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Arg21 is the preferred kexin cleavage site in parathyroid-hormone-related protein

Hanne DIEFENBACH-JAGGER[1], Charles BRENNER[2], Bruce E. KEMP[1], Will BARON[3], John MCLEAN[1], T. John MARTIN[1] and Jane M. MOSELEY[1]

[1] St Vincent's Institute of Medical Research and The University of Melbourne Department of Medicine, St Vincent's Hospital, Fitzroy, Victoria, Australia
[2] Department of Biochemistry, Stanford University Medical Center, Stanford, USA
[3] Genentech Inc, South San Francisco, USA

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Parathyroid-hormone-related protein (PTHrP) contains several potential sites for proteolytic processing. Although there is considerable evidence for the existence of cleaved products *in vivo*, little is known about the post-translational processing of PTHrP. We have used purified kexin (Kex2) protease to identify which cleavage sites in recombinant PTHrP(1–141) might be of physiological significance. Cleavage products were identified by N-terminal sequencing. Kex2 preferentially cleaved PTHrP(1–141) carboxy to the triplet arginine site Arg-Arg-Arg_{21} with a K_m of 3.3 ± 1.7 μM and a k_{cat} of 6 ± 1.2 s^{-1}. Substitution of alanine for Arg_{19} resulted in substantially reduced conversion, while no detectable cleavage occurred when alanine was substituted for either Arg_{20} or Arg_{21}. In contrast, the degree of Kex2 cleavage at Arg_{21} in PTHrP(1–34) was lower. No detectable cleavage occurred in an unrelated synthetic peptide containing both double and triple arginine sites. Low levels of cleavage also took place carboxy to Lys-Arg_{97}, Lys-Arg_{105}, Arg-Arg_{106} and Thr-Arg_{108}. Cleavage carboxy to Lys-Arg_{105}, the best of these minor sites, occurred with a K_m of 8.4 ± 2.7 μM and a k_{cat} of 0.8 ± 0.2 s^{-1}. These studies indicate that the preferred Kex2 cleavage site in PTHrP(1–141) is carboxy to Arg-Arg-Arg_{21}, which effectively destroys its parathyroid-hormone-like biological activity. Cleavage of this site by Kex2-related mammalian convertases *in vivo* may be an important mechanism for full elaboration of the non-parathyroid-hormone-like paracrine actions of PTHrP in a tissue-specific manner.

Keywords. Parathyroid-hormone-related protein; processing; Kex2; convertases.

Many biologically active proteins and hormones are synthesised as pro-proteins which require processing, commonly on the carboxy side of the dibasic sites Lys-Arg and Arg-Arg, to generate their active forms (Barr, 1991). Kexin, the Kex2 protease of *Saccharomyces cerevisiae*, a highly specific, calcium-dependent serine protease responsible for the cleavage of yeast pro-α-factor, was the first prohormone convertase to be cloned, characterized, purified in chemical quantities and analyzed kinetically (Julius et al., 1984; Fuller et al., 1989a; Brenner and Fuller, 1992; Brenner et al., 1993; Brenner et al., 1994). Kex2 cleaves on the carboxy side of Lys-Arg, Arg-Arg and Pro-Arg sites in its natural substrates which, in addition to the pro-α-factor, include pro-killer toxin (Bostian et al., 1984; Russo et al., 1992; Zhu et al., 1992) and a secreted exoglucanase (Basco et al., 1990).

Transfection studies in mammalian cells indicate that Kex2 correctly processes many vertebrate proprotein precursors, mimicking the actions of mammalian convertases (Thim et al., 1986; Thomas et al., 1988; Brennan et al., 1990). For example, it cleaves proopiomelanocortin (POMC) to produce ACTH (adrenocorticotropic hormone), β-lipotropin (β-LPH) and β endorphin, by cleavage carboxy to Lys-Arg sites (Thomas et al., 1988, 1991). *In vivo*, these sites in POMC are cleaved in a tissue-specific manner by prohormone convertase (PC) 1, which is in high concentration in the anterior pituitary, to give rise to ACTH, and by PC2, which is in higher concentration in the intermediate pituitary, to give rise to β-LPH (Thomas et al., 1991; Seidah et al., 1991; Benjannet et al., 1991). In *S. cerevisiae*, two sites within proinsulin are cleaved by Kex2 (Thim et al., 1986); in the mammalian pancreas, proinsulin is cleaved by the prohormone convertases PC1 (PC3) and PC2, each of which cleaves at only one site (Smeekens et al., 1992). In *S. cerevisiae*, Kex2 processes angler fish prosomatostatin I at three sites, only one of which (Arg-Lys_{-2}) is normally cleaved *in vivo* by the angler fish, giving rise to somatostatin-14. Cleavage by Kex2 also occurred after Arg_{-3}, giving rise to lysine-somatostatin-14. The third cleavage at a single arginine residue (Arg_{-15}) generates a product normally produced from a homologous precursor (Bourbonnais et al., 1993). Thus, *in vivo* cleavage at any one site will depend upon the tissue-specific expression of convertases; studies to date support the view that Kex2 correctly identifies sites of processing by mammalian convertases.

