REVIEW ARTICLE

Molecular biology of tissue kallikrein

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Introduction

Tissue kallikrein (or glandular kallikrein as it was called first) is a late bloomer. Sixty-three years after its discovery in urine as a hypotensive material and half-a-century after the description of its enzymic activity (Frey *et al.*, 1950, 1968), many of its functions are still being unravelled. Tissue kallikrein is a simple serine protease that is related in many regards to trypsin, but with a higher specificity for polypeptide substrate cleavage sites. Its principal known biochemical function is the highly selective cleavage of the plasma protein kininogen at two peptide bonds to release stoichiometrically the vasoactive and spasmogenic decapeptide kallidin (Lys¹-bradykinin) (Fiedler, 1979; Fig. 1).

Blood plasma contains a complex serine protease of high M_r that selectively cleaves kininogen to release bradykinin. Although both the plasma and tissue kallikreins are initially synthesized as inactive pro-enzymes, release a kinin and cleave ester and peptide bonds after basic amino acids, they differ in many other properties and are clearly distinctly different enzymes. The activation of the plasma kallikrein can involve the blood clotting and complement systems (Kaplan & Silverberg, 1987; Colman *et al.*, 1985), while the activation of tissue kallikrein appears to be less complex. It is also possible that we just know less about the activation of tissue kallikrein than about the cascade which activates plasma kallikrein.

Kallikreins have also been referred to as kininogenases (Webster, 1970). A number of other serine proteases

have been called kininogenases as well (Schachter, 1980), because they can release a bioactive kinin (although inefficiently and non-stoichiometrically) from kininogen. These proteases are closely related to tissue kallikrein; they have very similar enzymic and physical properties and similar amino acid sequences and often cross-react with polyclonal and occasionally monoclonal antibodies to kallikrein (Woodley *et al.*, 1985). However, these other proteases appear not to function physiologically as alternative kallikreins. Tissue kallikrein and these related proteases are members of a family of very similar genes. In this review we use the term kallikrein for only the *bona fide* tissue kallikrein, which is probably a single enzyme in most species. Related proteases are referred to as kallikrein-like (and their genes as kallikrein-related) or as their known functional identities (e.g., γ -NGF, EGF-BP).

Historically, tissue kallikrein was first named glandular kallikrein, because exocrine glands such as the salivary gland or pancreas contained the highest kallikrein concentration. As discussed below, in some species kallikrein or its closely related family members have a much wider distribution outside these glands, and are present in many organs ranging from the brain to genital tract.

The kallikrein gene family

The kallikrein gene family is a set of closely related genes named after the protein product of its most prominent and first sequenced member, tissue kallikrein

Fig. 1. Kinin generation by tissue kallikrein from the low- M_r kininogen precursor and inactivation by kininases

The relevant sequence shown for kininogen is for both the bovine (Nawa *et al.*, 1983) and human (Takagaki *et al.*, 1985) forms.

Abbreviations used: α - and γ -NGF, α and γ subunits of the mouse nerve growth factor complex; EGF-BP, epidermal growth factor binding protein; mGK, designation for a mouse genomic kallikrein gene; NEP, neutral endopeptidase 24.11, enkephalinase

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Fig. 2. Structure of preprokallikreins from several species

Key amino acid residues for catalysis by serine proteases (denoted by asterisks) and kallikrein-like preferences for substrate cleavage (Chen & Bode, 1983) are indicated. These crucial residues are conserved in the other identified true kallikreins, with the few exceptions shown. The positions of the signal (Pre) and activation (Pro) peptides are indicated; cleavage of the signal peptide occurs at the *N*-terminal side of alanine (residue -7) (Takada *et al.*, 1985; Takahashi *et al.*, 1986); the structure of the prepro region of the pig enzyme (broken line) is not known. Numbering is for the rat, mouse and human proteins; the sites of a two-residue insertion and a region deleted by protease action (broken section) for the pig enzyme are shown. See text and Table 1 for the sources of amino acid sequence information.

(Tschesche *et al.*, 1979). The family members are characteristically very closely related, so that long hybridization probes derived from one detect all or nearly all others. Estimates of the size of the family are based on the number of cross-hybridizing genes. The number of members may vary greatly among mammals: the mouse has 24 member genes (Evans *et al.*, 1987), the rat 10 (Ashley & MacDonald, 1985b) to 17 (Gerald *et al.*, 1986); and possibly the hamster as few as three (Howles *et al.*, 1984). The size of the human gene family appears smaller than that of mouse and rat (Fukushima *et al.*, 1985; Baker & Shine, 1985), but contains at least four distinct genes: kallikrein and an unidentified partial gene sequence (Fukushima *et al.*, 1985; Baker & Shine, 1985), prostate-specific antigen (Watt *et al.*, 1986; Lundwall & Lilja, 1987), and a characterized but unidentified gene termed hGK-1 (Schedlich *et al.*, 1987). Clearly, the number of kallikrein gene family members varies greatly and rapidly in mammalian evolution.

