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Kallikrein-gene expression in the rat gastrointestinal tract

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The serine proteinase glandular kallikrein has been demonstrated in the gastrointestinal tract, although there is some doubt as to whether it is synthesized there or derives from exocrine-gland secretions. Using a rat pancreatic kallikrein cRNA probe we have demonstrated kallikrein-like gene expression in the corpus, duodenum, jejunum, ileum, caecum and colon, and compared the pattern of expression with that of the gastrointestinal peptides somatostatin, gastrin and glucagon. In addition, using a panel of oligonucleotide probes specific for various members of the rat kallikrein-gene family, we have shown that the kallikrein-like gene expressed appears to be expressed as true kallikrein.

INTRODUCTION

Glandular kallikreins are arginine-bond-specific esteropeptidases involved in the processing of prohormone precursors to their bioactive end products [for reviews, see Schachter (1980), Fuller & Funder (1986), Isackson *et al.* (1987) and MacDonald *et al.* (1988)]. Kallikrein-like enzyme activity has been demonstrated in various regions of the rat gastrointestinal tract (Zeitlin *et al.*, 1986). This activity has been purified from rat and human gastric mucosa (Uchida *et al.*, 1980; Uetsuji *et al.*, 1982). More recently, kallikrein-like immunoreactivity and kallikrein-like (kininogenase) enzyme activity have been demonstrated in mucous cells of the colon of several species (Schachter *et al.*, 1983, 1986) as well as in mucous cells elsewhere in the gastrointestinal tract (Schachter *et al.*, 1986). In addition, the inactive pro-form of kallikrein has been previously demonstrated in the colon of various species (Seki *et al.*, 1972). Although such studies suggest that kallikrein is synthesized in the gastrointestinal mucosa, Skagen & Andersen (1986) found levels of kallikrein to be decreased in surgically isolated loops of bowel. Since experimentally administered porcine kallikrein has been shown to be absorbed in the rat gastrointestinal tract (Overlack *et al.*, 1983), Skagen & Andersen (1986) argued that kallikrein seen in the gut might be of submaxillary or pancreatic origin.

Swift *et al.* (1982) used a rat pancreatic kallikrein cDNA probe to demonstrate kallikrein-like gene expression in the pancreas, kidney, submaxillary gland, parotid, spleen and prostate. Clements *et al.* (1986) and, more recently, Pritchett & Roberts (1987) have extended these data to include the rat anterior pituitary gland. The rat kallikrein/arginine esteropeptidase family probably consists of eight (Ashley & MacDonald, 1985*b*) to 17 (Gerald *et al.*, 1986) genes compared with 24 in the mouse (Evans *et al.*, 1987) and possibly only three in the human (Baker & Shine, 1985) genome. Of the rat genes which have been characterized (Swift *et al.*, 1982; Ashley & MacDonald, 1985*a*,*b*; Gerald *et al.*, 1986; Chen *et al.*, 1988), only one would appear to be a pseudogene (Gerald *et al.*, 1986). mRNA sequences for six transcribed genes have been derived from submaxillary-gland and/or pancreatic cDNA libraries (S or PS series) and kidney (K) or prostatic (P) cDNA libraries (Swift *et al.*, 1982; Ashley & MacDonald, 1985*a*; Clements *et al.*, 1988). Thus, in addition to PS, which encodes true kallikrein, and S2, which encodes tonin, several other similar but distinct arginine-esteropeptidase genes have been sequenced. These have been designated kallikrein-like (S1, K1, P1) or tonin-like (S3) with respect to amino acid sequence, although their specific enzymic function is as yet unknown.

In the present study we have shown that the gastrointestinal tract is a site of kallikrein-like gene expression by demonstrating the presence of kallikrein-like mRNA throughout the gastrointestinal tract. In addition, we have used oligonucleotide probes specific for six rat arginine esteropeptidase genes, namely PS, S1, S2, S3, K1 and P1, to identify the specific gene expressed as true kallikrein.

