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SERPINB3 and B4: from biochemistry to biology

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Abstract

Human SERPINB3 and SERPINB4 are evolutionary duplicated serine/cysteine protease inhibitors. Genomic analysis indicates that these paralogous genes were encoded from independent loci arising from tandem gene duplication. Although the two molecules share 92% identity of their amino acid sequences, they are distinct in the Reactive Center Loop (RCL) including a hinge region and catalytic sequences which accounts for altered substrate specificity. Elevated expression of the two molecules have been reported to contribute to numerous pathological conditions such as inflammatory diseases and cancer. In this review, we focus on summarizing the biochemical features of SERPINB3/B4 and discussing the mechanistic basis for their biological functions and implications in human diseases.

1. Introduction

In the 1970s, Kato et al isolated a tumor specific antigen using tissues from squamous cell carcinoma (SCC) of the uterine cervix [1]. It was initially named TA-4 and was later on given the name squamous cell carcinoma antigen (SCCA) and was found to be a mixture of two isoforms with almost identical molecular weight (390 amino acids, 45 kDa). The two isoforms were separated by isoelectric focusing into a neutral form SCCA1 (pI=6.4) and an acidic form SCCA2 (pI=5.9) [2]. SCCA1 and SCCA2 belong to the family of Clade B serine protease inhibitors (SERPINs), and were later on assigned as SERPINB3 and SERPINB4, respectively, by the Human Genome Organization (HUGO). SERPINB3 and B4 are 98% and 92% identical at the nucleotide and amino acid sequences, respectively. Genomic analysis found that these paralogous genes were encoded from independent loci arising from tandem gene duplication [3-5]. In the initial study, 27 out of the 35 cervical SCC patient samples showed detectable levels of serum SERPINB3/B4 antigen activity [1]. All 27 cases presented with advanced stage of the disease, suggesting an association between elevated SERPINB3/B4 levels and more advanced cancer, which was further noticed in other cancer types [6, 7]. This forms the basis for SERPINB3/B4 to be considered as diagnostic and prognostic biomarkers [8]. In recent years, these two molecules have been reported to have important functions in several pathological conditions especially cancer. It is important to

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note that while SERPINB3 and B4 have distinct biochemical features and *in vitro* substrate protease specificity, their regulation and function in biological settings have not been well distinguished, partially due to the fact that these two proteins are often co-expressed in both normal and disease tissues and share high homology at both the mRNA and protein sequence levels.

2. Biochemical features

Human SERPINs are a family of endogenous protease inhibitors with broad biological functions. In the human genome, there are 36 protein-coding genes and 8 pseudogenes [9]. The majority of SERPINs are inhibitors to serine proteases, but some of them have cross-class inhibitory activity towards caspases or papain-like cysteine proteases. Some SERPINs are non-inhibitory and function as hormonal transporters and molecular chaperones [10]. Human SERPINs are phylogenetically grouped into nine clades (A-I) in which 13 ovalbumin SERPINs (ov-serpins) are classified as clade B SERPINs [11]. While most human serpins are secreted, clade B serpins lack a signal peptide and are therefore predominantly intracellular in distribution. Among them, three clade B genes (*SERPINB1, B6 and B9*) are located at chromosome 6p25, and the rest ten genes are clustered at chromosome 18q21.3 (*SERPINB2, B3, B4, B5, B7, B8, B10, B11, B12, and B13* [11, 12]. *SERPINB3* and *SERPINB4* have evolved by gene duplication and are tandemly arrayed. Both genes are approximately 10 kb and contain 8 exons flanked by 7 introns [5].

