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Review

Inter-α-inhibitor, hyaluronan and inflammation★

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Inter-α-inhibitor is an abundant plasma protein whose physiological function is only now beginning to be revealed. It consists of three polypeptides: two heavy chains and one light chain called bikunin. Bikunin, which has antiproteolytic activity, carries a chondroitin sulphate chain to which the heavy chains are covalently linked. The heavy chains can be transferred from inter-α-inhibitor to hyaluronan molecules and become covalently linked. This reaction seems to be mediated by TSG-6, a protein secreted by various cells upon stimulation by inflammatory cytokines. Inter-α-inhibitor has been shown to be required for the stabilization of the cumulus cell-oocyte complex during the expansion that occurs prior to ovulation. Hyaluronan-linked heavy chains in the extracellular matrix of this cellular complex have recently been shown to be tightly bound to TSG-6. Since TSG-6 binds to hyaluronan, its complex with heavy chains could stabilize the extracellular matrix by cross-linking hyaluronan molecules. Heavy chains linked to hyaluronan molecules have also been found in inflamed tissues. The physiological role of these complexes is not known but there are indications that they might protect hyaluronan against fragmentation by reactive oxygen species. TSG-6 also binds to bikunin thereby enhancing its antiplasmin activity. Taken together, these results suggest that inter-α-inhibitor is an anti-inflammatory agent which is activated by TSG-6.

In the 1960's a novel trypsin inhibitor was isolated from human plasma and found to occur at a concentration of about 0.5 mg/ml. Upon paper electrophoresis the protein ran in

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the middle of the α-region and was therefore given the name inter-α-trypsin inhibitor (IαI) (Heide *et al.*, 1965; Steinbuch & Loeb, 1961). However, since trypsin does not normally occur in plasma, the trypsin part of the name was later dropped. IαI was found to be a poor inhibitor of other more physiologically relevant proteases such as elastase and kallikrein (Potempa *et al.*, 1989), and its function remained unknown for many years after its discovery.

STRUCTURE OF INTER-α-INHIBITOR

IαI consists of three different polypeptides: two homologous heavy chains of about 75 kDa (HC1 and 2) and one light chain of 16 kDa (bikunin). There is another plasma protein closely related to IαI, called pre-α-inhibitor, which consists of one bikunin molecule and one heavy chain (Fig. 1); this heavy chain – HC3 – is homologous to those of IαI. Bikunin bears a chondroitin sulphate chain and the C-terminal α-carboxyl group of the heavy chains of both inter- and pre-α-inhibitor is esterified with an internal, unsulphated GalNAc residue of this polysaccharide (Fig. 2) (Morelle *et al.*, 1994). (For a review on IαI see Salier *et al.*, 1996). Bikunin, which also occurs in free form in plasma, accounts for the protease inhibitory activity of IαI (Wachter & Hochstrasser, 1981). As shown in Fig. 3, the polypeptide of bikunin contains two protease domains of the Kunitz type. Each of these has antiproteolytic activity but with different specificities. (For a review on bikunin see Fries & Blom, 2000). The crystal structure of bikunin has been determined and docking analysis has indicated that domain I sterically hinders binding of proteases larger than trypsin to domain II (Xu *et al.*, 1998). Indeed, proteolytically released domain II has a several-fold higher inhibitory activity against factor Xa and plasma kallikrein than intact bikunin (Morishita *et al.*, 1994).

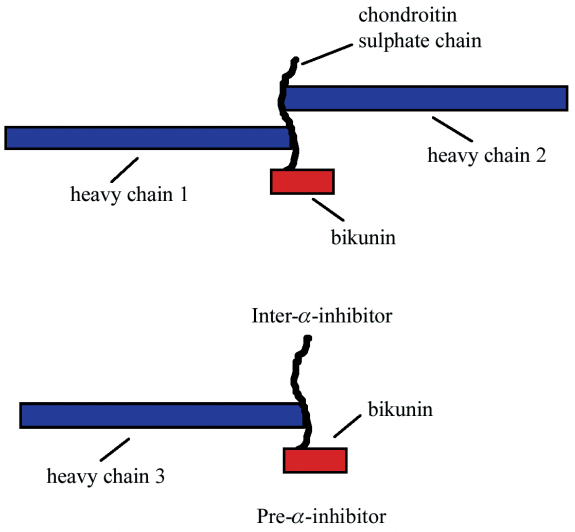

Figure 1. Schematic representation of the plasma proteins pre- and inter-α-inhibitor.

