by transporting hormones and nutrients but also directly by participating in the construction of the expanded cumulus oophorus. The results also provide a new insight into the structure of the cumulus matrix, and show directly the importance of the expanded cumulus oophorus in fertilization in vivo. The findings clarify a possible mechanism underlying some forms of female infertility and suggest a promising therapeutic method, namely supplementation with ITI during the pre-ovulatory period.

The protease inhibitory activity of bikunin has drawn most of the research attention to the ITI family. A successful example is the clinical application of UTI to the treatment of acute pancreatitis and shock. The inhibitory activities of UTI to cancer metastasis (25) and nephrolithiasis (26) have also been reported. Here, we showed that bikunin is necessary for the formation of the SHAP-HA complex that is essential for fertilization. The finding identifies an important "SHAP-presenting" role for bikunin, i.e., activating (esterifying), transporting and presenting the heavy chains to suitable recipients under suitable conditions, such as the newly synthesized HA in the expanding cumulus oophorus. Such a role is physiologically most important because bikunin deficiency itself did not significantly impair ontogenesis. The fact that most bikunin in plasma is linked with the heavy chains (ITI and Pδ1) (8) and the released bikunin in circulation is rapidly excreted into urine (9) supports this notion.

The influx of plasma into preovulatory follicles and the local formation of the SHAP-HA complex recalls similar situations in inflammatory sites, where cytokines stimulate local HA synthesis and induce capillary hyperpermeability to allow the efflux of plasma components. The SHAP-HA complex may also play roles in such inflammatory responses. Indeed, the SHAP-HA complex was found to accumulate significantly in the synovial fluid of patients suffering rheumatoid
arthritis (6,27). Studies of the SHAP-HA complex will help understand the pathogenesis of such
diseases.

The importance of cumulus oophorus in ovulation and fertilization has already been noticed (28-31). Our result confirmed the role of the cumulus oophorus in the capture of the oocyte by the fimbria of oviducts (29) and gave a more accurate assessment of its contribution in vivo. It also provided direct evidence showing that the expanded cumulus oophorus is indispensable to fertilization in vivo. In Bik⁻/⁻ mice, the naked oocytes appeared normal, and under the microscope the active attack of spermatozoa on the naked oocytes could be observed. However, it requires further investigation to determine the precise mechanism for the infertility at this stage.

The molecular mechanism for the construction and metabolism of the HA-rich cumulus matrix is still largely unknown. Its HA-rich nature has been manifested by many studies (28, 32-35). Here we demonstrate that the cumulus matrix contains the SHAP-HA complex as an essential component. Previous studies with scanning electron microscopy revealed many trypsin-sensitive granules along with the hyaluronan filament in the cumulus matrix (32). The N-terminal regions of the heavy chains of ITI have granule appearances (36). Therefore, it is very likely that the granule-filament structure represents the SHAP-HA complex. Our findings also help explain the previous observations, which showed that ITI and PδI stabilized the HA-rich matrix of cultured cells (37,38) and in vitro cumulus expansion (39), and that HA oligomers interfered with ovulation in vivo (40).

The cumulus matrix might include other components, such as proteoglycans (41,42), TSG-6 (43), and the link protein (44). The characterization of their interaction with the SHAP-HA complex is necessary for the completely delineation of the structure of cumulus matrix, for example the possible interaction between the SHAP and PG-M/versican (45). The cumulus matrix might share some structural features with the HA-rich matrices of cultured cells, which are organized by HA-binding proteoglycans like aggrecan and PG-M/versican and anchored onto cell surfaces by membrane HA receptors, such as CD44 (46). However, the anchoring of the cumulus matrix on the protein surface of the zona pellucida might be different. The complete shedding of the cumulus cells
in the absence of the SHAP-HA complex raised an interesting question about the role of SHAP in
the anchoring interactions.

We have clarified a molecular mechanism underlying a form of female infertility. It encourages us
to survey spontaneous genetic mutations in the infertile women population to identify possible
defects in ITI. So far no such case has been reported. However, a heritable null allele of the HC1
gene resulting from a deletion/frameshift has previously been identified (47).

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(Footnotes)

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1The abbreviations used are: AMBP, α-1-microglobulin/bikunin precursor gene; α1M, α-1-microglobulin; Bik, bikunin; dpc, days post coitus; HA, hyaluronan; HC, heavy chain; hCG, human chionic gonadotropin; H-E, hematoxylin-eosin; ITI, inter-α-trypsin inhibitor; neo, neomycin-resistance gene; pA, polyadenylation signal sequences; PaI, pre-α-inhibitor; SHAP, serum-derived hyaluronan-associated protein; tk, thymidine kinase gene; UTI, urinary trypsin inhibitor.
FIG. 1. Schematic representation of the synthesis of the SHAP-HA complex. Note the equivalent ester linkage of the C-terminal of a heavy chain/SHAP to the chondroitin sulfate (CS) of bikunin and hyaluronan (HA).

