REVIEW ARTICLE

The inter- α -inhibitor family: from structure to regulation

Jean-Philippe SALIER*, Philippe ROUET, Gilda RAGUENEZ and Maryvonne DAVEAU

INSERM Unit-78 and Institut Fédératif de Recherches Multidisciplinaires sur les Peptides, B.P. 73, 76233 Boisguillaume, France

Inter- α -inhibitor (I α I) and related molecules, collectively referred to as the I α I family, are a group of plasma protease inhibitors. They display attractive features such as precursor polypeptides that give rise to mature chains with quite distinct fates and functions, and inter-chain glycosaminoglycan bonds within the various molecules. The discovery of an ever growing number of such molecules has raised pertinent questions about their pathophysiological functions. The knowledge of this family has long been structure-oriented, whereas the structure/function and structure/regulation relationships of the family members and their genes have been largely ignored. These relationships are

now being elucidated in events such as gene transcription, precursor processing, changes in plasma protein levels in health and disease and binding capacities that involve hyaluronan as well as other plasma proteins as ligands. This review presents some recent progress made in these fields that paves the way for an understanding of the functions of I α I family members *in vivo*. Finally, given the wealth of heterogeneous, complicated and sometimes contradictory nomenclatures and acronyms currently in use for this family, a new, uniform, nomenclature is proposed for I α I family genes, precursor polypeptides and assembled proteins.

INTRODUCTION

In higher organisms, a delicate balance between proteases and their natural inhibitors participates in the control of activation and catabolism of many intra- and extra-cellular proteins. In mammals, the bloodstream is a major carrier for many glycoproteins that act as protease inhibitors. Inter- α -inhibitor (I α I) and related molecules, collectively referred to as the I α I family, are a fascinating group of such plasma protease inhibitors. As detailed below, they display unique and interesting features such as precursor polypeptides that give rise to mature chains with quite distinct fates and functions, and inter-chain glycosaminoglycan bonds within the various molecules. The discovery of an increasing number of such molecules has raised numerous questions about their pathophysiological functions. Until recently, the knowledge of the I α I family was mostly structure-oriented. Indeed, I α I was for many years described as a single entity, and a major breakthrough in the late 1980s was the emergence of a set of related molecules. Six years have now elapsed since a review on I α I summarized structural data about these molecules and outlined the emergence of what is now an acknowledged family of proteins [1]. However, detailed information about the relevant gene structures and mRNA/protein sequences was not available at that time. Moreover, the regulatory steps involved in the synthesis and processing of the chains and molecules had not been studied. Hence the structure/function and structure/regulation relationships of the members of this family, and their genes, were largely ignored. Recently these relationships have been elucidated in events such as gene transcription, precursor processing, changes in plasma protein levels in health and disease, and protein–ligand binding. Therefore this review presents some recent progress made in these fields, as the current research tendency to shift from structure to regulation paves the way for a final understanding of the functions of I α I family members *in vivo*.

As detailed below, an ever-growing number of proteins in the I α I family are being described by various research teams. Concomitantly, an ever-growing difficulty in finding further informative names that decipher the chain assemblies within these proteins has been encountered. At the moment, research into the I α I family is plagued by a wealth of heterogeneous, complicated and sometimes contradictory nomenclatures and acronyms. Clearly, the need for an improved nomenclature comes of age. Accordingly, we wish to take advantage of the present review to propose a uniform nomenclature. For the purpose of clarity some former protein names will be used at the beginning of this review, and the proposed new nomenclature will be introduced later on.

GENES, PRECURSORS AND GLYCOPROTEINS: A STRUCTURAL OVERVIEW

At least four genes, designated *H1*, *H2*, *H3* and *H4*, are involved in the synthesis of I α I family members (Figure 1). The *H1*, *H2* and *H3* genes and cDNAs have been known for some time [1], whereas the *H4* cDNA was cloned quite recently [2]. The cDNA sequences of these genes are clearly homologous [1–3], which implies the occurrence of several gene duplication events from a shared ancestral *H* gene [3,4]. As detailed in Figure 1, each of the *H* loci has been mapped to two homologous areas on human and mouse chromosomes, which indicates that all of the *H* gene duplication events took place prior to the human–rodent divergence [5]. On the basis of protein sequence homologies, the *H2* gene diverged from an *H* ancestor approx. 300 million years ago, while the *H1* and *H3* genes separated later, some 230 million years ago [3]. Indeed, *H1* and *H3* are still located in close physical proximity [4–6]: the human *H1* gene comprises 22 exons, the last one being only 2.7 kb away from exon 1 of *H3* [7,8]. The approximate timing of the separation of the *H4* gene is still

Abbreviations used: I α I, inter- α -inhibitor; α 1m, α 1-microglobulin; AMBP, α 1m/bikunin precursor; P α I, pre- α -inhibitor; I α LI, inter- α -like-inhibitor; PGP, protein–glycosaminoglycan–protein; HNF, hepatocyte nuclear factor; vWA, von Willebrand factor type-A; HA, hyaluronic acid.

* To whom correspondence should be addressed.

