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SerpinB6 is an Inhibitor of Kallikrein-8 in Keratinocytes

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SerpinB6 (Proteinase inhibitor 6/PI-6) is an intracellular serpin produced by leukocytes, platelets, endothelial cells, keratinocytes and other epithelial cells. It is a potent cathepsin G inhibitor thought to protect monocytes, neutrophils and bystander cells from ectopic cathepsin G during inflammation. Here we show that serpinB6 also inhibits the human serine protease kallikrein-8 (hK8) and that in human and mouse skin, serpinB6 and kallikrein-8 co-localize in differentiated keratinocytes. SerpinB6 inhibits hK8 with an association rate constant (k_{ass}) of $1.8 \pm 0.2 \times 10^5\,M^{-1}s^{-1}$ compared to $3.4 \pm 0.2 \times 10^6\,M^{-1}s^{-1}$ for the interaction between the mouse orthologue of serpinB6 (SPI3/serpinb6a) and mouse kallikrein-8 (mK8). Molecular modelling suggested that the lower efficiency of the serpinB6/hK8 interaction is partly due to the bulkier P2 methionine residue of serpinB6 compared to the smaller P2 valine in SPI3. Taken together, these results suggest that serpinB6 is a physiologically relevant inhibitor of hK8 in skin. We postulate that serpinB6 protects the intracellular compartment of keratinocytes from ectopic hK8.

Key words: kallikrein, keratinocyte, neuropsin, serpinB6, SPI3.

Abbreviations: CatG, cathepsin G; HEK, human epidermal keratinocytes; hK8, human kallikrein-8; k_{ass}, association rate constant; mK8, mouse kallikrein-8; RCL, reactive centre loop; SI, stoichiometry of inhibition; SCCA, squamous cell carcinoma antigen.

Serpins are important endogenous regulators of proteolysis, as indicated by diseases such as emphysema and angioedema which are due to serpin dysfunction and involve tissue damage arising from uncontrolled proteolysis (*1*). An inhibitory serpin is a metastable protein that undergoes a marked conformational change when the P1–P1' bond in the exposed reactive centre loop (RCL) is cleaved by a cognate protease (*2*). This conformational change results in translocation of the protease to the opposite pole of the serpin, induces structural deformation of the protease, and drives the formation of an irreversible serpin–protease complex (*3*). The RCL P1 residue essentially determines the inhibitory specificity of each serpin, although flanking residues also contribute to the affinity of protease binding (*4*).

Keratinocytes produce a number of different serpins, including serpinA5 (protein C inhibitor), serpinE1 (plasminogen activator inhibitor-1/PAI-1), serpinB2 (plasminogen activator inhibitor-2/PAI-2), serpinB3 (squamous cell carcinoma antigen-1/SCCA1), serpinB4 (squamous cell carcinoma antigen-2/SCCA2), serpinB5 (maspin/PI-5), serpinB6 (protease inhibitor 6/PI-6) and serpinB13 (hurpin/headpin/PI-13) (*5–12*). At present the physiological functions and proteases regulated by these serpins in keratinocytes are unknown, but a role in inflammation, cell-death regulation or in barrier function against microbial or viral proteases is likely (*9*, *11*, *13*, *14*).

Although it is present in keratinocytes, the nucleocytoplasmic serpinB6 is also expressed in most epithelial cells, endothelial cells, platelets, mast cells, monocytes and neutrophils (*15–18*). *In vitro*, it has a very broad and unusual inhibitory profile and can utilize alternate P1 residues in inhibiting either trypsin- or chymotrypsin-like proteases such as thrombin, trypsin, factor Xa, plasmin, chymotrypsin and cathepsin G [CatG; (*19–22*)]. The wide distribution, properties and inhibitory profile of serpinB6 suggests that it regulates a number of different proteases *in vivo*, although only one has been identified. In neutrophils and monocytes the target for serpinB6 is CatG, and here the serpin probably acts to protect cells from CatG leaking from azurophilic granules (*22*).