The lumenal moiety of the transmembrane Kex2 protease contains a domain homologous to subtilisin and the so-called 'P-domain' (Fuller et al., 1989b; Gluschankof and Fuller, 1994),

Correspondence to J. M. Moseley, St. Vincent's Institute of Medical Research, St. Vincent's Hospital, 41, Victoria Parade, Fitzroy 3065, Australia
Fax: +61 3 416 2676.

Abbreviations. PTHrP, parathyroid-hormone-related protein; ACTH, adrenocorticotropic hormone; Kex2, kexin; LPH, lipocortin; MLCK, myosin light chain kinase; PC, prohormone convertase; POMC, proopiomelanocortin; PTH, parathyroid hormone.

Enzyme. Kexin (EC 3.4.21.61).

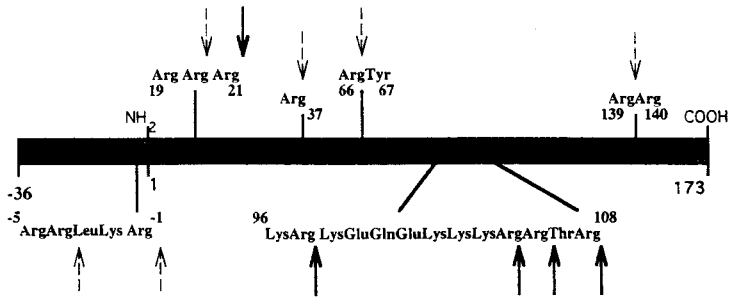

Fig. 1. Cleavage of prepro-PTHrP(-36-173). Arrows indicate potential convertase cleavage points. Solid arrows indicate Kex2 cleavages of PTHrP(1−141), demonstrated in the present study.

both of which are conserved in the recently discovered family of Kex2-related mammalian processing enzymes which includes PC1, PC2, PC4, PC6 and furin (Smeekens and Steiner, 1990; van der Ouweland et al., 1990; Seidah et al., 1990, 1991, 1992; Hatsuzawa et al., 1990; Nakayama et al., 1992; Nakagawa et al., 1993). Precursors with polybasic sequences such as Arg-Xaa-Lys-Arg and Arg-Xaa-Arg-Arg are found to be cleaved, especially in the constitutive secretory pathway of neuroendocrine cells (Watanabe et al., 1992) and the specificity and localization of furin are consistent with furin having a role in such cleavages in the constitutive pathway (Hatsuzawa et al., 1990; Barr, 1991; Hosaka et al., 1991; Misumi et al., 1991; Molloy et al., 1992).

Parathyroid-hormone-related protein (PTHrP) is the principal mediator of humoral hypercalcemia of malignancy which results from its production by certain tumors, particularly those of squamous origin (reviewed in Martin et al., 1992). In addition to its endocrine parathyroid-hormone-like N-terminus, which mediates hypercalcemia in cancer patients, different parts of PTHrP are also be responsible for other biological actions. Some of these actions are paracrine in nature, particularly in smooth muscle (Thiede et al., 1989; Paspaliaris et al., 1992; Yamamoto et al., 1991), skin (Hayman et al., 1989; Kremer et al., 1991) and early placenta (Rodda et al., 1988), but the extent of tissue-specific processing to elaborate them is unknown (Orloff et al., 1994). Physiological studies suggest that the placental calcium-transport activity lies carboxy to PTHrP(1−34) (Rodda et al., 1988; Abbas et al., 1989; Care et al., 1991) and an osteoclast inhibitory action has been proposed for the carboxy-terminal sequence PTHrP(107−111) (Fenton et al., 1991). Alternate splicing of the *PTHrP* gene predicts three isoforms of PTHrP of 139, 141 or 173 amino acids in length, each synthesized with a 36-amino-acid prepro-sequence. In addition to processing of the prepro-sequences, there is some evidence to indicate that isoform and tissue-specific processing may occur (Yang et al., 1994). Many PTHrP fragments have been detected (Moseley et al., 1987; Zajac et al., 1989; Burtis et al., 1992, 1994; MacIsaac et al., 1990; Soifer et al., 1994), although some of these may result from non-specific proteolytic degradation. The presence of multiple dibasic sites in PTHrP suggests that it is a target for convertase action (Fig. 1) and that, in addition to processing of the signal and pro-sequences, it is likely that PTHrP is subject to processing by specific convertases, possibly in a tissue-specific manner.