Figure 1 A current view of the IαI family, from genes to glycoproteins

This current view of the biosynthetic pathways in the IαI family is modified from a former proposal [1]. The homologous areas of the human and mouse chromosomes to which a given gene maps are indicated by the species (HSA, *Homo sapiens*; MMU, *Mus musculus*) and the chromosome band numbers (*H4* gene mapping: L. Jean, M. G. Mattei, F. Banine and J. P. Salier, unpublished work). Symbols read as follows. mRNAs: thin line, non-coding sequence; black box, open reading frame. Precursor polypeptides: crossed box, signal peptide; hatched box, α1m; double-hatched box, bikunin; black box, N-terminal cleavage product of H precursor; pink box, mature H chain; grey box, C-terminal cleavage product of H precursor. Glycoproteins: wavy line, covalent GPG cross-link. Only the major members of the IαI family are depicted; for the sake of clarity, their former, usual, acronyms as well as the newly proposed nomenclature (see Table 2) are given.

Table 1 Sizes of the H1 to H4 precursor polypeptides and mature chains

HxP, precursor polypeptide; Hx, mature chain found in some IαI-related plasma proteins. The sizes are expressed as the number of amino acid residues, as inferred from the cDNAs and protein sequences published and/or discussed in [2,3,9,10,11,63,64]. The signal peptides as defined in [2,3] are not included in the precursor sizes indicated. Note that the order H2P, H1P, H3P, H4P, as shown, reflects the most closely related polypeptide pairs: H1P and H3P; H3P and H4P [2–4]. A possible trimming of a 36-amino-acid propeptide at the N-terminus of H2P [3,9] is included in the calculations. The H4P polypeptide can undergo some cleavage steps after its secretion into plasma, eventually resulting in a major 633-amino-acid peptide (see text and [2]). n.a., not applicable; n.d., not determined.

	No. of amino acids							
	H2P	H2	H1P	H1	H3P	H3	H4P	H4
Human	928	648	882	643	868	630	902	n.a.
Mouse	928	648	881	642	868	628	n.d.	n.d.

unknown. The mRNAs of these genes are 2.9–3.3 kb in size [1,2,6].

The cDNA-deduced H1, H2 and H3 polypeptide sequences, but not the H4 sequence, are considered to represent precursors [2,3,9,10] (Table 1). Indeed, these precursors undergo extensive

trimming of their C-terminal ends, with removal of 240–280 amino acid residues (Figure 1; Table 1), as judged from the sequences of the mature H polypeptides found in the purified plasma proteins. Furthermore, the H2 precursor displays a stretch of 36 amino acids located between its signal peptide and the N-terminus of the mature H2 chain, which suggests a further trimming of a propeptide at the H2 N-terminus [3,9,11] (Figure 1; Table 1). Based upon the N-terminal amino acid sequences of the purified H chains [11] and alignment of the cDNA-deduced sequences, the presence of a similar propeptide in the H1, H3 and H4 precursors is unlikely, as discussed elsewhere [3].

In addition to the four *H* genes, another gene, designated the α1-microglobulin (α1m)/bikunin precursor (*AMBP*) gene, is involved in the synthesis of most IαI family members (Figure 1; Table 2). The *AMBP* mRNA encodes a precursor for two polypeptides, α1m and bikunin. The *AMBP* gene is comprised of six α1m-encoding exons and four bikunin-encoding exons separated by a large (7 kb) intron, suggesting that the modern *AMBP* gene resulted from the fusion of two ancestral α1m and bikunin genes [12]. As homologous *AMBP* mRNAs have been cloned in many vertebrates, including human, pig, rodents and fishes [13–20], this gene fusion event must have taken place prior to the teleost–tetrapod divergence that occurred some 400 million years ago. The *AMBP*-gene-encoded polypeptide undergoes a cleavage step (see below) that releases α1m and bikunin; these

Table 2 Acknowledged or suggested nomenclatures for the genes, polypeptides and proteins of the I α I family

1. Genes						
Accepted symbol ¹			Former symbols			
Human	Mouse	Encoded polypeptide	Human	Mouse	References	
<i>AMBP</i>	<i>Ambp</i>	α 1m/bikunin precursor	<i>ITIL</i>	<i>Intin-4</i>	[6,65]	
<i>ITIH1</i>	<i>Itih-1</i>	Heavy chain 1 precursor	<i>ITIH1</i>	<i>Intin-1</i>	[6,66a]	
<i>ITIH2</i>	<i>Itih-2</i>	Heavy chain 2 precursor	<i>ITIH2</i>	<i>Intin-2</i>	[6,66a]	
<i>ITIH3</i>	<i>Itih-3</i>	Heavy chain 3 precursor	<i>ITIH3</i>	<i>Intin-3</i>	[6,66a]	
<i>ITIH4</i>	<i>Itih-4</i>	Heavy chain 4	<i>ITIHL1</i>	None	[66b]	
2. Precursor polypeptides						
Proposed acronym		Polypeptide	Former designations		References	
AMBP		α 1m/bikunin precursor	α 1m/bikunin precursor		[5]	
H1P		Heavy chain 1 precursor	Heavy chain 1; H1		[4]	
H2P		Heavy chain 2 precursor	Heavy chain 2; H2		[4]	
H3P		Heavy chain 3 precursor	Heavy chain 3; H3		[4]	
H4P		Heavy chain 4 ²				
3. Mature polypeptides						
Proposed name/acronym		Polypeptide	Former designations		References	
α 1m		α 1m ³	HC protein ('heterogenous in charge'); α 1m		[21]	
Bikunin		Double-headed, Kunitz-type, protease inhibitor	HI-30; ITI light (L) chain; ITI subunit 1 or α ; bikunin		[4,27,67]	
H1		Mature heavy chain 1	Heavy chain 1; H1; HC1; ITI subunit 2 or β		[4,27,67]	
H2		Mature heavy chain 2	Heavy chain 2; H2; HC2; ITI subunit 3 or γ		[4,27,67]	
H3		Mature heavy chain 3	Heavy chain 3; H3; HC3; P α I subunit δ		[4,27,67]	
H4P		Heavy chain 4 ²	PK-120 ⁴ ; IHRP ⁴		[3,10]	
4. Proteins ⁵						
Proposed name/acronym		Combination of mature polypeptides	Former designations		References	
Bikunin		Free bikunin	HI-30; light chain (L); bikunin; trypstatin; urinary trypsin inhibitor		[1,67,68]	
$I\alpha I$ ⁶ or $I\alpha IH1,2,B$		H1 + H2 + bikunin	Inter- α -trypsin inhibitor ($I\alpha I$; ITI; $I\alpha TI$); extracellular matrix stabilizing factor		[1,49,67]	
P αI ⁶ or $I\alpha IH3,B$		H3 + bikunin	Pre- α -trypsin inhibitor (P αI); HC3/bikunin		[27,30]	
$I\alpha IH2,B$		H2 + bikunin	$I\alpha LI$; $I\alpha I$ -related HC2/bikunin; extracellular matrix stabilizing factor; p126		[27,31,46]	
$I\alpha IH2,3,B$ ⁷		H2 + H3 + bikunin	p236		[46]	
$I\alpha IH1,B$		H1 + bikunin	$I\alpha I$ -related HC1/bikunin		[27]	
$I\alpha IH1,2$		H1 + H2 (human)	SHAP ⁴		[47a]	
$I\alpha IH2,3$		H2 + H3 (bovine) ⁸	SHAP ⁴		[47a]	
$I\alpha IH4P$		Free H4P ²	PK-120 ⁴ ; IHRP ⁴		[3,10]	