We have shown previously that in differentiating human keratinocytes serpinB6 interacts with an endogenous serine protease that is not CatG (*10*). Here we show that this protease is kallikrein-8 (hk8/KLK8/neuropsin/PRSS19) a plasticity-related serine protease expressed in mouse brain, mast cells and skin (*23–25*). Kallikrein-8 and serpinB6 co-localize in human and mouse keratinocytes, and an SDS-stable complex between serpinB6 and hK8 can be immunoprecipitated from primary human epidermal keratinocytes. The kinetics and stoichiometry of hK8 inhibition by serpinB6 suggests that the interaction is physiologically relevant. Hence hK8 represents a second *in vivo* target of serpinB6, and is the first protease target identified for a keratinocyte serpin.

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MATERIALS AND METHODS

Cell Culture and Antibodies—Normal human epidermal keratinocytes (HEK) were isolated and cultured as previously described (*26*). Recombinant hK8, monoclonal rat anti-hK8 and polyclonal rabbit anti-hK8 antiserum were a kind gift from N. Yamaguchi (*27*). The rabbit anti-mK8 antiserum was a kind gift from K. Kato. The rabbit polyclonal anti-SPI3 antiserum was previously described (*28*). The monoclonal anti-serpinB6 antibody and polyclonal anti-serpinB6 antiserum have previously been described (*10*, *15*).

Immunohistochemistry—Normal human skin was obtained from volunteers during biopsy procedures for skin-graft preparation. Preparation of formalin-fixed paraffin-embedded tissue samples was as described (*29*). After dewaxing and rehydration, serial sections were blocked with 2% (w/v) BSA. All antibodies were diluted in PBS, 1% (w/v) BSA, 0.1% (v/v) Tween 20. Sections were incubated with either rabbit polyclonal or mouse monoclonal anti-serpinB6 antibodies, serpinB6 preimmune sera or rabbit anti-hK8 antisera, at room temperature for 18 h in a humidified chamber. After washing sections were incubated with biotinylated sheep anti-rabbit Ig or biotinylated sheep anti-mouse Ig (Silenus) for 1 h at room temperature. Following a further wash sections were incubated with streptavidin-HRP (Silenus) for 1 h at room temperature, washed again, developed with diaminobenzidine DAB (DAKO) and mounted with DPX mountant (BDH).

Indirect Immunofluorescence—Skin was obtained from the abdomen of a C57/BL6 mouse, snap frozen in liquid nitrogen and embedded in Tissue-Tek® OCT compound (Sakura) at –20°C. 7 μm sections were fixed in 4% (v/v) formaldehyde and incubated in 20 mM NH_4Cl for 30 min. All antibodies were diluted in PBS, 1% (w/v) BSA, 0.1% (v/v) Tween 20. Sections were incubated for 18 h with either rabbit anti-mouse kallikrein-8, rabbit anti-SPI3 or SPI3 preimmune serum. After washing, sections were incubated with FITC conjugated sheep anti-rabbit Ig (Silenus) and sections were analysed by confocal microscopy.

Immunoblotting—HEK cells were washed three times with PBS and lysed with 20 mM Tris-HCl, 0.15 M NaCl, 1% (v/v) Triton X-100, pH 7.6 containing 1 μg/ml leupeptin, 100 μM EDTA, 150 μg/ml PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin on ice. Lysates were cleared by centrifugation at 15 800*g* and ~500 μg of protein was boiled with an equal volume of Laemmli sample buffer containing 100 mM DTT (*30*). Samples were electrophoresed through a 12.5% SDS–PAGE slab gel and transferred to nitrocellulose. The membrane was incubated in blocking buffer [5% (w/v) skim milk powder in 10 mM Tris–HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.6] and cut into 0.5 cm strips. Strips were incubated with the indicated primary antibody, washed with 10 mM Tris–HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.6 and incubated with either HRP-conjugated sheep anti-rabbit, sheep anti-rat or sheep anti-mouse Ig (Silenus). Detection was performed with an enhanced chemiluminescence system (DuPont).