Both SERPINB3 and B4, similar to other clade B SERPINs, comprise an ovalbumin-like domain as the main body of the protein with nine α-helices and three antiparallel β-sheets, and a hydrophobic c-terminal reactive center loop (RCL) that is also termed reactive site loop (RSL) [13-15]. The RCL is a flexible stretch containing about 17 residues located between β-sheets A and C, which is essential for binding and inhibiting the target protease [16-20]. Like other protease substrates, amino acid residues in SERPINs are consecutively numbered outward from the protease cleavage site within the RCL as… -P3-P2-P1-P1′-P2′-P3′-… (the scissile bond is located between the P1 and P1′ positions), while the corresponding residues in the proteases catalytic sequence are labeled as …-S3-S2-S1-S1′-S2′-S3′-…[21]. Mutagenesis-based enzymatic activity and gel shift assays revealed that both RCL regions of SERPINB3 and B4 comprise two regions: the “hinge region” from P15 to P8 that is highly conserved between SERPINB3 and B4, and the “variable region” from P7 to P5′ corresponding to catalytic residues of their targeted proteases (Table 1). The variable region contains different residues around the scissile bond and dictates the target protease specificity [16-19]. *In vitro* enzymatic activity assays revealed that SERPINB3 mainly inhibits papain-like cysteine proteases including Cathepsin L, S and K, and papain, while SERPINB4 inhibits chymotrypsin-like serine proteases including Chymase, Cathepsin G, and Der P (Table 1). Swapping the RCL domains between SERPINB3 and SERPINB4 reversed their inhibitory substrate preference [16].

Most SERPINs inhibit their target proteases through a suicide inhibition mechanism. Initially SERPIN binds to protease through a non-covalent Michaelis-like complex by interactions with residues flanking the scissile bond (P1-P1′). Attack of the active site serine on the scissile bond leads to a covalent ester linkage between protease and the backbone

carbonyl of the P1 residue, resulting the cleavage of the peptide bond. Hinge region of the cleaved RCL then inserts into the β sheet A and transports the covalently bound protease with it. As a result, the protease gets translocated by over 70Å and its active site gets distorted. Distortion of the active site prevents the final hydrolysis events and results an irreversible covalent serpin-protease complex [22-26]. Like other SERPINs, SERPINB3 and B4 are believed to form a SDS-resistant complex via an acyl-oxyester bond with their proteases to inhibit their activities [16, 17, 19, 20]. An exception to this event is papain. Instead of forming the acyl-oxyester bond, it is believed that SERPINB3 and papain form an acyl-thioester bond that can be easily hydrolyzed [27].

Despite these intensive *in vitro* studies, however, the *bona fide* protease substrates of SERPINB3/B4 *in vivo*, and the molecular basis for their possible interaction and inhibitory functions remains largely unclear. Although SERPINB3/B4 protease substrates characterized in the *in vitro* systems are also inhibited by SERPINB3/B4 in cultured cells [28, 29], the structures of the interacting complex have not been crystallized and the interaction and related enzymatic kinetics study remain to be carried out under *in vivo* conditions.

3. Tissue distribution

The distributions of SERPINB3/B4 in tissues and intracellular localization have been studied at both transcript and protein levels. Two independent studies showed these two genes are frequently co-expressed in same tissues including bladder, uterus, esophagus, lung, prostate, testis, thymus, and trachea, except SERPINB4 was not detectable in bladder and thymus [14, 30]. The clarification of tissue distribution of these two duplicated proteins benefited from the development of specific antibodies, anti-SERPINB3 (clone 8H11) and anti-SERPINB4 (clone 10C12), which was validated using recombinant proteins [30]. These findings fit the expectations of the two closely-related gene duplicates being more likely to share cis-regulatory motifs and to display stronger co-expression patterns than two randomly selected genes [31]. The biological significance of SERPINB3 and B4 in normal tissue development and function remains largely unclear, partially due to the fact that their true functional homologs in other organisms including mouse have not been unequivocally defined.

