Endoproteolytic Processing of the Human Protein C Precursor by the Yeast Kex2 Endopeptidase Coexpressed in Mammalian Cells†

Donald C. Foster,* Rick D. Holly, Cindy A. Sprecher, Kathleen M. Walker, and A. Ashok Kumar

ZymoGenetics, Inc., 4225 Roosevelt Way, NE, Seattle, Washington 98105

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ABSTRACT: The human protein C precursor undergoes extensive co- and posttranslational modification during its biosynthesis in the liver. These modifications include glycosylation, γ-carboxylation, and β-hydroxylation of specific amino acids and endoproteolytic processing to remove the pre- and propeptides as well as the pair of basic amino acids which connect the light and heavy chains in the precursor. Previous studies with a recombinant mammalian expression system have indicated that the endopeptidase in several mammalian cell types which recognizes and cleaves this dibasic site has a substrate specificity for sites which also include a basic amino acid in the −4 position (Foster et al., 1990). Since the human protein C precursor has His154 in the −4 position, it is poorly and incompletely cleaved in BHK and several other mammalian cell lines and also apparently secreted from the liver as a mixed population of mature two-chain and precursor one-chain molecules. In the present study, a mammalian expression system has been used to study the effect of coexpressing the protein C precursor together with the yeast Kex2 endopeptidase which is known to recognize and process dibasic pairs within peptide precursors in yeast. Coexpression of the *KEX2* gene resulted in complete conversion of the protein C precursor to the mature two-chain form. Amino-terminal sequencing of the cleavage products has indicated that the cleavage occurs in the correct location and that this site is preferentially recognized by the yeast endopeptidase within the context of the mammalian cell secretory pathway.

Protein C is the zymogen form of a serine protease present in plasma which plays an important physiological role in the regulation of blood coagulation pathways (Esmon, 1987). Human protein C undergoes extensive co- and posttranslational modification during its biosynthesis in the liver. These modifications include the γ-carboxylation of nine glutamic acid residues near its amino terminus (Discipio & Davie, 1979; Fernlund & Stenflo, 1982), β-hydroxylation of aspartic acid-71 (Drakenberg et al., 1983; McMullen et al., 1983), glycosylation of four asparagine residues, proteolytic removal of a prepeptide and a propeptide (Foster et al., 1987), and endoproteolytic internal cleavage which converts the M_r 62 000 single-chain precursor to a light chain (M_r 21 000) and a heavy chain (M_r 41 000) which are held together by a disulfide bond (Kisiel et al., 1976; Stenflo, 1976).

The cDNA sequence for human protein C (Foster & Davie, 1984; Beckmann et al., 1985) indicates that human protein C is initially synthesized as a single-chain precursor in which the light chain and heavy chain are connected by a basic Lys-Arg dipeptide. Conversion of the precursor to the mature, two-chain form must involve some combination of endo- and exoproteolytic processing to remove these two amino acids. However, these processing steps are incompletely performed in the liver, as indicated by the presence of small amounts of unprocessed, single-chain protein C present in plasma (Miletich et al., 1983). This single-chain protein C cannot be converted to the two-chain form upon prolonged exposure to plasma, supporting the interpretation that this processing step occurs intracellularly prior to secretion of the protein.

We and others have also found a significant variation in the efficiency with which different transfected cell lines carry out the processing of recombinant protein C precursor of two-chain form. Human kidney 293 cells process approximately 80% of the precursor to two chains (Foster et al., 1987; Grinnell et al., 1987). Mouse epithelioid C127 cells process approximately 50% of the protein to the two-chain form (Oppenheimer & Wydro, 1988). Baby hamster kidney (BHK)[1] cells also perform this processing step with low efficiency, with approximately 70% of the protein being secreted as a single-chain form (Foster et al., 1990). Human liver-derived HepG2 cells also secrete a mixture of approximately 50% single-chain protein C (J. Miletich, personal communication). The reason for the discrepancy between HepG2 cells (~50% single chain) and human liver (~15% single chain) is not clear but may represent loss of fully differentiated phenotype in this cell line in culture.