FIG. 2. Generation of bikunin-deficient mice. A, bikunin gene-targeting strategy. Among the ten exons, exon 1 to 6 encodes for α1M and the others for bikunin (Bik). Restriction sites: C, ClaI; E, EcoRI; K, KpnI and P, PvuII. B, modification of exon 7 (capitals in bold). RARR is the conserved proteolytic cleavage site for separating α1M and Bik. Bik is truncated immediately before the glutamic acid residue at position 7, which is followed by the conserved chondroitin sulphate chain attachment site, EGSG, at position 8 to 11. A SV40-derived sequence provides the new stop codon (*) and polyadenylation signals (small cases in bold) for α1M gene. C, Southern blot analysis. KpnI digestion, the outer probe. D, RT-PCR analysis of liver total RNA. HC1 and HC2, the heavy chains 1 and 2 of the ITI family. E, Northern blot analysis of liver total RNA. The α1M cDNA probe reveals a short transcript from the truncated precursor gene. F, immunoblot analysis of mice sera using anti-α1M antibody under a non-reducing condition and anti-Bik or anti-ITI antibodies under a reducing condition. The new 110 kDa heavy chain-related protein in Bik−/− mice is presumably the unprocessed heavy chains with intact C-terminal extensions. G, test-tube assay for the formation of SHAP-HA complex. ITI in Bik+/- mice forms the SHAP-HA complex with exogeneous HA, but the unprocessed heavy chain in Bik−/− mice does not. The sera of Bik−/− mice includes the activity to catalyze the reaction between exogeneous ITI and HA (see FIG. 1).

FIG. 3. Impaired cumulus expansion and oocyte fertilization in Bik−/− female mice. A, uteri of Bik+/− and Bik−/− mice at 5.5 day post coitus (dpc). The implanted embryo (*) is rarely found in Bik−/− mice. Only one side of the utreus of Bik+/− mouse is shown. B, all oocytes in the oviducts of Bik−/−
mice at 0.5 dpc are devoid of cumulus masses. The naked oocytes are not fertilized and remain at the single-cell stage at 1.5 dpc. C, paraffin section of ovaries after hCG induction. In Bik\(^{-/-}\) mice, the cumulus cells are released during expansion of cumulus oophorus due to a defect of cumulus matrix formation. H-E staining.

FIG. 4. The SHAP-HA complex is the major component of the cumulus matrix. A, immunofluorescent localization of SHAP and HA in the cumulus-oocyte complex (COC). The SHAP co-localizes well with HA throughout the matrix network. The COCs show no bikunin immunoreactivity (Bik). DAPI stains the nuclei of the cumulus cells. B, 10 COCs from Bik\(^{+/}\) females or 10 naked oocytes from Bik\(^{-/-}\) females were digested with *Steptomyces* hyaluronidase (HAase), and the supernatants were subjected to immunoblot analysis with anti-ITI antibody. Only the heavy chain in the form of the SHAP-HA complex is detected. As a control, the heavy chains were released from ITI and P\(\alpha\)I by NaOH treatment. C, ITI administration rescues the cumulus matrix and oocyte fertilization in Bik\(^{-/-}\) females. proHC, the unprocessed heavy chain.
# TABLE 1

**Impaired fertility in Bikunin-deficient female mice**

| Genotype | Male Total No. of females | Genotype | Female Total No. of females | Male No. of females pregnant<sup>a</sup> | Female Total No. of offspring<sup>b</sup> | Mean litter size |
|-----------|--------------------------|-----------|-----------------------------|----------------------------------------|-------------------------------------------|-----------------|
| +/-       | 10                       | +/-       | 10                          | 79                                     | 7.9±2.7                                   |
| -/-       | 9                        | +/-       | 4                           | 5                                      | 0.6±0.7                                   |
| +/-       | 8                        | +/-       | 8                           | 68                                     | 8.5±1.2                                   |
| +/-       | 11                       | +/-       | 4                           | 9                                      | 0.8±1.5                                   |

<sup>a</sup>The females and males were caged continually. The data are collected after 1 month for Bik<sup>+-</sup> females and 2 months for Bik<sup>-/-</sup> females.  
<sup>b</sup>All offspring of Bik<sup>+-</sup> females survive. However, the neonatal pups of Bik<sup>-/-</sup> females died from starvation within 2 days. Two of the pregnant Bik<sup>-/-</sup> females delivered second litters (2 pups each), which are not included here.
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