4. Mouse homologues of SERPINB3 and SERPINB4

Mouse genome contains 62 functional SERPIN genes, many of which are orthologous to human *SERPIN* genes while some have expanded into multiple paralogous genes [9]. Mouse orthologs to *SERPINB3* and *SERPINB4* are represented by 4 copies, named Serpinb3a (SQN-5), b3b, b3c and b3d, which are located on chromosome 1E2.1 with 8 exons [32, 33]. Similar to their human counterparts, mouse Serpinb3a and Serpinb3b proteins are highly homologous to each other with 93% similarity of their amino acid sequences yet with different target protease profiles, whereas Serpinb3c and b3d share 82% similarity with Serpinb3a. Until now, among the 4 copies of mouse clade b3 serpins, only Serpinb3a and b3b have been described for their protease inhibitory functions [34]. Systemic Serpinb3a knockout mice are developmentally normal but display attenuated pro-inflammatory response in an asthma model [35]. This is in line with the elevated expression of

SERPINB3/B4 in human airway inflammatory diseases [36-38]. Therefore, while the lack of overt developmental phenotype may be due to the compensatory effect of other Serpins such as Serpinb3b and Serpinb3c, it is also possible that SERPINB3/B4 mostly function as a stress-responsive factor. The role of Serpinb3a in mouse models for other diseases such as cancer remains to be determined.

Mouse Serpinb3 homologues share relative low amino acid similarity to their human homology, only up to 60% of Serpinb3a with SERPINB3. In addition, mouse Serpinb3a and Serpinb3b have several distinct features (Table 1). 1) Unlike human SERPINB3 that predominantly inhibits papain-like cysteine proteases and SERPINB4 that predominantly inhibits chymotrypsin-like serine proteases, both mouse Serpinb3a and Serpinb3b are cysteine/serine dual protease inhibitors with only 2 different amino acid residues differing in their RCL variable regions. 2) The tissue distribution of their expression is different. Human SERPINB3 and B4 are co-expressed broadly in many tissues including the epithelium of tongue, tonsil, esophagus, uterine cervix, vagina, the conducting airways, Hassall's corpuscles of the thymus, and certain areas of the skin [30, 39, 40]. Mouse Serpinb3a is ubiquitously expressed with strongest expression in the thymus, whereas Serpinb3b is expressed more restrictively in keratinocytes [34]. 3) Their expression in response to inflammatory cytokines varies. IL-4 and IL-13 can induce the expression of SERPINB3 and B4 in human bronchial epithelial cells (HBECs) and keratinocytes. In mouse, these cytokines are able to induce Serpinb3a and b3b in bronchial epithelial cells but not in keratinocytes [17, 36, 37, 41, 42]. These differences in human and mouse SERPINB3/B4 expression and function may have evolved due to the different needs in inhibiting harmful proteases from pathogenic microbes in human and mouse. They also raise the caution that careful data interpretation is important when using the murine system to study SERPIN function and regulation. One such example is the use of genetically engineered mouse models (GEMM). While it has been reported that Ras oncoproteins can induce SERPINB3/B4 expression in human cells, they do not seem to induce Serpinb3a and b3b in mouse models of oncogenic Ras [43].

5. SERPINB3/B4 in inflammatory diseases

Elevated expression of SERPINB3/B4 have been detected in chronic inflammatory conditions involving the skin, especially atopic dermatitis (AD)and psoriasis, as well as in respiratory inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and tuberculosis [36, 38, 41, 44-52].

In skin, the epidermis is a rich source of proteases and protease inhibitors. For example, activation of transglutaminases, which is involved in cross-linking structural proteins of cornified epidermal cell envelope, is controlled by a protease cascade involving legumain, cathepsin L, and cathepsin D [53, 54]. Increased SERPINB3/B4 expression can modulate the activity of cysteine proteases, and subsequently the activation of transglutaminases and epidermal cell homeostasis.

