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# Prohormone processing in *Xenopus* oocytes: Characterization of cleavage signals and cleavage enzymes

(yeast α-factor/egg-laying hormone/furin/prohormone convertase/KEX2)

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ABSTRACT **In this study, we characterize the sequences required for the cleavage of prohormones in *Xenopus* oocytes. We demonstrate that the yeast α-factor and the *Aplysia* egg-laying hormone (ELH) precursors are not cleaved in oocytes following simple pairs of basic residues, such as Lys-Arg, but that the ELH precursor is cleaved following the consensus sequence Arg-Xaa-(Lys/Arg)-Arg. This motif is conserved among precursors that are cleaved in virtually all mammalian cell types. Mutations that generate this sequence in the α-factor prohormone also result in efficient processing within oocytes. Cleavage at this consensus sequence may be due to the action of the *Xenopus* homologues of mammalian furin.**

Proteolytic processing is frequently required to liberate biologically active products from inactive precursors (1, 2). Some precursor proteins are cleaved in virtually all cell types following the conserved motif Arg-Xaa-(Lys/Arg)-Arg [RX(K/R)R] (3–6). Since precursor proteins that undergo this cleavage are continuously transported to the cell surface, it is likely that a ubiquitous endoprotease capable of recognizing this motif is a component of the constitutive secretory pathway. Precursors that are processed at simple pairs of basic residues (e.g., KR or RR) are most often cleaved within the regulated secretory pathways characteristic of specialized secretory cells such as neurons or endocrine cells (7–9). In the regulated pathway, proteins are stored in granules that are released in response to appropriate stimuli (10). Gene transfer experiments have shown that proinsulin, for example, is cleaved at dibasic sites within the regulated pathways of different endocrine cells but is not processed when expressed in fibroblasts that only secrete proteins via the constitutive pathway (7). In contrast, the insulin receptor precursor is cleaved at the RX(K/R)R consensus motif in virtually all cell types (3). These observations suggest that vertebrates express an endoprotease activity common to all cells, as well as additional cleavage enzymes restricted to endocrine and neuronal cell types.

Gene cloning has led to the demonstration that these cleavage activities are probably encoded by a family of eukaryotic endoproteases homologous to the bacterial subtilisins. One member of this gene family, furin, is expressed in all cells examined (11), whereas two other members, prohormone convertase 1 (PC1, also known as PC3) and PC2, are expressed in specific populations of endocrine and neuronal cells (12–15). The enzymes encoded by this gene family cleave at different sites in protein precursors. Furin, for example, exhibits a preference for substrates containing the RX(K/R)R motif (16–19). In contrast, PC1 and PC2 cleave at simple pairs of basic residues and exhibit more subtle substrate specificities that are not apparent from mere examination of the cleavage site (12, 20, 21). Thus, furin is likely to represent the ubiquitous endoprotease activity within constitutive secretory pathways, and the PC1 and PC2 proteases may be responsible for cleavages within the regulated pathways of endocrine and neuronal cells.

We have combined gene cloning with assays for prohormone processing in *Xenopus* oocytes to characterize the functional properties of genes responsible for cleavage of protein precursors (12). In this study, we further define the sequences required for cleavage within constitutive secretory pathways. We demonstrate that the yeast α-factor and the *Aplysia* egg-laying hormone (ELH) precursors are not cleaved in oocytes following simple paired basic residues. Cleavage of the ELH precursor is observed following the motif RX(K/R)R, and mutations that generate this sequence in the α-factor prohormone also result in efficient processing. Moreover, processing at this site is likely to result from the action of the *Xenopus* homologues of mammalian furin.‡

