Selective Cleavage of Human Sex Hormone-Binding Globulin by Kallikrein-Related Peptidases and Effects on Androgen Action in LNCaP Prostate Cancer Cells

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Stimulation of the androgen receptor via bioavailable androgens, including testosterone and testosterone metabolites, is a key driver of prostate development and the early stages of prostate cancer. Androgens are hydrophobic and as such require carrier proteins, including sex hormone-binding globulin (SHBG), to enable efficient distribution from sites of biosynthesis to target tissues. The similarly hydrophobic corticosteroids also require a carrier protein whose affinity for steroid is modulated by proteolysis. However, proteolytic mechanisms regulating the SHBG/androgen complex have not been reported. Here, we show that the cancer-associated serine proteases, kallikrein-related peptidase (KLK)4 and KLK14, bind strongly to SHBG in glutathione S-transferase interaction analyses. Further, we demonstrate that active KLK4 and KLK14 cleave human SHBG at unique sites and in an androgen-dependent manner. KLK4 separated androgen-free SHBG into its two laminin G-like (LG) domains that were subsequently proteolytically stable even after prolonged digestion, whereas a catalytically equivalent amount of KLK14 reduced SHBG to small peptide fragments over the same period. Conversely, proteolysis of 5α-dihydrotestosterone (DHT)-bound SHBG was similar for both KLKs and left the steroid binding LG4 domain intact. Characterization of this proteolysis fragment by [^{3}H]-labeled DHT binding assays revealed that it retained identical affinity for androgen compared with full-length SHBG (dissociation constant = 1.92 nM). Consistent with this, both full-length SHBG and SHBG-LG4 significantly increased DHT-mediated transcriptional activity of the androgen receptor compared with DHT delivered without carrier protein. Collectively, these data provide the first evidence that SHBG is a target for proteolysis and demonstrate that a stable fragment derived from proteolysis of steroid-bound SHBG retains binding function *in vitro*. **(*Endocrinology* 153: 3179–3189, 2012)**

Prostate cancer is the most common newly diagnosed cancer in the United States and is the third leading cause of cancer-related death in men (1). Early stage prostate cancer is dependent on sex steroids, and hence, the disease is designated a "hormone dependent cancer" (2). The developing prostate is also reliant on the androgens testosterone and its reduced metabolite, 5α-dihydrotestosterone (DHT). Classical androgen signaling proceeds through the androgen receptor (AR), which resides intracellularly (3) and binds androgens that freely diffuse through plasma membranes (4, 5). After steroid binding, the androgen-AR complex translocates to the nucleus,

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Abbreviations: ADH7, Alcohol dehydrogenase 7; AR, androgen receptor; DHT, 5α-dihydrotestosterone; GST, glutathione S-transferase; k_{cat}, catalytic rate constant; K_d, dissociation constant; KLK, kallikrein-related peptidase; K_M, Michaelis constant; LG, laminin G like; *p*NA, *para*nitroanilide; PSA, prostate-specific antigen; q, quantitative; R_{SHBG}, SHBG cell-surface receptor; SHBG, sex hormone-binding globulin.

where it activates transcription of androgen-responsive genes (6). This in turn drives proliferation and differentiation of both the prostate stroma and epithelium. The androgen dependence of the prostate is highlighted by androgen deprivation, which leads to induction of apoptosis *in vitro* and *in vivo* (7, 8). Accordingly, treatments for recurrent hormone sensitive prostate cancer focus on androgen ablation.

Delivery of androgens (and other sex steroids) to hormone-responsive tissues is primarily mediated by sex hormone-binding globulin (SHBG), the principal specific carrier of sex steroids in blood (9). SHBG is secreted from the liver as a 373-amino acid glycoprotein that circulates as a homodimer. Each SHBG subunit consists of two laminin G-like (LG) domains termed the LG4 (N-terminal) and LG5 (C-terminal) domains. Individual steroid-binding pockets are located within the LG4 domain of each monomer (10, 11), which bind testosterone and estradiol with nanomolar affinities (9). In addition to steroid binding, the LG4 domain mediates dimerization (11, 12), divalent metal ion binding (13), and interaction with the putative SHBG cell-surface receptor (R_{SHBG}) (14). However, the biological function of the LG5 domain is yet to be elucidated.

Several interactions between SHBG and extracellular proteins have been identified which are suggested to aid efficient steroid transport and delivery. SHBG binds to matrix-associated proteins fibulin-1D and fibulin-2 within uterine stroma resulting in preferential sequestration of estradiol-bound SHBG from circulation (15). The nonspecific endocytotic membrane receptor for the vitamin A and D carrier protein (megalin) has also been demonstrated to bind and internalize SHBG-steroid complexes in mice (16). This is proposed to aid steroid entry into target cells, although activation of a secondary signaling response by this process remains to be confirmed in a physiological context. Additionally, nontransport functions have been postulated for SHBG. The high binding affinity of androgens and estrogens to SHBG sequesters these active steroids, thereby restricting steroid bioavailability (5) and modulating both stability and rate of clearance of steroids from the bloodstream (17–19). Further, glycosylation-variant SHBG shows cytoplasmic accumulation and may act as a reservoir of androgen in mouse proximal convoluted tubule cells (20). It has also been proposed that SHBG participates in nongenomic steroid signaling via R_{SHBG} as distinct from classical androgen and estrogen pathways (21, 22), although this remains a matter of debate.

Although it is clear that proteolysis can modulate the binding affinity of steroid carriers, such as corticosteroid binding globulin (23, 24), a similar mechanism involving SHBG has not been reported. A previous yeast two-hybrid screen examining interaction between prostatic proteins and human SHBG identified the kallikrein-related peptidase (KLK)4 as a potential interacting partner (25). KLK4 belongs to the 15-member tissue KLK family (26, 27) and is over expressed in prostate cancer compared with normal prostatic tissue (28–31). Because expression of KLK4 and a number of other KLKs are regulated by androgens (32), interaction between SHBG and KLK proteases may influence biological functions of SHBG and the androgen signaling axis within the prostate.