Inter- and pre-α-inhibitor consist of two and one 75–80 kDa polypeptide, respectively (the heavy chains, in blue), which is covalently linked to bikunin (in red) through its chondroitin sulphate chain.

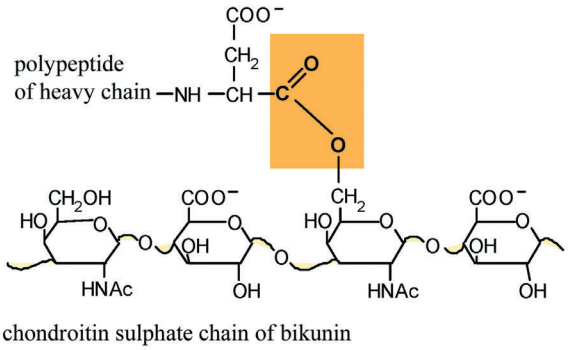

Figure 2. Structure of the link between heavy chains and the chondroitin sulphate chain of bikunin.

The α-carbon of the C-terminal amino-acid residue of the heavy chains is linked through an ester bond to C6 on an internal, unsulphated GalNAc residue of the chondroitin sulphate chain.

BIOSYNTHESIS OF INTER- AND PRE-α-INHIBITOR

Inter- and pre-α-inhibitor are synthesized by hepatocytes and their assembly has been stud-

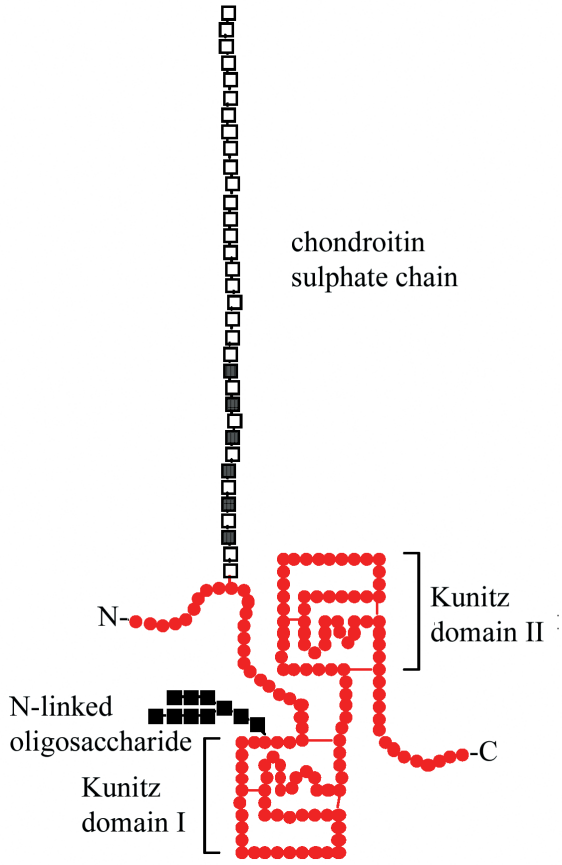

Figure 3. Schematic representation of the structure of bikunin.