## MATERIALS AND METHODS

**Plasmids and Site-Directed Mutagenesis.** cDNA clones were kindly provided by the following investigators: prepro-α-factor (22) in pSP65 (Promega) from David Julius (University of California, San Francisco) and ELH (clone U; ref. 23) in pBluescript KS(−) (Stratagene) from Linda Jung and Richard Scheller (Stanford University). KEX2 cDNA (24) was kindly provided by Robert Fuller (Stanford University) and Jeremy Thorner (University of California, Berkeley) and subsequently subcloned into pGEM-2 (Promega). Recombinant DNA manipulations were performed essentially as described (25). Mutagenesis of prepro-α-factor cDNA was performed with *dut⁻ung⁻ Escherichia coli* by using the Bio-Rad Muta-Gene kit (26). Synthetic oligodeoxynucleotides 5′-CTCTCTTTTATCCCGAGATACCCC-3′ and 5′-CTCTCTTTTAGACCGAGATACCCC-3′ were used to generate αF-R and αF-RS, respectively. Substituted nucleotides are underlined.

**Microinjection and Metabolic Labeling of *Xenopus laevis* Oocytes.** Oocytes were microinjected with RNA and metabolically labeled with [$^{35}$S]methionine as described (12). [$^{3}$H]Leucine (163 Ci/mmol; 1 Ci = 37 GBq) was also added to the incubation medium at 2.4 mCi/ml for radiosequence analysis of peptides.

**Immunoprecipitation, Electrophoresis, and Amino-Terminal Mapping.** Rabbit antiserum against mature ELH was a generous gift from Linda Jung and Richard Scheller. Rabbit antiserum raised against a synthetic 13-amino acid α-factor peptide was obtained from Peninsula Laboratories. Immunoprecipitates were normalized to an equivalent num-

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Abbreviations: ELH, egg-laying hormone; PC, prohormone convertase.

‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M80471 (Xen-14) and M80472 (Xen-18)].

ber of oocytes per experiment and applied to SDS/polyacrylamide gels (27). Procedures for immunoprecipitations, electrophoresis, and amino-terminal radiosequence analysis of cleavage products have been described (12). Each set of microinjections was repeated at least twice and yielded reproducible results.

**Cloning of *Xenopus* Furin cDNAs.** Degenerate primers were derived from amino acid sequences surrounding the Asp-153 [5'-GCTCTAGAGCGA(C/T)GA(C/T)GGIAT(C/T)GA(A/G)AA(A/G)AA(C/T)CA-3'], His-194 [5'-GCTCTAGAG-CAC(A/C/T)CA(A/G)ATGAA(T/C)GA(T/C)AA(T/C)(C/A)GICA-3'], and Ser-358 [5'-GGAATTCCI(C/G)(T/A)(T/G/A)GTICCIGT(A/G)TGI(C/G)(T/A)(T/C)TC(T/G/A)GT-3'] active-site residues of the human furin gene. Primers included either *Xba* I or *Eco*RI sites. cDNA template was synthesized using 2 μg of *X. laevis* oocyte poly(A)$^+$ RNA and Moloney murine leukemia virus reverse transcriptase in a volume of 200 μl. One-tenth the volume of the cDNA reaction mixture was used for the initial polymerase chain reaction (PCR) with Asp and Ser primers, followed by amplification with His and Ser primers using 1/100th volume of the initial reaction. Amplifications with *Taq* DNA polymerase were performed according to instructions provided by the vendor (Perkin–Elmer/Cetus). Amplifications were carried out in a Perkin–Elmer/Cetus thermal cycler for 30 cycles of denaturation (96°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 3 min). A 562-base-pair PCR product was purified by electrophoresis in a 1.5% agarose gel and used to screen a *Xenopus* oocyte cDNA library kindly provided by Doug Melton (Harvard University). Inserts from plaque-purified phage were subcloned into the *Eco*RI site of pBluescript and coding regions were sequenced in both directions (28). Nucleotide sequences were assembled using the IBI MacVector 3.0 program (29).