Here, we examine interactions between SHBG and four closely related members of the KLK family (KLK3, KLK4, KLK7, and KLK14), which show varying levels of androgen responsiveness, to establish whether SHBG is a substrate for these proteases. For KLKs found to cleave SHBG, protease cut sites were identified by N-terminal sequencing of proteolysis fragments, and the effect of occupying the steroid binding pocket on proteolysis was determined by assays with DHT-bound SHBG. Functional consequence(s) of KLK-mediated SHBG cleavage were determined by characterizing steroid binding affinity of full-length and cleaved SHBG in [^{3}H]-DHT binding assays and exploring the ability of each species to modulate transcriptional activity of the AR, an indicator of androgen bioavailability in cell culture. Collectively, these data suggest a selective cleavage and processing of SHBG by KLKs.

Materials and Methods

Reagents

SHBG purified from human serum was obtained from Fitzgerald Industries (Acton, MA). Recombinant KLK7 was sourced from R&D Systems (Minneapolis, MN). Thermolysin was obtained from Calbiochem (San Diego, CA), and active prostate-specific antigen (PSA) from human seminal plasma, DHT, BSA, and phosphoramidon were from Sigma-Aldrich (Castle Hill, Australia). Antibodies for Western blot analysis were rabbit antihuman SHBG polyclonal antisera (33), mouse anti-V5 primary antibody (Invitrogen, Mount Waverly, Australia), Alexa Fluor 680-conjugated goat antirabbit secondary antibody (Invitrogen), and Alexa Fluor 488-conjugated goat antimouse secondary antibody (Invitrogen). All reagents for cell culture and reverse transcriptase PCR were from Life Technologies (Carlsbad, CA) unless otherwise stated.

Recombinant protein expression

Recombinant pro-KLK4 and pro-KLK14 were produced in Sf9 insect cells as described previously (34, 35). Recombinant human SHBG-glutathione S-transferase (GST) fusion proteins (full length or SHBG-LG4 comprising residues 1–205) were produced in *Escherichia coli* as described previously (36). For androgen signaling assays, recombinant SHBG-LG4 was liberated from GST by overnight cleavage with thrombin and purified

using a two-step chromatography method according to Grishkovskaya *et al.* (37).

GST pull-down interaction analyses

Full-length SHBG-GST (20 μg) and an equimolar quantity of GST were each immobilized on glutathione Sepharose resin (100 μl) for 1 h at 4 C (protein loading for GSH beads was normalized using Bradford assay). Steroid-bound SHBG-GST was prepared by preincubation with DHT (100 nM). SHBG-GST interaction analyses were performed according to Ng *et al.* (15). Briefly, pro-KLK4 or pro-KLK14 (1.25 μg) was incubated with SHBG-GST or GST resin overnight at 4 C in binding buffer (100 μl) containing 20 mM Tris-HCl (pH 8.0), 2.5 mM $CaCl_2$, 0.02% Nonidet P-40, and 0.2 mg/ml BSA. Sepharose-bound complexes were sedimented by centrifugation, and resin was washed three times with binding buffer (without BSA). Interacting protein was extracted by boiling in reducing SDS-PAGE loading buffer, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by Western blot analysis against the V5 epitope on both pro-KLK4 and pro-KLK14.

Activation of pro-KLK

Pro-KLK4, pro-KLK7, and pro-KLK14 were activated with thermolysin, which was inhibited by 40 μM phosphoramidon immediately after activation. Active KLK4 for [^{3}H]-DHT binding assays was purified from thermolysin by anion exchange chromatography using a Pharmacia Resource Q anion exchange column (Amersham Biosciences, Piscataway, NJ).

Proteolysis of SHBG by KLK3, KLK4, KLK7, and KLK14

Active KLKs with trypsin-like specificity (KLK4, 7 nM; KLK14, 10 nM) or chymotrypsin-like specificity (KLK3, 130 nM; KLK7, 120 nM) was incubated with SHBG (350 nM) for 2 h at 37 C in buffer (10 μl) containing 0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1.5 mM EDTA, and 0.02% Tween 20. Reactions were quenched by boiling in reducing SDS-PAGE loading buffer, after which fragments were separated by SDS-PAGE and examined by Western blot analysis for SHBG. To examine kinetics of SHBG proteolysis by KLK4 and KLK14, six concentrations of human SHBG (100–2000 nM) were incubated with a constant concentration of either KLK4 (7 nM) or KLK14 (10 nM) at 37 C for 2 h (KLK14) or 4 h (KLK4). Samples were taken at intervals (15 min for KLK14, 30 min for KLK4), diluted in reducing loading buffer, and heated at 95 C to quench protease activity. Triplicate blots for each SHBG concentration were subjected to densitometric analysis using a LI-COR Odyssey infrared imaging system for undigested SHBG (48 kDa). Data were compiled using GraphPad Prism 5.01 (GraphPad, San Diego, CA) and K_M (Michaelis constant), k_{cat} (catalytic rate constant), and k_{cat}/K_M values determined by nonlinear regression.

N-terminal sequencing of SHBG proteolysis fragments

Human SHBG (1 μg) was incubated with KLK4 or KLK14 for 45 or 120 min at 37 C as described above. Samples from each KLK digestion were pooled, lyophilized, and resuspended in reducing SDS-PAGE loading buffer. Proteolysis fragments were resolved by SDS-PAGE, transferred to a polyvinyl difluoride membrane in N-cyclohexyl-3-aminopropanesulfonic acid buffer, and stained with Coomassie R-250. After membranes had been destained, proteolysis fragments were excised and analyzed by Edman degradation N-terminal sequencing carried out at the Australian Proteomic Analysis Facility (Sydney, Australia). To assess whether identified cleavage sites were high-affinity substrates for KLK4 or KLK14, putative P4-P1 residues (FNLR and TSLR) were synthesized as peptide-*para*nitroanilides (*p*NA) according to previously published methods (35, 38) and incubated with each protease according to Swedberg *et al.* (35), with kinetic parameters (K_M, k_{cat}, and k_{cat}/K_M) determined by nonlinear regression as described above.