Since Kex2 reliably identifies sites for the action of mammalian convertases, we have examined cleavage of pure recombinant full-length PTHrP(1−141) by purified secreted soluble Kex2 protease (Brenner and Fuller, 1992; Brenner et al., 1994). This has allowed us to investigate both the potential for convertase action on PTHrP and also the specificity of the Kex2 protease in a full-length protein. Native PTHrP and mutant PTHrP species with substitutions adjacent to two cleavage sites were used as substrates.

METHODS

Peptide synthesis and protease purification. PTHrP(1−34) and a myosin light chain kinase (MLCK) peptide analogue spanning residues 787−807 [MLCK (798−807)Leu_{800}, $Arg_{788,792,793,799,802}$] were synthesised as described (Kemp et al., 1987). Single-stranded Kex2 was expressed and purified as described (Brenner and Fuller, 1992; Brenner et al., 1994).

Construction of mutant PTHrP plasmids. Mutations were generated using PCR (Higuchi et al., 1989) with pairs of reverse-complementary primers to introduce mutations (primary reactions) and amplified using a common set of sense and antisense primers (secondary reactions).

Primary reactions (100 µl) contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.0001 % gelatin, 0.2 mM each dNTP, plus 100 ng template (pTrpMLE.brf3.1; Hammonds et al., 1989), 33 ng primer and 5 U *Taq* polymerase (Perkin Elmer Cetus). The template for the [Ala108]PTHrP(1−141) primary reactions was [Ala106]PTHrP(1−141). Primers were removed by centrifugal filtration (Centricon 30) with three 2-ml washes of 10 mM Tris/HCl, pH 7.6, 1 mM EDTA, pH 8.0 (TE), and the volumes were reduced to approximately 50 µl. Secondary reactions were carried out using 5 µl primary reactions as templates (conditions as described above).

Pairs of primers were as follows, sequences 5′ to 3′ (sense, antisense):

[Ala19] PTHrP(1−141), AGTCCATCCAAGATTTAGCGC-GACGATTCTTCCTTC, GAAGGAAGAATCGTCGCGCTAA-ATCTTGGATGGACT; [Ala20]PTHrP(1−141), ATCCAAGA-TTTACGGGCACGATTCTTCCTTCA, TGAAGGAAGAATC-GTGCCCGTAAATCTTGGAT; Ala]21PTHrP(1−141), TCCA-AGATTTACGGCGAGCATTTCCTTCACCATC, GATGGTG-AAGGAAGAATGCTCGCCGTAAATCTTGGA; [Ala106]-PT-HrP(1−141), GGAAAAGAAAAAACGGGCAACTCGCTGC-CTTGG, CCAGGCAGAGCGAGTTGCCCGTTTTTTCTTTT-CC; [Ala108]PTHrP(1−141), GAAAAAACGGCGAACTGCC-TGCCTGGTTAGAC, GTCTAACCAGGCAGAGGCAGTTC-GCCGTTTTTTC; [Ala106, Ala108]PTHrP(1−141), GAAAA-GAAAAAACGGGCAACTGCCTCTGCCTGGTTAGAC, GT-CTAACCAGGCAGAGGCAGTTGCCCGTTTTTTCTTTTC; common sequence ATTGCCACCGCHGCATCATGCACAG. TCAGAAGCTTGGAAGGTCTCTGCTGAAAATTTCA. PCR was initiated by 5 min at 94 °C and performed in 25 cycles of 55, 72 and 96−98 °C, each temperature being applied for 30 s, except that the final 72 °C extension was continued for 10 min.

Secondary PCR reactions were digested with restriction enzymes *Eco*RI and *Hin*dIII, gel purified, and ligated into a version

of plasmid pTrpMLE.brf3.1 in which nucleotides 1043–1557 had been deleted. Ligations were transformed into *Escherichia coli* strain W3110. The sequences of clones were confirmed by the dideoxy method with Sequenase v2.0 (US Biochemical).

Expression and purification of PTHrP mutants. Recombinant PTHrP(1–141) was expressed and purified as described (Hammonds et al., 1989). Overnight cultures (10 ml) of *E. coli* strain W3110 transformants were grown in Luria-Bertani broth containing 10 μg/ml tetracycline (Sigma) were used to innoculate 200 ml cultures. After 90 min growth at 37 °C, cells were induced with 150 μl indolacetic acid (Sigma, 25 mg/ml) and were harvested 24 hours later. Cell paste from 50 ml culture was suspended in 4 ml 25 mM Tris/HCl, 5 mM EDTA, pH 7.2. Following sonication, inclusion bodies were removed by centrifugation and suspended in 3.0 ml 70 % formic acid containing 100 mg/ml cyanogen bromide (Merck). After 2 hours at room temperature, the solution was clarified by centrifugation and diluted with 55 ml distilled water followed by 1 ml equilibrated S-Sepharose (Pharmacia). Resin was removed after 45 min stirring and washed with 12 ml water, then 25 ml 0.1 M NaCl, 20 mM sodium acetate, pH 4.5, and finally suspended in 10 ml 0.25 M NaCl in 20 mM sodium acetate, pH 4.5. The resin was poured into a column and washed stepwise with 2 ml each of 0.3, 0.4, 0.5 and 0.6 M NaCl containing 20 mM sodium acetate, pH 4.5. PTHrP was eluted with 5 ml 1 M NaCl, 20 mM sodium acetate, pH 4.5, and further purified by reverse-phase HPLC in a 60-min gradient of acetonitrile (28–60 %) in 0.1 % trifluoroacetic acid, using a 0.46 cm×25 cm C_8 Bakerbond column (J. T. Bakerbond). The main peak was collected and rechromatographed under the same conditions. The purity of the PTHrP preparations was confirmed by amino-acid analysis and SDS/PAGE.