## RESULTS

**Processing of the ELH Precursor in Oocytes.** The *Aplysia* ELH precursor is cleaved at eight internal sites to generate multiple peptides, including ELH, required to elicit the behavioral array associated with egg laying (Fig. 1*B*; refs. 31 and 32). In *Aplysia* neurons, the initial cleavage of this precursor occurs after the tetrabasic site RRKR at position 155 within the precursor molecule (30). Subsequent cleavages then occur at paired basic residues to generate the collection of active peptides. We and others have shown that most precursors that contain simple pairs of basic residues are not processed in the oocyte (12, 33, 34). If oocytes express an endopeptidase with a specificity similar to that observed in the constitutive secretory pathway of mammalian cells, we would expect a single cleavage at the consensus sequence after residue 155 of the ELH polyprotein.

When RNA encoding the ELH precursor is injected into oocytes and immunoprecipitated with antiserum directed against mature ELH, the 34-kDa ELH precursor is observed (Fig. 1*A*, lane 2). Two additional immunoreactive products are detected at 7.7 kDa and 6.3 kDa. Only the 7.7-kDa product is observed in the supernatant (lane 5). Upon isolation and radioactive sequence analysis, both the 7.7-kDa and 6.3-kDa fragments exhibit an amino-terminal sequence consistent with cleavage after the tetrabasic signal at position 155 within the precursor molecule (data not shown). The molecular mass of the larger product suggests that this peptide extends from the tetrabasic cleavage site to the carboxyl terminus of the polyprotein. The 6.3-kDa product must result from an additional cleavage near the carboxyl terminus. Unlike the 7.7-kDa product, the 6.3-kDa peptide is not observed in *Aplysia* neurons (30).

We next asked whether KEX2, a yeast serine protease of the subtilisin family (24, 35), can cleave the ELH precursor at simple pairs of basic residues. Coinjection of RNA encoding the ELH precursor with RNA encoding KEX2 results in the disappearance of the 6.3-kDa fragment, diminution of the 7.7-kDa fragment, and appearance of a new 4.4-kDa cleavage product in the oocyte extract (Fig. 1*A*, lane 3). This product is also detected in the supernatant on longer exposures of the original gel (data not shown). The 4.4-kDa cleavage product shares an amino-terminal sequence with mature ELH, consistent with cleavage after the Lys-Arg pair at position 176, and comigrates with synthetic ELH in SDS/polyacrylamide gels (data not shown). A 14-kDa immunoreactive product is also detected in the presence of KEX2 and is probably the