Increased SERPINB3/B4 expression may also facilitate a feed forward mechanism to modulate immune response. In psoriatic skin lesions, a proteolytically cleaved product of

SERPINB3, Pso p27, was identified in mast cells [55, 56]. Pso p27 can complex with IgG as well as complement factor C1q and form large aggregates in skin scale lesions and serum in psoriasis patients, suggesting an immunogenic role of Pso p27 [57]. High concentrations of Pso p27 specific antibodies can be extracted from psoriatic scales, suggesting Pso p27 is an immunogen [58]. While the biochemical nature of Pso p27 production under physiological circumstances remains to be determined, *in vitro*, Pso p27 can be generated by incubating SERPINB3 with chymase [59]. On the contrary, SERPINB3 did not inhibit chymase activity in a peptide probe-based enzymatic activity assay [17], raising the possibility that it may not be a natural proteolytic substrate for chymase.

The induction of SERPINB3/B4 expression in psoriasis and AD may be caused by elevated cytokine produced by infiltrated immune cells. Psoriasis and AD are chronic and relapsing inflammatory diseases associated with various immunologic abnormalities. Histologically, psoriasis is distinguished from AD by hyper-proliferation of keratinocytes [60]. Inflammatory cells invading the dermis and epidermis in psoriasis are Th1 cells, macrophages, dendritic cells, and neutrophils while infiltrating inflammatory cells in AD are Th2 cells, eosinophils, and mast cells [60]. Despite differences of proliferation status of keratinocytes and infiltrated inflammatory cells, elevated SERPINB3/B4 transcripts in epidermis were detected in both diseases. At least in AD, SERPINB3/B4 expression is likely induced by Th2 cytokines IL-4 and IL-13, which can be observed in cultured human keratinocytes and bronchial epithelial cells. The regulation of SERPINB3/B4 expression in psoriasis remains to be determined as Th1 cytokines, such as IFN-gamma or TNF, fail to induce their expression [37, 41].

In asthma patients, elevated SERPINB3/B4 is detected in the serum of patients with asthma. SERPINB3 protein is also elevated in epithelial lining fluid from the chronic obstructive pulmonary disease (COPD) patients after smoking [38, 42]. Similar as in the skin, Th2 cytokines IL-4 and IL-13 significantly induce SERPINB3/B4 expression in bronchial epithelial cells in a STAT6-dependent fashion [37, 42]. Mouse homologue Serpinb3a knockout mice have been valuable to study the function of SERPINB3 and B4 response to inflammatory stimuli in skin and respiratory system. In an asthma model, wild type and Serpinb3a KO mice were challenged with house dust mite or IL-13. The knockout mice were more resistant to asthma due to the inhibition of goblet cell hyperplasia and mucus production [35]. In another conventional cutaneous inflammation model, wild type and Serpinb3a KO mice were challenged with *Aspergillus fumigatus* extract epicutaneously [47]. The allergen exposure induced Serpinb3a expression in the skin, along with increased transepidermal water loss (TEWL), epidermal thickness, and skin inflammation, all of which were attenuated in Serpinb3a KO mice. The rescued phenotype was correlated with decreased expression of the pro-inflammatory marker S100A8. Therefore, SERPINB3/B4 and their mouse homologues can promote progression of inflammation when cells are exposed to cytokines, microbes, or allergens. As IL-4 and IL-13 have been shown to induce cathepsin expression [61], and cathepsins may play an important role in emphysema induced by IL-13 [62], it is conceivable that SERPINB3/B4 function to suppress the inflammatory reaction by inhibiting cathepsins.

6. SERPINB3/B4 as biomarkers in cancer

The levels of SERPINB3/B4 transcripts or proteins from biological samples, mostly sera or tumor tissue, have been used in numerous studies to predict disease stage and response to therapy [63-65]. Detailed histological analysis revealed that SERPINB3/B4 are normally co-expressed in squamous epithelial cells of tongue, esophagus, tonsils, epidermal hair follicles, lung and uterus, while becoming highly up-regulated in squamous carcinomas of these organs [30, 39, 40]. SERPINB3/B4 levels have also been shown to coincide with tumor infiltration and frequency of lymph node metastasis in both cervical and esophageal squamous cell carcinomas [66-68]. Recent evidence also suggests that SERPINB3/B4 expression is not limited to cancers of squamous origin but also extends to adenocarcinoma of the lung, breast, and pancreas, as well as hepatocellular carcinoma [69-73]. Elevated SERPINB3/B4 expression correlates with resistance to platinum combined treatment in non-small cell lung carcinoma (NSCLC) and poor prognosis of anthracyclin based chemotherapy in breast cancer patients [71, 74].