Bikunin consists of a polypeptide of 16 kDa, a chondroitin sulphate chain of 7 kDa and an N-linked oligosaccharide of 2 kDa. The amino-acid residues are depicted as circles in red and sugar residues as open squares; sulphated sugar residues are in gray. The polypeptide contains two proteinase inhibitor domains of the Kunitz type.

ied by pulse-chase experiments with isolated cells (Sjöberg & Fries, 1992; Thøgersen & Enghild, 1995). Both the heavy chains and bikunin are synthesized as precursors with C- and N-terminal extensions, respectively (Fig. 4). Upon arrival in the Golgi complex, the chondroitin sulphate chain of the bikunin precursor is elongated and the C-terminal extension of the heavy chains cleaved off. The new C-terminal carboxyl group of the heavy chains then immediately becomes covalently linked to the chondroitin sulphate chain of the bikunin precursor. Coupling can also be achieved with recombinant proteins expressed in COS-cells (Blom *et al.*, 1997). Experiments with this system indicate that the C-terminal extension mediates the coupling reaction (Thuveson & Fries, 1999). The N-terminal extension of the bikunin precursor is released by proteolytic cleavage late in the Golgi complex and/or secretory vesicles (Bratt *et al.*, 1993). This part of the precursor, which gives rise to the plasma protein α_1-microglobulin, is not essential for coupling (Kaczmarczyk *et al.*, 2002) and there is no known functional relationship between the two proteins.

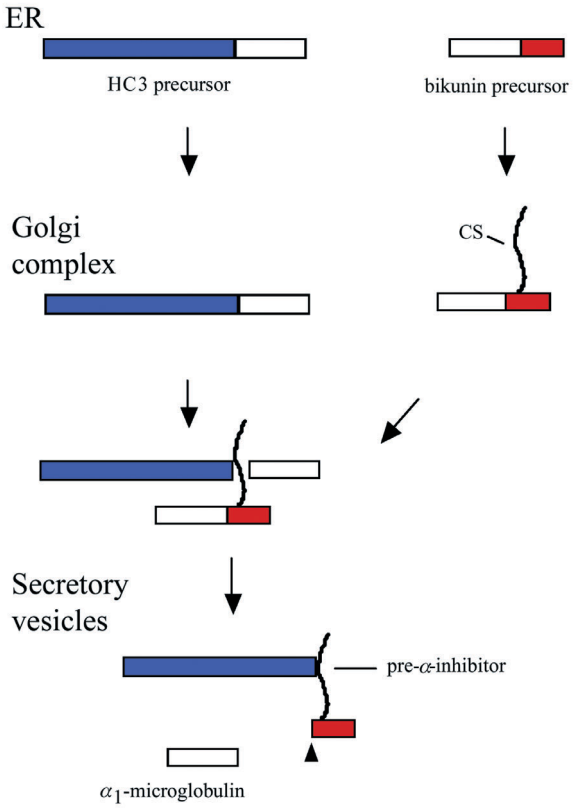

Figure 4. Biosynthesis of pre-α-inhibitor.

Bikunin (in red) is synthesized as a precursor with an N-terminal extension containing the plasma protein α_1-microglobulin. In the Golgi complex, the precursor acquires a chondroitin sulphate chain (indicated with CS). The heavy chain (in blue) is also synthesized as a precursor with a C-terminal extension, which is cleaved off in the Golgi complex. Concurrently, the chondroitin sulphate chain of the bikunin precursor is covalently linked to the new C-terminus of the heavy chain yielding the structure shown in Fig. 2. When the complex reaches the secretory vesicles, the bikunin precursor is proteolytically cleaved resulting in the release of the α_1-microglobulin moiety and the formation of mature pre-α-inhibitor. Inter-α-inhibitor is assembled in a similar way.

