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Current perspectives on the molecular biology of the renal tissue kallikrein gene and the related tissue kallikrein gene family

JUDITH A CLEMENTS*

Centre for Molecular Biotechnology, School of Life Sciences,
Queensland University of Technology, Brisbane, Queensland, Australia

*Renal tissue kallikrein is a member of the multigene family of serine proteases called the tissue kallikrein (**KLK**) gene family. This is a highly conserved family of genes, with a genomic structural organization that is identical for all these genes and other genes in the larger serine protease family, such as trypsin and chymotrypsin. These genes exhibit high sequence similarity both within and between species. However, there are clearly areas of sequence variability, which is most apparent in regions that form the substrate binding pocket of each enzyme and confers the substrate specificity of each individual enzyme. These genes are also often expressed in the same tissue, although each gene can have an individual tissue-specific pattern of expression. Similar patterns of diversity yet identity are also apparent in the regulation of kallikrein gene expression or enzyme activity. These similarities, and the fact that several of these gene families are located in tight clusters in the genome, support the notion that they have arisen by gene duplication. In this review, an overview of the molecular biology of the renal tissue kallikrein (**KLK1**) gene and the larger **KLK** gene family is given, highlighting the similarities yet diversity that is the hallmark of this family of genes, and how this knowledge has, and will, impact on our understanding of the role these enzymes play in normal physiological events and disease.*

Key terms: *kallikrein gene family, molecular biology, renal tissue kallikrein, tissue kallikreins*

INTRODUCTION

The crucial importance of the serine protease, renal tissue kallikrein, in the generation of bradykinin and the involvement of the tissue kallikrein-kinin system in cardiovascular and renal function has been appreciated for many decades. It is only in the last 10-15 years, however, that the gene encoding renal or pancreatic tissue kallikrein was isolated in several species and shown to be part of a larger family of highly related genes. Given their high degree of sequence conservation to tissue kallikrein, these genes were designated to be the tissue kallikrein gene family of serine proteases. This family has been recently given the descriptor, *KLK* gene family, and the renal tissue kallikrein gene is called *KLK1* (Berg *et al*, 1992). In the following sections, I will briefly review the current knowledge of the molecular

* **Correspondence to:** Assoc Prof Judith Clements, Centre for Molecular Biotechnology, School of Life Sciences, Queensland University of Technology, GPO Box 2434, Brisbane, QLD, Australia 4001. Tel: (61-7) 3864-1899. Fax: (61-7) 3864-1534. E-mail: j.clements@qut.edu.au

biology of renal tissue kallikrein or *KLK1* and the larger tissue kallikrein (*KLK*) gene family, and how it has impacted on our current understanding of the function and regulation of kallikrein action and what questions still need to be answered in order to fully understand the roles of these enzymes in health and disease.

TISSUE KALLIKREIN - cDNA AND GENE

The complementary (c) DNA sequence of tissue kallikrein/*KLK1* was first reported in the early 1980s in rat, mouse and human pancreas, kidney or salivary glands (Ashley & MacDonald, 1985; Baker & Shine, 1985; Fukushima *et al*, 1985; Swift *et al*, 1982; Van Leeuwen *et al*, 1986). That this cDNA sequence did indeed encode tissue kallikrein was substantiated by several facts. First, these three tissues were known as a source of tissue kallikrein enzyme activity (Bhoola & Dorey, 1971; Lemon *et al*, 1979; Orstavik *et al*, 1980; Schachter *et al*, 1980; Vio & Figueroa, 1985). It was also clearly a serine protease, having the classical trio of amino acids (His^{41}-Asp^{90}-Ser^{180}) indicative of the catalytic site of a serine protease (Neurath, 1989). Moreover, the deduced amino acid sequence was highly similar to the previously reported pig pancreatic protein sequence that was obtained on biochemical purification (Fiedler, 1979), retaining all amino-acid residues in the substrate binding pocket suggested to be crucial for kininogenase activity (Tyr^{93}, Ser^{135}, His^{164}, Asp^{183}, Trp^{205}, Gly^{206} (Bode *et al*, 1983; Chen & Bode, 1983). The only exception was that it had an extra 8 amino acids at residues 81-88, in the so-called kallikrein loop region, that was missing in the biochemically purified 2 chain pancreatic enzyme. This kallikrein loop region is a feature of all other tissue kallikreins and members of the larger gene family so far studied. The validation of these deductions has been only recently confirmed with the expression of a human *KLK1* cDNA in baculovirus and mammalian cell expression systems and the observation that the recombinant tissue kallikrein had identical biochemical and enzymatic properties to the previously described biochemically purified enzymes (Angermann *et al*, 1992; Lu *et al*, 1996; Wang *et al*, 1992).