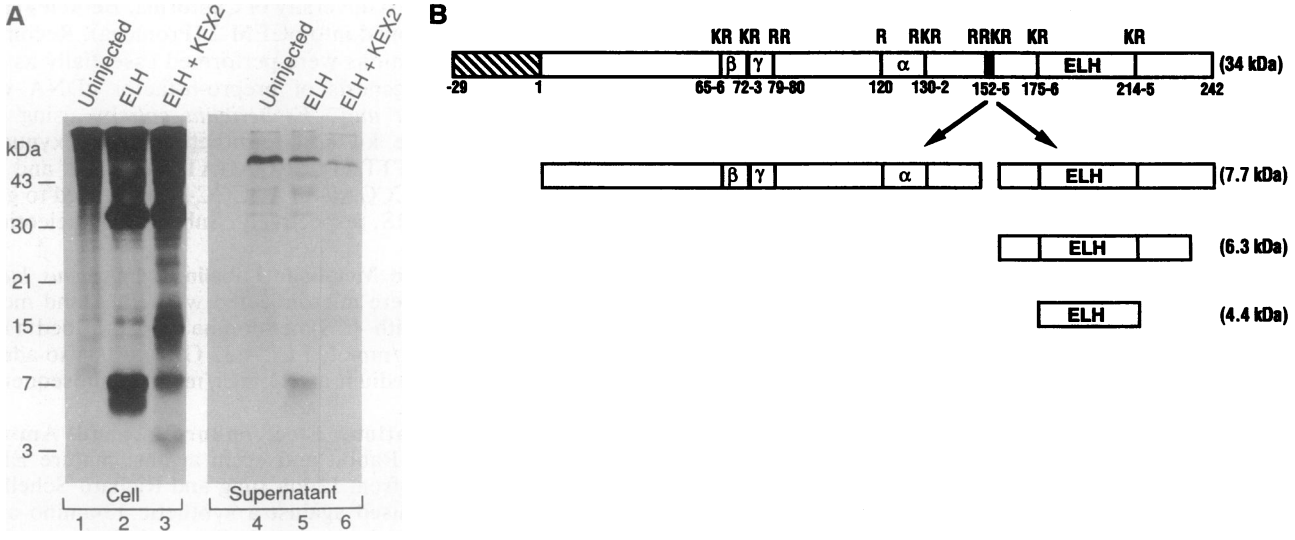


FIG. 1. Proteolytic processing of the ELH polyprotein. (*A*) RNA encoding the ELH precursor was synthesized *in vitro* and microinjected into oocytes alone (lanes 2 and 5) or with RNA encoding KEX2 (lanes 3 and 6). Cells were metabolically labeled with [$^{35}$S]methionine for 23 hr. Incubation medium (supernatant) and cell extracts were immunoprecipitated with antiserum raised against a synthetic 36-amino acid ELH peptide and then fractionated by SDS/18% PAGE. (*B*) Schematic representation of the ELH precursor (30). The signal sequence is indicated at the amino terminus and basic residues surrounding known physiologically active peptides are denoted by vertical bars. The amino and carboxyl termini of cleavage products containing the ELH peptide were deduced from amino-terminal radiosequence analysis and migration in SDS/polyacrylamide gels. Products are indicated schematically.

result of cleavage at one of several potential upstream sites (Fig. 1A, lane 3). Thus, the ELH precursor is processed in oocytes at a tetrabasic site also cleaved in *Aplysia* neurons, but the subsequent cleavages at paired basic residues observed in neurons do not occur in oocytes. The introduction of an exogenous endoprotease, KEX2, is required for cleavage at these paired basic residues to generate mature ELH.

***Xenopus* Oocytes Cleave Mutant α-Factor Precursors That Contain an RXKR Motif.** We have performed *in vitro* mutagenesis experiments on the yeast α-factor precursor gene to further examine the specificity of cleavage by the endogenous endopeptidases in *Xenopus* oocytes. The α-factor precursor is cleaved in yeast by KEX2 at the carboxyl side of four Lys-Arg pairs to generate four intermediates, each containing an α-factor sequence (Fig. 2A; refs. 24 and 36). We previously showed that the α-factor prohormone was not processed when expressed in *Xenopus* oocytes; however, cleavage was observed upon coinjection of RNA encoding KEX2 (12). We therefore asked whether mutation of the paired basic residues to the consensus sequence (RXKR) would render the precursor sensitive to cleavage by endogenous oocyte endopeptidases. The sequence including the first pair of basic residues, LDKR, was mutated *in vitro* to generate either RSKR (αF-RS) or RDKR (αF-R) at this site (Fig. 2A). Cleavage at this site would generate an 8-kDa carboxyl-terminal fragment containing the four α-factor peptide sequences.