¹ These gene symbols are currently recommended by the international nomenclature committees for human and mouse genes.² Apart from its signal peptide cleavage, no maturation step of the H4P polypeptide prior to its secretion into plasma has been observed. The single-chain H4P protein can undergo kallikrein-induced cleavages in plasma [2].³ Originates from AMBP but is not to be included in the I α I family.⁴ PK-120, plasma kallikrein-sensitive glycoprotein 120; IHRP, inter- α -trypsin inhibitor family heavy-chain-related protein; SHAP, serum-derived, hyaluronan-associated, protein.⁵ Including some proteins that might be natural or *in vitro* by-products of other I α I family members.⁶ Given the current lack of information about which enzyme(s) are physiological target(s) for this molecule, the former indication 'trypsin' is currently omitted [67].⁷ Described in bovine only.⁸ Mistakenly designated H1 + H2 in [47a].

two proteins are structurally and functionally unrelated. $\alpha 1m$ belongs to a large set of hydrophobic ligand carriers called lipocalins [21–23], and is found in a free state as well as complexed with plasma proteins such as IgAs in the human or $\alpha 1$ -inhibitor-3 and fibronectin in the rat [24]. Bikunin is a Kunitz-type protease inhibitor found in most I α I family molecules (Figure 1). Kunitz-type inhibitors, whose chief member is the well known pancreatic trypsin inhibitor, usually have a low relative molecular mass, a basic isoelectric point and one or several inhibitory domain(s) with a typical disulphide bond arrangement and a broad spectrum of activity towards serine proteases. Two such tandemly arranged, Kunitz-type, inhibitory domains are present in bikunin, the target enzymes of which include trypsin, chymotrypsin, cathepsin G, leucocyte elastase, acrosin and plasmin. However, these enzymes are more efficiently inhibited by other, quantitatively major, plasma inhibitors, which makes the genuine target(s) of bikunin, and hence the biological significance of I α I family members as protease inhibitors, still elusive (reviewed in [1]).

The I α I family of plasma proteins is made up of various structurally related molecules that were initially designated I α I, pre- α -inhibitor (P α I), inter- α -like-inhibitor (I α LI) etc., based on their behaviour in non-denaturing electrophoresis at pH values greater than 8. The major members of this family are depicted in Figure 1, and a full list of the molecules described so far is provided in the final section of Table 2. All evolutionarily related members in the family contain one or more H chains that identify them. For instance, I α I and P α I result from the processing and assembly of H1 + H2 + bikunin and H3 + bikunin respectively. Notably, most proteins in the I α I family contain the bikunin moiety and are protease inhibitors. Bikunin is also found in a free state in plasma, urine and hepatocyte cultures [1,25]. For practical reasons the free bikunin molecule is quoted as a member of the I α I family, although it is not evolutionarily related to the H chains.

The inter-chain link in the molecules is quite unusual. It is covalent and made up of a chondroitin 4-sulphate glycan bond that has been termed a protein–glycosaminoglycan–protein (PGP) cross-link [26]. The involvement of this PGP in the cross-linking of bikunin to mature H chains in I α I, P α I and I α LI is proven [26–28]. The anchor sites on the chains for this PGP bond will be described further below. The PGP cross-link is resistant to reduction but it can be cleaved by chondroitinase, hyaluronidase or mild treatment with NaOH [26,27,29–31]. The complete structure of this chondroitin 4-sulphate glycan bond has been recently reported to be Δ HexA- $\alpha 1$ -3GalNAc(4-sulphate) $\beta 1$ -4GlcA $\beta 1$ -3Gal(4-sulphate) $\beta 1$ -3Gal $\beta 1$ -4Xyl-ol, where Δ HexA is 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid and Xyl-ol is xylitol [32]. Elucidation of the number and sequence of other, more usual, N- or O-linked glycan moieties on the chains of I α I family members still awaits detailed analysis.