The genes and/or cDNAs have now been described for monkey and dog (Gauthier *et al*, 1994; Lin *et al*, 1993) as well as mouse, rat and human tissue kallikreins. The *KLK1* gene is 5-6 kilobases long and consists of 5 exons with 4 introns, a genomic organizational structure that is typical of many other members (trypsin, chymotrypsin) of the larger serine protease family (Neurath, 1989; Wines *et al*, 1991). Exons 1 and 2 encode the N terminal prepro- region and exons 2-5 encode the mature enzyme. From this gene, a *KLK1* mRNA of 850-900 base pairs is transcribed. These cDNAs encode preprokallikreins of 257-265 amino acids. Generally the pre-region or signal peptide, necessary for the secretion of the zymogen is 17 amino acids, with a zymogen pro-peptide of 7 residues, although rat preprokallikrein has a longer pro-region (Wines *et al*, 1989). The mature enzyme generated by cleavage of the pro-peptide is generally 237 amino acids long. The exceptions are the human (238 residues) which has an extra threonine at codon 108 (Evans *et al*, 1988) and monkey tissue kallikrein which is 5 amino acids shorter (Lin *et al*, 1993). The degree of identity between species varies, ranging from 59-92% at the protein level, with the human and monkey enzymes most similar.

More recently, tissue kallikreins have been biochemically identified in lower vertebrates such as fish, frogs and moles (Lipke & Olsen, 1990; Richards *et al*, 1995, 1996). This suggests that the enzyme tissue kallikrein, and probably the kallikrein-kinin system, have important functional roles throughout evolution. It will be interesting to see whether tissue kallikrein's primary evolutionary function is as a kininogenase and if kinins are generated, whether these peptides in lower vertebrates are as multifunctional as their mammalian counterparts. It is interesting to note that mammalian tissue kallikreins can, at least *in vitro*, process many other polypeptides in addition to LMW kininogen, such as pro-renin, atrial natriuretic factor, matrix metalloproteases and pro-insulin,

suggesting that tissue kallikrein may also be a multifunctional enzyme (Currie *et al*, 1984; Ole-Moiyoi *et al*, 1979; Sealey *et al*, 1978; Tschesche *et al*, 1989). However, there is no evidence to date that these events can occur *in vivo*.

The 5' and 3' flanking regions of the human, mouse and rat *KLK1* genes also show considerable homology (Wines *et al*, 1991). They share similar TATA (TTTAAA) and CAAT (CATCT) box sequences (Evans *et al*, 1988; Van Leeuwen *et al*, 1986; Wines *et al*, 1989, 1991), two sites in the 5' region necessary for the transcription of the *KLK1* gene. The polyadenylation signals at the 3' end are different between rodents (AATAAA) and man and dog (AGTAAA) (Evans *et al*, 1988, Gauthier *et al*, 1994; Van Leeuwen *et al*, 1986; Wines *et al*, 1989). The sequences responsible for the molecular events, which regulate tissue-specific expression and hormonal regulation of the *KLK1* gene, must also be present in the 5' flanking region. The majority of this information is yet to be determined and this is clearly an area that has lagged behind. Molecular cell biologists and molecular physiologists are still to unravel the precise mechanisms behind the ubiquitous expression and regulation of *KLK1*/tissue kallikrein activity, data which was provided by earlier physiological studies and is still an area of considerable activity.