Because all family members are so closely related, individual genes must be distinguished by very discriminating means such as the nucleotide sequence of the gene or mRNA, or by specific oligonucleotide hybridization probes complementary to short regions that vary among family members (Ashley & MacDonald, 1985b; van Leeuwen *et al.*, 1986). The protein products of individual genes can only be distinguished by monoclonal antibodies of proven specificity (Woodley *et al.*, 1985).

The identification of the mRNA (Swift *et al.*, 1982) and the gene (Pritchett & Roberts, 1987) for the rat true kallikrein has been made by comparison of the amino acid sequence derived from the nucleotide sequences with the amino acid sequence of the purified enzyme (Lazure *et al.*, 1981). Identification of the gene for *bona fide* kallikrein of mouse is more problematic, because the kallikrein protein of mouse has not been sequenced for comparison with the gene-derived amino acid sequence. Rather, the identification of the mouse gene (designated mGK-6) encoding true kallikrein has been based on the characteristic spectrum of tissue-specific expression (pancreas, submaxillary gland, and kidney) for true kallikrein. Moreover, mGK-6 is the only mouse kallikrein family member expressed in the kidney (van Leeuwen *et al.*, 1986).

The identification of human kallikrein mRNA is based upon its expression in kidney and pancreas (Fukushima *et al.*, 1985; Baker & Shine, 1985), but has not been confirmed. Key amino acid residues proposed to be primary determinants of true kallikrein polypeptide cleavage specificity (Chen & Bode, 1983) are conserved among pig, rat, mouse and human kallikreins (Fig. 2).

It appears that only a single member (mGK-6) of the mouse multigene family encodes *bona fide* tissue kallikrein (van Leeuwen *et al.*, 1986). Although this probably applies to human, rat and hamster as well, it cannot be stated conclusively yet, because all the kallikrein gene family members have not been analysed for these species. The other genes of the kallikrein family encode closely related enzymes which distinctly differ from kallikrein; some release lysyl-bradykinin from kininogen inefficiently and non-stoichiometrically. They may be called kininogenases to distinguish them from genuine tissue kallikrein. The protein products of these genes often differ at key amino acid residues that would likely alter the cleavage specificity for polypeptide substrates. Some of these kallikrein-related enzymes have been identified and characterized in detail, as described below.

The gene or mRNA for any individual kallikrein family member cannot be identified by comparing amino acid sequences across species, because sequences differ more for the same gene across species than for most of the family members within a single species. For example, the amino acid sequence identity among the five known active members of the rat kallikrein family (74–89%) is greater than the sequence identity among the four known true kallikreins from different species (ranging from 59 to 70% in pair-wise comparisons; see Table 1). [However, the kallikreins from different species retain the key amino acid residues tyrosine-99 and tryptophan-215, aspartate-189, and glycine-216 and serine- or alanine-226 proposed to be primary determinants of the characteristic cleavage specificity of the kininogen substrate (Fig. 2).] Sequence conservation within the family holds true for mouse as well (Evans *et al.*, 1987). The basis for this phenomenon is the concerted evolution (homogenization) of closely linked gene family members by any of a variety of genetic

Table 1. Amino acid sequence relationships among the identified true kallikreins of pig, rat, mouse and human

	Amino acid sequence identity (%) ^a		
	Rat ^b	Mouse ^c	Human ^d
Porcine ^e	61	61	65
Rat	—	70	59
Mouse	—	—	63

^a Comparisons are for the amino acid sequences of the proposed mature, active enzymes.

^b From the mRNA (Swift *et al.*, 1982).

^c From gene mGK-6 (van Leeuwen *et al.*, 1986).

^d From the mRNA (Fukushima *et al.*, 1985; Baker & Shine, 1985).

^e From the purified enzyme (Tschesche *et al.*, 1979; Bode *et al.*, 1983).

mechanisms (Dover, 1986; Dover & Tautz, 1986). Therefore, whereas the mouse and rat families have evolved independently, the family members within each species tended to co-evolve. In contrast, the relative lower sequence conservation among human kallikrein family members indicates that the genes either are separated by greater distances or perhaps dispersed within the genome.