In lung cancer, the association of SERPINB3 with disease progression seems to be cancer subtype dependent. A tumor transcriptome study in NSCLC revealed that high level of SERPINB3 transcript expression, although invariably associates with chemoresistance, has contrasting prognostic impact on untreated two most common NSCLC subtypes, squamous cell carcinomas (SCC), or adenocarcinomas (AC). High level of SERPINB3 indicates a poor prognosis in AC but a favorable prognosis in SCC [69]. In liver cancer, SERPINB3/B4 are overexpressed in liver tumor tissues, also detectable in serum from patients with HCC coupled to IgM to form circulating immune complex (IC). Compared with another liver cancer biomarker, alpha-fetoprotein (AFP), which is detected in 42% of patients, SERPINB3/B4-IgM IC levels measured by ELISA were elevated significantly in a greater proportion of patients (70%). Interestingly, unlike in cervical SCC patients where the serological level of SERPINB3/B4 correlates with the severity of the cancer [75], there was no serum SERPINB3/B4 detected in HCC patients [76]. In addition, elevated mRNA ratio of SERPINB4 vs B3 was documented during the process of cervical carcinogenesis that indicated a higher risk of recurrence in early-stage uterine cervical cancer [77]. A diagnostic ELISA kit has been developed for the detection of serological SERPINB3/B4 in pathological conditions of inflammation and cancer [78]. Nonetheless, detection methods with better specificity and higher sensitivity are needed for a more empirical practice of using SERPINB3/B4 as biomarkers.

7. SERPINB3/B4 as driving factors in oncogenesis

In addition to being a biomarker, SERPINB3/B4 have been found to associate with several oncogenic processes, suggesting that they are *bona fide* oncoproteins. Ectopic expression of SERPINB3 leads to oncogenic transformation of the non-tumorigenic mammary epithelial MCF10A cells [79]. Suppression of SERPINB3 using antisense RNA or short-hairpin RNA leads to decreased growth of a SCC cell line and a number of breast and pancreatic cancer cells [43, 79, 80]. A well characterized oncogenic feature of SERPINB3/B4 is the induction of epithelial-mesenchymal transition (EMT). In cultured cells, ectopic expression of SERPINB3 leads to an epithelial-mesenchymal transition (EMT)-like phenotype in

hepatocellular carcinoma cell line HepG2, mammary epithelial cell line MCF10A, and a baby mouse kidney (BMK) epithelial cell line, characterized by a number of morphological and molecular changes [29, 79, 81]. As EMT has been mostly implicated in increased proliferation, metastasis, and therapy-resistance, SERPINB3/B4 can promote cell transformation, migration, and resistance to a number of cell death inducing agents. Expression of SERPINB3 leads to anchorage-independent cell growth, increased cell migration, resistance to DNA alkylating damage and hypo-osmotic stress, and xenograft tumor growth [29, 79, 81]. Conversely, knockdown of SERPINB3/B4 in established breast and pancreatic cancer cell lines leads to cellular senescence and decreased xenograft tumor growth [43, 79]. As the precise meaning of EMT in human cancer is under debate, it remains to be elucidated whether the EMT-like phenotype induced by SERPINB3/B4 plays a functional role in the increased oncogenic transformation. Nonetheless, the EMT-promoting activity of SERPINB3/B4 is of particular clinical relevance given that SERPINB3/B4 positivity correlates with high grade, poorly differentiated cancer. Associating with its EMT-promoting function, SERPINB3 induces pro-inflammatory/pro-tumorigenic cytokines such as IL-6, which is a major EMT-driving cytokine. Elevated SERPINB3/B4-dependent IL-6 production has been found in numerous cultured cell lines, as well as in the transgenic models of the mouse mammary gland simultaneously expressing SERPINB3 and wild-type Erbb2 (also called Neu) and in the liver of SERPINB3 transgenic mice after partial hepatectomy [43, 79, 82]. Further, oncogenic Ras-induced inflammatory cytokine production, including IL-6, IL-8, CXCL4 and GM-CSF was also found to be mediated by SERPINB3/B4 [39]. In addition to EMT promotion, SERPINB3/B4 has been found to up-regulate the expression of c-Myc [83]. Positive correlation between c-Myc and SERPINB3 was detected in hepatoma cell line and HCC, and in SERPINB3 transgenic and Serpinb3a knockout mice [83], which may also contribute to the oncogenic potential of SERPINB3.