OVULATION AND INTER-α-INHIBITOR

In fully grown follicles the oocytes form a complex with a compact layer of surrounding cells called cumulus cells. Upon ovulatory stimulation, the cumulus cells start producing a hyaluronan-rich extracellular matrix, which leads to a many-fold expansion of the volume of the cumulus cell-oocyte complex (COC). This expansion seems to facilitate the detachment of the COC from the follicle wall and its extrusion upon ovulation. Furthermore, the expanded extracellular matrix seems crucial for the capture of the COC by oviductal fimbriae as well as for successful fertilization (Chen *et al.*, 1993). It was found many years ago that in the presence of serum and follicle stimulating hormone, the preovulatory expansion of the COC could occur also *in vitro* (Chen *et al.*, 1990; Eppig, 1980). In 1992 it was reported that IαI (or pre-α-inhibitor) is the essential component of the added serum (Chen *et al.*, 1992). It was subsequently shown that the permeability of the capillaries surrounding the follicles increases upon ovulatory stimulation leading to an influx of IαI (Powers *et al.*, 1995). In addition, immunofluorescence microscopy showed the appearance of heavy chains in the COCs indicating a direct structural role for this protein (Chen *et al.*, 1994). These findings were recently corroborated through the study of mice lacking bikunin: female animals were found to be infertile with small, non-expanded COCs, and the defect could be alleviated through injection of IαI (Zhuo *et al.*, 2001).

HYALURONAN AND IαI

Various cells in culture, as for example fibroblasts, have been found to produce a pericellular, gel-like structure, or coat, which can be visualized by the addition of particles, such as erythrocytes, to the medium (Clarris & Fraser, 1968). This coat contains hyaluronan, as shown by the fact that it will disappear upon treatment with hyaluronidase. If the culture medium contains serum, the coat will reform after removal of the enzyme. IαI has been shown to be the component of serum that is essential for coat formation (Blom *et al.*, 1995). When analyzing hyaluronan from fibroblasts in culture, Sugahara and coworkers found covalently linked heavy chains which were derived from the medium (Zhao *et al.*, 1995). Heavy chains covalently linked to hyaluronan have also been detected in the synovial fluid of rheumatoid arthritis patients (Hutadilok *et al.*, 1988). Analysis of this material by mass spectrometry showed that the heavy chains were linked to hyaluronan through the same kind of ester bond as had earlier been shown to exist between chondroitin sulphate and heavy chains in inter-α-inhibitor (Zhao *et al.*, 1995) and pre-α-inhibitor (Enghild *et al.*, 1991).

TSG-6 AND IαI

The *TSG-6* gene (the tumour necrosis factor-stimulated gene 6) codes for a 35 kDa protein secreted by various cells, such as fibroblasts, upon stimulation by the proinflammatory cytokines tumour necrosis factor or interleukin 1. (For a review see Wisniewski & Vilcek, 1997). The N-terminal half of the TSG-6 protein (the link module) binds hyaluronan, and its amino-acid sequence shows 36–40% identity with members of the hyaladherin family, including the lymphocyte homing/hyaluronan receptor CD44, cartilage link protein, aggrecan, and versican (Lee *et al.*, 1992). High levels of TSG-6 have been found in synovial fluid of patients with rheumatoid arthritis. When analyzing the TSG-6 protein in this material by immunoblotting, Wisniewski *et al.* (1994) detected not only a 35 kDa band but also one of 120 kDa. Amino acid sequencing indicated that this band contained equimolar amounts of TSG-6, bikunin and one of the IαI heavy chains (HC2). Furthermore, they found that the 120 kDa complex would

form when recombinant TSG-6 and isolated IαI were incubated at 37°C. Based on these observations they suggested that TSG-6 displaces HC1, possibly through a transesterification reaction. They noted, however, that the apparent molecular mass of the complex was not consistent with it containing both bikunin, TSG-6 and one heavy chain. They therefore proposed that it had undergone proteolytic modification. In a later study of the extracellular matrix of COCs, Mukhopadhyay *et al.* (2001) detected a 125 kDa complex, which, based on mass spectrometry, was found to contain HC1, HC2 and TSG-6, but not bikunin. They concluded that the presence of both heavy chains in the analyzed material probably reflected the occurrence of two different TSG-6-containing complexes: one with HC1 and another with HC2. Since TSG-6 binds hyaluronan, the complex between TSG-6 and heavy chain could act as a cross link between different hyaluronan molecules thereby stabilizing the extracellular matrix of the COCs. Furthermore, some of the hyaluronan molecules could be anchored to the cells by binding to proteins such as CD44 (Fig. 5).