The many kallikrein gene family members encode structurally and functionally homologous serine proteases with similar preferences for cleavage of synthetic peptide substrates after arginine residues. The natural polypeptide substrates for some of the kallikrein-like enzymes have been identified, and suggest important physiological roles. Rat tonin (Ashley & MacDonald, 1985a; Lazure *et al.*, 1987) can cleave angiotensinogen directly to angiotensin II *in vitro* (Boucher *et al.*, 1974). Three epidermal growth factor binding proteins (EGF-BPs A, B, and C) are encoded by different genes (mGK-22, -13 and -9, respectively) (Drinkwater *et al.*, 1987; Blaber *et al.*, 1987). At least one, but possibly all three are thought to cleave the precursor of epidermal growth factor to the active form (Frey *et al.*, 1979). The γ subunit of nerve growth factor (γ -NGF), which appears to cleave the precursor of the β subunit of nerve growth factor to yield the active form (Berger & Shooter, 1977; Thomas *et al.*, 1981), is a typical member of the gene family (mGK-3) (Ullrich *et al.*, 1984; Evans & Richards, 1985). The EGF and NGF processing enzymes remain bound as part of the growth factor complexes secreted by the submaxillary gland (Taylor *et al.*, 1970; Varon *et al.*, 1968; Burton *et al.*, 1978). The third subunit (α -NGF) of the NGF complex is an enzymically inactive kallikrein family member encoded by gene mGK-4 (Isackson & Bradshaw, 1984; Isackson *et al.*, 1984; Evans & Richards, 1985). Because many of the identified members of the gene family participate in prohormone processing (Taylor *et al.*, 1970; Bothwell *et al.*, 1979; Fiedler & Fritz, 1981) it has been proposed that other family members may process precursors of other bioactive peptides.

Mutations in some family members produce altered proteins with interestingly modified functions. As mentioned above, at least one member encodes an inactive serine protease remnant, α -NGF, that still binds a

potential polypeptide substrate, β -NGF, without cleavage. The mutations responsible for the inactivation of the protease function have been determined by sequence analysis of the protein (Ronne *et al.*, 1984; Isackson & Bradshaw, 1984) and the cloned mRNA (Isackson *et al.*, 1984) and gene (Evans & Richards, 1985). A principal alteration prevents the cleavage and release of the activation peptide, which is necessary for the formation of an active protease.

Not all genes of the family express proteins, however. More extensive mutations in 10 of the 24 mouse genes (Evans *et al.*, 1987) and at least one rat gene (Gerald *et al.*, 1986) have generated inactive pseudogenes. Pseudogenes are recognized by extensive alterations that destroy either the potential to be transcribed (extensive mutations within regulatory regions) or to produce functional mRNA or protein (e.g., gross disruption of exon and intron organization or the accumulation of multiple stop codons in all reading frames). The evolutionary degeneration of active, functional genes to pseudogenes is a natural consequence of the presence of redundant genes within gene families. The existence of multiple pseudogenes in gene families suggests the possibility that there are other redundant members that remain active and may have accumulated mutations that alter enzyme activity, including substrate specificity. Thus, biochemists and molecular biologists may face the disconcerting possibility that some of the expressed enzymes may have no biochemical or physiological role. It is in the nature of the process of the evolution of large gene families with very closely related and closely spaced genes that makes this a strong possibility for individual family members.

The kallikrein gene family may be viewed as a very tightly clustered group of genes encoding very similar enzymes that are disparately expressed in a variety of tissues. The mouse kallikrein genes are only about 5 kbp in length and often separated by 5 kbp or less (Mason *et al.*, 1983; Evans *et al.*, 1987); thus they are among the most tightly clustered protein-coding gene families yet described for mammals. In contrast to this tight linkage and very high sequence similarities, the individual gene members often are expressed dissimilarly (Ashley & MacDonald, 1985b; van Leeuwen *et al.*, 1986). Whereas at least one member of the kallikrein gene family is represented in many tissues, individual members often are expressed in one or a very few tissues. These properties make the kallikrein gene family an interesting paradigm for the study of gene regulation.

Future directions

Three important areas of the molecular biology of the kallikrein gene family demand further investigation.

(1) The mechanisms involved in the concerted evolution of the tandemly linked gene family members. The mouse kallikrein genes have been shown to be tightly clustered on chromosome 7 (Mason *et al.*, 1983; Howles *et al.*, 1984). The rat genes appear clustered as well (D. Wines, J. Brady & R. MacDonald, unpublished work). The close linkage of the family members plus the great homology among family members within a species suggest extensive gene correction via gene conversion, slippage replication or nonreciprocal recombination. Moreover, the large apparent fluctuation in family size among mammals suggests the deletion or duplication of

genes by nonreciprocal recombination. Clues to the mechanisms of the co-evolution of this gene family lie within the nucleotide sequences of the genes and adjacent regions.

(2) The genetic and molecular mechanisms for the differential expression of individual family members in various tissues. The fundamental mechanisms of cell-specific gene expression are only recently being investigated successfully (e.g., Maniatis *et al.*, 1987). The kallikrein gene family offers certain advantages that stem from the conserved gene sequences. In contrast with the remarkably divergent regulation of individual family members, the nucleotide sequence of the genes of a given species are highly conserved throughout exonic, intronic and nearby 5' and 3' flanking domains. Differences in the 5' flanking sequences, which likely harbour cell-specific regulatory sequences, are limited to modest base pair substitutions, insertions and deletions (van Leeuwen *et al.*, 1986), so that relatively minor sequence differences appear to cause the disparate regulation of individual genes. Comparative analysis of these sequences may reveal the general nature of cell-specific regulatory elements and the nature of subtle sequence differences that can alter cell-specific transcription.