8. Mechanisms of action

Inhibition of cell death

The positive association of elevated SERPINB3/B4 with more advanced malignancy as well as poor prognosis suggests that SERPINB3/B4 may have a positive impact on tumorigenesis and/or tumor progression. The pro-tumorigenic role of SERPINB3/B4 has been largely attributed to their anti-cell death function. SERPINB3 has been shown to protect cancer cells from apoptosis induced by UV irradiation and anti-cancer therapy [84]. In response to UV irradiation, SERPINB3 protects cells through a directly inhibitory effect on mitogen-activated protein kinase (MAPK) family members JNK or p38 [85, 86]. SERPINB3 has also been found to localize to the mitochondrial inner membrane to interact with Complex I and suppress mitochondrial ROS generation [87].

Another anti-cell death function of SERPINB3/B4 may be through their protective effect against lysosomal injury. Srp-6, the SERPINB3/B4 homolog in *C. elegans*, protects cells from several stress conditions including hypoxia, hyperoxia, hypotonic, oxidative stress, and hyperactivation of the degenerin/epithelial sodium channel Mec-4 that induce intracellular calcium imbalance, lysosomal membrane permeability (LMP), and necrosis [88]. Similarly, human SERPINB3 and mouse SERPINb3b, maintain lysosomal integrity in response to

DNA alkylating reagent and hypotonic shock [29]. Although SERPINB3/B4 and Srp-6 do not share the exact same protease targets, they both inhibit cysteine proteases, which are the major group of evolutionarily conserved lysosomal proteases. These studies indicate that SERPINB3 and Srp-6 can help maintain lysosome integrity and protect cells from cell death resulting from LMP, which is often induced by stress conditions encountered by cancer cells during tumor development and anti-cancer therapy. It is important to note that while Srp-6 is predominantly localized in the cytoplasm and protects cells downstream of LMP by inhibiting proteases leaked out of the lysosomal lumen [88], SERPINB3 and SERPINb3b can localize to the lysosomes and suppress lysosomal proteases and lysosomal damage [29].

Induction of pro-tumorigenic unfolded protein response (UPR)

The endoplasmic reticulum (ER) is the organelle where proteins and lipids are synthesized and modified. It is also the main storage site of intracellular calcium and is the entrance to the protein secretory pathway. In response to a number of stimuli that disrupt ER function, such as Ca^{2+} depletion from the ER lumen, inhibition of asparagine (N)-linked glycosylation, reduction of disulfide bonds, or overexpression of some proteins, protein misfolding occurs and unfolded proteins accumulate and aggregate in the ER. The misfolded and aggregated proteins then trigger the unfolded protein response (UPR) that involves the activation of three signaling pathways, PERK-ATF4, ATF6, and IRE1-XBP1 [89-91]. While it is well appreciated that excessive misfolded protein stress triggers apoptosis, UPR signaling under more physiological conditions plays an important adaptive role in helping cells to cope with stress and to restore homeostasis. The connection between elevated UPR and cancer has been well appreciated in light of cancer cells' highly increased growth rate and exposure to growth-limiting conditions such as nutrient deprivation and hypoxia [89, 92]. While over-activating UPR in cancer cells can lead to cell death and has been regarded as a therapeutic opportunity using proteotoxic agents such as the proteasome inhibitor Velcade (Bortezomib) [93, 94], the UPR signaling pathway has been implied in promoting tumorigenesis by increasing tumor cell survival and proliferation [89, 95].