Proteolysis of SHBG-DHT by KLK4 and KLK14

Human SHBG (200 nM) was incubated with KLK4 or KLK14 for 2 h at 37 C (as above) in presence or absence of DHT (1 μM) or an equivalent volume of ethanol (steroid vehicle control). Proteolysis fragments were examined by Western blot analysis for SHBG. To confirm that effects proceeded through SHBG-steroid interaction, control incubations were carried out with mouse recombinant amelogenin (M179) produced in *E. coli* (39). Purified amelogenin (4 μM) ± DHT (1 μM) or ethanol was incubated with KLK4 and KLK14 for 1 h at 37 C. Samples were separated by SDS-PAGE, stained with Coomassie R-250, and visualized using a LI-COR Odyssey infrared imaging system.

[^{3}H]-DHT binding assays and Scatchard analysis

Human SHBG was cleaved by incubation with purified KLK4 as described above except that reactions proceeded for 16 h to maximize production of cleaved SHBG. Samples were examined by anti-SHBG Western blot analysis after separation by native and reducing SDS-PAGE to verify proteolysis of SHBG and generation of distinct fragments. Steroid-binding capacity of full-length human SHBG, KLK4-cleaved human SHBG, and recombinant SHBG-LG4 was examined by ligand-saturation analysis (40). SHBG species were incubated with [^{3}H]-DHT (133.4 Ci/mmol; GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada) followed by separation of bound and free steroid with dextran-coated charcoal. Equilibrium dissociation constants (K_d) were determined by Scatchard analysis (41) on measured levels of SHBG-bound and free [^{3}H]-DHT.

Androgen signaling assays

LNCaP cells were grown at 37 C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA), penicillin (100 U/ml), and streptomycin (100 μg/ml). LNCaP cells were seeded into six-well tissue culture plates 1 d before treatment in 2 ml phenol red-free RPMI 1640 medium containing 2% dextran charcoal-treated fetal bovine serum. Cells were treated with DHT (5 nM) in the absence or presence of SHBG (5 nM) or SHBG-LG4 (5 nM) for 24 h; 6 nM SHBG-LG4 was used in the alcohol dehydrogenase 7 (ADH7) analysis. mRNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, followed by RT performed at 42 C for 50 min using 3 μg of total RNA and 200 U of Superscript II. Quantitative (q)RT-PCR for PSA, ADH7, and 18S mRNA (internal control) was carried out in 25 μl containing 12.5 μl of 2× SYBR Green PCR master mix, 1 μl of each forward (PSA, 5′-CAACCCTGGACCTCACACCTA; ADH7, 5′-AGTTGCCCCACC

AAAGACTAA; and 18S, 5′-CGCCGCTAGAGGTGAAATT CT) and reverse primer (PSA, 5′-GGAAATGACCAGGCCAA-GAC; ADH7, 5′-AACTTGGACACCATTGTTCCTT; and 18S, 5′-CGAACCTCCGACTTTCGTTCT), 2.5 μl of RT product (diluted 1:5) and 8 μl of distilled H_2O. Amplification was performed using an ABI Prism 7000 Sequence 10 detection system (Life Technologies) equipped with a 96-well optical reaction plate. Negative controls, containing H_2O instead of sample cDNA, were included in each plate. All experiments were run in triplicate, and mRNA values were calculated based on cycle threshold and monitored for an amplification curve.

Androgen bioavailability assays

LNCaP monolayers established in 24-well plates were deprived of androgens by culture in serum-free media for 72 h. Before treatment, SHBG (final concentration 35 nM) was incubated with DHT (3 nM) for 60 min at 4 C as described in Ref. 42. Cells were treated with fresh serum-free media containing either DHT (3 nM), steroid-bound SHBG (35 nM), untreated SHBG (35 nM), or steroid vehicle control. Samples of media were taken at 0, 8, 16, and 24 h to monitor free androgen by DHT ELISA (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions.

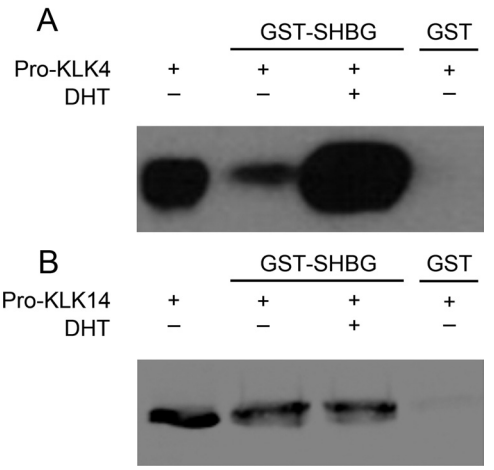

FIG. 1. GST interaction analyses between SHBG and pro-KLKs. Pro-KLK4 (A) or pro-KLK14 (B) interacting with GST and SHBG-GST fusion protein. Interacting protein was analyzed by Western blot analysis against the V5 epitope on recombinant pro-KLK4 and pro-KLK14 after overnight binding to immobilized GST or SHBG-GST. Additionally, interaction with SHBG-GST preloaded with DHT was examined to determine the effect of steroid binding on the SHBG-KLK interaction. GST alone controls confirmed that the binding interaction was specific for SHBG. Western blots are representative of three independent experiments.

Results

Pro-KLK4 and pro-KLK14 interact with SHBG

GST pull-down assays were used to assess interaction between SHBG and pro-KLKs. Zymogen KLKs were produced recombinantly with inclusion of a V5 epitope tag allowing postinteraction analysis by Western blotting visualized with V5-specific antibody conjugates. Neither pro-KLK4 nor pro-KLK14 interacted with immobilized GST, whereas both zymogens interacted with the SHBG-GST fusion protein as shown in Fig. 1. These data indicate that both pro-KLK4 and pro-KLK14 bind to SHBG. To examine effects of androgen on SHBG binding, incubations were performed in the presence of DHT. Interestingly, pro-KLK4, but not pro-KLK14, binding to SHBG was markedly accentuated by the presence of androgen (Fig. 1). Nonspecific interaction with GST-only appeared not to be affected by DHT treatment (data not shown). Differential effects of DHT on pro-KLK/SHBG binding suggest that despite a high degree of sequence and structural similarity, pro-KLK4 and pro-KLK14 interactions with SHBG potentially involve enzyme-specific SHBG contacts.