Cleavage conditions. Cleavage experiments were carried out in 200 mM Tris/HCl, pH 7.0, with 1 mM $CaCl_2$ at 37 °C. Control incubations contained 1 mM EDTA instead of $CaCl_2$ and the same concentration of enzyme. Controls without enzyme contained $CaCl_2$.

Kinetic analysis of amino-terminal cleavage. Three separate experiments with single points were carried out. Reaction volumes were 80 μl cleavage buffer, the substrate concentration ranged over 0.67–50 μM PTHrP(1–141). Reactions were started at 37 °C with 66 fmol enzyme and stopped after 40 s with 100 μl 0.23 % trifluoroacetic acid. Digests were kept frozen until HPLC analysis. At low substrate concentrations, it was necessary to scale up the reaction volume to allow reliable measurement of peak heights of the PTHrP(1–21) cleavage product. Results (13 points) were analyzed by the ENZFITTER program (Fig. 4). Conversion of peak heights at 214 nm to pmol PTHrP(1–21) was via a standard curve ranging over 5–100 pmol, based on quantitative amino-acid analysis of the peptides. k_{cat} and K_m were calculated with ENZFITTER, version 103.

Kinetic analysis of carboxy-terminal cleavages. A single experiment with single points was carried out in 0.5 ml cleavage buffer. The substrate concentration ranged over 2–25 μM PTHrP(1–141) and 0.6–12.3 μM [Arg20]PTHrP(1–141). The reaction was started at 37 °C with 330 fmol enzyme, stopped after 5 m with 80 μl 1 % trifluoroacetic acid and analyzed by HPLC. The amount of product was determined by peak-height measurements at 214 nm, which were compared to the peak height of 30 pmol product as determined by amino-acid analysis. Total cleavage was 5–6 % at the lowest substrate concentration. k_{cat} and K_m were calculated with ENZFITTER, version 103.

Amino-terminal cleavage of native and substituted recombinant PTHrP(1–141). Substrates in 80 μl volumes, concentrations as indicated in Table 1, were incubated with 66 fmol enzyme at 37 °C for 2 hours. Reactions were stopped with 100 μl 0.23 % cold trifluoroacetic acid and frozen at −20 °C until analysed by HPLC. The three recombinant mutants of PTHrP(1–141) with Ala_{19}, Ala_{20} or Ala_{21} were compared under identical conditions to determine the relative cleavage efficiency of Kex2. Synthetic PTHrP(1–34) and an unrelated peptide MLCK(787–807)Leu_{800},$Arg_{788,792,793,799,802}$ (Ser Arg Asp Met Arg Arg Tyr Met Ala Arg Arg Arg Leu Gln Arg Thr Gly His Ala Val) with both a doublet and a triplet of arginine residues, were included for comparison. The amount of cleavage was determined from peak heights before and after cleavage. Values are means of three determinations.

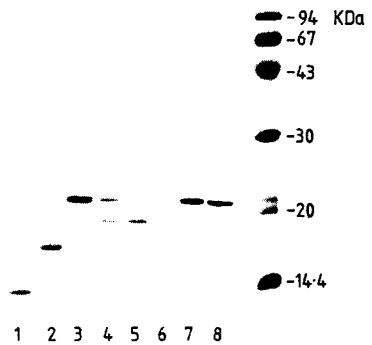

Fig. 2. Coomassie-blue-stained SDS/PAGE of PTHrP(1–141) cleavage by Kex2 protease as described in Materials and Methods. Lane 1, recombinant PTHrP(1–84) standard; lane 2, recombinant PTHrP(1–108) standard; lane 3, recombinant PTHrP(1–141) standard; lane 4, incubation of PTHrP(1–141) with Kex2 for 20 min; lane 5, incubation of PTHrP(1–141) with Kex2 for 40 min; lane 6, incubation of PTHrP(1–141) with Kex2 for 80 min; lane 7, incubation for 80 min in the presence of EDTA; lane 8, control incubation for 80 min without enzyme.