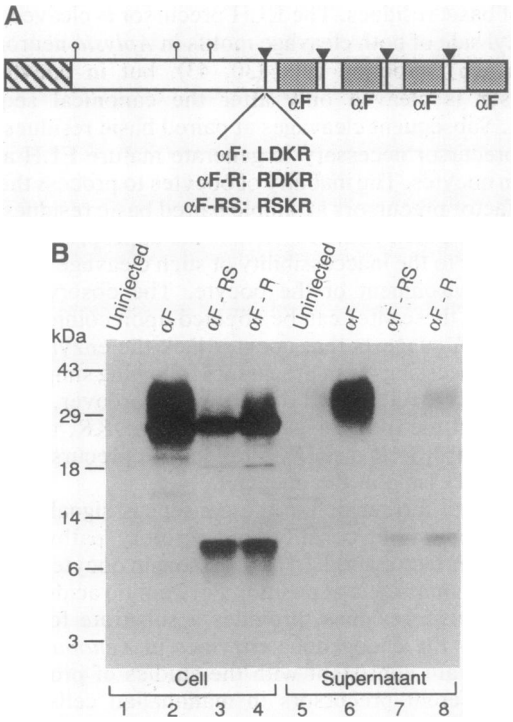


FIG. 2. Cleavage of pro-α-factor mutants in *Xenopus* oocytes. (*A*) The α-factor precursor encodes four copies of the 13-amino acid hormone. In addition, a signal sequence (hatched box), three sites for addition of asparagine-linked carbohydrate (○) (22), and the sites of cleavage at KR pairs by the KEX2 endoprotease (▼) are indicated. The sequence at the most amino-terminal cleavage site is LDKR in the wild-type prohormone. This site was mutagenized to encode RDKR (αF-R) or RSKR (αF-RS). (*B*) *In vitro* synthesized RNA encoding prepro-α-factor (αF) or RNAs encoding two different cleavage-site mutants (αF-RS or αF-R) were microinjected into oocytes. After a 26-hr incubation in the presence of [$^{35}$S]methionine, samples were immunoprecipitated with antiserum against mature α-factor. Immunoprecipitates of cell lysates (lanes 1–4) or incubation medium (lanes 5–8) from the equivalent of 10 oocytes were loaded into each well of an SDS/18% polyacrylamide gel.

RNA was transcribed from the wild-type and mutant α-factor templates and injected into *Xenopus* oocytes. Metabolically labeled oocytes, as well as the incubation medium, were analyzed by immunoprecipitation with antiserum directed against the mature 13-amino acid α-factor peptide. As shown in Fig. 2B (lane 2), wild-type α-factor RNA directs the synthesis of two unprocessed forms of the α-factor precursor: a 26-kDa product as well as higher molecular mass products between 26 and 32 kDa. These products correspond to the α-factor precursors observed in yeast. Glycosylation in the endoplasmic reticulum produces a 26-kDa product, and further glycosylation in the Golgi generates the larger precursors (37). Only precursors larger than 26 kDa are detected in the oocyte medium (lane 6). In contrast, injection of either αF-R or αF-RS RNA results in the synthesis of precursors that are cleaved to generate an 8-kDa immunoprecipitable product present in both the oocyte extract and the supernatant (lanes 3, 4, 7, and 8). Amino-terminal radiosequence analysis of the 8-kDa cleavage products confirms that cleavage occurs immediately following the sequence RXKR in both mutants (data not shown). The appearance of the 8-kDa cleavage product is associated with the loss of the diffuse high molecular mass precursors but not with the loss of the 26-kDa form. These data suggest that the 26-kDa precursor either is an inappropriate substrate or is not colocalized with an active processing enzyme. The continued presence of precursors larger than 26 kDa in oocytes injected with the RDKR mutant suggests that this site is less efficiently cleaved than RSKR. These results indicate that paired basic residues in the α-factor precursor are not cleaved in *Xenopus* oocytes. Mutations at these sites to the consensus sequence RXKR render these precursors susceptible to cleavage by oocyte enzymes. Thus the *Xenopus* oocyte expresses an endopeptidase with a substrate specificity similar, if not identical, to that deduced for the ubiquitous cleavage activity in mammalian cells.

**Cloning and Expression of *Xenopus* Processing Enzymes.** The enzyme furin is ubiquitously expressed in mammalian cells and enhances the cleavage of protein precursors after the consensus sequence RX(K/R)R (16–19). These observations suggest that the furin gene encodes this ubiquitous processing activity in mammalian cells. Since oocytes exhibit a cleavage activity with similar substrate specificity, we attempted to clone a *Xenopus* homologue of mammalian furin from oocyte RNA. Degenerate oligonucleotides encompassing the sequences of the putative catalytic sites of human furin were used as primers to amplify the *Xenopus* homologue from a population of oocyte mRNA. A 562-base-pair PCR product was obtained and subsequently used to screen a *Xenopus* oocyte cDNA library.