SHBG is a substrate for KLK4 and KLK14 but not KLK3 or KLK7

Having shown that zymogen KLK4 and KLK14 bind to SHBG, we were interested in whether active forms of these trypsin-like serine proteases cleave SHBG. Additionally, we assessed the SHBG cleaving ability of two other members of the KLK family, KLK3 and KLK7, which have chymotrypsin-like substrate specificity. Low concentrations of active KLK4 (7 nM) and KLK14 (10 nM) and greater than 10-fold higher concentrations of KLK3 (130 nM) and KLK7 (120 nM) were incubated with human SHBG and analyzed by Western blot analysis using a rabbit anti-SHBG polyclonal antibody. This showed that although KLK4 and KLK14 cleave SHBG (Fig. 2), this protein is not efficiently cleaved by KLK3 and KLK7, which show only low level proteolysis of SHBG after a 2-h incubation (data not shown). Given that all recombinant KLKs were proteolytically activated with thermolysin, thermolysin/SHBG control digests were performed, and no cleavage was observed (see Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In addition, distinct proteolysis fragments generated by KLK4 and KLK14 indicated that each protease has individual SHBG cleavage preferences. KLK4 proteolysis generated a predominant fragment of 25 kDa in addition to a second band of slightly higher molecular weight (Fig. 2A), whereas KLK14 produced fragments of 30, 25, and 20 kDa (Fig. 2B).

Kinetics of SHBG proteolysis by KLK4 and KLK14 were examined by Michaelis-Menten plots of data from anti-SHBG Western blot analyses from three independent experiments. Densitometry analysis was performed on uncleaved SHBG (48 kDa) with the reduction in signal intensity over time used as a measure of proteolytic activity. Plots of degradation against time followed pseudo first

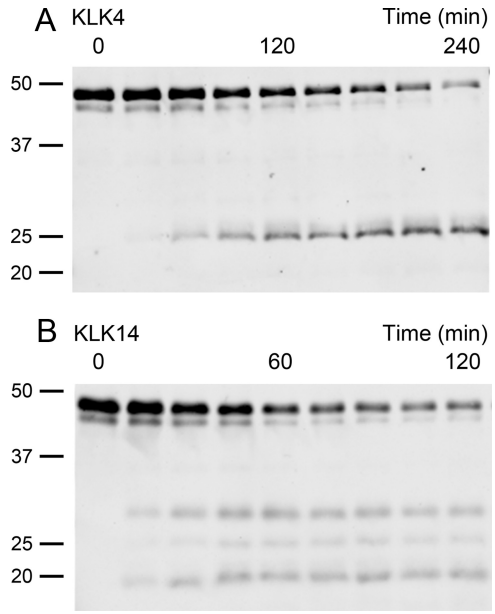

FIG. 2. SHBG is cleaved by KLK4 and KLK14. Purified human SHBG digested with active KLK4 (A) or KLK14 (B). Varying concentrations of SHBG were digested with a constant concentration of protease to calculate semiquantitative kinetic constants (1000 nM SHBG digestion is shown). Protease activity was quenched by heating to 95 C in reducing SDS-PAGE loading buffer, after which SHBG digestion products were separated by SDS-PAGE and analyzed by Western blotting. Blots were visualized using the LI-COR Odyssey infrared imaging system and are representative of three independent experiments.

order kinetics providing kinetic constants for proteolysis of SHBG (Table 1), which yielded k_{cat}/K_M values of 1.6×10^4 M^{-1} sec^{-1} and 3.8×10^4 M^{-1} sec^{-1} for KLK4 and KLK14, respectively.

KLK4 and KLK14 cleave SHBG between the LG4 and LG5 domains with unique specificities

Fragments liberated by KLK4 and KLK14 cleavage of SHBG (Fig. 3A) were analyzed by Edman degradation N-terminal sequencing of material electroblotted onto PVDF immobilon to determine cut sites for each protease. Sequence data (Table 2) identified two predominant cut sites: one generated immediately after Arg186 (fragment 1) and one commencing after Arg209 (fragment 2). Fragment 3 corresponded to the N terminus of full-length SHBG (undefined C terminus) as determined by Edman degradation of material from KLK14 digests. The comigrating band from KLK4-digested material did not provide N-terminal sequence data. However, it is logical to assume that this KLK4-derived fragment also constitutes the N-terminal domain on the basis that it binds steroid (see below), and this activity is N-terminal domain dependent (11, 36). Both cut sites were located on the region between the two LG domains of SHBG, illustrated in Fig. 3B. To confirm that identified cut sites were high-affinity substrates for KLK4 or KLK14, the four nonprime residues (P4–P1) of each cut site were synthesized as peptide-*p*NA substrates. Kinetic constants were determined for cleavage of these substrates corresponding to cut site preference of each protease (KLK4/FNLR-*p*NA and KLK14/TSLR-*p*NA). Calculated k_{cat}/K_M values (Table 1) verified cleavage with high efficiency.

Generating sufficient amounts of each KLK14 proteolysis fragment for sequencing proved difficult, because the products after the initial cleavage events were rapidly digested further. Consistent with this, abundance of KLK14 proteolysis fragments did not noticeably change in previous time-course digestions despite continuing proteolysis of full-length SHBG (Fig. 2B). In contrast, fragments produced by KLK4 seemed to accumulate over the duration of the assay (Fig. 2A). This suggested that not only did KLK4 and KLK14 cleave SHBG at distinct sites, but additionally, the fate of proteolysis fragments generated by each enzyme was different. To examine this further, longer duration SHBG digestions were carried out with KLK4 and KLK14. Fragments were detected by silver stain to overcome any bias in epitope recognition when visualizing proteolysis products by Western blot analysis. For KLK4 digestions, two bands of similar size were apparent and persisted even after 16 h of digestion (Fig. 3C), suggesting that cleavage was limited to a single cut site and products were resistant to further degradation. This doublet becomes more apparent when higher protein loadings are visualized by Western blot analysis (see Supplemental Fig. 2). In contrast, prolonged incubations with KLK14 resulted in loss of all detectable fragments, indicating that proteolysis of SHBG by this enzyme was a degradative processing event.