Carboxy-terminal cleavage of native and substituted PTHrP(1–141). Under conditions where amino-terminal cleavage of parent PTHrP(1–141) was optimal, a cluster of minor peaks eluted on reverse-phase HPLC with retention times similar to PTHrP(107–139) (Fig. 3, peaks D). Western-blot transfer confirmed the presence of two peptides of 6.5 kDa and 3.5 kDa, respectively, which stained positively with a polyclonal antibody raised against PTHrP(107–139) (data not shown). By increasing the amount of substrate to 1 mg and maintaining the enzyme/substrate ratio, sufficient product was generated to allow sequence analysis. Extension of the reaction time to 6 hours, or increasing the enzyme/substrate ratio twofold did not increase the amount of these peptides. Cleavage at these carboxy-terminal sites was then compared in the mutants [Ala106]PTHrP(1–141), [Ala108]PTHrP(1–141), [Ala106, 108]PTHrP(1–141), [Ala20, His94]PTHrP(1–141) and [Ala20]PTHrP(1–141).

HPLC analysis of digestion products. Digestion products were analysed on a model 440 Waters liquid chromatograph with a gradient of 9–60 % acetonitrile in 0.1 % trifluoroacetic acid (by vol.) over 85 min. The detection system was a single-wavelength detector at 214 nm with an Omniscribe recorder (Texas Instruments).

Amino-terminal sequencing. Cleavage products were identified by amino-terminal sequencing. The samples were sequenced on an Applied Biosystems model 471A protein sequencer.

Polyacrylamide gel analysis. SDS/PAGE was by the method of Laemmli (1970) using gels which consisted of 17 % acrylamide crosslinked with 0.6 % bisacrylamide. Equal aliquots of the cleavage digest were added at the various time points to the sample buffer (including 2-mercaptoethanol) to stop reactions. Samples were boiled for 3 min. The gel was stained with Coomassie blue G (Pharmacia South Seas Pty Ltd) in 7 % acetic

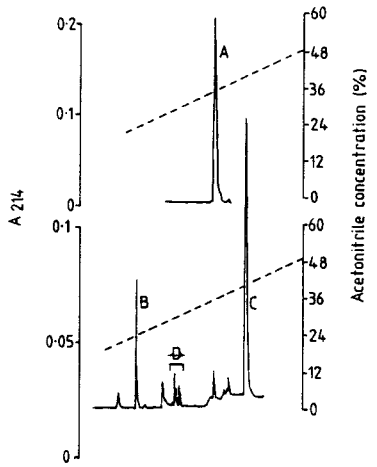

Fig. 3. Separation of PTHrP cleavage products by HPLC in acetonitrile/trifluoracetic acid. Peak A, PTHrP(1−141) prior to cleavage with Kex2; peak B, PTHrP(1−21); peak C, PTHrP(22−141). Products were identified by N-terminal sequencing. Peak D, C-terminal cleavage products identified by scaled up digestion (see Fig. 5).

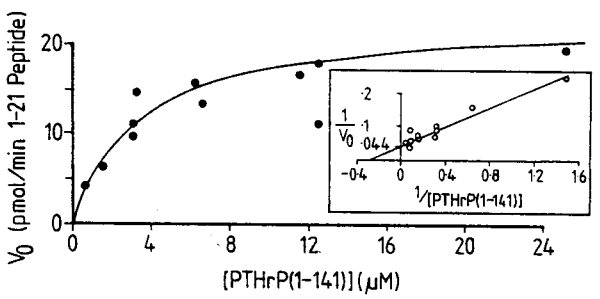

Fig. 4. Initial velocity and K_m for production of PTHrP(1–21) by Kex2 protease cleavage of PTHrP(1–141) at Arg_{21}. Calculations were made from peak heights produced at each substrate concentration after 40 s incubation.

acid (by vol.), 25 % methanol (by vol.), and was destained in the same solution without added stain. Western-blot analysis and immunoblotting were carried out with antibodies raised in rabbits to PTHrP(1−16) (Danks et al., 1989), in goats to PTHrP(50−69) (MacIsaac et al., 1990) and to PTHrP(107−139) (unpublished results).

RESULTS

Kex2 cleavage of PTHrP(1–141) at the Arg_{19}-Arg-Arg_{21} site. Incubation of PTHrP(1−141) with Kex2 protease at 37°C (pH 7.0) resulted in the rapid conversion of PTHrP(1−141), which resolves on SDS/PAGE at 21.5 kDa, to a 19.2-kDa fragment, shown in Fig. 2. Substantial conversion took place within the first 20 min, but required 80 min for quantitative cleavage. Conversion was dependent upon the presence of calcium and no conversion was detectable in the absence of enzyme (Fig. 2). No smaller fragments were resolved and it is likely that the concentration of the carboxy-terminal fragments subsequently identified was below the limit of detection of this technique.