One cDNA clone, Xen-14, encodes a 783-amino acid open reading frame with 72% identity to human furin (Fig. 3) and mouse furin (ref. 39; comparison not shown). The identity is especially notable within the catalytic domain (amino acids 118–418) characteristic of the subtilisin serine protease family (40). A second clone, Xen-18, is also homologous to human furin yet is distinct from Xen-14. Xen-18 encodes a partial coding region of 596 amino acids with 97% identity to Xen-14 (Fig. 3). The isolation of two distinct furin cDNA species is not surprising considering the tetraploid nature of the *X. laevis* genome (41).

Northern blot analysis indicates that *Xenopus* furin is expressed in all *Xenopus* tissues analyzed, including ovary, liver, muscle, and skin (Fig. 4A). The major RNA species is 7.5 kilobases long; minor transcripts are detected at 4.4 and 3.7 kilobases. Since the PCR product used as probe in these experiments derives from coding-region sequences, we cannot at present determine whether the individual transcripts originate from the Xen-14 or the Xen-18 gene.

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XEN-14    MDLSPSLLLML--WTLLSVLVEEITGQKVYTNTWAAHISGGSAEADRLCKKYGFINHGLIFEDHYHFSHRAVMKRSLTPKRTRQVLLKREPQVHWLEQQV   98
XEN-18    .....T.....--.......FK....H........V...........SR........Q.....................................   98
hFURIN    .E.R.W..WVVAATGT.VL.AADAQ....F.....VR.P..P.V.NSVAR.H..L.L.Q..G.Y...W..G.T....S.H.P.HSR.Q.....Q.....  100
                                                          ▼                                        ▼

XEN-14    AKKRKKRDIYTDPTDPKFMQQWYLLDTNRHDLHVKEAWEQGFTGKGIVVSILDDGIEKNHPDLQANYDPAASYDVNDQDPDPQPRYTQLNDNRHGTRCAG  198
XEN-18    .........FI.....................................................................................  198
hFURIN    ..R.T...V.QE......P.....SGVTQR..N..A..A..Y..H.................AG....G..F..............M.....  200
                                                                                                    ▼

XEN-14    EVAAVANNGICGVGIAYNANIGGVRMLDGEVTDAVEARSLGLNPNHIHIYSASWGPEDDGKTVDGPAKLAEEAFYRGVTQGRGGLGSIYVWASGNGGREH  298
XEN-18    ........D.....................................................R.....................Q.  298
hFURIN    .........V....V....R.....................................R......F...S.........F.....  300
                                                                   ▼

XEN-14    DSCNCDGYTNSIYTLSISSTTQMGNVPWYSEACSSTLATTYSSGNQNEKQIVTTDLRQKCTDSHTGTSASAPLAAGIIALALEANKNLTWRDMQHLVVQT  398
XEN-18    .....................T.....................................................................  398
hFURIN    ..................A..F.....................................E.................T.....  400

XEN-14    SNPAGLNANDWITNGVGRKVSHSYGYGLLDAGAMVAMAKTWVTVGPQRKYVIDILSEPKDIGKALEVRRKVEPCAGMSNYISTLEHVQARLSLSYNCRGD  498
XEN-18    ....S.....................................N.....................................................  498
hFURIN    .K..H......A.....................L.QN.T..A....CI....T.......R....KT.TA.L.EP.H.TR...A....T....R...  500

XEN-14    LAIYLTSPMGTRSCLLAPRPHDYSADGFNDWSFMTTHSWDEDPAGEWVLEIENVSNNNNYGTLTQFVLVLYGTASE--GLSRKFDGDGSRNVASSQSCIV  596
XEN-18    ..................L.....................................K.....................TP....Q.....Y.........  596
hFURIN    ...H.V.......T...A.............A...........S.........T.EA.......K.T.......P.--..PVPPESS.CKTLT...A.V.  598