FROM STRUCTURE TO NOMENCLATURE REGULATIONS

In Table 2, we propose a uniform nomenclature for I α I-related genes, gene products and proteins. It has been elaborated with advice from others (see the Acknowledgements section) and with the following considerations in mind. (1) Electrophoretic migration as part of a protein name is of limited usefulness, and this parameter has not been evaluated for all I α I family members. Protein designations should instead be informative in terms of structure/assembly, and yet they should remain simple. (2) A new nomenclature should preferably take into account all I α I-related polypeptides and molecules whether or not they are physiologically relevant, as it may be useful to quote a non-

physiological by-product. (3) Ambiguous acronyms such as ‘HC’, which can currently stand for $\alpha 1m$ (synonym: HC protein) as well as a mature H chain, must be avoided; however, a polypeptide precursor and its mature by-products should be distinguished. (4) As exemplified by H4, not all proteins in the family contain bikunin, and therefore a nomenclature of ‘bikunin proteins’, as recently proposed [33], cannot encompass all I α I family members. (5) Addition of further genes, polypeptides and proteins in the future should not compromise the internal consistency of this new nomenclature.

The proposed nomenclature regulations rely in particular on structure. The proposed protein acronyms clearly identify the H chain(s) involved by their number(s) and indicate whether precursors or mature H chains are involved. Indeed, the H chain precursors are designated ‘HxP’ whereas the mature H chains are designated ‘Hx’ (a C-terminal by-product from an HxP could be quoted ‘cHx’ if required in the future). The letter H applies to all subsequently listed H numbers (i.e. x), and these H numbers are separated by commas (in case more than ten H chains are discovered in future investigations). In the protein acronyms the presence of bikunin is marked ‘B’ whenever appropriate. The I α I symbol is retained for the family name and is further included in all acronyms, which allows the reader to immediately identify any protein as an I α I family member. For instance I α LI, comprising H2 + bikunin, is now I α IH2,B. Only in two instances, namely the I α IH1,2,B and I α IH3,B proteins that are historical leaders in the family, do we propose to also use their original, complete, names, i.e. inter- α -inhibitor and pre- α -inhibitor respectively, when appropriate.

FROM GENE REGULATION TO STRUCTURE

Various physiological or pathological phenomena such as the tissue-restricted expression of some proteins, and their quantitative changes during development and organogenesis or an acute inflammatory response, allow one to observe concomitant changes in gene expression and to infer some structural hints about how the transcription of a given gene is controlled. These aspects have been recently investigated within the I α I family.

Gene expression in liver and brain

The pattern of expression of the AMBP and H mRNAs in various tissues has been studied in detail in primates, pigs and rodents [1,3,7,10,15,16,34], and the overall conclusion is clear-cut. The AMBP and H genes are primarily transcribed in the liver, which is the source of all plasma proteins of the I α I family. Transcription of the AMBP gene is tightly regulated, as no other tissue has been found to express the AMBP mRNA, apart from its possible weak expression in pig stomach [15]. The pattern of expression of the H genes is also tightly regulated, but it involves two different organs, namely the liver and, to a lower extent, the brain (and cerebellum). The quantitative expression of the H1, H2 and H3 mRNAs is roughly similar in the liver [3,34,35], whereas an obvious H3 > H2 hierarchy is seen in the brain, where the H1 mRNA is undetectable [3,34]. The transcription of the H4 gene in brain has yet not been studied. The implications of these observations in terms of gene structure are severalfold.

First, the liver-restricted expression of the AMBP and H genes strongly suggests the presence of similar regulatory elements in these genes. Addressing this issue requires a functional analysis of the 5' flanking regions in all I α I family genes. A first, major, advance in this field has been reported recently [36,37]. Indeed, the tissue-specific pattern of expression of the AMBP gene is accounted for by a potent and liver-specific enhancer that drives

Figure 2 Regulation of *AMBP* gene transcription by a liver-specific enhancer

The 5' flanking region of the *AMBP* gene contains an enhancer located 2.7 kb from the promoter. This promoter displays a ubiquitous activity and therefore does not account for the liver-specific transcription of the gene. The enhancer comprises six 'boxes', numbered 1–6, namely two HNF-1-, two HNF-3- and two HNF-4-binding sites, which together increase by about 50-fold the basal activity of the promoter when the gene is in a hepatic context. The various HNF sites act to a variable extent: the quantitative participation of a given box/factor unit in the overall enhancer activity is roughly proportional to the thickness of the line bounding the corresponding rectangle. Within the enhancer, a positive (+) or negative (–) influence of a box/factor unit on another one is proven (solid red arrow) or suspected (broken red arrow). X is an unidentified nuclear protein that binds box 5. The cognate factor of box 6 may be a member of the HNF-4 family but has not been completely identified. The black arrow indicates the start site of gene transcription. After [36,37].

a weak promoter of ubiquitous activity. The *AMBP* enhancer is made up of six functional binding sites for transcription factors, the so-called hepatocyte nuclear factors (HNFs) (Figure 2). Three major HNF proteins, designated HNF-1, -3 and -4, are currently known and a family of related proteins exists for each of them [38]. The HNF-1, -3 and -4 proteins are enriched in the liver and are known to be activators of numerous genes in this organ [38]. A tight cluster of six binding sites for three distinct HNF proteins, as seen in the *AMBP* enhancer (Figure 2), has not been reported for other genes. This enhancer is functional exclusively in a liver cell context, which accounts for the liver-restricted expression of the *AMBP* gene [36,37].