DHT alters KLK4 and KLK14 proteolysis of SHBG

Given SHBG's high affinity for sex steroids, we considered the possibility that DHT binding might influence

TABLE 1. Kinetic constants for SHBG and peptide-*p*NA KLK digestions

Protease/substrate	K_M	k_{cat}	k_{cat}/K_M
KLK4/SHBG	1.2±0.7μM	0.02±0.01sec^{-1}	1.6±0.5×$10^4$$M^{-1}$$sec^{-1}$
KLK4/FNLR-*p*NA	111.6±6.6μM	5.07±0.09sec^{-1}	4.6±0.7×$10^4$$M^{-1}$$sec^{-1}$
KLK14/SHBG	2.1±0.6μM	0.08±0.01sec^{-1}	3.8±0.5×$10^4$$M^{-1}$$sec^{-1}$
KLK14/TSLR-*p*NA	181.6±9.8μM	18.80±0.42sec^{-1}	10.4±1.1×$10^4$$M^{-1}$$sec^{-1}$

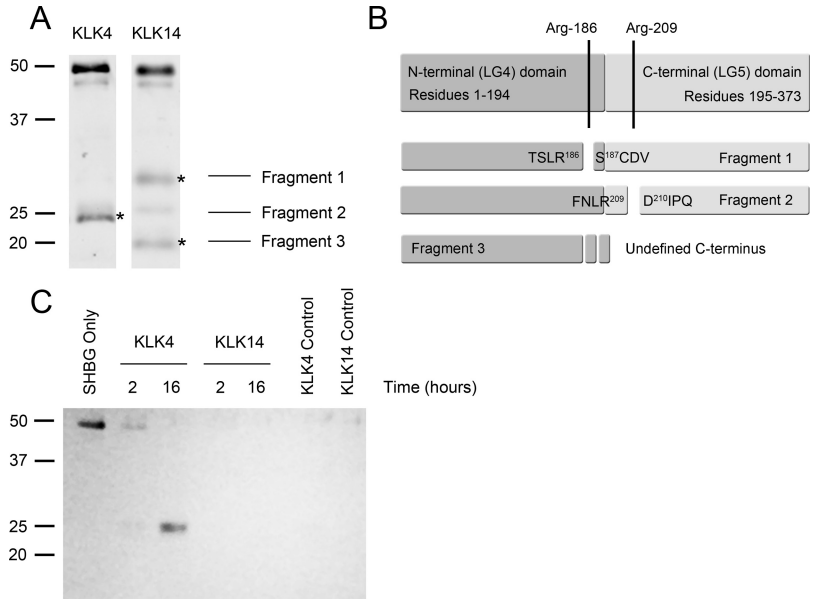

FIG. 3. N-terminal sequencing of SHBG proteolysis fragments reveals distinct cut sites for KLK4 and KLK14. A, SHBG proteolysis fragments generated by KLK4 and KLK14 visualized by Western blot analysis from Fig. 2 (120 min, KLK4; 60 min, KLK14). Fragments excised for Edman degradation N-terminal sequencing are indicated by an *asterisk*, and sequence data returned for fragments 1–3 are shown in Table 2. B, Schematic representation of human SHBG showing the N-terminal (LG4) and C-terminal (LG5) LG domains with fragments generated by proteolysis shown below. Sequence analysis indicated that the cleavage sites occurred between the two LG domains. C, Comparison of the specificity of SHBG cleavage by KLK4 and KLK14. SHBG (50 ng) was digested with 7 nM KLK4 or 10 nM KLK14 for 2 or 16 h at 37 C. Fragments were resolved on 4–12% gradient gels and detected by silver stain. Incubation with KLK4 resulted in a single cleavage event with generated fragments resistant to further digestion, whereas no detectable fragments were visible after 2 h of digestion with KLK14. KLK4 and KLK14 control lanes contain proteases only.

KLK-mediated proteolysis. Accordingly, SHBG proteolysis assays were carried out with and without exogenously added androgen. DHT-bound SHBG showed noticeably reduced rates of proteolysis by KLK4 (Fig. 4B) and KLK14 (Fig. 4C), suggesting that steroid binding exerts a stabilizing influence on SHBG. Further, ligand-bound SHBG showed a shift in cut site preference when digested by KLK14 being cleaved predominantly at Arg209. In control digestions with amelogenin, a substrate common to KLK4 and KLK14, DHT did not influence proteolysis by either enzyme (Fig. 4, E and F), confirming that altered proteolysis of SHBG was driven by steroid binding to SHBG rather than a KLK-DHT interaction.

SHBG-LG4 domain proteolysis fragment retains identical androgen binding affinity

Previous studies have indicated that the LG4 (N-terminal) domain is responsible for the majority of biological functions of SHBG, including steroid binding (11). Because proteolysis of DHT-bound SHBG appeared to liberate an intact LG4 domain from the LG5 domain, we assessed its androgen binding affinity to determine whether it was functional. Initially, digestion fragments were analyzed under native conditions to confirm that proteolysis resulted in separation of LG4 and LG5 domains to discount the presence of any substantial noncovalent interdomain interactions. Western blot analysis verified production of distinct fragments as well as a lack of detectable full-length SHBG (Fig. 5A). Steroid binding capacities of full-length and cleaved human SHBG were measured by saturation binding assays, from which binding affinities were determined by Scatchard plot analysis. Remarkably, despite proteolytic separation from the LG5 domain, the [^{3}H]-DHT binding curve for cleaved SHBG overlayed precisely with full-length SHBG. Consistent with this, calculated K_d from Scatchard analysis (Fig. 5C) were identical (K_d SHBG-DHT = 1.92 nM *vs.* K_d SHBG/KLK4-DHT = 1.92 nM). Collectively, these findings indicate that the ability of the SHBG cleaved by KLK4 to interact with steroid is undiminished.

