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At the Cutting Edge

Anterior pituitary glandular kallikrein: a putative prolactin processing protease

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Introduction

Prolactin (PRL) has long been considered to be synthesized as a pre-hormone needing only signal sequence removal for generation of its secreted form (Cooke et al., 1980); trypsin-like processing typical of preprohormones has appeared unnecessary. Indeed, intact PRL clearly exhibits potent lactogenic activity and is a physiological inducer of milk production. Similarly, intact PRL has marked mitogenic actions on Nb2 lymphoma cells (Tanaka et al., 1980), suggesting a role in immune function. PRL has also been considered to function in mammary growth based on classic in vivo studies by Lyons et al. (1958). However, in vitro studies have provided equivocal support for this role, since PRL exhibits only modest mitogenic effects on mammary cells at levels hundreds to thousands of times higher than those which markedly induce lactogenesis or Nb2 lymphoma cell growth (see Imagawa et al., 1990 for review of PRL actions on mammary cells).

Such disparities have led to the hypothesis that PRL may require proteolytic maturation analogous to that of other hormones and growth factors for full expression of mammary mitogenic effects (Mittra, 1980). Indeed, many groups have reported novel PRL variants which seem likely to arise from proteolytic processing (see Sinha, 1992 for review). However, only limited progress has been made in the characterization of such variants, and the production mechanisms and products identified in some cases have raised serious questions of artifactual generation. Nonetheless, the hypothesis that PRL may undergo specific proteolytic processing to novel hormonal forms has recently received strong support from studies of rat pituitary glandular kallikrein (GK). This review surveys findings indicating that anterior pituitary GK functions as a processing enzyme that specifically cleaves intact PRL to produce PRL_{1-173} — an estrogen-dependent secretory product of the female rat pituitary.

Discovery of anterior pituitary glandular kallikrein (GK)

Endoproteolysis is a critical step in bioactive peptide maturation that is a product of diverse groups of enzymes. Subtilisin-related proteases appear to process many hormones (Seidah and Chretien, 1992). Renin, an aspartyl protease, forms angiotensin. Plasma kallikrein and complement factors (serine proteases of the chymotrypsin superfamily) produce vasoactive kinins and anaphylatoxins. Glandular kallikrein (GK; a chymotrypsin-related protease) is also linked to peptide processing; it exhibits high substrate specificity and generates kinins (potent vasodilator peptides) from kininogens by trypsin-like cleavages at Arg and Lys residues (Schachter, 1980). GK is also the prototypical member of a serine protease subfamily linked to peptide processing (MacDonald et al., 1988; Clements, 1989). Other members of the family include the γ-subunit nerve growth factor and epidermal growth factor-binding protein, which have been linked to growth factor processing (Server and Shooter, 1977; Isaackson et al., 1987). The submandibular gland and exocrine pancreas contain the highest GK levels but secrete the enzyme into nonvascular spaces lacking kininogen, suggesting other substrates. Indeed, GK cleaves some precursors in vitro as expected of a processing enzyme (Ole-MoiYoi et al., 1979; Mindroui et al., 1991). Together, the above properties of GK suggested that related enzymes may function in prohormone processing, and led to the screening of pituitary tissue for GK-like enzymes as a probe for likely processing proteases.

Two proteases were found when pituitaries were screened for kinin-producing activity. Pig anterior pitu-

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itary microsomes contained a protease resembling plasma kallikrein that largely existed as a latent zymogen (Powers and Nasjletti, 1982, 1984a). A similar enzyme was found in pig neurointermediate lobes (Cromlish and Seidah, 1986), and in rat pituitaries (Powers, 1986a,b). However, the resemblance of this protease to plasma kallikrein and a lack of tissue-specific distribution did not support a role as a processing enzyme. Indeed, Seidah et al. (1988) have identified this protease as plasma kallikrein which probably arises in pituitary from plasma contamination. Rat pituitaries were found to contain a second kinin-generating enzyme with enzymatic and immunologic properties identical to GK (Powers and Nasjletti, 1983, 1984b; Powers, 1986a,b). GK displayed tissue-specific regulation and distribution in the rat pituitary, being negligible in the neural lobe but at substantial levels in the intermediate lobe of both sexes. However, anterior lobe GK displayed a striking sex difference with levels 20-fold higher in females than in males. A pituitary origin for GK was proven by detection of true GK mRNA in the pituitary with expected patterns of regulation (Fuller et al., 1985, 1988; Clements et al., 1986; Chao et al., 1987; Pritchett and Roberts, 1987). Pituitaries of mice also contain GK mRNA (Penschow et al., 1991).