(3) The biochemical and physiological roles of the serine proteases encoded by the multiple kallikrein-related genes that have been discovered, sequenced and characterized by gene cloning techniques. Cloning and sequencing multiple gene family members has uncovered a wealth of new serine proteases closely modelled on kallikrein structure and enzyme action, but with unknown biological functions. Since many of these kallikrein-related genes (and cloned cDNAs) are available, expression in suitable prokaryotic or eukaryotic hosts would permit characterization of their encoded enzyme products as an approach to identifying their natural polypeptide substrates. Several rat submaxillary kallikrein-like enzymes have been characterized biochemically (Khullar *et al.*, 1986; Chao, 1983). It would be informative to correlate the individual enzymes with cloned mRNAs and genes (Ashley & MacDonald, 1985a,b). If this gene family does encode a collection of proteases that process polypeptide hormone precursors, then the identification of their biological substrates would be an exciting endeavour indeed.

Regulation of kallikrein gene expression

Studies of the regulation of kallikrein gene expression have thus far been confined to some of the rat or mouse tissues where kallikrein concentration is known to be relatively large (10 ng/mg of protein or more), such as in the major salivary glands, kidney and pituitary. Salivary gland kallikrein-like enzymes respond to androgen by changes in tissue levels of immunoreactive enzyme or rate of synthesis of kallikrein (Chao & Margolius, 1983; van Leeuwen *et al.*, 1987; Miller *et al.*, 1984). There is also sexual dimorphism in this gland; enzyme concentrations and RNA levels are roughly 2-fold higher in the male than in the female gland. Of more recent interest is the work which has begun to dissect the relative expression of individual kallikrein gene family members in the mouse salivary glands (van Leeuwen *et al.*, 1987). One kallikrein gene, designated mGK-6 and considered to encode the mouse renal kallikrein (van Leeuwen *et al.*,

1986), is expressed constitutively. It does not show sexual dimorphism, and is unresponsive to, or even repressed by, testosterone. The cellular distribution of mGK-6 gene expression, assessed by *in situ* hybridization histochemistry, appears to differ from that of the hormonally-inducible mGK-3 (γ -NGF), -4 (α -NGF) and -5 genes. Testosterone induction of mGK-3, -4 and -5 gene expression appears to accompany a differentiation of the striated ductal cells to granular convoluted tubular cells. The time course of induction of these genes generally confirms the impression gathered in several previous studies, that large doses of hormone, administered for up to 7 days, are required to observe the concomitant changes in gene expression and tissue morphology. These results suggest strongly that hormonal regulation of the salivary kallikrein-like enzymes is not mediated by direct hormone action on the genes or their regulatory elements. Nevertheless, the tissue and gene specificity of this androgen response suggests that the induction of these enzymes might play some role in the associated cell proliferation and cytodifferentiation.

At least one kallikrein gene is expressed in the mouse, rat, or human kidney (van Leeuwen *et al.*, 1986; Fuller *et al.*, 1986; Ashley & MacDonald, 1985b; Fukushima *et al.*, 1985; Baker & Shine, 1985). It is quite clear that the concentrations of protein encoded by the gene(s) are very sensitive to multiple influences such as several steroid and polypeptide hormones, anatomical interventions (e.g., renal artery constriction), and induced or heritable diseases (Fuller & Funder, 1986). The earliest studies of a hormonal influence showed that mineralocorticoids could increase urinary or renal kallikrein levels or synthesis (Miller *et al.*, 1984), but it is now clear that the rate of renal kallikrein synthesis does not respond acutely to mineralocorticoids, lagging behind the renal actions (e.g., increased Na⁺ reabsorption) of these steroids (Miller *et al.*, 1985). Even in rats chronically treated with mineralocorticoids for up to 14 days, there is no detectable change in renal kallikrein mRNA (Fuller *et al.*, 1986). Changes in the rate of prokallikrein activation, processing, or compartmentalization are likely to account for the relatively late effects of mineralocorticoids upon the renal enzyme.

There are other hormonal candidates for more direct roles in the regulation of renal kallikrein gene expression. Vasopressin produces very rapid changes in urinary kinin excretion in man, dog and in Brattleboro rats with hereditary diabetes insipidus (Kauker *et al.*, 1984). Although alteration in kallikrein gene expression may be unlikely to account for these rapid changes, no experiments were reported to examine the effects of vasopressin on renal kallikrein synthesis or mRNA levels. Other hormones of interest in this regard include insulin and glucocorticoids, as they very rapidly affect tissue kallikrein levels and synthesis rates. Physiologically relevant dosages of a glucocorticoid can reduce both kallikrein synthesis and activation within 2–3 h (Jaffa *et al.*, 1987). Whether this change reflects an alteration in kallikrein gene transcription is an interesting possibility.