Native SHBG and SHBG-LG4 both enhance DHT-mediated stimulation of the AR

SHBG's high affinity for DHT and testosterone will influence local androgen bioavailability and, thus, access of signaling molecules to intracellular receptors (43). Observation of equivalent binding affinities for native SHBG

TABLE 2. Amino acid sequence from Edman degradation N-terminal sequencing of fragments generated by KLK4 and KLK14 proteolysis of SHBG

Proteolytic incubation	Fragment	Sequence	Cleavage site residues P4 P3 P2 P1/P1′ P2′ P3′ P4′
KLK14-SHBG	1	^{187}SCDVES	^{183}T S L R/S C D V
KLK4-SHBG	2	^{210}DIPQPH	^{206}F N L R/D I P Q
KLK14-SHBG	3	^{1}LRPVLP	N-terminus of mature SHBG

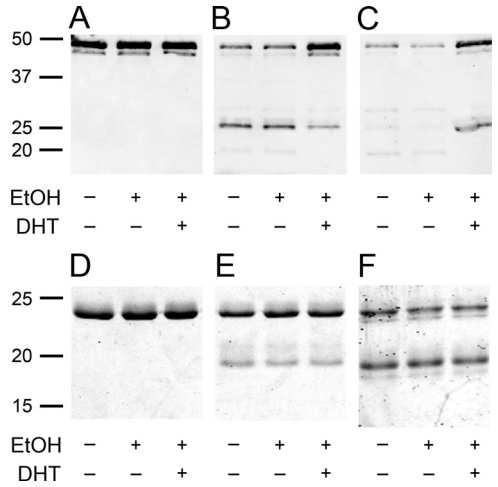

FIG. 4. DHT modulates KLK proteolysis of SHBG. A–C, Western blot analysis for buffer only, ethanol (EtOH) vehicle, or DHT-treated SHBG, which was not digested (A), digested with KLK4 (B), or digested with KLK14 (C). D–F, Coomassie R-250-stained SDS-PAGE analysis for buffer only, ethanol vehicle, or DHT-treated recombinant mouse amelogenin, which was not digested (D), digested with KLK4 (E), or digested with KLK14 (F). Gels were visualized using the LI-COR Odyssey infrared imaging system. All figures are representative of three independent experiments.

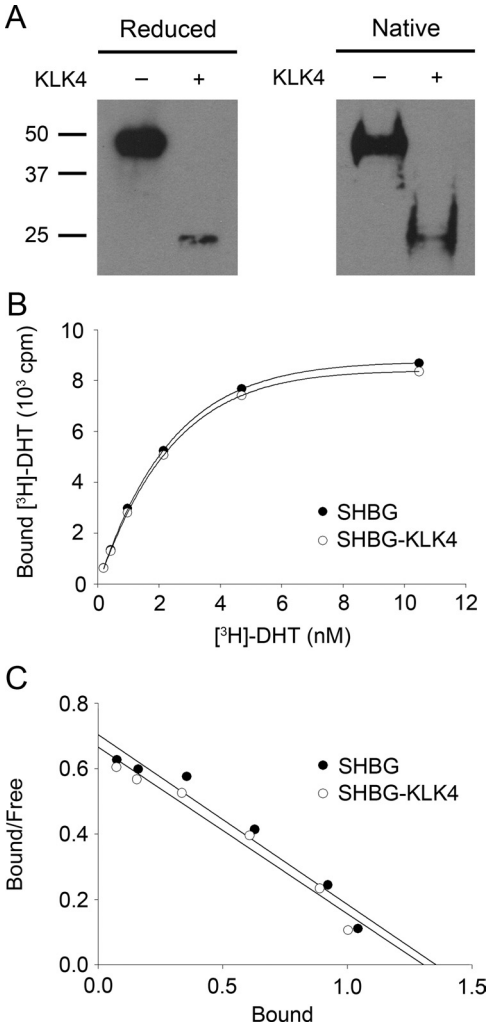

FIG. 5. High steroid-binding affinity of SHBG is maintained after KLK4 proteolysis. A, Western blot analysis of intact and KLK4-cleaved SHBG (16 h at 37 C) after reducing (*left*) and native (*right*) PAGE confirming proteolysis of SHBG produces separate fragments. B, Saturation curve for binding of various concentrations of [^{3}H] DHT to a constant concentration of full-length SHBG (*closed circles*) or KLK4-cleaved SHBG (*open circles*), the vertical axis shows [3H]-DHT binding as indicated by counts pre minute (cpm). C, Scatchard analysis transformed from saturation curve data indicating that the steroid-binding affinities of native SHBG (K_d = 1.92 nM) and KLK4-cleaved SHBG (K_d = 1.92 nM) are identical. A representative plot is shown from three independent experiments.

and the SHBG-LG4 domain prompted us to assess their impact on androgen-driven transcription. Transcriptional activity of the AR was measured by expression of PSA and ADH7 in qRT-PCR assays after DHT stimulation. Steroid-binding capacities of SHBG and recombinantly expressed SHBG-LG4 were first determined in cell culture media to confirm equivalence with previous experiments. Scatchard analysis (Fig. 6, A and B) revealed similar binding affinity for SHBG-LG4 and SHBG (K_d = 1.23 and 0.97 nM, respectively). These values are lower than those of the previous assays reflecting the change in assay conditions from simple buffer to cell culture medium. The small difference in affinity of native SHBG and SHBG-LG4 was consistent in three separate experiments and reflects the inherent difference in affinity between full-length native SHBG and the recombinant SHBG-LG4 domain expressed in *E. coli*, as observed previously (36).

The ability of each SHBG species to influence DHT-stimulated androgen signaling was examined in cultures of the androgen-responsive cell line, LNCaP (Fig. 6, C and D). Addition of 5 nM DHT elevated PSA and ADH7 expression compared with vehicle-treated cells ($P < 0.05$). However, this effect was significantly increased by delivering steroid in complex with SHBG compared with steroid alone ($P < 0.05$), irrespective of whether DHT was bound to native SHBG or recombinant SHBG-LG4. This suggested that binding DHT to SHBG enabled more steroid to stimulate the AR, potentially by provision of a reservoir of steroid that could be gradually released over time while simultaneously protecting it from breakdown in the extracellular environment. Further, this demonstrated that the SHBG-LG4 domain was sufficient to achieve this DHT-dependent effect. To ascertain whether steroid-free SHBG alone could stimulate PSA production, we treated LNCaP cells with SHBG protein but could not detect increased PSA expression (see Supplemental Fig. 3).