GK has classically been postulated to control local blood flow and capillary permeability through kinin generation, even though pituitary vessels lack smooth muscle and a high pressure arterial supply, suggesting that GK may have novel roles in the pituitary.

Anterior pituitary GK regulation and localization in lactotrophs

It is now established that estrogens induce the sex difference in anterior pituitary GK protein and mRNA levels (Clements et al., 1986, 1989; Powers, 1986a; Chao et al., 1987; Hatala and Powers, 1987). GK induction by estrogens is tissue-specific: GK in the intermediate lobe and other organs is not induced. Estrogen induction of GK is powerfully inhibited by tamoxifen (Powers et al., 1989). Indeed, tamoxifen acts as a pure estrogen antagonist on GK whereas it acts as a partial agonist on other estrogen targets in the rat.

Anterior pituitary GK is also subject to inhibitory dopaminergic control (Powers and Hatala, 1986; Pritchett and Roberts, 1987; Hatala and Powers, 1988a). Haloperidol and reserpine (which block dopaminergic neurotransmission) increase GK enzyme and mRNA levels, whereas bromocriptine (a dopamine agonist) has the opposite effects. Haloperidol has little effect on GK in the absence of estrogen, indicating that estrogen does not induce GK by antagonism of dopaminergic control, but rather that dopamine modulates estrogen effects. Dopamine also represses intermediate lobe GK (Powers, 1985, 1986c; Pritchett and Roberts, 1987) consistent with a GK localization in intermediate lobe melanotrophs.

The estrogen-induction and dopaminergic repression of anterior pituitary GK uniquely parallels PRL, suggesting a localization in lactotrophs. Moreover, GK and PRL parallel each other during pregnancy and the development of sex differences at puberty (Powers and Westlin, 1987; Hatala and Powers, 1988b, Clements et al., 1990). GK is also elevated in lactotroph tumors (Powers, 1987; Fuller et al., 1988; Hatala and Powers, 1988a), and is produced by GH_3 cells, a PRL-secreting cell line (Chao and Chao, 1988). Proof that anterior lobe GK originates from lactotrophs was provided by immunocytochemical studies showing GK in lactotrophs but not other anterior lobe cell types (Kitagawa et al., 1990; Kizuki et al., 1990; Vio et al., 1990). GK immunoreactivity has also been detected in human prolactinoma cells (Jones et al., 1990, 1992).

GK mRNA encodes a preproenzyme targeted to the secretory pathway (MacDonald et al., 1988). Cell fractionation (Hatala and Powers, 1989) and immunocytochemical studies (Kitagawa et al., 1990; Vio et al., 1990) have shown anterior lobe GK to be located in the Golgi apparatus and secretory granules. Indeed, GK is secreted both from GH_3 cells (Chao and Chao, 1988), and from rat pituitaries (Anthony et al., 1993). Bromocriptine inhibits GK release from pituitaries, showing that GK is targeted to the regulated secretory pathway.

Pituitary GK predominantly exists as a latent zymogen (proGK) that can be trypsin-activated (Powers, 1986a,b; Chao and Chao, 1988; Hatala and Powers, 1989; Kitagawa et al., 1990). GK also exists as a zymogen in the pancreas, but is active in submandibular glands. The activation mechanism of pituitary proGK is unknown: it is unaffected by estrogen, dopamine blockade, secretion, or organelle localization. It seems unlikely that proGK would be so powerfully regulated without having a function; accordingly, nonproteolytic activation mechanisms appear likely, precedents for which can be found in the coagulation, fibrinolytic and complement cascades (Meier et al., 1977; Castellino, 1979; Loos, 1982; Manchanda and Schwartz, 1991; Lui et al. 1992).