The other tissue of interest here is the rat anterior pituitary. The presence of kallikrein-like activity at this site (Powers & Nasjletti, 1982) is known to reflect gene expression of authentic tissue kallikrein as assessed by radioimmunoassay of the enzyme, identification of kallikrein mRNA using different tissue kallikrein cDNA probes (Fuller *et al.*, 1985; Clements *et al.*, 1986; Chao

et al., 1987; Pritchett & Roberts, 1987), and sequence analysis of pituitary-derived cDNA clones (Pritchett & Roberts, 1987). There is a clear dimorphism here with mRNA levels 10–15-fold higher, and immunoreactive enzyme levels about 4-fold higher, in the female anterior lobe. Treatment of intact or gonadectomized male or ovariectomized female rats with oestrogen markedly increases both RNA (approx. 50-fold) and enzyme (20–90-fold) levels in the anterior lobe (Chao *et al.*, 1987). As in the case of the salivary gland and androgen, this clear-cut induction of kallikrein gene expression appears to require several days of hormone administration.

Dopamine represents the second neuroendocrine influence on kallikrein gene expression in the pituitary (Powers, 1985; Pritchett & Roberts, 1987). In both anterior and neurointermediate lobes, bromocryptine, the dopamine agonist, decreases tissue kallikrein mRNA of male rats. It affects only the anterior lobe of females where, as noted above, the level of kallikrein gene expression is much higher than in the male. In contrast, the dopamine antagonist haloperidol causes a marked increase in kallikrein mRNA in the neurointermediate lobe of both sexes and in the anterior lobe of female rats. These changes are due, in part, to changes in transcription of the kallikrein gene. Altered rates of transcription were shown to parallel changes in pro-opiomelanocortin mRNA in neurointermediate lobe nuclei using a run-on assay of transcription (Pritchett & Roberts, 1987). Thus, there is now a modest array of tissue sites, hormonal tools and techniques available to examine the expression of tissue kallikrein genes and the relations of regulation of such expression to hypotheses about the destination, processing and functional responsibilities of the encoded proteins.

Activation of prokallikrein

Kallikrein, as other secretory proteins, is synthesized on the rough endoplasmic reticulum and glycosylated and processed in the Golgi apparatus. Kallikrein contains carbohydrates which contribute to the microheterogeneity of human kallikrein in electrophoresis (Morichi *et al.*, 1984; Takada *et al.*, 1985). Pig pancreatic kallikrein contains fucose, mannose, galactose, glucosamine and sialic acid (Fiedler *et al.*, 1977; Moriya *et al.*, 1983). Human urinary kallikrein has a similar composition, but a relatively higher total carbohydrate content (Moriya *et al.*, 1983).

The activation process was characterized in experiments *in vitro* (Takada *et al.*, 1985). Judging from the reported sequence of the propeptide and the known cDNA sequence, the signal peptide consists of 17 amino acids in human kallikrein and an Ala⁻⁸–Ala⁻⁷ bond is cleaved to release the proenzyme. Alternatively, a 16-amino-acid signal peptide could be liberated by cleavage at the Gly⁻⁷–Ala⁻⁸ bond. Then Ala⁻⁸ could be removed rapidly by an aminopeptidase and this action would stop due to the resistance of the next peptide bond (Ala⁻⁷–Pro⁻⁶) to hydrolysis.

After the signal peptide is cleaved off, the resulting prokallikrein is inactive. Prokallikrein has a very short activation peptide of seven amino acids. In purified prokallikrein, the *N*-terminal amino acid is Ala, and after the activation peptide is cleaved, Ile is the newly revealed *N*-terminal amino acid (Takada *et al.*, 1985). This is in agreement with the finding that Ile⁺¹ forms the *N*-terminus of purified human urinary active kallikrein

(Lottspeich *et al.*, 1979). Thus, the amino acid sequence of the activation peptide as derived from the nucleotide sequence of the human cDNA is Ala-Pro-Pro-Ile-Gln-Ser-Arg (Baker & Shine, 1985; Fukushima *et al.*, 1985).