To confirm that binding of steroid to SHBG resulted in prolonged androgen presence *in vitro*, the residual concentration of bioavailable DHT was quantified by DHT ELISA (Fig. 6E) in LNCaP culture media over 24 h. In treatments of DHT without SHBG, bioavailable DHT was abundant at 0 h but was rapidly depleted within 8 h. In

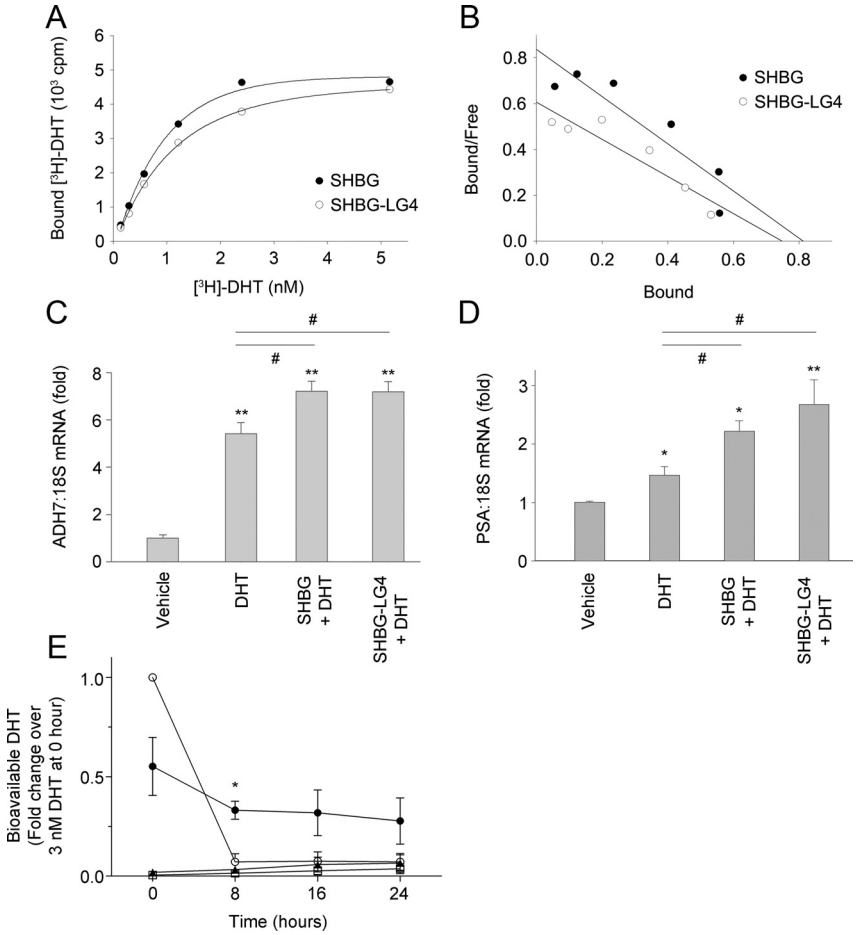

FIG. 6. Native SHBG and recombinant SHBG-LG4 both increase androgen action in LNCaP cells by sustaining androgen bioavailability in cell culture. A, Saturation binding curve for intact SHBG (*closed circles*) and SHBG-LG4 domain (*open circles*) in culture medium, using [^{3}H]-DHT as a labeled ligand, the vertical axis shows [^{3}H]-DHT binding as indicated by counts per minute (cpm). B, Scatchard plot showing the steroid-binding affinities of SHBG (K_d = 0.97 nM) and SHBG-LG4 (K_d = 1.23 nM) in culture medium are similar. Analyses were performed on samples taken from triplicate experiments, which all showed the same results, and a representative plot is shown. C, Human ADH7 mRNA was measured by qRT-PCR in LNCaP cells grown in phenol red-free RPMI 1640 medium supplemented with 2% charcoal-treated fetal bovine serum, after treatments with DHT (3 nM) in the absence or presence of SHBG (3 nM) or SHBG-LG4 (6 nM) for 24 h. 18S mRNA was also measured as an internal control. Values represent mean ± SD of three separate experiments. Significant differences are **, $P < 0.01$; *, $P < 0.05$ comparing DHT, SHBG plus DHT, and SHBG-LG4 plus DHT-treated cells to vehicle-treated cells. #, $P < 0.05$ for SHBG plus DHT and SHBG-LG4 plus DHT *vs.* DHT-treated cells. D, Human PSA mRNA was measured by qRT-PCR in LNCaP cells as described for the previous panel except concentrations used were SHBG (5 nM), SHBG-LG4 (5 nM), and DHT (5 nM). E, DHT ELISA for bioavailable androgen in cell culture media over 24 h. Serum-starved LNCaP cells were treated with serum-free media containing 3 nM DHT (*open circles*), 35 nM untreated SHBG (*triangles*), or 35 nM SHBG preloaded with 3 nM DHT (*closed circles*). Data were expressed as a fold-change over DHT measured at 0 h in 3 nM free DHT treatments as mean ± SEM of three independent experiments. *, $P < 0.05$ for SHBG plus DHT *vs.* DHT only.

contrast, steroid delivered in complex with SHBG, free DHT was lower at 0 h, because a proportion of steroid was bound to the carrier protein. However, it was more efficiently retained with detectable levels present at every time interval, and only a gradual decline was observed by 24 h. No steroid was detected in media containing untreated SHBG, confirming a lack of contaminating androgen. This reinforced that binding to SHBG greatly increased retention of DHT in the *in vitro* extracellular milieu.

Discussion

Prostate physiology is dominated by androgen signaling, which dictates prostatic development and is the prime point of therapeutic intervention in prostate cancer (44). Delivery of androgens to target tissues, enhancement of their stability, and regulation of their bioavailability are primary functions of the steroid-binding protein, SHBG (45). Therefore, SHBG determines the ability of steroids to effect androgen-responsive gene expression with potential significance to tumorigenesis within the prostate. To date, knowledge of SHBG binding proteins is fairly limited. Further, interaction validation and characterization are confined to a sparse subset of targets, such as uterine stroma matrix-associated proteins fibulin-1D and fibulin-2 (15) and the nonspecific cell-surface receptor megalin (16). Moreover, proteolytic modulation of SHBG steroid carriage and delivery has not been investigated, despite identification of this phenomenon in other steroid binding proteins, such as corticosteroid binding globulin (23, 24). Hence, this study is the first to establish that SHBG is a target for proteolysis.