PRL proteolysis by GK in vitro

PRL is synthesized as a prehormone requiring only signal peptide cleavage to generate lactogenic hormone (Cooke et al., 1980). However, PRL contains an Arg-Arg pair typical of prohormone processing sites, and several groups have reported novel processed forms of PRL in the pituitary (see below). The coordinate expression and regulation of GK and PRL in the lactotroph secretory pathway suggested that GK may

Fig. 1. C-terminal domain of rat PRL showing GK cleavage sites (arrows). Asterisks indicate basic residues removed by carboxypeptidase B or E following GK cleavage. CT-peptide corresponds to the synthetic peptide used to raise a specific antiserum for the 22K PRL variant (CT-antiserum).

function to process PRL to forms with novel hormonal activity. To test this hypothesis, the ability of active GK (purified from rat urine) to cleave rat PRL in vitro was studied. GK failed to cleave standard rat PRL but cleavage was elicited with moderate levels of thiols such as dithiothreitol, and thiol effects were enhanced by Triton X-100, a nonionic detergent (Powers and Hatala, 1990). Under such conditions, GK specifically cleaved PRL from a 25 kDa (25K) form to a 22K form as determined by gel electrophoresis. In contrast, trypsin rapidly degraded PRL to small fragments. Cleavage by GK occurred at three sites clustered in a highly conserved domain near the PRL C-terminus (Arg_{174}-Arg_{175}, Lys_{185}-Phe_{186}, Arg_{188}-Cys_{189}) (Fig. 1). This domain was bracketed by Cys residues, and had features resembling GK cleavage sites in kininogens. Thiols did not affect GK activity but markedly altered PRL conformation. Thus, thiols seem to favor cleavage by altering PRL disulfides and producing novel folding isomers that are GK substrates.

Synthetic thiols (dithiothreitol, mercaptoethanol) are the most potent in eliciting PRL cleavage by GK in vitro. Biological thiols are much less potent, mainly due to their tendency to rapidly oxidize (Hatala et al., 1991). However, the biological thiols glutathione and thioredoxin exhibit marked increases in potency when coupled to metabolic pathways that shuttle reducing equivalents to oxidized thiols, thus permitting physiological levels of glutathione and thioredoxin to support PRL cleavage (Hatala et al., 1991). Intriguingly, the dependence of biological thiols on reducing shuttles permits reaction control by enzymes and substrates making up the shuttle (Hatala et al., 1991), suggesting that PRL cleavage in vivo might be regulated by extracellular signals altering lactotroph metabolism and redox status.

Evidence of PRL processing by GK in vivo

Thiol-elicited PRL cleavage by GK presents a unique mechanism of protein processing. Thus, it is of note that several groups have reported evidence indicating that disulfide bonds in PRL are labile and undergo changes in their redox status in vivo, including reports of PRL "transformation" during secretory events (Grosvenor et al., 1982; Mena et al., 1986), the presence of disulfide-linked oligomers in secretory granules (Lorenson and Jacobs, 1982), and the ability of cysteamine (a biological thiol) to selectively decrease PRL immunoreactivity in vivo (Millard et al., 1982). Though the significance of these findings has been obscure, the discovery of thiol-elicited PRL cleavage by GK suggests that such events may enable PRL processing in vivo.

Direct evidence indicating biological significance for PRL cleavage by GK was provided by reports of 21–22K PRL variants in rat pituitary (detected by Western blot analysis). Oetting and Walker (1985) reported the synthesis and secretion of a 21K variant by cultured pituitary cells which did not reflect lysosomal degradation, and arose from C-terminal processing (consistent with GK cleavage). Later work (Oetting et al., 1989) showed that levels of the 21K PRL variant were increased by cysteamine, prefiguring of our own results on thiol-elicited PRL cleavage by GK. Recently, Ho et al. (1991) reported that products of thiol-elicited PRL cleavage by GK comigrated with the 21K PRL variant during two-dimensional gel electrophoresis, and 21–22K PRL variants have also been reported by other groups (Sinha and Jacobsen, 1988; Shah and Hymer, 1989; Mena et al., 1992).