The amino acid sequence of the propeptide was also recently confirmed by sequencing (Takahashi *et al.*, 1986). This short propeptide sequence is in contrast to the 43 amino acids in the activation peptide of human prorenin (Nakanishi *et al.*, 1985). The peptide bonds connecting the propeptide to the active enzyme are quite similar; it is Arg⁻¹–Leu⁺¹ in renin and Arg⁻¹–Ile⁺¹ in kallikrein. The same type of basic-to-hydrophobic amino acid link was also found in the proenzyme form of other serine proteases and hormones (Takada *et al.*, 1985). In rat pancreatic kallikrein this bond is formed by Arg⁻¹–Val⁺¹ (Swift *et al.*, 1982). Thus, the activation process of tissue kallikrein may be similar to that of other serine proteases, such as chymotrypsin, where the newly formed *N*-terminal Ile⁺¹ links up via an ionic bond with Asp¹⁹⁴ (Kraut, 1971), altering the three-dimensional structure to expose the active site. The same mechanism is probably applicable to kallikrein, and this subsequently makes the active domain accessible to substrates. Thus, prokallikrein can be activated by either trypsin-type enzymes cleaving after Arg⁻¹ or by an enzyme that cleaves at the *N*-terminus of hydrophobic amino acids such as Ile⁺¹. Trypsin has been used as a standard activator *in vitro* (Frey *et al.*, 1950, 1968), although obviously this enzyme, except in the pancreas, would not be the activator *in vivo*. Plasmin, activated plasminogen (Yamada & Erdős, 1982) and plasma kallikrein (Takada *et al.*, 1985) all activate purified prokallikrein *in vitro*. Thermolysin, a bacterial metalloprotease which cleaves substrates at the *N*-terminus of hydrophobic amino acids, was about 5 times more active than trypsin in activating human urinary prokallikrein (Takada *et al.*, 1985).

Although the steps involved in the enzymic activation of prokallikrein *in vitro* have been characterized, the intracellular processing of prokallikrein is still undefined. The situation of prokallikrein in this respect resembles prorenin, because in spite of the very extensive work on the molecular biology of renin, prorenin activation *in vivo* still remains unexplained (Takada *et al.*, 1987).

Inactive prokallikrein was found in many tissues, for example in the pancreas (Frey *et al.*, 1950), in erythrocytes (Chao *et al.*, 1984b), in the colon (Frey *et al.*, 1968; Seki *et al.*, 1972) or in the pituitary gland (Powers & Nasjletti, 1983; Powers, 1986). In the porcine pancreas, kallikrein is in the form of prokallikrein, which during extraction is very rapidly converted to active kallikrein, probably by trypsin (Fiedler, 1979).

Renal prokallikrein has been investigated extensively, probably because of the reported decreased renal excretion of kallikrein in hypertension (Margolius *et al.*, 1971). In addition, urine is a readily available material for the purification of human kallikrein and prokallikrein. Prokallikrein has been purified from human urine (Takada *et al.*, 1985; Takahashi *et al.*, 1986). Human urinary or rabbit renal kallikrein (Silver *et al.*, 1980; Oza *et al.*, 1981; Omata *et al.*, 1983) consist of about equal portions of active and inactive prokallikrein. Rat urinary kallikrein, on the other hand, is mostly in active form (Noda *et al.*, 1983), although prokallikrein was isolated

from rat kidney and urine (Takaoka *et al.*, 1982; Nishimura *et al.*, 1983; Okamura *et al.*, 1985). Dexamethasone, given in pharmacological doses, increases prokallikrein concentrations in the basolateral membrane-enriched fraction of kidneys from adrenalectomized rats. The results were interpreted to mean that dexamethasone may induce an inhibitor of the prokallikrein converting enzyme (Noda *et al.*, 1983). A serine protease was isolated and partially purified from rat kidney, which can convert prokallikrein to kallikrein *in vitro* (Nishimura *et al.*, 1983). Renal cortical extract can also activate prokallikrein at acid pH, probably by the action of a thiopeptidase (Takaoka *et al.*, 1985).

Substrates

Plasma kininogen, the so-called low molecular weight kininogen (M_r 50 000–68 000) (Müller-Esterl *et al.*, 1986) is the primary physiological substrate of tissue kallikrein. It contains a coding sequence identical with the cysteine proteinase inhibitors; thus it can inhibit cathepsins.

Although studies *in vitro* would group tissue (glandular) kallikrein with other trypsin-type serine proteases, which cleave after basic amino acids, its best known function *in vivo* involves an anomalous cleavage specificity (Webster, 1970; Fiedler, 1979). To release kallidin (Lys¹-bradykinin) from kininogen, kallikrein cleaves a Met-Lys bond in this protein as well as the C-terminal Arg-Ser. The resulting kallidin is easily converted to bradykinin by an ubiquitous arginine aminopeptidase-type enzyme cleaving the Lys-Arg bond (Erdős, 1979). Rat urinary (Alhenc-Gelas *et al.*, 1981) and kidney (Yamada & Erdős, 1982) kallikrein as well as kallikrein of human colonic mucosa (Seki *et al.*, 1972) release bradykinin directly from kininogen.

Kinins are easily catabolized after their release, by enzymes in blood, biological fluids and tissues, called collectively, and not too imaginatively, kininases. The best known kininases cleave to release either the C-terminal Arg or Phe-Arg. Kininase I-type enzymes such as carboxypeptidase N of plasma (Erdős, 1979) or carboxypeptidase M of cell membranes (Skidgel & Davis, 1986; Skidgel *et al.*, 1984; Johnson *et al.*, 1984) release arginine. The C-terminal dipeptide (Phe-Arg) is liberated by kininase II (angiotensin I converting enzyme; Erdős, 1979) and by the neutral endopeptidase 24.11 (enkephalinase; Gafford *et al.*, 1983).