Immunoprecipitation of SerpinB6/hk8 Complexes from Human Keratinocytes—HEK cells were washed three times with PBS and lysed with 1 ml of ice cold 20 mM Tris–HCl, 0.15 M NaCl, 1% (v/v) Triton X-100, pH 7.6 containing 1 μg/ml leupeptin, 100 μM EDTA, 150 μg/ml PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin. The lysate was cleared by centrifugation at 15 800*g* and mixed with 1 ml of ice cold 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, 0.05% (v/v) Nonident P-40, pH 7.4 (NETGEL) containing the above-mentioned protease inhibitors. Samples were immunoprecipitated for 4 h at 4°C with 100 μl of 10% (w/v) protein A-Sepharose (Pharmacia Biotech Inc.) with or without monoclonal anti-serpinB6 antibody. Immunoprecipitates were washed twice with ice-cold NETGEL containing 250 mM NaCl and 0.025% (w/v) SDS and once with ice-cold 10 mM Tris–HCl, pH 8.0. Immune complexes were eluted by resuspending the pellet in Laemmli sample buffer on ice. Precipitated proteins were resolved by 12.5% SDS–PAGE under non-reducing conditions and transferred to nitrocellulose. The membrane was blocked, incubated with or without the indicated primary antibody and washed with 10 mM Tris–HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.6. The secondary antibody was HRP-conjugated sheep anti-rabbit Ig (Silenus) and detection was performed with an enhanced chemiluminescence system (DuPont).

Kinetic Analysis—SerpinB6 was prepared with the *Pichia pastoris* expression system (*21*). A constant amount of recombinant hK8 (0.2 nM) was mixed with different concentrations of recombinant serpinB6 and excess substrate (40 μM of Boc-Val-Pro-Arg-methylcoumaryl-7-amide; Sigma) in a final volume of 200 μl in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% PEG8000. The reactions were monitored at 37°C for 2 h with a Perkin–Elmer LS50B spectrofluorometer using an excitation wavelength of 370 nm and an emission wavelength of 450 nm, determining residual activity periodically. The interactions of serpinB6 with hK8 were determined with the progress curve method followed by slow binding inhibition kinetics (*31*):

$$Y = v_s t + \frac{(v_0 - v_s)^*\left(1 - e^{-kt}\right)}{k} \quad (1)$$

Y is the amount of product at time t, k is the apparent first order rate constant, and v_0 and v_s are the initial and steady-state velocities, respectively. The association rate constant (k_{ass}) was calculated using the following equation:

$$k_{ass} = k_{obs}\left(\frac{1 + [S]}{K_M}\right) \quad (2)$$

[S] is the concentration of substrate and K_M is the Michaelis constant. Stoichiometry of inhibition (SI) was determined essentially as described in Hopkins and Stone, except hK8 was fixed at 12 nM with 0, 2.5, 5, 10, 20 nM serpinB6, and residual enzyme activity was measured after the inhibitor and enzyme were incubated together for 4 h (*32*). Kinetic assays were performed in triplicate and the mean and SE are presented.