MATERIALS AND METHODS

Tissue preparation

Male Sprague–Dawley rats weighing 120–180 g from a pathogen-free colony bred in the Central Animal House of Monash University were used in all experiments. Rats were maintained on water and standard rat chow *ad libitum*. Animals were killed by cervical dislocation, and the relevant tissues were dissected, snap-frozen in liquid N_2 and stored at −70 °C. The specific regions examined were the corpus of the stomach, antrum, duodenum, jejunum (a 2 cm segment 20 cm from the duodenum), terminal ileum, caecum and the ascending, transverse and descending colon.

mRNA analysis

Total RNA was isolated by the method of Chirgwin *et al.* (1979). Polyadenylated RNA was prepared by the method of Aviv & Leder (1972). Northern-blot analysis was performed as previously described (Clements *et al.*, 1986; Fuller *et al.*, 1986). Briefly, 12.5 μg of total RNA was denatured in 1 M-glyoxal/50% dimethyl sulphoxide, electrophoresed in a 1.2%-agarose gel and transferred to Hybond nylon membranes (Amersham International) (Thomas, 1983). The membranes were baked at 80 °C for 2 h, u.v.-cross-linked for 10 min and prehybridized at 42 °C in hybridization solution [50% formamide/5 × SSPE (1 × SSPE is 0.15 M-NaCl/10 mM-sodium phosphate/1 mM-EDTA, pH 7.4), 5 × Den-

hardt's solution (1 × Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll 400/0.02% polyvinylpyrollidone), herring sperm DNA (100 μg/ml) and *Escherichia coli* tRNA (200 μg/ml)] before hybridization at 65 °C for 20 h with the cRNA probes or at 42 °C for 24–36 h with the cDNA probe. The blots were then washed once at room temperature in 2 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0), 0.1% SDS and then twice for 15 min at 65 °C (cRNA probes) or 50 °C (cDNA probe) in 0.2 × SSC/0.1% SDS. They were then blotted dry and exposed to Kodak X-AR (Eastman–Kodak, Rochester, NY, U.S.A.) or Fuji X-ray film with a Cronex Lightning-Plus intensifying screen (du Pont, Wilmington, DE, U.S.A.) at −80 °C. For hybridization with oligononucleotide probes, blots were prehybridized at 42 °C in 5 × SSC/50 mM-sodium phosphate (pH 8.0)/10 × Denhardt's solution/0.1% SDS/herring sperm DNA (100 μg/ml), hybridized for 24–48 h at 37 °C, washed in 0.1 × SSC/0.1% SDS at room temperature and then at 37 °C. Before rehybridization with a different probe, blots were placed in boiling distilled water for 2 min, re-exposed to determine the adequacy of removal of the previous probe, then prehybridized and hybridized as before.

^{32}P-labelled probes

The kallikrein cRNA probe was constructed by subcloning a 300 bp restriction-endonuclease-*Hin*dIII–*Bgl*II fragment of the rat pancreatic kallikrein cDNA clone pcXP39 (Swift *et al.*, 1982) into the plasmid pGEM-4 (Promega Biotech, Madison, WI, U.S.A.) with the 3′-end of the sense strand of the cDNA contiguous with the SP6 promoter. This construct was linearized with *Hin*dIII, enabling synthesis of an ~ 300-base ^{32}P-labelled cRNA probe in a reaction mixture containing 1 μg of linearized plasmid DNA, 10 units of SP6 polymerase (Promega), 40 mM-Tris/HCl, pH 7.5, 6 mM-$MgCl_2$, 1 mM-spermidine, 10 mM-NaCl, 500 mM-ATP, -GTP and -CTP, 12 μM-UTP, 10 mM-dithiothreitol, RNasin (an RNAase inhibitor; 1 unit/μl; Promega) and [α-^{32}P]UTP (> 400 Ci/mmol; BRESA, Adelaide, South Australia, Australia) (Melton *et al.*, 1984). The somatostatin, gastrin and glucagon cRNA probes have been described previously (Fuller *et al.*, 1987; Brand & Fuller, 1988). Relative levels of kallikrein mRNA between tissues was determined by scanning the autoradiograph with a densitometer (ISCO gel scanner 1312).

The rat tubulin cDNA probe (Lemischka *et al.*, 1981) was labelled by nick translation (Amersham Nick Translation Kit; Amersham International) with [α-^{32}P]dCTP (1800 Ci/mmol; BRESA).