Although the salivary gland, pancreas and kidney are the three most abundant sites of *KLK1* expression (Ashley & MacDonald, 1985; Fukushima *et al*, 1985; Gauthier *et al*, 1994; Lin *et al*, 1993; Swift *et al*, 1982; Van Leeuwen *et al*, 1986), it is clear that *KLK1* is also expressed at varying levels in almost every tissue or organ in the body. *KLK1* expression has been reported in gastrointestinal and reproductive tract, adrenal, heart and vasculature, pituitary and brain of different species, as was shown by MacDonald *et al* (1996) in a comprehensive study in the rat (also reviewed in Clements, 1997). These findings support the earlier evidence of tissue kallikrein activity in these tissues (reviewed in Bhoola *et al*, 1992; Clements, 1997). Tissue kallikrein activity has also been found in bronchio-nasal secretions, sweat glands, synovial fluid and neutrophils (reviewed in Bhoola *et al*, 1992) suggesting these tissues and/or perhaps invading neutrophils are also sites of *KLK1* expression. The overall regions (800 base pairs to 5 kilobases) in the promoter of the rat and human *KLK1* gene directing salivary gland, pancreas, kidney, brain or spleen expression have been determined from transgenic studies (Simson *et al*, 1994; Southard-Smith *et al*, 1992). Although the precise sequences directing these events have not yet been elucidated, an element at -177 to -157 in the rat *KLK1* promoter (Wines *et al*, 1989) is similar to a sequence in the elastase gene that is known to direct pancreatic specific expression (Swift *et al*, 1984). Of interest, although there is considerable homology between rat and human *KLK1* promoters, there are also major differences. The sequences directing pancreatic expression appear to be in the first 800 base pairs of both these genes. Those sequences directing salivary gland and renal expression, however, are present in 800 base pairs of the human *KLK1* promoter, but do not appear to be present in the first 5 kilobases of the rat *KLK1* promoter (Simson *et al*, 1994; Southard-Smith *et al*, 1992).

Similarly, the elucidation of the genomic sequences and molecular events that are involved in the hormonal regulation of *KLK1* gene expression and tissue kallikrein activity are essentially unknown. Again, from cell biology and physiological studies, we know that salt, mineralocorticoids, glucocorticoids and insulin variously affect tissue kallikrein activity in the rat kidney (Bascands *et al*, 1987; Jaffa *et al*, 1987, 1990; Miller *et al*, 1984). Both glucocorticoids and insulin are known to act at the biosynthetic level (Jaffa *et al*, 1987, 1990), but similar studies on mineralocorticoid regulation at the mRNA level have not been conclusive (Fuller *et al*, 1986; Miller *et al*, 1985). Estrogen and dopamine regulate rat pituitary *KLK1* expression and activity (Clements *et al*, 1986; Powers, 1986; Pritchett & Roberts, 1987). Changes in rat uterine tissue kallikrein activity across the estrous cycle

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or during pregnancy (Corthorn & Valdés, 1994; Valdés *et al*, 1993), urinary kallikrein excretion (Albano *et al*, 1994) and *KLK1* expression in the human endometrium (Clements *et al*, 1994) across the menstrual cycle are also suggestive of estrogen regulation. The action of dopamine is clearly at the transcriptional level although the precise mechanism is not known (Pritchett & Roberts, 1987). Putative hormone binding elements for estrogen, progesterone, glucocorticoids and cAMP have been identified in the promoter of the human *KLK1* gene (Murray *et al*, 1990) but these elements have not been functionally characterized. A putative cAMP responsive element (at -235 to -214) is also well conserved between the rodent and human *KLK* genes (Evans *et al*, 1988; Wines *et al*, 1989), containing a sequence similar to an AP-2 binding site, a transcription factor implicated in the regulation of many genes. No studies have formally linked these putative sequence elements in the *KLK1* promoter with hormonal regulation of tissue kallikrein gene expression and activity.

THE LARGER TISSUE KALLIKREIN (*KLK*) GENE FAMILY

Tissue kallikrein is clearly a member of a larger gene family of highly conserved and related enzymes (Evans *et al*, 1987; Murray *et al*, 1990; Wines *et al*, 1989). Although there are just three *KLK* genes in man (reviewed in Clements, 1997) and just 1-2 genes in most other species (Berg *et al*, 1992; Murray *et al*, 1990), the rodent families are very large. The mouse and rat families comprise 26 and 13 genes, respectively (Evans *et al*, 1987; Wines *et al*, 1989), of which several genes (10 in the mouse and 3 in the rat) are pseudogenes and are not expressed. The enormous conservation of sequence, identical structural arrangement and their close proximity in the genome in gene clusters suggest that they have evolved by gene duplication. The mouse and rat *KLK* gene families are clustered in 300-440 kilobase regions on chromosomes 7 and 1, respectively (Evans *et al*, 1987; Kurtz & StLezin, 1992, Southard-Smith *et al*, 1994). Similarly, the 3 human *KLK* genes are tightly linked over a 60 kilobase region on chromosome 19q.13.3-13.4, a region which is analogous to that of the mouse *Klk* locus on chromosome 7 (Riegman *et al*, 1992).