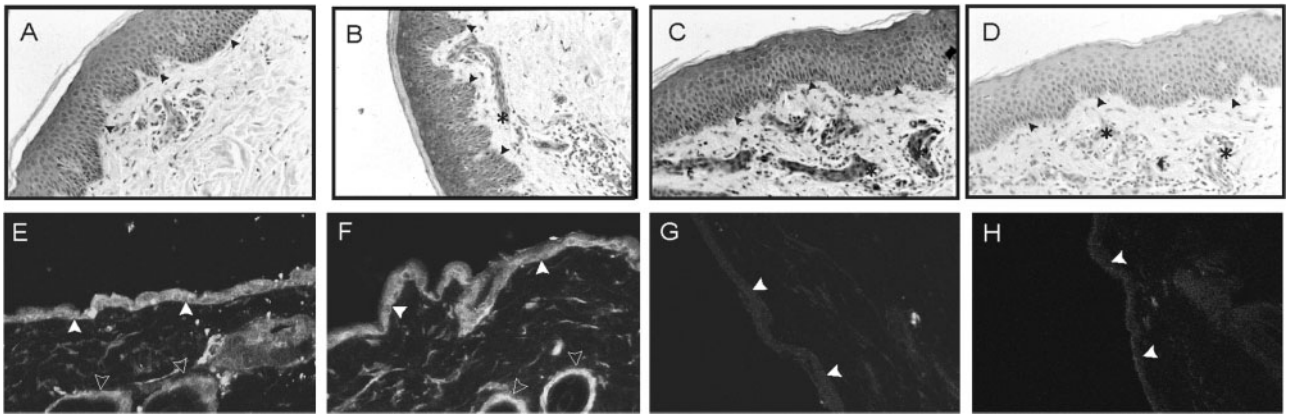

Fig. 1. **Distribution of kallikrein-8 and serpinB6 in human and mouse skin.** Serial sections of formalin fixed, paraffin-embedded human skin were dewaxed, blocked and immunoreacted with the indicated primary antibody. Detection was with a biotin–streptavidin–HRP system and sections were developed with DAB. (A) Monoclonal anti-serpinB6, (B) polyclonal rabbit anti-hK8, (C) polyclonal rabbit anti-serpinB6 or (D) serpinB6 preimmune sera. *Solid black arrowhead*—basal cell layer; *asterisk*—endothelial cells. Mouse skin was excised from the abdomen, snap frozen in liquid nitrogen and embedded in OCT compound. Frozen mouse skin sections were fixed in formalin and stained with either (E) rabbit anti-SPI3, (F) rabbit anti-mK8, (G) SPI3 preimmune sera or (H) no primary antibody. The secondary antibody was FITC-conjugated sheep anti-rabbit Ig and sections were analysed by confocal microscopy. *Solid white arrow head*—epidermis; *open white arrow head*—hair follicle.

Molecular Modelling—Human and mouse kallikrein-8 share 75% sequence identity. We used the X-ray crystal structure of mK8 [PDB identifier 1NPM; (*33*)] as a template to model hK8 using the program MODELLER (*34*). To build a peptide into the active site we superposed mK8 onto trypsin from the X-ray crystal structure of the Michaelis complex between trypsin and serpin1 from Manduca sexta (PDB identifier 1I99) using a program by Arthur Lesk (*35*). The two proteases superposed extremely well, with an rms deviation of 0.382 Å/Atom over 90% of Cα atoms. We removed the trypsin molecule and everything apart from the P4–P4′ of the RCL of serpin1 using the delete facility in Quanta (M.S.I. Inc.), leaving mK8 with the P4–P4′ region of serpin1 in the active site. Using the 'mutate' facility within Quanta we changed the peptide sequence IVPKSLIL (the P4–P4′ of serpin1) to the P4–P4′ region of the RCL of the mouse SPI3 (MTVRCMRF). We then subjected the model to CHARMm minimization, firstly with the mK8 molecule and the peptide backbone of the modelled peptide constrained, and later with no constraints. Minimization was performed to convergence. The model between hK8 and the same peptide derived from the RCL of serpinB6 (MMMRCARF) was built in a similar fashion. The stereochemistry of the model was checked and all residues found to be in allowed conformations. To investigate the P2 and P3 positions we used the 'mutate' facility in Quanta to change the P2 from Val to Met, or P3 from Thr to Met, then used the rotamer libraries available within Quanta in conjunction with CHARMm minimization to investigate possible conformations that the sidechains could adopt. Contacts were investigated using a program by Arthur Lesk (*35*).

RESULTS

Kallikrein-8 and SerpinB6 Co-Localize in Human and Mouse Keratinocytes—SerpinB6 is found in the cytoplasm of human epidermal keratinocytes in the stratum spinosum and granulosum, and is a potent inhibitor of trypsin-like serine proteases (*10*, *21*). We have previously shown that serpinB6 interacts with an endogenous protease in human keratinocytes. A potential candidate for this protease is hK8, a keratinocyte serine protease with trypsin-like specificity that cleaves substrates at Arg-X (*36*, *37*). Human kallikrein-8 mRNA has been demonstrated in cultured keratinocytes and terminal differentiated keratinocytes of the skin but the cellular distribution of hK8 protein in human epidermis has not been studied (*37*, *38*). Mouse kallikrein-8 protein is expressed in keratinizing stratified squamous epithelia of the skin (*39*) and may have a role in keratinocyte differentiation (*40*) and desquamation (*41*). It is found in the cytoplasm of keratinocytes in the stratum spinosum, but more differentiated keratinocytes of the stratum granulosum release mK8 into the intercellular space. It is proposed that kallikrein-8 is produced by cells of the stratum spinosum and stored for secretion as they migrate through the stratum granulosum towards the epithelial surface (*39*).