Oligonucleotide probes specific for the previously described (Swift *et al.*, 1982; Ashley & MacDonald, 1985*a*) rat kallikrein genes (PS, S1, S2 and S3), together with probes specific for two additional rat kallikrein-like cDNAs recently cloned from kidney (K1) and prostate (P1), which have been described in detail elsewhere (Clements *et al.*, 1988), were provided by Dr. Ray MacDonald (Southernwestern Medical Center, Dallas, TX, U.S.A.). To ensure hybridization and wash conditions were sufficiently stringent to distinguish the different kallikrein-gene-specific mRNAs, dot-blots of 5 ng of denatured cDNA (PS, S1, S2, S3, P1 and K1) were included in each Northern hybridization. Probes were end-labelled with [γ-^{32}P]ATP (~ 2000 Ci/mmol; BRESA) to a specific radioactivity of ~ 10^9 c.p.m./μg.

RESULTS

As Fig. 1(*a*) shows, the kallikrein cRNA probe hybridizes to kidney RNA as reported previously (Swift *et al.*, 1982; Fuller *et al.*, 1986). Hybridization to transcripts of equivalent size is also seen in the various regions of the gastrointestinal tract examined, with the probable exception of the gastric antrum. The level of kallikrein mRNA is highest in the caecum (~ 64% of the level in the kidney), slightly lower in the corpus (~ 50%) and colon (ascending ~ 60%, transverse ~ 56% and descending ~ 43%), much lower in the duodenum (~ 23%) and very low, but clearly detectable, in the jejunum (~ 3%) and terminal ileum (~ 6%). Although a very faint band can be seen in the gastric antrum (Fig. 1),

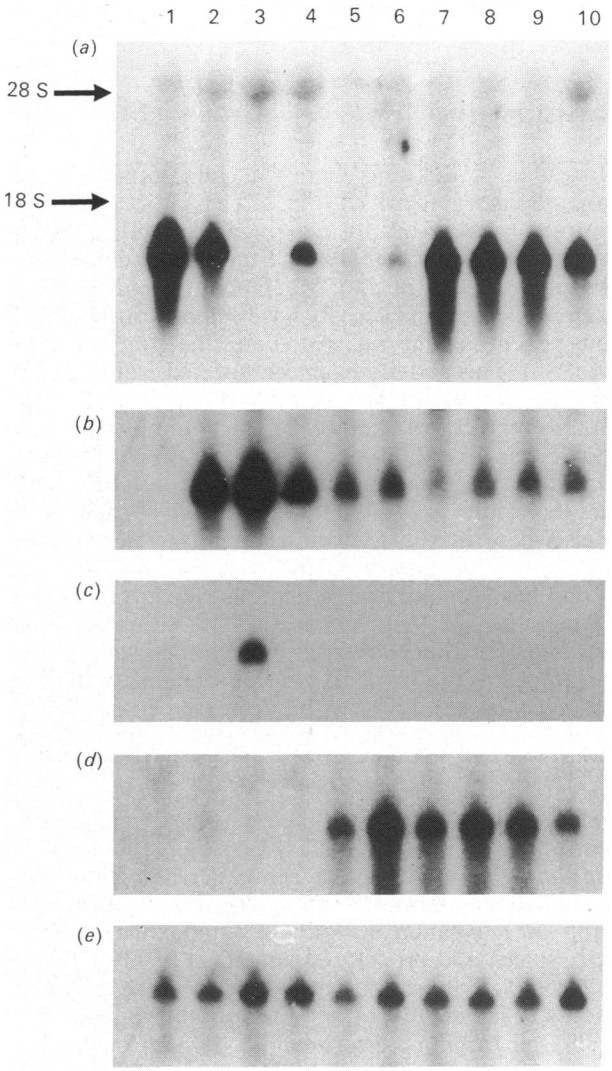

Fig. 1. Hybridization of ^{32}P-labelled kallikrein cRNA (*a*), somatostatin cRNA (*b*), gastrin cRNA (*c*), glucagon cRNA (*d*) and tubulin cDNA (*e*) probes sequentially to the same Northern blot of 12.5 μg of kidney (lane 1), corpus (lane 2), antrum (lane 3), duodenum (lane 4), jejunum (lane 5), ileum (lane 6), caecum (lane 7), ascending colon (lane 8), transverse colon (lane 9) and descending colon (lane 10) total RNA

The position of the 18 S and 28 S ribosomal bands in (*a*) is indicated. Autoradiography was for 4 (*a*), 5 (*b*), 2 (*c*), 4 (*d*) and 3 (*e*) days.