SERPINB3 was found to induce a constitutive chronic UPR response, possibly via its inhibition of the lysosomal proteases [79]. As a consequence of activated UPR, SERPINB3 induces NF-κB and the expression of pro-inflammatory cytokines, predominantly IL-6, which leads to an epithelial-mesenchymal transition (EMT)-like phenotypical change and oncogenic transformation in the mammary epithelial MCF10A cells. The elevated UPR induced by SERPINB3/B4 plays a critical role in the activation of NF-κB, IL-6 production, and EMT. As all three arms of UPR are activated by SERPINB3/B4, the precise contribution of each arm to SERPINB3/B4's biological function remains to be determined. SERPINB3/B4-induced cytokine production is abrogated upon silencing of ATF6α, and IL-6 neutralizing antibody prevents the EMT phenotype in MCF-10A cells [43, 79]. In pancreatic cancer models, silencing of ATF6 or XBP1 abolishes Ras-induced cytokine production, whereas ectopic active spliced XBP1 restored Ras-induced cytokines following SERPINB3 silencing. Silencing endogenous SERPINB3 in K-ras activated pancreatic cells inhibited xenograft tumor growth. The UPR-regulated inflammatory phenotype was also observed in a transgenic mammary gland tumor model where transgenic human SERPINB3 and Neu were simultaneously expressed [79].

9. Regulation of SERPINB3/B4 expression

Transcription control of SERPINB3/B4 in normal epithelial tissues is not well understood. In cancer, several oncogenic events have been shown to induce SERPINB3/B4 expression. Oncogenic mutant Ras isoforms (KrasG12D, HrasG12V, NrasQ61R) and B-Raf V600E can induce SERPINB3/B4 in mammary epithelial cells, lung fibroblasts, and pancreatic epithelial cells [43]. This Ras-driven upregulation of SERPINB3/B4 is dependent on the MAPK/ERK pathway, which leads to the activation of the ETS family transcription factor PEA3 via its sumolyation [96]. PEA3 binds to SERPINB3/B4 promoter and activates their expression [97]. Silencing of PEA3 in Ras^{V12}-expressing cells results in a drastic decrease of SERPINB3/B4 expression at both transcript and protein levels. The elevated SERPINB3/B4 expression leads to increased UPR, NF-κB activation, and production of inflammatory cytokines such as IL-6, independent of Ras-induced cellular senescence. Correlating with the high rate of Kras mutation in human pancreatic cancer, SERPINB3/B4 positivity within pancreatic intraepithelial neoplasia (PanIN) lesions and pancreatic ductal adenocarcinoma (PDA) increases with disease progression. Therefore, SERPINB3/B4 may play a critical role in inflammatory cytokine production upon Ras activation.

It has also been described that SERPINB3/B4 gene expression is regulated by the transcription factor STAT3. ChIP analysis reveals that STAT3 occupies the promoter of SERPINB3/B4 [98]. Suppression of the STAT3 signaling using gp-130 antibody diminishes SERPINB4/B4 expression [98]. This STAT3-mediated expression of SERPINB3/B4, together with the notion that SERPINB3/B4 can induce the expression of IL-6 [43, 79], which activates STAT3, indicate a feed-forward loop involving SERPINB3/B4, IL-6, and STAT3 that may play an important role in IL-6 signaling during tumor initiation and differentiation.

Another cancer promoting condition that induces SERPINB3/B4 expression is hypoxia. This is though hypoxia-induced stabilization of hypoxia-inducing factor-2α (HIF-2α), which was found to bind to the promoter of SERPINB3/B4 by Chromatin immunoprecipitation (ChIP) analysis, and activates SERPINB3/B4 transcription [99].