If serpinB6 is a physiologically relevant regulator of kallikrein-8, the two proteins should exhibit identical or overlapping distribution in skin. As shown in Fig. 1A and C, serpinB6 is present in the cytoplasm of human suprabasal keratinocytes in the stratum spinosum and stratum granulosum. Using a polyclonal anti-hK8 antibody, hK8 was also detected in suprabasal keratinocytes (Fig. 1B), showing a cytoplasmic distribution in keratinocytes in the stratum spinosum and granulosum.

Indirect immunofluorescence analysis of frozen sections revealed that SPI3 and mK8 are expressed in identical keratinocytes within mouse abdominal epidermis (Fig. 1E and F). Cytoplasmic staining was evident for both mK8 and SPI3 and appeared in all cell layers of the epidermis. Keratinocytes within the hair follicles were also positive for both mK8 and SPI3. In addition to

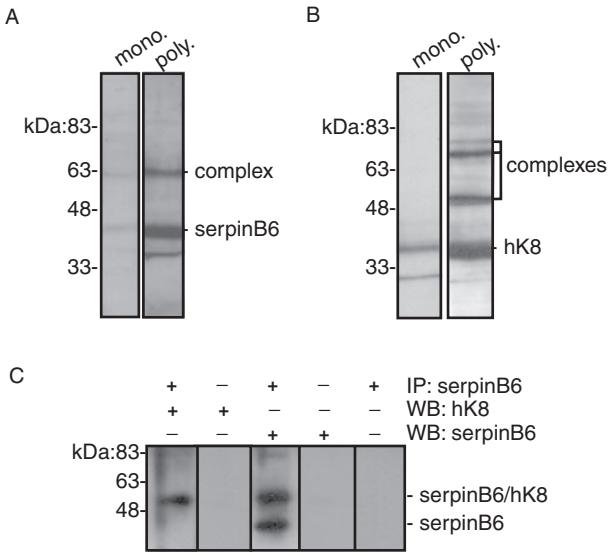

Fig. 2. **hK8, serpinB6 and serpinB6/hK8 complexes are present in differentiated human keratinocytes.** Differentiated HEK cells were lysed on ice with Tris–NaCl buffer containing 1% Triton X-100 and a cocktail of protease inhibitors. Equal amounts of extracts were separated via 12.5% SDS–PAGE and immunoblotted using monoclonal (mono.) or polyclonal (poly.) antibodies against (**A**) serpinB6 or (**B**) hK8. Alternatively (panel C) lysates were immunoprecipitated with or without monoclonal anti-serpinB6 antibody in the presence of protein A-Sepharose and an equal volume of NETGEL containing protease inhibitors. Immune complexes were separated by 12.5% SDS–PAGE under non-reducing conditions, and immunoblotted using rabbit polyclonal antibodies against serpinB6, hK8 or no blotting antibody. The secondary antibody was HRP-conjugated sheep anti-rabbit Ig and detection was via ECL.

keratinocytes, mK8 and SPI3 were detected in cells scattered throughout the dermis. These were identified as neurons and dermal macrophages, respectively. Thus SPI3 and mK8 co-localize in keratinocytes of hair follicles and epidermis in mouse abdominal skin.

To confirm the presence of hK8 and serpinB6 in keratinocytes, we used immunoblotting to examine extracts of primary human epidermal keratinocytes (HEKs). Both polyclonal and monoclonal anti-serpinB6 antibodies detected a 42 kDa protein in HEK cell lysates, which is the size of serpinB6 (Fig. 2A). An additional species of ~63 kDa was also detected by both antibodies. This probably represents a serpinB6/protease complex, similar to the SDS-stable complex formed *in vitro* between serpinB6 and thrombin or trypsin (*21*, *42*), and the serpinB6/protease complex formed on differentiation of the immortalized keratinocyte cell line, HaCat (*10*). The 35 kDa species detected by the polyclonal anti-serpinB6 antiserum represents serpinB6 proteolytically cleaved within the RCL. This form of serpinB6 is not detected by the monoclonal antibody (*10*).