In search for a function of the protease inhibitory activity of IαI, Wisniewski *et al.* (1996) discovered that TSG-6 potentiates its antiplasmin activity. They also found that injection of TSG-6 suppresses infiltration of neutrophils into inflamed tissue and suggested that the cause of this effect was inhibition of plasmin by bikunin. In a subsequent study by Day and coworkers based on the analysis of recombinant link modules mutated at different sites, it was found, however, that potentiation of antiplasmin activity did not correlate with inhibition of neutrophil migration (Getting *et al.*, 2002).

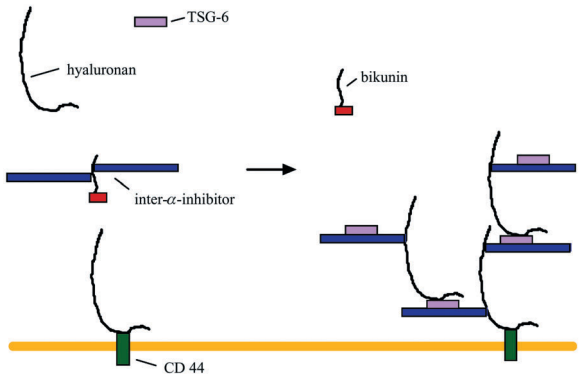

Figure 5. Proposed role of IαI and TSG-6 in stabilizing the hyaluronan-containing extracellular matrix of the complex between cumulus cells and oocytes.

The heavy chains of IαI become covalently coupled to hyaluronan molecules that are either free or bound to a cell surface receptor for hyaluronan (CD44). In this process bikunin is released. In an unknown fashion, TSG-6 becomes linked to the heavy chains. Since TSG-6 can bind hyaluronan (non-covalently), it will cross-link hyaluronan molecules giving rise to a stable structure.

CONCLUSIONS

The identification of a complex between the heavy chains of IαI and TSG-6 in the hyaluronan-rich extracellular matrix of COCs provides a simple explanation for the role of IαI in the stabilization of the COCs. However, the general function of IαI, whose plasma level is stable and equal in males and females, is still unclear. Ovulation has a number of characteristics typical of an inflammatory reaction (Espey, 1994) and it is therefore conceivable that IαI is in fact an inflammatory agent. Certain observations suggest that its activity is regulated by TSG-6, whose expression is clearly controlled by inflammatory signals. Thus, it was recently reported that in a model system, TSG-6 mediates the transfer of heavy chains from IαI to hyaluronan (Jessen & Ødum, 2003). The physiological significance of this transfer is unclear, but *in vitro* experiments have shown that the heavy chains protect hyaluronan against cleavage by reactive oxygen species (Hutadilok *et al.*, 1988). Therefore, the role of the heavy chains could be to contain the destructive effect of activated neutrophils. Plasmin plays an important role in inflammation, and the fact that the anti-

plasmin activity of IαI is enhanced by TSG-6 lends further support to the idea that IαI is an antiinflammatory agent.

Analysis of proteins expressed by fibroblasts cultured on plastic has led to the conclusion that these cells are in a state resembling inflammation (Iyer *et al.*, 1999). It is possible that the hyaluronan-containing coat seen on fibroblasts and other cells also exists in inflamed tissue where it could facilitate cell migration (Camenisch *et al.*, 2000). Unlike the hyaluronan of the COCs, that extracted from fibroblasts has not been reported to contain TSG-6. It is therefore not clear how the heavy chains could stabilize the pericellular coat on these cells. Possibly, they might bind to other proteins yet to be discovered. Interestingly, comparative sequence analysis has indicated that the heavy chains contain a von Willebrandt factor A domain, which in other proteins has been shown to bind to collagens (Bork & Rohde, 1991).

REFERENCES

Blom A, Pertoft H, Fries E. (1995) Inter-α-inhibitor is required for the formation of the hyaluronan-containing coat on fibroblasts and mesothelial cells. *J Biol Chem.*; **270**: 9698–701.