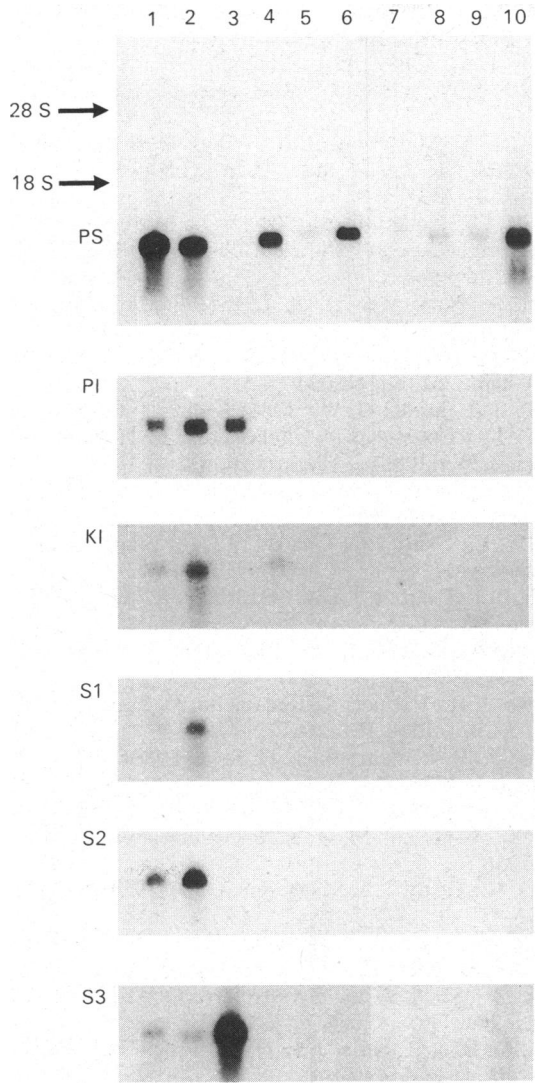

Fig. 2. Hybridization of ^{32}P-labelled kallikrein-gene-specific oligonucleotide probes sequentially to the same Northern blot of 12.5 μg of female-rat submaxillary-gland (lane 1), male-rat submaxillary-gland (lane 2), prostate (lane 3), kidney (lane 4), corpus (lanes 5 and 6), duodenum (lanes 7 and 8) and colon (lanes 9 and 10) total RNA (except lanes 6, 8 and 10, which relate to ~12.5 μg of polyadenylated RNA)

The probe used for panel PS is specific for the true glandular kallikrein gene (PS). The other probes are specific for P1 (a kallikrein-like gene), K1 (a kallikrein-like gene), S1 (a kallikein-like gene), S2 (true tonin gene) and S3 (a tonin-like gene). The position of the 18 S and 28 S ribosomal bands is indicated in the top panel. Autoradiography was for 3 (PS), 1 (P1), 4 (K1), 3 (S1), 3 (S2) and 3 (S3) days.

this band has been absent from blots of other preparations (results not shown) and may thus represent a small amount of contamination by corpus RNA.

The somatostatin cRNA probe (Fig. 1*b*) yields a very different pattern of hybridization along the gastrointestinal tract; note particularly the high levels observed in the gastric antrum. The gastrin cRNA probe (Fig. 1*c*) hybridizes almost exclusively with the antral RNA (lane 3). The glucagon cRNA probe (Fig. 1*d*) yields a distinct pattern of expression, being localized predominantly to terminal ileum, caecum and colon. The similar levels of non-specific binding to the 28S ribosomal RNA seen with the various cRNA probes demonstrates the relatively uniform loading of RNA. This is reinforced by equivalent hybridization with the tubulin cDNA probe (Fig. 1*e*) to the various gastrointestinal-tract RNAs, except the jejunal RNA, which may be loaded at slightly lower levels.

Hybridization with the kallikrein-specific oligonucleotide probes (Fig. 2) shows that the kallikrein gene expressed in the gastrointestinal tract is true kallikrein, since only the PS-specific oligonucleotide hybridizes to the corpus, duodenal and colonic RNA. Whereas all of the probes hybridize with the submaxillary-gland RNA, only K1 and PS hybridize with the kidney RNA; only S3 and P1 hybridize with the prostate RNA, as has been previously described (Ashley & MacDonald, 1985*b*; Clements *et al.*, 1988).