Immunoblotting of HEK cell lysates with polyclonal and monoclonal antibodies specific for hk8 demonstrated the presence of a 35 kDa species, as expected (Fig. 2B). The polyclonal anti-hK8 antibody also detected a number of higher molecular weight species. These included two species of ~70 and 55 kDa and less-abundant species of ~63, 73 and 80 kDa (Fig. 2B). The identity of these species is unknown but they are consistent with the predicted size of the serpinB6/hK8 complex (80 kDa) and commonly observed serpin–protease complex breakdown products (63 and 73 kDa). Due to the substantial deformation of the protease in a serpin–protease complex, it is extremely prone to proteolytic degradation (*43–46*). This deformation may also disrupt of the epitope recognized by the monoclonal anti-hK8 antibody, thus explaining why it does not detect the serpinB6/hK8 complexes.

Taken together, these results show that hK8 and serpinB6 are co-expressed in differentiated primary human keratinocytes. The fact that the anti-hK8 antibody and anti-serpinB6 antibody both detect a 63 kDa species suggests that hK8 and serpinB6 interact in human keratinocytes.

SerpinB6 and Kallikrein-8 Complex in Differentiated Keratinocyte Extracts—To establish whether hK8 and serpinB6 interact in keratinocytes, HEK cell extracts were immunoprecipitated with a monoclonal anti-serpinB6 antibody and the resulting immune complexes were separated by non-reducing SDS–PAGE and analysed for the presence of hK8. Immunoprecipitation of HEK cell extracts with a monoclonal antibody against serpinB6 resulted in the precipitation of 42 kDa native serpinB6 and a 55 kDa species (Fig. 2C, lane 3). When these immune complexes were immunoblotted with an anti-hK8 antibody, the 55 kDa species was also detected (Fig. 2C, lane 1). Therefore serpinB6 and hK8 form an SDS-stable complex in differentiated human keratinocytes that can be detected as a 55 kDa complex under non-reducing conditions. The discrepancy between the predicted size of the complex (~80 kDa) and the detected size (55 kDa) is due to degradation to a (stable) 55 kDa form and/or aberrant mobility under non-reducing conditions. Degradation of a serpinB6/protease complex has been observed previously in the interaction between CatG and serpinB6 (*22*).

SerpinB6 is an Efficient Inhibitor of Kallikrein-8—The observations that serpinB6 and kallikrein-8 are co-expressed in human and mouse keratinocytes, and that serpinB6 and hK8 interact in extracts of differentiated primary keratinocytes, suggest that serpinB6 is a physiologically relevant inhibitor of hK8 in skin. It is generally accepted that kinetic analysis of a physiologically significant serpin/protease interaction should yield a rate constant of between 10^5 and $10^7\,M^{-1}s^{-1}$ with a SI close to one (*47*).

To confirm the hK8 and serpinB6 interaction and assess its efficiency, the SI and association rate constant (k_{ass}) were investigated under pseudo first-order conditions using the progress curve method (*31*). SerpinB6 was estimated to inhibited hK8 with a k_{ass} of $1.8 \pm 0.2 \times 10^5\,M^{-1}s^{-1}$ and approximate SI of 2.2. (Table 1). This is slightly slower than $3.4 \pm 0.2 \times 10^6\,M^{-1}s^{-1}$ and 1.0 for the interaction between mK8 and SPI3. These values are well within the physiological range expected for protease inhibition by serpins, and are comparable to the rate of thrombin and factor Xa inhibition by serpinB6 (1.25×10^5 and $1.3 \times 10^5\,M^{-1}s^{-1}$,

Table 1. **Kinetics of Kallikrein 8 Inhibition.**

Serpin	Protease	k_{ass} ($M^{-1}s^{-1}$)	SI
SerpinB6	hK8	$1.8 \pm 0.2 \times 10^5$	2.2
SerpinB6 M340V	hK8	$5.7 \pm 0.4 \times 10^5$	1.5
SPI3	mK8	$3.4 \pm 0.2 \times 10^6$ (*48*)	1