HPLC analysis of the reaction mixture after 2 hours at 37°C (Fig. 3) revealed two major product peaks B and C, eluting at 25 % and 40 % acetonitrile, repectively, in addition to the uncleaved PTHrP(1−141) peak A, which eluted at 38 %. Peak B was identified by amino-acid analysis as the amino-terminal fragment PTHrP(1−21) and peak C as the fragment PTHrP(22−141) with the corresponding amino-terminal sequence Phe-Phe-Leu-His-His. Two smaller peaks (D in Fig. 3) were identified as carboxy-terminal cleavage products by cross-reactivity with PTHrP(107−139) antibodies, but not with antibodies to PTHrP(1−16). For kinetic analysis, initial rates of formation of peak B, PTHrP(1−21), were measured under conditions in which less than 6 % of the initial substrate concentration was cleaved and rates remained linear at high substrate concentrations for several times longer than the endpoints employed. Least squares fitting of the 13 points shown in Fig. 4 to the Michaelis-Menton equation gave a K_m of 3.3 ± 1.7 μM and k_{cat} of 6 ± 1.2 s^{-1}.

Table 1. Comparison of cleavage of PTHrP analogues by Kex2 at Arg_{21}. The cleavage was calculated as a mean of the reduction in the amount of substrate and the amount of PTHrP(1−21) product. 5 % represents the smallest detectable change.

Substrate	Initial concentration	Cleavage
	μM	%
PTHrP(1−141)	6.03	90
[Ala19]PTHrP(1−141)	10.1	55
[Ala20]PTHrP(1−141)	7.25	< 5
[Ala21]PTHrP(1−141)	5.62	< 5
PTHrP(1−34)	8.11	50
Unrelated peptide [Arg788, Arg792, Arg793, Arg799, Leu800, Arg802] MLCK (787−807)	6.6	< 5

Influence of residues adjacent to the cleavage site Arg_{19}-Arg-Arg_{21}. Comparison of the extent of cleavage of the substituted PTHrP(1−141) species is shown in Table 1. Replacement of Arg_{19} in PTHrP(1−141) with alanine reduced the conversion by approximately 50 %, and no cleavage was detectable when either Arg_{20} or Arg_{21} was replaced with alanine. PTHrP(1−34) was a weaker substrate for Kex2 than PTHrP(1−141), being cleaved to a similar extent as [Ala19]PTHrP(1−141). Interestingly, the basic-residue-rich MLCK peptide was not cleaved, despite the presence of both a double and a triple arginine site. This latter control was repeated four times with different substrate/enzyme concentrations, but on no occasion was cleavage observed.

Kex2 cleavage of PTHrP(1–141) at carboxy-terminal sites. Initial analysis of the cleaved products of PTHrP(1−141) revealed, in addition to the amino-terminal cleavage products (see above), at least four small peaks eluting at 30.5−32.5 % acetonitrile (Fig. 3). There were insufficient amounts of these products to allow more detailed analysis and further investigation of the carboxy-terminal cleavages was carried out in a scaled-up analysis, as described in Materials and Methods. In these experiments, four carboxy-terminal cleavage products (peaks A−D in Fig. 5) were identified. Amino-terminal sequence analysis identified the amino-terminal amino-acid residues of the products to be residues 98, 106, 107 and 109 of the PTHrP sequence (Fig. 4, Table 2). These sites were cleaved to lesser extents (< 10 %) than the Arg_{21} site, at 6 μM substrate concentration. The preferred cleavage site was carboxy to Lys-Arg_{105}. The elimination of the amino-terminal cleavage using [Ala20]PTHrP(1−141) as substrate resulted in very little change in the degree of conversion at these minor sites, although the additional substitution of histidine at position 94 increased cleavage after Arg_{97} (data not shown).

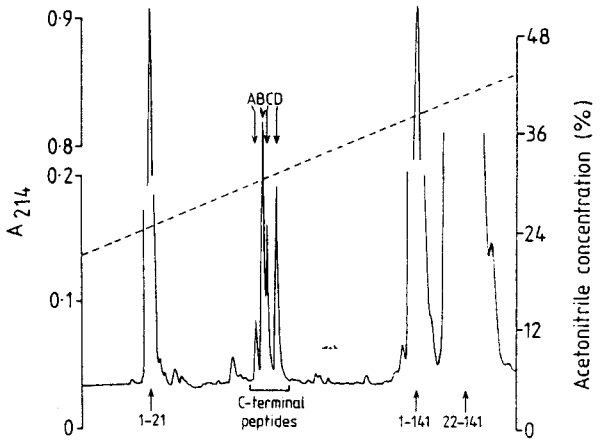

Fig. 5. Separation of carboxy-terminal cleavage products by HPLC in acetonitrile/trifluoracetic acid. Cleavage sites were identified by amino-terminal sequencing. Peak A represents the product of cleavage after Lys-Arg_{97}; peak B represents the product of cleavage after Lys-Arg_{105}; peak C represents cleavage after Arg-Arg_{106}; peak D represents cleavage at Thr-Arg_{108}.