Secondly, the transcription of the *H3* and *H2* genes in the brain suggests that one or more homologous sites for the binding of brain-specific transcription factor(s) to the *H3* and *H2* regulatory regions are likely to mediate the activation of these genes in this tissue. Alternatively, one or more binding sites for a brain-specific factor might be located in both the *AMBP* and *H1* sequences and involved in a brain-specific 'turn-off' of these two genes. Given the shared ancestry of the *H* genes (see above), the first situation is more likely; nevertheless, it implies a late mutation in the *H1* gene with a concomitant loss of a binding site for an activator brain protein.

Gene expression and development

The developmental pattern of expression of the *AMBP* mRNA was first investigated in pig liver [15] but the results should be regarded with caution, as the changes in *AMBP* mRNA levels were normalized against the albumin mRNA level, which itself is particularly prone to development-associated changes. More recently, the developmental pattern of expression of the *AMBP*, *H1*, *H2* and *H3* genes has been investigated in the mouse [34,39]. In the mouse embryo, all four mRNAs are clearly seen in the liver in the late gestation period. mRNA levels peak 4–5 days after birth and eventually return to a significantly lower value in adulthood. The transient and co-ordinated increase in these

mRNAs seen after birth is transcriptionally regulated, which suggests that all four genes are perinatally triggered at the level of similar control elements by development-associated nuclear factors [34]. This conclusion is supported by the strongly impaired perinatal expression of these four genes in a mutant mouse strain that is deficient in transcription factors such as HNF-1 and HNF-4 [34]. Therefore our current view is that similar HNF-binding boxes in the *AMBP* and *H* genes might participate, whether directly or indirectly via interactions with other box/factor units, in a co-ordinated expression of the I α I family genes during development.

Gene expression and acute inflammation

The acute inflammatory condition is known to up- and down-regulate transcription of the so-called positive and negative acute-phase genes respectively in liver. The behaviour of I α I family members in inflammation has been debated for years and a prevailing but false view has long regarded free bikunin as a positive acute-phase protein. Indeed, many contradictory results were published when (i) molecules such as I α IH3,B were still unknown and likely to be confused with free bikunin due to their similar electrophoretic migration and a lack of H-chain-specific antisera, and (ii) cDNAs for the various H mRNAs remained to be cloned. Therefore only recently have we been able to undertake a detailed analysis of the behaviour of the whole set of I α I family mRNAs and proteins (apart from H4) in acute inflammation using appropriate probes [31,35]. Using human serum samples and liver biopsies, as well as hepatoma cell cultures, we demonstrated that the *AMBP* and *H2* genes are down-regulated and the corresponding molecules (bikunin, I α IH1,2,B and I α IH2,B) are negative acute-phase proteins, whereas the *H3* gene is up-regulated and the corresponding I α IH3,B molecule is a positive acute-phase protein. The *H1* gene does not seem to be affected by acute inflammation. Finally, recent data obtained in the rat and pig suggest that the *H4* gene, too, is up-regulated in acute inflammation [40]. The major pro-inflammatory cytokines

interleukin-1 and interleukin-6 mediate the concomitant changes in gene transcription [35]. Our results provided the first example in humans of positive and negative acute-phase plasma proteins that are encoded by evolutionarily related genes [35]. The opposite regulation of the evolutionarily related *H2* and *H3* genes in acute inflammation does not contradict our proposal of homologous elements in the transcriptional control regions of these genes. Indeed, in different genes similar box/factor units can undergo opposite regulation by virtue of further neighbouring (in the present case, interleukin-1- or interleukin-6-driven) box(es) (e.g. [41]).

Overall, our current view is that homologous regulatory elements, e.g. some HNF-binding boxes, allow for a spatially (liver) and temporally (development) co-ordinated expression of the set of genes that synthesize the various chains that are ultimately assembled as I α I family molecules. In unusual circumstances (e.g. acute inflammation), where the amount of a particular precursor is increased compared with the others (e.g. H3P, which drives I α IH3,B synthesis), further, opposite and therefore gene-specific regulations takes place and modulate the basal HNF-mediated transcriptions by virtue of neighbouring box/factor units that differ from gene to gene.

FROM PROTEIN STRUCTURE TO REGULATION

In contrast to the situation for the I α I family genes, a great deal of structural data is available for the corresponding polypeptides and proteins; however, comparatively little is known about how the primary structures participate in precursor processing or protein activities. Indeed, the unusual cleavages of the H precursors, the resulting chain assemblies via a PGP cross-link and the numerous possibilities of such assemblies that are a basis for

diversity in the I α I family raise provocative questions: What are the sequence/function relationships within a chain? How are the precursor cleavages controlled? How are some preferred chain assemblies controlled? What is the purpose of diversity in sequences and molecules within the family? Some of these issues are currently being investigated by various groups.