Blom AM, Thuveson M, Fries E. (1997) Intracellular coupling of bikunin and the heavy chain of rat pre-α-inhibitor in COS-1 cells. *Biochem J.*; **328**: 185–91.

Bork P, Rohde K. (1991) More von Willebrand factor type A domains? Sequence similarities with malaria thrombospondin-related anonymous protein, dihydropyridine-sensitive calcium channel and inter-α-trypsin inhibitor. *Biochem J.*; **279**: 908–10.

Bratt T, Olsson H, Sjöberg EM, Jergil B, Åkerström B. (1993) Cleavage of the α_1-microglobulin-bikunin precursor is localized to the Golgi apparatus of rat liver cells. *Biochim Biophys Acta.*; **1157**: 147–54.

Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest.*; **106**: 349–60.

Chen L, Wert SE, Hendrix EM, Russell PT, Cannon M, Larsen WJ. (1990) Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol Reprod Dev.*; **26**: 236–47.

Chen L, Mao SJ, Larsen WJ. (1992) Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-α-trypsin inhibitor family. *J Biol Chem.*; **267**: 12380–6.

Chen L, Russell PT, Larsen WJ. (1993) Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev.*; **34**: 87–93.

Chen L, Mao JT, McLean LR, Powers RW, Larsen WJ. (1994) Proteins of the inter-α-trypsin inhibitor family stabilize the cumulus extracellular matrix through their direct binding with hyaluronic acid. *J Biol Chem.*; **269**: 28282–7.

Clarris BJ, Fraser JRE. (1968) On the pericellular zone of some mammalian cells *in vitro*. *Exp Cell Res.*; **49**: 181–93.

Enghild JJ, Salvesen G, Hefta SA, Thogersen IB, Rutherfurd S, Pizzo SV. (1991) Chondroitin 4-sulfate covalently cross-links the chains of the human blood protein pre-α-inhibitor. *J Biol Chem.*; **266**: 747–51.

Eppig JJ. (1980) Role of serum in FSH stimulated cumulus expansion by mouse oocyte-cumulus cell complexes *in vitro*. *Biol Reprod.*; **22**: 629–33.

Espey LL. (1994) Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol Reprod.*; **50**: 233–8.

Fries E, Blom AM. (2000) Bikunin — not just a plasma proteinase inhibitor. *Int J Biochem Cell Biol.*; **32**: 125–37.

Getting SJ, Mahoney DJ, Cao T, Rugg MS, Fries E, Milner CM, Perretti M, Day AJ. (2002) The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter-α-inhibitor-independent manner. *J Biol Chem.*; **277**: 51068–76.

Heide K, Heimburger N, Haupt H. (1965) An inter-α-trypsin inhibitor of human serum. *Clin Chim Acta.*; **II**: 82–5.

Hutadilok N, Ghosh P, Brooks PM. (1988) Binding of haptoglobin, inter-α-trypsin inhibitor and α_1-proteinase inhibitor to synovial fluid hyaluronate and the influence of these proteins on its degradation by oxygen derived free radicals. *Ann Rheum Dis.*; **47**: 377–85.

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson JJr, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO. (1999) The transcriptional program in the response of human fibroblasts to serum. *Science.*; **283**: 83–7.

Jessen TE, Ødum L. (2003) Role of tumour necrosis factor stimulated gene 6 (TSG-6) in the coupling of inter-α-trypsin inhibitor to hyaluronan in human follicular fluid. *Reproduction.*; **125**: 27–31.

Kaczmarczyk A, Thuveson M, Fries E. (2002) Intracellular coupling of the heavy chain of pre-α-inhibitor to chondroitin sulfate. *J Biol Chem.*; **277**: 13578–82.

Lee TH, Wisniewski HG, Vilcek J. (1992) A novel secretory tumor necrosis factor inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. *J Cell Biol.*; **116**: 545–57.

Morelle W, Capon C, Balduyck M, Sautiere P, Kouach M, Michalski C, Fournet B, Mizon J. (1994) Chondroitin sulphate covalently cross-links the three polypeptide chains of inter-α-trypsin inhibitor. *Eur J Biochem.*; **221**: 881–8.