In some cases, cleavage of the minor sites was altered by the substitution of alanine at positions 106, 108 or both 106 and 108 (Table 2), although the preferred and only significant cleavage site was following Arg_{105} and the level of cleavage at other sites was very low. Substitution of alanine for Arg_{106} also greatly increased cleavage only at Lys-Arg_{105}. In contrast, [Ala108] PTHrP(1−141) generated two major products identified by sequence analysis to represent cleavage after Lys-Arg_{105} and Arg-Arg_{106}. Both sites were cleaved equally well and to a greater degree than in the parent molecule. The double substitutions at positions Arg_{106} and Arg_{108} resulted in a major peak representing cleavage after Lys-Arg_{105}, which was substantially greater than in native PTHrP(1−141). Thus, the major effect of these substitutions was on the cleavage after Lys-Arg_{105}, which was increased by substitution of alanine for arginine at positions 106 and 108, and cleavage at Arg_{106} which was significant in the [Ala108]PTHrP(1−141) mutant. Cleavage after Lys-Arg_{97}, Thr-Arg_{108} or Arg-Arg_{106} (except in the Ala_{108} mutant) were all at a very low level and the influence of the alanine substitutions could not readily be assessed.

Kinetic analysis of cleavage carboxy to Lys-Arg_{105} indicated a K_m of 8.4 ± 2.7 μM and a k_{cat} of 0.8 ± 0.2 s^{-1}.

DISCUSSION

Specific posttranslational processing of PTHrP is likely to be of great physiological importance in view of its multiple actions. Our study of Kex2 cleavage of PTHrP *in vitro* has identified Arg_{19}-Arg-Arg_{21} as a potentially important site for posttranslational processing by mammalian convertases *in vivo*. Recent identification of small amino-terminal fragments has suggested that amino-terminal processing may be important (Burtis et al., 1992, 1994; Yang et al., 1994), although in most cases fragments have not been obtained in sufficient quantities to allow full characterization. Sequence analysis indicates that a PTHrP fragment, cleaved at Arg_{37} and lacking carboxy-terminal epitopes, is a major product (Soifer et al., 1992; Burtis et al., 1994) but no fragment corresponding to the cleavage identified in this study has yet been described. A minor carboxy-terminal site Lys-Arg_{105}, cleaved by Kex2, may also be significant in the light of evidence for carboxy-terminal processing of PTHrP, which is indicated by immunological detection of PTHrP-derived peptides from conditioned medium of PTHrP-producing cells, found in tumors and in the circulation of cancer patients (Burtis et al., 1990, 1992; Zajac et al., 1989; Danks et al., 1989, 1990; Imamura et al., 1991; Brandt et al., 1991; Soifer et al., 1992; Kasahara et al., 1992; Yang et al., 1994). However, it is not yet clear whether the fragments identified represent specific post-translational processing or secondary proteolytic degradation.

Most specificity studies of Kex2 enzyme action have been carried out with short peptide substrates considered to have random structure (Brenner and Fuller, 1992; Brenner et al., 1994). In our study, we have used full-length recombinant PTHrP species in order to identify potential convertase cleavage sites in the native protein. Cleavage carboxy to Arg-Arg-Arg_{21} suggests that this sequence is an important target for the action of convertases and substitutions for each of the arginine residues indicated that all three were essential for maximum cleavage. Kex2 protease cleaved this site with a k_{cat}/K_m ratio of 2×10^6 s^{-1} M^{-1}, a value within sixfold of the specificity constant for the best identified synthetic substrate of Kex2 (Brenner and Fuller, 1992; Brenner et al., 1994). Sites carboxy to Lys-Arg_{97}, Lys-Arg_{105}, Arg-Arg_{106} and Thr-Arg_{108} were cleaved at reduced rates. Of these minor sites, cleavage occurred predominantly at Lys-Arg_{105} with a k_{cat}/K_m ratio of 1×10^5 s^{-1} M^{-1}, a value 20-fold lower than for the Arg_{21} site. It is noteworthy, however, that the yeast killer toxin is normally cleaved by Kex2 protease *in vivo* carboxy to a Pro-Arg site (Zhu et al., 1992), despite the fact that a short synthetic substrate containing Pro-Arg is cleaved with a k_{cat}/K_m

Table 2. Comparison of minor C-terminal cleavage products from mutant PTHrP(1–141). Peak heights were calculated relative to the amount of PTHrP (1−21) generated in each case (designated as 100). Peaks asigned as described in Fig. 5. Peak heights shown in parentheses refer to peaks identified by mobility and not confirmed by N-terminal sequencing.