As was seen with the *KLK1* genes across species, other *KLK* genes both within and across the 3 major *KLK* gene families (rat, mouse and human) are also highly conserved. Their coding regions are 70% identical at the nucleotide level (Wines *et al*, 1991) with similar conservation observed for the 5' and 3' flanking regions of the rodent genes (73-90%). Similarly, the human *KLK2* and *KLK3* genes are >90% identical in the 5' flanking region, although less similar to *KLK1* (Evans *et al*, 1988; Riegman *et al*, 1989; Schedlich *et al*, 1988). The minor differences in these transcriptionally important regions suggest that small sequence differences must determine the different transcriptional regulation and tissue-specific expression patterns observed for all the *KLK* genes in each species.

The hallmark of the *KLK* gene families is that all of the *KLK* genes are expressed in the salivary gland, as has been most extensively studied in the mouse and rat (MacDonald *et al*, 1996; Penschow *et al*, 1991a). A distinct tissue-specific pattern of expression in a variety of other tissues is then apparent for each individual *KLK* gene. Although many studies have shown aspects of this tissue-specificity for individual genes (as reviewed in Clements 1997), the most comprehensive study for the rat *KLK* family was published by MacDonald *et al* (1996). Using the reverse transcriptase-polymerase chain reaction (RT-PCR), they demonstrated essentially ubiquitous expression of the *KLK1* gene as was known previously, but disparate and different patterns of expression for the other 9 rat *KLK* genes in kidney, spleen, gastrointestinal and male reproductive tract, pituitary and brain. To date, similar extensive studies have not been performed in the mouse, although mouse *KLK* expression has been detected in the Leydig cells of the testis (*mKLK* 21; Penschow *et al*, 1991b) and in mouse mesenteric lymph nodes (Summers & Hume,

1985). Similarly, in man, the *KLK2* and *KLK3* genes are primarily expressed in the prostate (Chapdelaine *et al*, 1988a), although recent studies at the RT-PCR level have identified *KLK3* expression in the pituitary, breast, uterus and ovary (Clements & Mukhtar, 1994; Clements *et al*, 1996; Monne *et al*, 1994). The dog and monkey families also have a second gene *KLK* gene that is predominantly expressed in the prostate (Chapdelaine *et al*, 1991; Gauthier *et al*, 1993).

Transgenic studies have again indicated some regions in the 5' promoter of the *rKLK2* and *rKLK8* genes that may confer some of the tissue-specific differences in expression observed for these genes (MacDonald *et al*, 1996) compared to *rKLK1*, but the regions conferring salivary gland expression were not found (Southard-Smith *et al*, 1992). These authors concluded that there might be a region conferring salivary gland expression on all the *KLK* genes several kilobases upstream of the whole *KLK* gene locus (Southard-Smith *et al*, 1994). Similar experiments to determine the region that determines the prostate specific expression of human *KLK3* or prostate specific antigen have been variously successful (Schaffner *et al*, 1995; Pang *et al*, 1995). The importance of these kinds of experiments is reflected in the potential to target gene therapies to specific cell types using these promoter regions, such as for *KLK3*/prostate specific antigen in prostate cancer or perhaps *KLK1*/tissue kallikrein to a particular cell type important in hypertension.