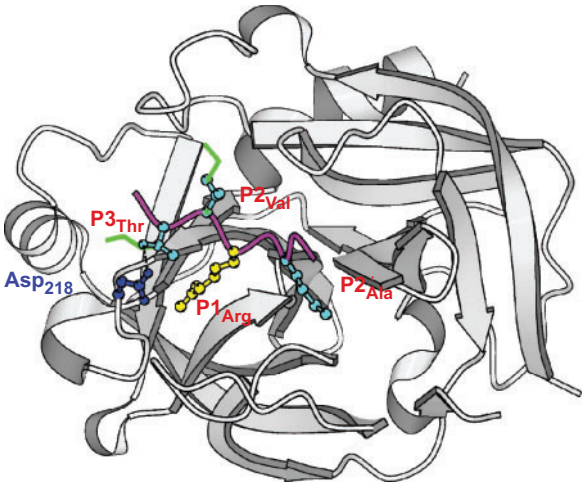

Fig. 3. **Structure of mK8/serpin RCL complex.** Shown is a ribbon structure of mK8 with the P4–P4′ residues of SPI3 modelled into the catalytic pocket. The serpin P1 residue (Arg) is shown in yellow ball-and-stick, whereas the P2 (Val), P3 (Thr) and P2′ (Ala) residues are in cyan ball-and-stick. The P3 Thr is predicted to form a hydrogen bond (dashed line) to Asp218 of the enzyme (dark blue ball-and-stick). In human serpinB6 the RCL differs at the P3 (Met), P2 (Met) and P2′ (Ala) residues. The predicted positions of the serpinB6 P2 Met and P3 Met in a serpinB6/hK8 complex are shown in green stick.

respectively), and faster than the rates observed for serpinB6 inhibition of urokinase ($4 \times 10^4 M^{-1}s^{-1}$) and activated protein C ($7.5 \times 10^3 M^{-1}s^{-1}$) (*19–21*). However, it is lower than the rates observed for serpinB6 inhibition of chymotrypsin and CatG [6×10^6 and $>10^7 M^{-1}s^{-1}$, respectively (*20*, *22*)].

Modelling the SerpinB6/Kallikrein-8 Interaction—The interaction between murine serpinB6 (SPI3) and mK8 is at least 10-fold faster than that observed for serpinB6 and hK8. To examine the basis for this difference, we used molecular modelling to compare the structures of hK8 and mK8. Using the published crystal structure of mK8 (*33*) we built a model of hK8, and compared the active site clefts of the two enzymes. These are essentially identical, suggesting that differences in the rates of inhibition of kallikrein-8 by serpinB6 and SPI3 are due to differences in the serpin RCLs (*data not shown*).

To examine the interaction between the serpins and kallikrein-8, we then produced models of mK8 complexed to the P4–P4′ region of the RCLs of both SPI3 and serpinB6 (Fig. 3). The RCLs differ at the P3, P2 and P2′ positions. Our model showed that the P2′ residue packs into a hydrophobic pocket comprising Leu40, Phe357 and Phe151. This pocket easily accommodates the P2′ methionine of SPI3 and the less bulky P2′ alanine of serpinB6, hence it is unlikely that the difference in the P2′ residue contributes to differences in the inhibitory capacity of SPI3 and serpinB6. In contrast, interactions between the P2 and P3 residues of each serpin and the S2 and S3 pockets of kallikrein-8 are likely to be different (even though the residues lining the S2 and S3 pockets are identical in the mouse and human enzymes). Specifically, the P3 threonine of SPI3 is able to form a hydrogen bond with the sidechain of Asp218, which the P3 methionine in serpinB6 would be incapable of making. Furthermore, the P2 valine of SPI3 packs tightly into the S2 pocket, forming van der Waal contacts with His99 and His57 (the active site His). Although the P2 methionine of serpinB6 could also fit into the S2 pocket without significant steric clashes or shifts in backbone residues, an electrostatic potential map of mK8 (*33*) reveals that the S2 pocket is extremely negatively charged (Tyr94 and the active site residue Asp102 line the base of the S2 pocket) and is unlikely to accommodate a bulky hydrophobic methionine as easily as it would accommodate a smaller valine. Thus the P2 methionine in the RCL of serpinB6 contributes to the reduced inhibitory capacity of serpinB6 towards kallikrein-8, compared to SPI3.

To test the latter hypothesis, the P2 methionine in serpinB6 was mutated to valine and the resulting mutant (serpinB6 M340V) was assessed for its ability to inhibit hK8. The *k*ass increased approximately 3-fold to $5.7 \pm 0.4 \times 10^5 M^{-1}s^{-1}$and the SI changed from 2.2 to 1.5 (Table 1). Thus changing the P2 residue in serpinB6 to Val improves its ability to inhibit hK8 and the resulting kinetics approach those observed for the interaction between mK8 and SPI3.