10. Subcellular localization

SERPINB3/B4 are localized predominantly in the cytosol. However, they have also been detected in other subcellular compartments including lysosomes, mitochondria, the nucleus, and may function extracellularly. Their precise subcellular localization under various physiological and pathological conditions remains obscure, adding to the lack of precise understanding of their biological function at that site. Based on the study of Srp-6, the homolog in C. elegans [88], in the cytosol, SERPINB3/B4 may function to guard against the proteases leaked out of the lysosome via lysosomal membrane permeability (LMP), which can be detrimental to the cell despite their reduced enzymatic activity in the suboptimal higher pH environment in the cytosol than in the lysosomal lumen. The other function of SERPINB3/B4 in the cytosol is to inhibit proteases that naturally reside in the cytosol or secreted by infectious pathogens [46, 100]. SERPINB3/B4 may also exist in lysosomes to directly inhibit lysosomal proteases. This is at least the case in cells with ectopically

overexpressed SERPINB3 and the murine form Serpinb3b, as they co-localize with the lysosomal markers and inhibited lysosomal protein degradation [29]. A fraction of SERPINB3 was also found to localize in mitochondria to interact with Complex I and mitigate the generation of reactive oxygen species upon chemotherapeutic drug treatment [87].

Another major localization for SERPINB3/B4 is the nucleus. While SERPINB3/B4 were not detected in the nucleus in certain cells at basal state [101], they have been found in the nucleus in response to UV irradiation [85] and in mouse tumor models with transgenic SERPINB3 [79, 83]. In clinical samples, nuclear SERPINB3/B4 has been commonly reported in various cancers [43, 70, 73, 102], psoriasis [44], and in idiopathic pulmonary fibrosis [103]. In response to UV irradiation, SERPINB3 was found to interact and inhibit the kinase activity of c-Jun-NH_2-terminal kinase-1 (JNK1) in the nucleus, through an unknown mechanism, to suppress apoptosis. The function, regulation, and biological significance of SERPINB3/B4 in many human cancers and other diseases remain to be studied.

As SERPINB3/B4 were initially discovered in the circulatory system of SCC patients, whether extracellular SERPINB3/B4 are a result of the passive release from dead cells or the active secretion from live cells is controversial [101, 104]. It has been suggested that extracellular SERPINB3 inhibits natural killer (NK) cell infiltration into the tumor tissue [80], while the mechanism underlying this observation remains to be elucidated.

In conclusion, since their discovery about 40 years ago, it has become clear that SERPINB3/B4 are important players in a large number of physiological/pathological conditions. However, although their cysteine/serine protease inhibitory function is well established in test tube enzymatic kinetics studies, there is only limited amount of evidence supporting that the biological functions of SERPINB3/B4 are mainly accounted for by their serine/cysteine protease inhibitory function. Identification of SERPINB3/B4 interacting partners and/or targeted enzymes *in vivo* will largely facilitate our understanding of the biological functions of SERPINB3/B4. Furthermore, as SERPINB3 and B4 are highly homologous yet may exert distinct biological functions, the development their inhibitors and detection methods with high selectivity and sensitivity will benefit the translational implication of SERPINB3 and B4 in human diseases such as cancer and inflammation.

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Table 1

	hinge											Variable							target proteases
position human	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1′	2′	3′	4′	
SerpinB3	G	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	T	S	Papain, Cathepsin L, S, K
SerpinB4	*	V	*	*	*	*	*	*	*	*	*	V	V	E	L	*	S	P	*	Chymase, Cathepsin G, Der P, Granzyme M
Mouse																				
serpinb3a	G	T	E	A	A	A	A	T	G	V	E	V	S	L	T	S	A	Q	I	Cathepsin K, L, S, V, G, papain, Der P, HMC
serpinb3b	*	*	*	*	*	*	*	*	*	*	*	*	*	V	R	*	*	*	*	Cathepsin L, G