From chain sequences towards functional domains

The well-known pair of Kunitz-type inhibitory domains are still the only biologically active sites that have been identified in the bikunin sequence. More recently, some progress has been made in the search for putative functional domains within the H1P to H4P sequences (Figure 3). First, all four H precursors and the resulting mature H chains harbour a so-called von Willebrand type-A (vWA) domain that is conserved in the human and mouse [3,42]. A heterophilic binding capacity for proteins with one or more vWA domain(s) seems to be the rule, and the targets for proteins with a vWA domain include such varied molecules as integrins, collagen, proteoglycans and heparin [43]. Secondly, H1P and H3P harbour a multicopper oxidase domain located within their C-terminal segment that is trimmed off during chain assembly [3]. The blue copper-containing oxidases and related proteins form an extremely ancient and diverse group of quite distantly related molecules that have retained or lost the ability to bind copper. Of the plasma proteins, caeruloplasmin and coagulation factors V and VIII are examples of such molecules. Whether the multicopper oxidase domain in H1P and H3P actually binds copper is currently unknown. This domain is absent from H2P and H4P, which is in keeping with the evolutionary distance between H sequences as mentioned above.

Figure 3 Structural comparison of the four H chain precursors and their putative functional domains

The four polypeptides are listed in the order H2P, H1P, H3P and H4P as a function of sequence similarities (see Table 1). A vertical broken line separates the N-terminal two-thirds of H4P which is homologous to the H3P, H1P and H2P counterparts from the C-terminal one-third of H4P that is not homologous to the other three polypeptides. Arrowheads indicate cleavage sites: black arrowhead, processing of H1P, H2P and H3P; red arrowhead, plasma kallikrein-sensitive site. Other symbols are as follows: stippled box, N-terminal propeptide [3,9]; pink box, vWA domain [3,42]; black box, Asp-(Pro-His-Phe-Ile-Ile) anchor site for a PGP cross-link (see text); crossed box, multicopper oxidase domain [3]; double-hatched box, plasma kallikrein-released, bradykinin-like, peptide (sequence: Pro-Gly-Val-Leu-Ser-Ser-Arg-Glu-Leu-Gly-Leu-Pro-Gly-Pro-Pro-Asp-Val-Pro-Asp-His-Ala-Ala-Tyr-His-Pro-Phe-Arg) [2]; hatched box, ATP-dependent-protease-like domain [10]. The relative sizes of all polypeptides and domains are drawn to scale, but minor differences in the numbers of amino acids (a.a.) between polypeptides or between species are not taken into account. The signal peptides are omitted.

Thirdly, the H4P C-terminal sequence harbours (i) a proline-rich, bradykinin-like domain (see the legend to Figure 3) bracketed by two Arg residues that allow it to be released by plasma kallikrein [2], and (ii) a domain with some similarity to an ATP-dependent protease [10]. The significance of this last observation remains to be confirmed, as the putative active site of the ATP-dependent protease appears to lie outside the region of similarity (as inferred from a similarity search made in our laboratory). More generally, it must be stressed that all the domains delineated so far in the H precursors are inferred from sequence similarity searches made against protein data banks or published sets of consensus amino acid sequences for domains with a given biological function. Therefore, even though the domains present in the H polypeptides are significant from an evolutionary viewpoint, they are only suggestive of functional activities that remain to be demonstrated experimentally.

From precursor structure to regulation of processing and assembly

The series of events that leads to the maturation, glycosylation and assembly of AMBP, H precursors and mature chains thereof takes place intracellularly in the liver [25,33,44,45]. Given that $\alpha 1m$ and bikunin have no known functional connection, their co-synthesis as a single precursor, i.e. AMBP, is unique compared with other pro-proteins such as pro-complement components or pro-hormones. Hence whether or not the co-expression of $\alpha 1m$ and bikunin is of functional significance is an important issue. The cleavage of AMBP into $\alpha 1m$ and bikunin takes place at the C-terminal end of a connecting tetrapeptide comprising a conserved motif including three basic amino acids [13–20]. As this motif is known to be a typical target for pro-protein processing proteases such as furin, this protease was suspected to be responsible for AMBP processing [13,44]. However, although the cleavage of AMBP by furin is possible *in vitro*, the regulatory protease involved *in vivo* is unlikely to be furin; indeed, a recombinant AMBP expressed in a furin-deficient mammalian cell line is still cleaved into $\alpha 1m$ and bikunin [45]. The cleavage step takes place during AMBP transport in the Golgi apparatus of liver cells [44]. Most importantly, recombinant bikunin molecules that are cleaved from recombinant AMBP or are synthesized alone display a difference in size that is accounted for by a difference in glycosylation and chondroitin 4-sulphate content [45]. Therefore the glycosylation and sulphation of bikunin, which are known to take place prior to AMBP cleavage in the Golgi [25], might be modulated by the adjacent $\alpha 1m$ sequence, as recently proposed [45]. Furthermore, the H-PGP-bikunin assemblies that follow bikunin sulphation and provide the I α I family members seem to be formed prior to AMBP cleavage [25]. Therefore the regulation of assemblies containing bikunin by the adjacent $\alpha 1m$ sequence, in as much as it controls the chondroitin 4-sulphate attachment, is an attractive possibility.