DISCUSSION

We have shown here that kallikrein-8 is inhibited by serpinB6 with physiologically significant kinetics, and that the two proteins co-localize in human and mouse keratinocytes. On the basis of these results, we propose that serpinB6 is an endogenous regulator of kallikrein-8 in keratinocytes. Kallikrein-8 is an extracellular serine protease with trypsin-like specificity originally cloned from mouse hippocampus (*24*). In adult mouse brain, SPI3 co-localizes with kallikrein-8 in pyramidal cells of the CA1-3 subfields of the hippocampus (*48*), which supports the hypothesis that serpinB6 is an endogenous kallikrein-8 inhibitor.

At present the function of kallikrein-8 in keratinocytes is unknown although a function in terminal differentiation and desquamation have been proposed (*38*, *40*, *41*). It may be produced in early stages of keratinocyte differentiation and stored for later release into the extracellular milieu. This would be analogous to other serine proteases, particularly those produced by pancreatic and cytotoxic cells. Initially, mK8 deficient mice (mK8−/−) show no overt skin phenotype (*49*, *50*). More recent studies of these mice show delayed recovery from UVB-induced or TPA-induced inflammation, excessive proliferation and reduced corneocyte shedding (*40*, *41*).

SerpinB6 is part of an emerging group of intracellular serpins that resemble chicken ovalbumin (*51*). The physiological roles of these ov-serpins remain unclear

but evidence is emerging that they are cytoprotective. For example, overexpression of serpinB2, serpinB3 or serpinB4 protects cells from TNFα, cytotoxic drug or radiation induced cell death (*14*, *52–55*), whereas expression of serpinB9 (protease inhibitor 9; PI-9) protects cells against granzyme B induced apoptosis (*56*).

A cytoprotective model is also proposed for the function of serpinB6 in monocytes and neutrophils (*22*). These cells produce the cytotoxic serine proteases CatG, elastase and protease 3, which are stored as active enzymes in azurophilic granules and released into phagosomes or outside the cell during inflammation. SerpinB6 is a very efficient CatG inhibitor and is expressed in the nucleocytoplasm of monocytes and neutrophils (*17*, *22*). We have proposed that serpinB6 protects these leukocytes from cytoplasmic CatG resulting from rupture of azurophilic granules within the cell, or from misdirected CatG arising from phagocytosis of microbes (*22*).

In keratinocytes serpinB6 is unlikely to regulate the extracellular function of kallikrein-8 because serpinB6 is not actively secreted from cells (*16*). Rather we propose that serpinB6 protects keratinocytes (and possibly neurons, astrocytes and mast cells) from any kallikrein-8 that inadvertently enters the cytoplasm. Secretion of kallikrein-8 from neurons is regulated and is induced by K^+ in a calcium-dependant manner (*57*). Whether kallikrein-8 secretion from keratinocytes is regulated in a similar way, or whether it is stored as an active enzyme intracellularly prior to secretion, is currently unknown. It remains to be determined whether kallikrein-8 is likely to leak from an intracellular storage compartment or re-enter the cell following secretion.

Finally, our finding that hK8 is less efficiently inhibited by serpinB6 than mK8 by SPI3 probably reflects physiological differences between humans and mice. It is known for instance that both serpinB6 and SPI3 are produced in chymotrypsinogen-producing pancreatic acinar cells (F.L. Scott, M. Buzza and P.I. Bird, *unpublished data*), and that the P2 methionine of serpinB6 is used as an alternate P1 residue in the inhibition of chymotrypsin (*20*). Hence serpinB6 and SPI3 may also protect the pancreas from aberrant chymotryptic activity, but we have shown that SPI3 is a much less effective chymotrypsin inhibitor than serpinB6 due to its P2 valine (k_{ass} $2.0 \pm 0.4 \times 10^4\,M^{-1}\,s^{-1}$; SI 2.3—J. Sun and P.I. Bird, *unpublished data*). It is likely, therefore, that control of chymotrypsin by serpinB6 in humans is more important than control of hK8, whereas the opposite applies to mice. This type of difference in the behaviour of serpin orthologues suggests that caution should be exercised when interpreting the results of studies in which heterologous (cross-species) components have been used.

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