Kallikrein hydrolyses, at least *in vitro*, other biologically important peptides and proteins at a basic amino acid residue. Low density lipoprotein (Cardin *et al.*, 1984), atrial natriuretic factor (Currie *et al.*, 1984), prorenin (Sealey *et al.*, 1980), proinsulin (Ole-Moiyoi, 1979), vasoactive intestinal peptide (VIP; Mutt & Said, 1974) and procollagenase (Eeckhout & Vaes, 1977) are substrates *in vitro*.

Kallikrein activity has been assayed by employing N-terminally protected arginine esters, such as benzoyl-arginine ethyl ester. Lysine-containing substrates are not cleaved as well (Fiedler, 1979; Webster, 1970). Tosyl-arginine [³H]methyl ester (Margolius *et al.*, 1971) has been used to assess the activity of urinary kallikrein. Protected tripeptides coupled to a chromophore group offer high sensitivity, and probably an improved specificity, for assaying tissue kallikrein. For example, a very sensitive fluorometric assay is based on cleaving D-Val-L-Leu-L-Arg-7-amino-4-trifluoromethylcoumarin after the Arg residue (Morita *et al.*, 1977; Takada *et al.*, 1985).

By analysing the hydrolysis of a series of di- and tripeptide methyl esters by porcine pancreatic kallikrein, Fiedler (1987) found that the P₂ residue of the substrate greatly affects the K_m . In the best substrates Phe or Leu was in the P₂ position. These results support the proposal by Chen & Bode (1983) that a principal determinant of the specificity of kallikrein is a hydrophobic sandwich of Tyr⁹⁹ and Trp²¹⁵ that binds the amino acid side chain at P₂ of the kallikrein substrate. Indeed, Leu and Phe are in the P₂ position (Leu-Met and Phe-Arg) in kininogen, where tissue kallikrein releases kallidin ([Lys]bradykinin; Fig. 1).

Tissue kallikrein localizations

To the discoveries of the expression of true tissue kallikrein at known sites we can add those which revealed the expression of kallikrein genes and products at some unanticipated tissue sites. In rat and human, immunoreactive tissue kallikrein is present in plasma (Rabito *et al.*, 1982; Shimamoto *et al.*, 1984). Amongst its possible origins is the vascular wall itself; some experiments showed kallikrein and/or closely related enzymes within the mesenteric, tail or renal arterial walls (Nolly *et al.*, 1985). Kallikrein-like enzymic activities and mRNA were found in spleen (Chao *et al.*, 1984a), skeletal muscle (Shimojo *et al.*, 1987), in rat splenic T-lymphocytes (Iwata *et al.*, 1983) and as mentioned above as prokallikrein on the human red cell (Chao *et al.*, 1984b).

In the salivary gland, kallikrein is in secretory granules, which are symmetrical, spherical granules resembling the secretory granules of the pancreas (Greene *et al.*, 1963). Intact granules, containing a high concentration of kallikrein, were isolated from the richest source, the rat submaxillary gland, and then subsequently from the guinea pig and mouse salivary gland (Erdős *et al.*, 1968; Bhoola & Heap, 1970; Chiang *et al.*, 1968). In glands, kallikrein is localized in the apical region of duct cells (Schachter *et al.*, 1980; Hojima *et al.*, 1977; Maranda *et al.*, 1978).

In the kidney, kallikrein enters the nephron in the distal tubules (Scicli *et al.*, 1978), probably from granulated cells of the collecting tubules and ducts (Omata *et al.*, 1982, 1983; Fuller & Funder, 1986). Renal kallikrein, at the Golgi apparatus, may be directed either towards the luminal or the basolateral membrane. [A Golgi-enriched fraction of rat kidney was also rich in kallikrein; Brandan *et al.* (1982).] Kallikrein enters the urine from the luminal side, but it can enter the renal circulation (Roblero *et al.*, 1976) from the basal side of the cells (Yamada *et al.*, 1981; Misumi *et al.*, 1983; van Leeuwen *et al.*, 1984). The basolateral membrane-bound kallikrein may have a more hydrophobic portion than luminal membrane kallikrein, as indicated by gel filtration after extraction with a detergent (Yamada & Erdős, 1982).

Renal tissue kallikrein localization with electron microscope immunohistochemistry (Figuroa *et al.*, 1984; Vio & Figuroa, 1985) has affirmed earlier conclusions (Yamada & Erdős, 1982) about the distribution of renal kallikrein(s) into both apical and basolateral membranes of cortical distal tubular cells. Whether this represents distribution of the same gene product subject to dissimilar post-translational processing to alternate surfaces of these polarized epithelial cells, or dissimilar gene products destined for different

roles at one or the other surface, remains to be determined.