We also found a similar 22K variant in rat pituitaries (Powers and Hatala, 1990), which was estrogen-dependent (like GK), and was induced by cysteamine in vivo; cysteamine also elicits PRL cleavage in vitro (Hatala et al., 1991). The 22K PRL variant comigrated with a PRL band generated in vitro with GK and carboxypeptidase B (Powers and Hatala, 1990). Carboxypeptidase B cleaves C-terminal Arg and Lys residues and mimics actions of carboxypeptidase E, the pituitary peptidase acting at this step of prohormone processing (Mains et al., 1990). The results suggested that the 22K PRL variant corresponds to PRL_{1-173} (cleavage at Arg_{174}-Arg_{175} followed by removal of Arg_{174}).

Cysteamine decreases PRL immunoreactivity by direct pituitary actions that alter its conformation (Scammell and Dannies, 1984; Sagar et al., 1985). Thus, cysteamine has provided a useful probe for exploring the linkage between thiol-induced transformation of PRL conformation and increased production of 22K PRL in vivo (Anthony and Powers, 1992). Dose–response studies showed that the 10–15-fold increases in 22K PRL with cysteamine were highly correlated with changes in PRL conformation, and conformational changes caused by cysteamine in vivo parallel those seen in vitro by thiol levels that elicit PRL cleavage. Time-course studies showed changes in PRL conformation in vivo to precede increases in 22K PRL, indicating a precursor–product relationship. Also, cys-

teamine transformed PRL in both untreated and estrogen-treated rats, but yielded large increases in 22K PRL only in estrogen-treated rats (Anthony and Powers, 1992); this parallels anterior lobe GK levels which are also estrogen-dependent. Together, such findings provided strong support for the hypothesis that the 22K PRL variant arises from thiol-dependent PRL processing by GK. The results also indicate that the rate of PRL processing in vivo is likely to be controlled by the supply of reducing equivalents for PRL transformation.

We have recently generated an antiserum that is highly specific for the endogenous 22K PRL variant by immunizing rabbits against a synthetic peptide corresponding to the C-terminus arising from sequential cleavage with GK and carboxypeptidase B (Fig. 1). This C-terminal directed antiserum (CT-antiserum) did not bind intact PRL or PRL cleaved by GK alone, but bound PRL cleaved with GK and carboxypeptidase B or E (Anthony et al., 1993). CT-antiserum binding appears to require Cys_{172}-Leu_{173} at the C-terminus since the presence of Arg_{174} prevented binding, and alkylation of Cys residues with iodoacetamide markedly impaired binding. CT-antiserum specifically bound a 22K band in rat pituitaries that was estrogen and cysteamine-induced, and comigrated with 22K PRL produced in vitro with GK and carboxypeptidase B (Anthony et al., 1993). This matched the properties of 22K PRL detected with standard PRL antiserum and identified the 22K variant as PRL_{1-173}, the proposed product of processing by GK (trypsin-like cleavage) and carboxypeptidase E. Such processing is typical of prohormone maturation and is unlikely to reflect a tissue handling artifact or a degradative process. In addition, alternative exon splice sites that might yield PRL_{1-173} are not evident in the rat PRL gene (Cooke and Baxter, 1982).

22K PRL is concentrated in secretory granule enriched fractions (Anthony et al., 1993), consistent with work by Oetting and Walker (1985) reporting release of 22K PRL from cultured pituitary cells. We have also detected 22K PRL release during short-term incubations of rat pituitaries (Anthony et al., 1993); female pituitaries released substantial amounts of 22K PRL whereas no release was detected from male pituitaries. 22K PRL release was potently blocked by bromocriptine, indicating release through the regulated secretory pathway. As expected of a processing enzyme, 22K PRL release was associated with proGK release which was also female-specific and inhibited by bromocriptine (Anthony et al., 1993).