Androgens and thyroid hormone are important regulators of *KLK* gene expression or the activity of the enzymes they encode in the rat and mouse salivary gland (Chao & Margolius, 1983; Clements *et al*, 1988, 1990; Penschow *et al*, 1991a,b). Thus, male rodents have higher levels of salivary gland *KLK* mRNA than females and castration depletes but androgen administration restores *KLK* expression in this tissue. Similarly, thyroid hormone administration increases *KLK* expression in rodent salivary glands. This is in stark contrast to *KLK1* expression in salivary glands which is not androgen or thyroid hormone dependent, as noted above. Prostatic expression of *KLK* genes in man, dog and rat are also androgen dependent (Chapdelaine *et al*, 1988b; Clements *et al*, 1988; Riegman *et al*, 1991a). The promoter regions conferring androgen or thyroid hormone regulation on these *KLK* genes in the rodent have not been elucidated. However, androgen-responsive elements have been described in the promoters of the human *KLK2* and *KLK3* genes (Riegman *et al*, 1991b; Wolf *et al*, 1992). One of these elements is similar to the reverse complement of a consensus sequence for the binding of glucocorticoid and progesterone receptors. *KLK3* or prostate specific antigen expression in breast tumour cell lines is regulated by progesterone, glucocorticoids and androgens (Zarghami *et al*, 1997), an event that is presumably also occurring through this same promoter element.

What has been surprising is that the function of many of these genes is yet to be established. The second *KLK* gene expressed in the prostate of the monkey, dog and Guinea pig is presumed to encode the homologue of the human enzyme, prostate-specific antigen, or the similar human K2 enzyme that is also predominantly expressed in the prostate, but this has not been proven. Prostate specific antigen degrades the seminal vesicle proteins, seminogelin and fibronectin, which leads to the dissolution of the seminal clot (Lilja, 1985). It can also degrade the extracellular protein, laminin (Webber *et al*, 1995), activate epidermal growth factor (Parries & Cohen 1996) and degrade insulin like growth factor binding protein -3 (Cohen *et al*, 1992). The human K2 enzyme can activate pro-urinary plasminogen activator (Frenette *et al*, 1997). These enzymic actions occur *in vitro* and are not known to also occur *in vivo*, however, they are the basis of current suggestions that these enzymes in the prostate may be important in proliferation and invasion of cancer cells by the activation of growth factors or extracellular proteases, or degradation of the extracellular matrix. Other enzymes that have been identified include the mouse growth factor processing enzymes, γ-nerve

growth factor, β-nerve growth factor endopeptidase and epidermal growth factor binding protein (Fahnestock *et al*, 1991; Frey *et al*, 1979; Greene *et al*, 1968). Others encode enzymes that generate vasoactive (poly)peptides such as tonin, submandibular enzymatic vasoconstrictor and T-kininogenase in the rat (Boucher *et al*, 1974; Gutman *et al*, 1988; Yamaguchi *et al*, 1991) and mouse renin (g-renin, pro-renin converting enzyme) processing enzymes (Kim *et al*, 1991; Poe *et al*, 1983). However, there are still 7 genes, in both mouse and rat *KLK* gene families, for which the enzymic functions of the enzymes they encode are unknown. Similarly, if there are homologues of these genes in the smaller human dog and monkey families they have yet to be identified. Given the suggested varied substrate specificity of prostate specific antigen above and perhaps tissue kallikrein, *in vitro*, it may be that the 3 human genes may be more multi-functional that their rodent counterparts and perhaps be found to encompass several, if not all, of the enzymic functions represented by the rodent kallikreins.

PERSPECTIVES

Overall, with respect to the molecular biology of *KLK1* or tissue kallikrein and the larger *KLK* gene families, there is a vast body of knowledge on the cDNA and genomic sequences and genomic organization. Less is known about the precise sequences or elements in the promoters of these genes that regulate their diverse patterns of tissue-specific expression that cell biologists, physiologists and pharmacologists have observed for some time. Similarly, except for the human *KLK2* and *KLK3* genes, little is known of the elements that might regulate hormonal expression in these tissues, by hormones such as glucocorticoids, mineralocorticoids or estrogen for *KLK1* tissue kallikrein or even precisely whether this regulation occurs transcriptionally or at what level post-translationally. Perhaps the most important regulatory event may be the activation of the kallikrein enzymes, but we have yet to elucidate how this precisely occurs or what regulatory event(s) triggers such activation in many tissues. The complex issue of enzyme specificity and the real physiological function of the individual kallikreins also needs to be addressed. The elucidation, at the molecular level, of the mechanisms behind these events are some of the important questions in the kallikrein field that still remain un-answered.

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