XEN-14    CEEGYFLHQKSCIKSCPQGFTSSIQNIHYTLDNNIEPLLVNVCVPCHVSCATCKGTTINDCLTCPAHSHYNLLDYSCTHQTQRSRESPTLKDSSH-----  691
hFURIN    ....FS......VQH..P..APQVLDT..STE.DV.TIRAS..A...A.....Q.PALT...S..S.ASLDPVEQT.SR.S.S.....PQQQPPRLPPEV  698

XEN-14    DYVAR------TSNLPFIVAILSCLFIIVVFGSIFLFLQLRSGGVLGRKRLYMLDSGIISYKGIPSGAWQEEGFSESETEETAAHSERTAFLKQQSTL*  783
hFURIN    EAGQ.LRAGLLP.H..EV..G...A..VL..VTV..V.....FSFRGVKV.TM.R.L.....L.PE.....CP.D..ED.--GRG.....I.D..A.*  794
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FIG. 3. Alignment of the deduced amino acid sequences of *Xenopus* (Xen-14 and Xen-18) and human (h) (38) furins. Dots represent amino acids identical to Xen-14. Gaps (dashes) were introduced for maximum alignment. The putative active-site residues are indicated (▼). Potential transmembrane domains are underlined. Stars at ends of sequences indicate termination codons.

By PCR, we have also obtained cDNA clones that encode *Xenopus* homologues of the mammalian PC1 and PC2 genes (J.K., J.C., and R.A., unpublished data). In contrast to the ubiquitous expression of *Xenopus* furin, Northern blot analysis demonstrates that PC1 is not expressed in oocytes, muscle, or liver (Fig. 4*B*). Expression is detected in *Xenopus* skin, an abundant source of secreted peptides (42). The pattern of expression of PC2 resembles that observed for PC1 except that the levels of PC2 RNA in skin are much lower (data not shown). These data suggest that the processing activity we detect in *Xenopus* oocytes is likely to result from the action of the *Xenopus* homologues of the furin gene.

## DISCUSSION

Recent data suggest that the specificity of protein processing in different cell types results from the regulated expression of different members of a family of endopeptidases that act on distinct target sites. *Xenopus* oocytes, for example, efficiently process precursor molecules at the canonical sequence RX(K/R)R but are unable to cleave after most simple pairs of basic residues. The ELH precursor is cleaved on the carboxyl side of both cleavage motifs in *Aplysia* neurons and mammalian endocrine cells (30, 43), but in oocytes this precursor is cleaved only after the canonical sequence RRKR. Subsequent cleavages at paired basic residues in the ELH precursor necessary to generate mature ELH are not made in oocytes. The inability of oocytes to process the ELH and α-factor precursors at simple paired basic residues could be due to the lack of expression of the appropriate cleavage enzymes or to the inaccessibility of such cleavage sites in the ectopic environment of the oocyte. The observation that cleavage at these sites can be effected upon coinjection with KEX2 RNA suggests that oocytes lack the enzymes necessary for processing these precursors following simple pairs of basic residues (ref. 12 and this study). Moreover, mutations that convert the α-factor cleavage site, LDKR, to the consensus motif RSKR or RDKR render the precursor susceptible to processing in the oocyte.

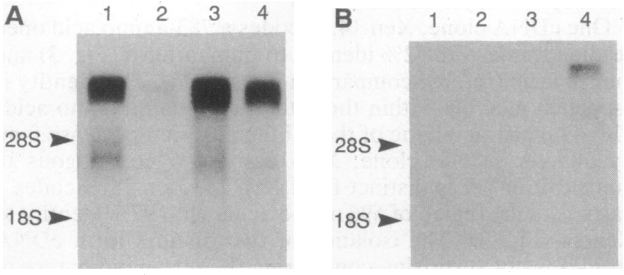


FIG. 4. Northern blot analysis of *Xenopus* furin and PC1 expression. Poly(A)$^+$ RNAs (4 μg per lane) from the following *Xenopus* tissues were electrophoresed in 1% agarose/formaldehyde gels and transferred to nylon membranes: lanes 1, defolliculated oocytes; lanes 2, muscle; lanes 3, liver; lanes 4, skin. Positions of *Xenopus* 28S and 18S ribosomal RNAs are indicated. Blots were hybridized with random-primed $^{32}$P-labeled PCR products encoding partial catalytic domains of *Xenopus* furin (*A*) and *Xenopus* PC1 (*B*). Longer exposures more clearly reveal the hybridization signal in lane 2 of *A*.