Conclusions

Fig. 2 shows the current hypothesis on GK processing of PRL. Evidence supporting the hypothesis that

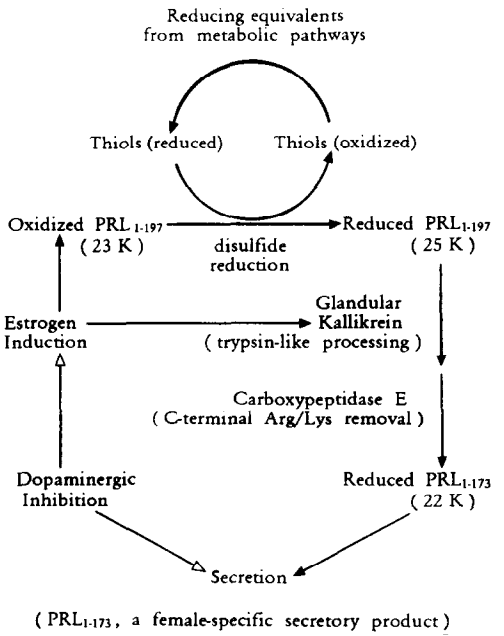

Fig. 2. Diagram summarizing the present hypothesis regarding GK production of the 22K PRL variant (PRL_{1-173}). Glutathione and thioredoxin are examples of biological thiols which potently elicit PRL cleavage by GK in vitro when coupled to metabolic pathways that shuttle reducing equivalents to the oxidized thiol (Hatala et al., 1991). 22K, 23K and 25K denote the apparent molecular weights of various PRL forms during sodium dodecyl sulfate-gel electrophoresis.

GK functions in PRL processing is as follows: (1) Anterior pituitary GK is coregulated with PRL and located in the lactotroph secretory pathway, a site suited for such a role. (2) GK specifically cleaves PRL in vitro to yield a 22K product (PRL_{1-174}) in a thiol-dependent reaction. (3) The pituitary contains PRL_{1-173}, the product of GK and carboxypeptidase E processing. (4) The estrogen and thiol-dependence of 22K PRL in vivo matches the estrogen induction of GK and thiol-dependence of PRL cleavage. (5) The secretory granule storage and regulated release of 22K PRL identifies it as a natural secretory product. Together, the evidence strongly argues for a role of GK in PRL processing. Proof of this role requires evidence that blockade of GK activity or synthesis inhibits PRL processing. The possibility that proteases other than GK might process PRL must be considered given the puzzling existence of pituitary GK as a zymogen. Conversely, proGK activation mechanisms and the pathways modulating PRL redox state in vivo require elucidation. Nonetheless, study of pituitary GK has successfully led to identification of PRL_{1-173} as a natural secretory product of the female rat pituitary. The 22K PRL content of female pituitaries is only a small fraction of intact PRL ($< 4\%$). However, PRL levels are 10- to 30-fold higher than many other pituitary hormones (LH, FSH, TSH, ACTH), and 22K PRL levels may thus be adequate for a hormonal function.

Study of the biological activity of 22K PRL has just begun but classic PRL actions on Nb2 lymphoma cells seem to be greatly diminished by GK processing (unpublished results). A similar event occurs during ACTH and β-lipotropin processing to α-MSH and β-endorphin, respectively. The precursor peptides lose most of their original activity but acquire different ones with processing. The novel biological activities possessed by 22K PRL remain to be elucidated but other sex-dependent members of the GK family have been linked to growth factor processing (Server and Shooter, 1977; Isaackson et al., 1988). The estrogen induction of GK and 22K PRL suggests a role in female reproductive biology, and the rise in GK levels during puberty would be appear to be well suited for a role in mammary growth. In addition, the GK processing site is among the most highly conserved regions of PRL, implying major functional significance. Finally, it should be noted that 22K PRL variants have been reported in the serum of women (Sinha et al., 1985; Lui et al., 1990), and GK is expressed in human lactotrophs (Jones et al. 1990, 1992). Thus, the PRL processing pathway described in the rat may also occur in humans. Further analysis of pituitary GK and its novel cleavage product (PRL_{1-173}) thus clearly appears warranted.

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