Morishita H, Yamakawa T, Matsusue T, Kusuyama T, Sameshima-Aruga R, Hirose J, Nii A, Miura T, Isaji M, Horisawa-Nakano R, Nagase Y, Kanamori T, Nobuhara M, Tanaka R, Koyama S, Naotsuka M. (1994) Novel factor Xa and plasma kallikrein inhibitory activities of the second Kunitz-type inhibitory domain of urinary trypsin inhibitor. *Thromb Res.*; **73**: 193–204.

Mukhopadhyay D, Hascall VC, Day AJ, Salustri A, Fulop C. (2001) Two distinct populations of tumor necrosis factor-stimulated gene-6 protein in the extracellular matrix of expanded mouse cumulus cell-oocyte complexes. *Arch Biochem Biophys.*; **394**: 173–81.

Potempa J, Kwon K, Chawla R, Travis J. (1989) Inter-α-trypsin inhibitor. Inhibition spectrum of native and derived forms. *J Biol Chem.*; **264**: 15109–14.

Powers RW, Chen L, Russell PT, Larsen WJ. (1995) Gonadotropin-stimulated regulation of blood-follicle barrier is mediated by nitric oxide. *Am J Physiol.*; **269**: E290–8.

Salier J-P, Rouet P, Raguenez G, Daveau M. (1996) The inter-α-inhibitor family: from structure to regulation. *Biochem J.*; **315**: 1–9.

Sjöberg EM, Fries E. (1992) Biosynthesis of bikunin (urinary trypsin inhibitor) in rat hepatocytes. *Arch Biochem Biophys.*; **295**: 217–22.

Steinbuch M, Loeb J. (1961) Isolation of an α_2-globulin from human plasma. *Nature.*; **192**: 1196.

Thøgersen IB, Enghild JJ. (1995) Biosynthesis of bikunin proteins in the human carcinoma cell line HepG2 and in primary human hepatocytes. *J Biol Chem.*; **270**: 18700–9.

Thuveson M, Fries E. (1999) Intracellular proteolytic processing of the heavy chain of rat pre-α-inhibitor. The COOH-terminal propeptide is required for coupling to bikunin. *J Biol Chem.*; **274**: 6741–6.

Wachter E, Hochstrasser K. (1981) Kunitz-type proteinase inhibitors derived by limited proteolysis of the inter-α-trypsin inhibitor, IV: the amino acid sequence of the human uri-

nary trypsin inhibitor isolated by affinity chromatography. *Hoppe-Seyler's Z Physiol Chem.*; **362**: 1351–5.

Wisniewski HG, Vilcek J. (1997) TSG-6: an IL-1/TNF-inducible protein with anti-inflammatory activity. *Cytokine Growth Factor Rev.*; **8**: 143–56.

Wisniewski HG, Burgess WH, Oppenheim JD, Vilcek J. (1994) TSG-6, an arthritis-associated hyaluronan binding protein, forms a stable complex with the serum protein inter-α-inhibitor. *Biochemistry.*; **33**: 7423–9.

Wisniewski HG, Hua J-C, Poppers DM, Naime D, Vilcek J, Cronstein BN. (1996) TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-α-inhibitor and exerts a strong anti-inflammatory effect *in vivo*. *J Immunol.*; **156**: 1609–15.

Xu Y, Carr PD, Guss JM , Ollis DL. (1998) The crystal structure of bikunin from the inter-α-inhibitor complex: a serine proteinase inhibitor with two Kunitz domains. *J Mol Biol.*; **276**: 955–66.

Zhao M, Yoneda M, Okashi Y, Kurono S, Iwata H, Ohnuki Y, Kimata K. (1995) Evidence for the covalent binding of SHAP, heavy chains of inter-α-trypsin inhibitor, to hyaluronan. *J Biol Chem.*; **270**: 26657–63.

Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y, Kawamura K, Suzuki T, Kimata K. (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. *J Biol Chem.*; **276**: 7693–6.