That a protease is able to cleave a given protein is not in itself a remarkable finding. The potential significance lies in the efficiency of proteolysis and the resulting biological effects. Kinetic analysis of *p*NA substrate hydrolysis by both KLK4 and KLK14 indicated that sequences identified by N-terminal sequencing were cleaved with high efficiency. Recent studies on KLK4 found a second-order rate constant of 2.31×10^4 M^{-1} sec^{-1} for its most preferred tetrapeptide-*p*NA substrate (35), which is very similar to that measured for FNLR-*p*NA in this study (4.61×10^4 M^{-1} sec^{-1}). Efficiency for cleavage of TSLR-*p*NA by KLK14 (1.04×10^5 M^{-1} sec^{-1}) is more distant from its

most preferred peptide substrate identified to date (3.81×10^6 M^{-1} sec^{-1}) (46). Proteolysis of SHBG yielded second-order rate constants of 1.67×10^4 M^{-1} sec^{-1} with KLK4 and 3.81×10^4 M^{-1} sec^{-1} with KLK14. These values are comparable with those seen for a variety of proteases for turnover of *in vivo* substrates, such as processing of von Willebrand factor by ADAMTS-13 (3.4×10^4 M^{-1} sec^{-1}) (47), Gag-Pol polyprotein by HIV protease (2.9×10^4 M^{-1} sec^{-1}) (48), or hydrolysis of mucin by StcE (2.61×10^4 M^{-1} sec^{-1}) (49). Comparison of catalytic efficiency of KLK4 and KLK14 to these enzymes suggests that SHBG may be an *in vivo* substrate.

Also striking is the nature of the substrate and effecting protease(s). SHBG is the principal specific carrier and distributor of androgens and estrogens, whereas expression patterns for KLK proteases are renowned for being highly influenced by sex steroids (32). This suggested KLK proteolysis of SHBG may be analogous to the previously identified proteolytic regulation of corticosteroid binding protein (23, 24). Ligand-free SHBG was cleaved by KLK4 neatly into its two separate domains, which were highly resistant to further proteolysis even after prolonged incubation. This is reminiscent of proteolytic processing of the structurally related laminin 5 by bone morphogenetic protein 1 (50), where the laminin G domain (a motif shared by SHBG) is precisely cleaved to produce proteolytically resistant fragments. KLK14 activity on the other hand seems to be far more promiscuous. Although we were able to gather N-terminal sequencing data on the larger fragments produced by KLK14, these products were very rapidly degraded to residual peptides, abrogating SHBG's biological activity.

Proteolysis of unoccupied SHBG contrasted with androgen-bound SHBG, which was cleaved by both KLKs at noticeably reduced rates and only at sites within the LG5 domain, thus leaving the steroid-binding LG4 domain intact. Previous structural analyses of SHBG in complex with DHT (10) and estradiol (51) have identified several LG4 domain conformational shifts that are dependent on bound steroid, including across a region homologous to a macromolecular interaction domain in structurally related proteins (52). Such an effect may explain both the distinct change in KLK14 cleavage site preference observed upon DHT binding from predominantly Arg186 (within the LG4 domain) to exclusively Arg209 (LG5 domain) and the markedly higher binding of pro-KLK4 to SHBG-GST in the presence of androgen. Ligand-dependent changes in conformation may be common in mechanisms regulating SHBG function. Indeed, steroid-specific effects are evident in several of SHBG's previously characterized binding interactions. The interaction between SHBG and members of the fibulin family of extracellular matrix-associated proteins occurs more efficiently in the presence of estradiol than in the presence of DHT (15), whereas SHBG cannot bind to its putative cell-surface receptor when bound with steroid (21).

However, unlike corticosteroid binding globulin, where proteolysis results in release of the bound steroid (23, 24), subsequent characterization of SHBG-LG4 revealed identical steroid binding affinity compared with native SHBG (Fig. 5). Because cleavage of SHBG at Arg209 would leave the LG4 domain intact, this is in accordance with existing studies, which have suggested that the LG4 domain of SHBG is solely responsible for steroid binding (10, 11). Additionally, the LG4 domain alone is capable of dimerization (11, 12) and interaction with R_{SHBG} (14), indicating that the SHBG proteolysis fragment identified in this study is functional. Consistent with indistinguishable steroid-binding properties, removal of the C-terminal LG5 domain did not alter SHBG's influence on the androgen signaling axis in cell culture. Androgen-responsive PSA and ADH7 expression was significantly increased by delivering DHT in complex with either full-length SHBG or SHBG-LG4, indicating a gradual release of DHT by SHBG or SHBG-LG4 as opposed to rapid internalization and metabolism as described previously (42).

Lack of knowledge of the structure and function of the LG5 domain hampers understanding the full significance of SHBG proteolysis. Unaltered steroid binding and bioavailability findings from this study indicate that SHBG proteolysis by KLK4 is not aimed at changing the interaction between SHBG and its bound steroid. Rather, a yeast two-hybrid screen using the SHBG-LG5 domain identified several hits within a prostate cDNA library, suggesting that the primary function of the LG5 domain may be to mediate protein-protein interactions (53). The latter study also identified KLK4 as an LG5 domain interactor, confirmed here because KLK4 cleaves SHBG at Arg209. Resolving the function of the LG5 domain will be pivotal to understanding the potential significance of SHBG proteolysis. In the meantime, the distinct endocrine regulation of the two KLKs found to cleave SHBG offers a promising lead. Although both KLK4 (28) and KLK14 (54) are moderately expressed in the prostate, their expression is not equally influenced by androgens: only KLK4 is highly upregulated after testosterone stimulation (55), not KLK14 (32). However, KLK14 is highly expressed under the influence of estrogens, including estradiol (56), and more abundantly expressed in the breast (54). Testosterone and estradiol are the two principle steroid ligands of SHBG (9), hence differential processing of SHBG by KLK4 and KLK14 might contribute to parallel mechanisms with significance to the androgen and estrogen signaling axes in

separate hormone sensitive tissues. Consistent with KLK4's association with prostate cancer, several reports have found KLK14 expression to be linked with prognosis of breast cancer patients (56, 57). This provocative finding warrants further investigation.

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