The processing of H precursors and the formation of their assemblies with bikunin is quite unusual as it involves the shedding of a large C-terminal peptide (240–280 amino acid residues) from the H precursors and the attachment of an inter-chain covalent PGP cross-link, as mentioned above. While the fate and biological function of the C-terminal peptide released from the H precursors remain unknown, other requirements and events involved in these chain assemblies have been investigated in detail. In the HepG2 hepatoma cell line, which is defective in the synthesis and assembly of most I α I family members, the abnormal presence of a mature but PGP-free H2 chain, in addition to the expected I α IH2,B protein, indicates that the mechanism responsible for the C-terminal cleavage of H pre-

cursors and the formation of the PGP cross-link is divided into two discrete steps, involving proteolytic cleavage followed by carbohydrate attachment [33]. This PGP cross-link is mediated by a chondroitin 4-sulphate chain attached to Ser¹⁰ of bikunin by an O-glycosidic link and esterified to the C-terminal Asp of H1, H2 or H3 by an N-acetylgalactosamine in the chondroitin 4-sulphate chain [26,27]. The C-terminal Asp residue on the mature H chains becomes so located by shedding of the C-terminal peptide from the H precursors. It has been observed that this Asp residue is part of a homologous Asp-Pro-His-Phe-Ile-Ile sequence that is found in H1P, H2P and H3P from humans and rodents [2,26,27]. Therefore this conserved sequence in H precursors appears to be a good candidate as a recognition unit and/or a target for one or more processing enzymes involved in C-terminal cleavage at the Asp-Pro junction and addition of PGP/bikunin to the Asp residue. This view has been supported by the recent discovery of a natural mutant of an H precursor, namely H4P. Indeed, the Asp-Pro-His-Phe-Ile-Ile sequence is absent from H4P [3,10], which correlates with the physiological lack of H4P C-terminal cleavage and H4/bikunin assembly in liver cells (Figure 1; Table 2).

If the chain assemblies were not controlled further, the I α I family would comprise a large variety of molecules. A theoretical possibility exists that some or all of the plasma molecules listed in Table 2 are by-products induced *in vitro* from a (fragile) super-molecule such as I α IH1,2,3,B that could give rise to all possible chain combinations. This proposal is quite unlikely, however, given (i) the unequal amounts of I α IH1,B, I α IH2,B and I α IH3,B in plasma [27,30], (ii) some differences between species, as exemplified by the I α IH2,3,B molecule that has been described in bovine plasma [46] but is unknown in the human (Table 2), and (iii) the simultaneous decrease in plasma I α IH1,2,B and increase in I α IH3,B in inflammation (see above) that cannot be accounted for by degradation of a super-molecule as a shared precursor. Therefore it is reasonable to consider that the limited variety of molecules in the I α I family is because certain chain assemblies (e.g. I α IH1,2,B and I α IH3,B) are preferred to others (e.g. I α IH1,3,B, which is as yet unknown) in the biosynthetic pathways. In this respect, an unequal synthesis of H1P, H2P and H3P in the liver is a simple way to favour some assemblies and, hence, the relative amounts of I α I family members. Indeed, a limited number of studies suggest that H2 mRNA and H2 may predominate slightly in liver and plasma respectively [31,34,35], which is in keeping with I α IH1,2,B and I α IH2,B being the quantitatively predominant plasma molecules, as seen in both human [30,31,35] and mouse (J. P. Salier and L. Jean, unpublished work). Apart from this relatively simple, quantitative, control of H precursors that in turn regulates the relative amounts of plasma molecules, it remains to be determined whether some sequence-borne information that differs between the various H precursors participates in the fine tuning of chondroitin 4-sulphate chain attachment and further regulates the occurrence of preferred assemblies.

From protein structure to regulatory functions: the hyaluronic acid (HA) connection

Significant progress has recently been made in the search for the biological functions of some I α I family members. Notably, a HA-binding capacity has been ascribed to some of them. Although serum I α IH1,2,B is not bound to HA under normal circumstances, adding HA to human or bovine serum promotes the occurrence of a bikunin-free, covalent, HA-protein complex that involves H1 + H2 in human [47a,48] and H2 + H3 in bovine [47a] serum (in [47a] an H3 sequence was incorrectly described as

H1). Likewise a natural, bikunin-free, complex comprising HA and H chains, including H1, can be observed *in vivo* in HA-rich media, e.g. normal human follicular fluid or synovial fluid from rheumatoid arthritis patients [48]. It has been proposed that these complexes originate from human I α IH1,2,B or bovine I α IH2,3,B from which bikunin has been shed and replaced by HA [47,47b].

The physiological relevance of a HA-binding capacity for I α I family members has been established with various cell types that display a HA-containing pericellular coat. Notably, the involvement of I α I family members in the process of oocyte maturation has been studied. During the pre-ovulatory period the cumulus cells of the so-called cumulus–oocyte complex secrete large amounts of a HA-enriched extracellular matrix and dissociate from each other as a consequence of the endogenous gonadotropin surge (reviewed in [49,50]). One or more I α I family members (e.g. I α IH1,2,B as well as I α IH3,B, I α IH2,B or bovine I α IH2,3,B) are absolutely required to limit this cumulus–oocyte complex expansion, which otherwise results in a deleterious loss of HA and cumulus cells [46,49–51]. A charge-mediated, non-covalent, binding of HA to I α I family members is likely to participate in this control of cumulus–oocyte complex expansion [50,52], whereas a major role for the enzyme inhibitory activity of bikunin has been ruled out [50]. The I α I family members involved in this physiological control of cumulus–oocyte complex expansion most likely originate from the blood. Indeed, I α IH1,2,B and I α IH3,B are present in the follicular fluid and ovarian follicles in both human and mouse after chorionic-gonadotropin-stimulated ovulation [49], and I α IH1,2,B molecules injected intravenously into the mouse are exclusively localized to the vasculature until an experimental surge of luteinizing hormone allows them to pass the blood–follicle barrier within seconds [51]. These results outline the importance of blood-borne I α I family molecules in the regulation of extracellular matrices that may lie outside the blood vessels. This regulatory function of I α I as a stabilizer of HA-containing pericellular coats has also been seen *in vitro* with other cell types, namely fibroblasts and mesothelial cells in culture [53].