It is also quite clear that a kallikrein family gene is being expressed in the prostate (Lazure *et al.*, 1984; Lilja, 1985; Lundwall & Lilja, 1987) and possibly in the adrenal medulla (Mizuno *et al.*, 1985). The immunohistochemical localization of tissue kallikrein in the ependymal cells lining of the cerebral ventricles (Simson *et al.*, 1985), as well as in cerebrospinal fluid, is raising questions about roles for the enzyme and kinins in closely adjacent areas of the central nervous system, as well as in the lining cells themselves. It seems very likely that additional tissue sites will be discovered where the kallikrein gene is expressed, as a kallikrein-like enzymic activity and kinin receptors are being found in new locations, for example in porcine retina, choroid and ciliary body (Igic, 1985) or in osteoclasts (Lerner *et al.*, 1987).

New associations with cellular functions and disease

The notion that tissue kallikrein might have functions other than kinin production finds an analogy in plasma kallikrein, which acts upon either high- M_r kininogen, to which it is complexed, or on Factor XII (Colman *et al.*, 1985; Kaplan & Silverberg, 1987). Rat kallikrein contracts the rat uterus apparently independent of detectable kinin formation (Chao *et al.*, 1981). This is an earlier example of a physiological event that is not kinin-related.

Other studies describe activities, that might be relevant to cardiovascular homeostasis. Tissue kallikrein does not seem to be involved in prorenin activation (Inagami *et al.*, 1982), but kallikreins and kinins can stimulate renin release from its synthesis and storage sites (Beierwaltes *et al.*, 1985). Participation in the activation (Currie *et al.*, 1984) or degradation of atrial natriuretic factors (Thibault *et al.*, 1984) was suggested, but the evidence thus far is weak. Either plasma or tissue kallikreins can cleave *in vitro* the principal protein of low-density lipoprotein, apoB₁₀₀, at the bonds hydrolysed *in vivo* (Cardin *et al.*, 1984) but this finding, although confirmed (Hardman *et al.*, 1986), is still of uncertain biological significance.

The roles of kallikreins in cellular electrolyte and water transport are being defined. These efforts began with descriptions of the effects of kallikrein inhibitors to inhibit sodium transport in amphibian bladder (Orce *et al.*, 1980), and stimulation of electrogenic chloride secretion by kinin in the kallikrein-synthesizing epithelium of the rat colon (Cuthbert & Margolius, 1982). The work has been extended to many tissues, primary cell cultures and even some epithelial cell lines (Cuthbert *et al.*, 1987). Kinin-induced chloride movements and co-transport events in epithelia, and relations of these phenomena to eicosanoids, phosphoinositides, cyclic nucleotides, and calcium, are being studied. An acute change in epithelial structure occurs concomitantly with kinin-induced ion transport (Baron *et al.*, 1986) and is providing new questions about relations between these structural changes and kinin-induced epithelial transporting events. However, there is still limited understanding of the cellular locations and regulation of kinin generation by tissue kallikrein as an autocrine or paracrine event.

Epilogue

Kallikrein has puzzled investigators for a very long

time. For decades its activity has been attributed to nothing more than contamination by other agents. Much of this enigma is disappearing with intensive research, but still quite a bit remains. For example, why are there 24 kallikrein-related genes, many unexpressed, in the mouse (Mason *et al.*, 1983; Evans *et al.*, 1987). Initially, prior to writing this review, some of us were inclined to offer a theological explanation. Divine force did not know what to do with the genes and thus kept making them in the hope that somebody in the laboratory would find a definite use for them! After reviewing the field, we feel that a more secular explanation would be appropriate. Half of the multiple mouse genes are inactive pseudogenes; of the remaining active genes there is likely one gene for 'bona fide' kallikrein. The other genes encode closely related enzymes with similar functions in cleaving dormant precursors.

The actions of tissue kallikrein may depend on its localization. These could be direct or indirect via the kinin it releases, as discussed above. For example, owing to its restricted specificity on high- M_r substrates, kallikrein can be a 'convertase' of proenzymes and prohormones. Finally, in vital organs, kallikrein may affect blood flow. For example, by increasing the circulation in the kidney, it may prevent a shutdown of renal function in shock and related conditions.

Advances in the study of relations between tissue kallikrein and kinins and disease are being made at an accelerating rate. The most notable areas of current interest are studies in hypertension, diabetes mellitus, allergic and inflammatory disorders. However, advances in understanding of the roles of tissue kallikrein-regulated systems in disease have been modest compared with those made in molecular, biochemical and biophysical studies. This may change rapidly as significant progress is being made in the design and synthesis of new molecules capable of inhibiting tissue kallikrein more specifically (Okunishi *et al.*, 1986) or blocking kinin receptors (Schachter *et al.*, 1987). These tools will lead to a very quick increase in our appreciation of the functional responsibilities of this interesting mammalian serine proteinase and its family of related proteins.

Some of the studies described here were supported in part by NIH grants: HL36081, HL36473, HL36082, GM31689 and DK27430.

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