Type of site	Peak	Site	Relative peak height for			
			PTHrP(1−141)	[Ala106]PTHrP	[Ala108]PTHrP	[Ala106,Ala108]PTHrP
Major (N-terminal)						
	PTHRP(1−21)	Arg-Arg_{21}	100	100	100	100
Minor (C-terminal)						
	A	Lys-Arg_{97}	2.5	(2.3)	(2.5)	(2.1)
	B	Lys-Arg_{105}	11.9	96.8	29.3	43.4
	C	Arg-Arg_{106}	6.8	(6.2)	30.7	(4.3)
	D	Thr-Arg_{108}	8.5	(1.5)	(1.5)	(6.5)

of 2×10^5 s^{-1} M^{-1}. On the basis of these criteria, Arg-Arg-Arg_{21} would appear to be a site of certain physiological significance and Lys-Arg_{105} a site of possible physiological significance. Furthermore, cleavage at this latter site was influenced by the substitutions of alanine for arginine at both position 106 and position 108, as also was cleavage at Arg-Arg_{106} when alanine was substituted at position 108. The low levels of cleavage elsewhere within the sequence PTHrP(97−108) in native and mutant PTHrP, depicted in Table 2, did not allow for meaningful evaluation of their potential relevance or specificity. In human cells, the degree of cleavage of the sites identified by Kex2, will depend upon the specificity of the convertases in those cells, the concentration of PTHrP and the convertases in the constitutive and secretory pathways, aspects of PTHrP biosynthesis that are currently under investigation.

In cleavage of synthetic substrates, Kex2 protease showed an approximately fourfold preference for Lys-Arg sites over Arg-Arg sites (Brenner and Fuller, 1992). In the context of folded PTHrP, however, Arg-Arg-Arg_{21} was preferred over Lys-Arg_{97} and Lys-Arg_{105}, only the latter of which was cleaved at a measurable rate. This is consistent with higher orders of protein structure influencing substrate recognition. The low level of cleavage of synthetic PTHrP(1−34) and the lack of cleavage of an unrelated peptide with double and triple arginine sites also supports this conclusion.

Initial nuclear magnetic resonance studies (Barden and Kemp, 1989; MacFarlane et al., 1992) have indicated that synthetic PTHrP(1−34) has a compact structure with a hinge in the region of the three arginine residues at positions 19−21, and that in PTHrP peptides of lengths less than that of PTHrP(1−34), the integrity of the hinge region conformation may be compromised. The differential rates of cleavage at Arg_{21} for PTHrP(1−34) and PTHrP(1−141) by Kex2 suggests that, in the full-length protein, there may be subtle changes in the shape of the hinge region.

PTHrP(1−34) also exhibits other properties characteristic of convertase substrates. There is an amphipathic helix between residues 4 and 14 in PTHrP(1−34) and the arginine at position 19 is part of a type-1 β helix spanning residues 16−19. Evidence suggests that conformation and, in particular, a type-1 β turn amino-terminally situated to cleavage sites could determine the exposure and accessibility of the processing site (Rholam et al., 1992) and, in general, the subtilisin family of proteases appears to bind substrates in an extended β conformation (Kraut, 1987).

By analogy with the ability of Kex2 to mimick the action of specific convertases which cleave POMC (Thomas et al., 1988, 1991) and insulin (Thim et al., 1986; Smeekens et al., 1992) *in vitro* cleavage of PTHrP by Kex2 at Arg_{21} raises the possibility that this cleavage could occur *in vivo*. Proteolytic disruption at Arg_{21} effectively destroys the parathyroid-hormone-like biological activity, which depends upon the full sequence PTHrP(1−34) (Kemp et al., 1987) and neither the PTHrP(1−21) nor the PTHrP(22−141) generated in this study were biologically active, as shown by their inability to stimulate adenylate cyclase in parathyroid-hormone-responsive cells (data not shown). The functional role of PTHrP(22−141) remains to be elucidated, but identification of this cleavage site now allows the development of reagents to identify the products generated from such a cleavage *in vivo*. Little is known of the post-translational processing of PTHrP or the extent to which processing occurs to elaborate its non-parathyroid-hormone-like actions. The amino-terminal cleavage could have some relevance for PTHrP as a paracrine regulator in tissues where it may be important to reduce the amount of parathyroid-hormone-like activity to favour other actions of the protein.

The cleavage of PTHrP(1−141) by Kex2 is highly specific and strongly dependent upon the native structure. For this reason, it will be important, in transfection studies in mammalian cells, to evaluate the specificities of the other members of the convertase family of proteases (i.e. PC1, PC2, furin, etc.) on the processing of PTHrP(1−141). It is notable that the sequence Arg-Arg-Thr-Arg_{108} resembles a site cleavable by furin (Hosaka et al., 1991). The tissue specificity of PTHrP processing will be of particular importance in view of the growing evidence for multiple tissue specific paracrine actions of PTHrP (Thiede et al., 1989; Kremer et al., 1991; Rodda et al., 1989; Moseley and Gillespie, 1993).

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