The HA-binding capacity of I α I family members has also been demonstrated to be significant in inflammatory conditions. In patients with rheumatoid arthritis and some other arthritic diseases, the so-called TSG-6 glycoprotein, an anti-inflammatory molecule and a member of the hyaladherin family of HA-binding proteins, is found at high levels in the synovial fluid and, at a lower concentration, in the plasma [54–56]. A unique feature of TSG-6 is its propensity to form a reduction-resistant complex with I α IH1,2,B; this complex involves the glycosaminoglycan bond harboured by I α IH1,2,B [53]. When TSG-6 and purified I α IH1,2,B are mixed *in vitro*, the resulting complex has been shown to comprise TSG-6, H2 and bikunin, which suggests shedding of an H1 chain [55]. Likewise, within complete plasma the involvement of I α IH1,2,B in a complex with TSG-6 is clear, even though the number and identity of other I α I family member(s) possibly participating in further complex(es) with TSG-6 *in vivo* should be borne in mind [55]. Indeed, in acute inflammation, plasma I α IH3,B is replaced by a heavier molecule that is resistant to reducing conditions and may carry a ligand [35]. If so, the ligand could well be TSG-6. Therefore whether I α IH3,B and TSG-6, two up-regulated acute-phase proteins [35,54], are indeed a source for a covalent TSG-6 + H3 + bikunin complex *in vivo* remains an important issue. Finally, the biological significance of the complex between TSG-6 and I α IH1,2,B is being deciphered, as this complex enhances the plasmin-inhibitory activity of bikunin, a very weak plasmin inhibitor [56]. Also, in a model of experimentally induced inflammation in the

mouse, TSG-6 mutants that have lost their anti-inflammatory activity are unable to potentiate plasmin inhibition, although they still form a complex with I α IH1,2,B [56]. These data suggest the involvement of several acute-phase plasma proteins, namely TSG-6 and I α I family members, in a negative feedback loop whereby plasmin, itself a central molecule in the activation of inflammation-associated enzyme cascades, is inhibited [56]. Such a regulatory loop may prove to be important in a number of tissues, given for instance the high contents of HA, TSG-6 and I α I family members in the synovial fluid of inflamed joints [54,57].

Finally, finding a link between one or more of the potential domains in the H precursors and/or mature chains (Figure 3) and a proven biological function of some I α I family members is a major aim of current studies, yet for the most part such links still remain to be established. However, in the *in vitro* induced H1 + H2/HA complex detailed above [47a], the HA-bound region has been tentatively located within the C-terminal half of H1 [47a], i.e. in the middle of the vWA domain (Figure 3). As targets for proteins with a vWA domain include proteoglycans and heparin [43], we have proposed that the vWA domain in H chains is involved in the HA-liganding activity [3]. If this is true, all H chains are likely to bind HA by virtue of their vWA domain (Figure 3), which is consistent with: (i) the diversity of complexes induced *in vitro*, i.e. H1 + H2/HA and H2 + H3/HA [47a], and (ii) the diversity of I α I family members that are able to stabilize HA-containing cell coats, as detailed above. This vWA domain-mediated binding of HA to H chains, if it occurred, would be transient, charge-mediated and non-covalent (see above) and followed *in vivo* by a final, covalent linkage. Indeed, such a covalent linkage between HA and the H chains, as recently seen in arthritic joints, has been tentatively localized to the C-terminal Asp residue of H chains [47b]. This suggests that a complex of HA with members of the I α I family may be formed by transferring some H chains from their PGP cross-link to HA by a substitution reaction [47b]. This last event is likely to be enzymically controlled (L. Chen, H. Zhang, R. W. Powers, P. T. Russell and W. J. Larsen, unpublished work).

CONCLUSIONS AND FUTURE TRENDS

In the last 6 years of research into I α I family members, shifting from a mostly structural description of a new family to an analysis of the fine-tuned regulation associated with genes and molecules thereof has proved rewarding. We now know, at least partly, where, when and why the *AMBP* and *H* genes are co-expressed. We are gaining some preliminary hints as to how the precursor polypeptides are processed and assembled. The precursors and molecules of the I α I family harbour some previously unexpected regulatory functions that have shed light on the importance of the H chains, in addition to the well-known protease-inhibitory capacity of bikunin. Finally, some of the above mechanisms have now been evaluated under pathological conditions.

Not surprisingly, as a spin-off of these recent studies further complexity has been brought to the field. While previous questions are not yet fully resolved, we are now facing new ones. Among these the need, if any, for combined HA-binding activity and protease-inhibitory activity within a molecule is an interesting one. Other regulation-oriented issues that warrant investigation include: (i) possible control of the activity of genes, mRNAs or proteins by sequence polymorphisms, as the latter have been described in detail by several groups (e.g. [6,7,58]); (ii) a role for bikunin-free H chains in the blood [2,10], brain [3] and possibly other tissues; (iii) the fate and functions of the H chain C-

terminal peptides released during formation of H+ bikunin assemblies; (iv) the fate and functions of blood-borne bikunin after it enters tissues such as normal skin [59] or brains of Alzheimer patients [60]; and (v) the capacity of bikunin to inhibit a plasmin-driven proliferation of tumour cells that harbour surface receptors for bikunin (reviewed in [61,62]). Some of these forthcoming research topics will undoubtedly prove beneficial to the elucidation of major pending issues that include the co-regulation of genes, the likely differences in biological activity between chains and between members of the I α I family and, ultimately, the precise biological function(s) of all I α I family members *in vivo*.

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