These data further define the consensus signals required for cleavage within constitutive secretory pathways. The analysis of α-factor and ELH processing in oocytes indicates that a basic amino acid at position P4, 2 amino acids upstream of paired basic residues, provides a substrate for efficient cleavage by the endogenous enzymes in *Xenopus* oocytes. These data are consistent with the studies of processing of several different precursors in mammalian cells. A basic amino acid at P4 in factor IX (44), the insulin receptor (45), the cytomegalovirus glycoprotein B (46), and the influenza hemagglutinin (47) is essential for proteolytic processing of these precursors. In some precursors, the precise nature of the basic residue at P4 is also important. Mutation of the P4 arginine residue at the human immunodeficiency virus envelope processing site to lysine abolishes cleavage (48), whereas a similar mutation in the murine leukemia virus envelope glycoprotein has no effect on cleavage (49). Two precursors, proalbumin and proparathyroid hormone, processed in constitutive secretory pathways do not have basic residues at P4 (50, 51). The presence of a basic residue at P6 in both precursors raises the possibility of alternative cleavage signals that encompass the P6 residue.

A basic residue at P2 may not be a stringent requirement if Arg is present at P4. For example, a Lys → Gln mutation at P2 of the cytomegalovirus glycoprotein B blocks cleavage (46), whereas mutations of the P2 lysine to an uncharged residue in either the insulin receptor precursor or the human immunodeficiency virus envelope precursor have no effect upon cleavage (45, 48). Furthermore, the pro-[Arg$^8$] vasopressin–neurophysin II precursor is processed in oocytes following the sequence RXXR (34). Although a basic residue is not required at P3 (45, 47), our data suggest that the specific amino acid at this position can influence the efficiency of cleavage.

The mammalian furin gene is a strong candidate to encode the ubiquitous endoprotease responsible for cleavage at the RX(K/R)R motif. (*i*) This gene is expressed in all mammalian tissues examined (11). (*ii*) High levels of furin expression result in a significant enhancement of processing of the $\beta$ nerve growth factor (17) and von Willebrand factor (18, 19) precursors at the canonical RX(K/R)R sequence. In addition, prorenin is not processed at a simple dibasic site within the constitutive secretory pathways of cells expressing high levels of furin. Mutation of this site to RXKR results in cleavage mediated by furin (16). (*iii*) Immunocytochemistry indicates that furin may be localized to the Golgi compartment (17), which correlates with the subcellular cleavage site of the human immunodeficiency virus envelope protein (52) and of the tetrabasic signal in the ELH precursor (43, 53). Finally, in cells such as *Xenopus* oocytes, which preferentially process precursors at this sequence motif, we detect the expression of furin but not the other members of this endopeptidase gene family. Although these experiments do not prove that the furin gene encodes the constitutive endoprotease or that furin is the only enzyme responsible for this activity, all of the properties of furin delineated to date coincide with those of a ubiquitous endopeptidase capable of cleavage at the RX(K/R)R consensus motif.

Neurons and endocrine cells express additional endoprotease activities encoded by the PC1 and PC2 genes that display different preferences for simple pairs of basic residues presented in different contexts (12, 20, 21). Thus, the availability of multiple endoproteases that cleave after basic amino acids allows each individual member of the family to exhibit a stringent target-site specificity. The restricted expression of these specialized enzymes perhaps ensures that only distinct sequences are cleaved in the appropriate cell type.

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