DISCUSSION

By using a kallikrein cRNA probe derived originally from rat pancreas (Swift *et al.*, 1982), we have obtained hybridization, on Northern blots, with a species of mRNA in the gastrointestinal tract of equivalent size to that seen in the kidney. In a previous report (Swift *et al.*, 1982) in which kallikrein mRNA was not detected with a cDNA probe in the 'intestine', only the small intestine was studied. Even with the highly sensitive cRNA probe, levels of kallikrein mRNA are found to be very low in both jejunum and ileum.

The kallikrein-like activity previously detected in the stomach (Uchida *et al.*, 1980; Schachter *et al.*, 1986; Kobayashi & Ohata, 1984) has been suggested to be derived from the salivary glands (Fuller & Funder, 1986; Skagen & Andersen, 1986). The present results, however, leave no doubt that synthesis of kallikrein does occur in the corpus of the stomach, though not in the gastric antrum. Schachter *et al.* (1986) have localized gastric kallikrein by immunocytochemistry and enzyme histochemistry to the surface epithelial mucous cells of the rat stomach. The histochemical studies by Schachter *et al.* (1986), and our mRNA data, clearly indicate local synthesis of kallikrein in the small intestine, albeit at lower levels than those observed in the corpus, caecum or colon. Miller *et al.* (1984) have demonstrated incorporation of [^{35}S]methionine into kallikrein-like immunoreactivity in the rat colon; Seki *et al.* (1972) have shown that the inactive zymogen (precursor) form of kallikrein is present in the colon of various species. Schachter *et al.* (1983, 1986) have localized kallikrein-like enzymes in the colon to the goblet cells, as is the case in stomach and intestine. The demonstration of local synthesis does not preclude the possibility that some portion of the kallikrein-like activity reported in the gastrointestinal tract (Uchida *et al.*, 1980; Uetsuji *et al.*, 1982; Schachter *et al.*, 1983, 1986; Skagen & Andersen, 1986; Zeitlin *et al.*, 1986), particularly the upper region, is derived from exocrine-gland secretions.

The seven members of the rat kallikrein-gene family so far characterized show extensive similarity in their nucleotide sequences. Though the cRNA probe used in these studies encodes true kallikrein, it will readily cross-hybridize with the other genes (Ashley & MacDonald, 1985*a*). The mRNAs for the various genes may be

distinguished on blots or by '*in situ*' hybridization (van Leeuwen *et al.*, 1987) by the use of short synthetic oligonucleotide probes directed at regions where the sequences are maximally different between family members. A panel of oligonucleotide probes specific for the six members of the rat kallikrein-gene family characterized so far (PS, S1, S2, S3, K1, P1) detected expression in the gut of only PS, true kallikrein (Ashley & MacDonald, 1985*a*).

In vitro, kallikrein and/or tonin have been shown to cleave prorenin to yield renin (Sealey *et al.*, 1978), pro-opiomelanocortin to yield adrenocorticotrophic hormone (Seidah *et al.*, 1979) and atriopeptinogen to yield atrial natriuretic factor (Currie *et al.*, 1984). The different pattern of expression of the kallikrein gene compared with that for the precursors of somatostatin, gastrin, glucagon and cholecystokinin (results not shown) suggests that they are unlikely to be necessarily subject to processing by kallikrein in the gastrointestinal tract. Schachter *et al.* (1986) postulate that kallikrein may have a role in goblet cells, processing the mucoprotein peptide segments, and that abnormal kallikrein function may contribute to the pathogenesis of cystic fibrosis. Similar speculation has related the possible role of kallikrein in epithelial chloride-ion transport to the pathogenesis of cystic fibrosis (Fuller & Funder, 1986). Since the only proven physiological substrate for true kallikrein is kininogen, the role of gastrointestinal kallikrein is probably to process circulating kininogen [kininogen mRNA has not been detected in the rat gastrointestinal tract (G. Schreiber, unpublished work)], to yield bradykinin. Kinins are thought to be involved in the regulation of local blood flow (Overlack *et al.*, 1983) and/or chloride-ion transport (Musch *et al.*, 1983; Cuthbert & MacVinish, 1986).

Identification of the expression of the kallikrein gene in the gastrointestinal tract establishes local synthesis as a source of the observed kallikrein-like activity; this should enable further studies of the regulation of its expression. Though the physiological substrate is yet to be identified, it is likely to be circulating kininogen, resulting in the generation of kinins.

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