Earlier studies from this laboratory (Foster et al., 1990) have shown that, at least for the case of BHK cells and 293 cells, the incomplete cleavage was attributable to the substrate specificity of the mammalian endoprotease, which was demonstrated to have a marked preference for substrates with a basic amino acid in the −4 position relative to the cleavage site. Human protein C has His154 in this position and is therefore a relatively poor substrate for the endoprotease. However, a mutant protein C with Arg in position 154 was completely converted to the two-chain form.

Such cleavage at or after Lys-Arg sequences to yield mature peptide products bears striking similarity both to the processing of polypeptide precursors to mature peptides in specialized endocrine mammalian cells and also to the processing of mating-type α-factor precursor to yield mature α-factor peptides in yeast. In both cases, the liberation of bioactive

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* To whom correspondence should be addressed.

[1] Abbreviations: BHK, baby hamster kidney; CP-B, carboxypeptidase B; DHFR, dihydrofolate reductase; POMC, preproopiomelanocortin; Kex2, killer expression; MTX, methotrexate.

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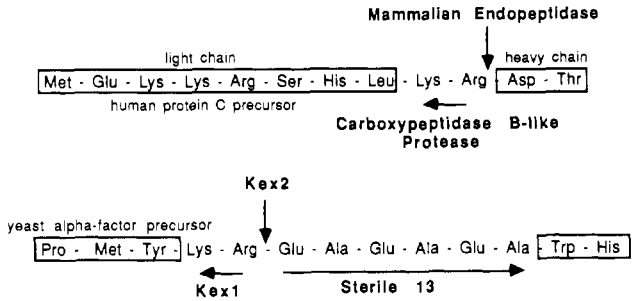

FIGURE 1: Diagram of the roles of Kex2 and the mammalian endopeptidase in proteolytic maturation of protein precursors. The cleavage of the α-factor precursor by Kex2 is from Julius et al. (1984). The cleavage of the protein C precursor by mammalian cell endopeptidases has been shown by Foster et al. (1990).

peptides is thought to occur via a two-step process (Steiner et al., 1980): (i) the precursor is cleaved on the carboxy-terminal side of the pair of basic residues by a trypsin-like endopeptidase followed by (ii) the removal of the pair of basic residues from the C-terminal end of the resulting fragment by a carboxypeptidase B like (CP-B) protease.

In yeast, the two enzymes responsible for these consecutive processing steps have been identified, respectively, as the protein products of the *KEX2* (endoprotease) and *KEX1* (carboxypeptidase) genes (Julius et al., 1984; Dmochowska et al., 1987). These processing peptidases are involved in proteolytic maturation of killer toxin (Wickner, 1974; Leibowitz & Wickner, 1976) and also of the mating-type α-factor precursor (Julius et al., 1984). The *KEX2* gene product is an endopeptidase which appears to have no substrate specificity beyond the requirement for basic residues in the −1 and −2 positions. The *KEX1* gene product is a carboxypeptidase with specificity for basic amino acids (Figure 1).

The yeast *KEX2* gene product has been demonstrated to be functionally active in mammalian cells in the proteolytic maturation of preproopiomelanocortin (POMC) (Thomas et al., 1988). We reasoned therefore that the Kex2 endopeptidase may recognize and cleave the dibasic site on the protein C precursor and lead to secretion of a fully processed form of protein C. If so, this cleavage would demonstrate functional analogy between proteolytic maturation of liver-derived plasma proteins and the very similar processing of polyhormone precursors.

In order to test this hypothesis, we have constructed a mammalian cell expression vector encoding the yeast *KEX2* gene and transfected it into cells (BHK) which process the wild-type protein C precursor poorly. Introduction of the *KEX2* gene caused complete conversion of the protein C precursor to the two-chain form, and subsequent protein characterization showed that the endoproteolytic maturation liberated the correct N-terminus of the protein C heavy chain.

Materials and Methods

Mutagenesis. An 840 bp *Sst*I fragment of the human protein C cDNA which corresponds to amino acids 9 of the light chain through 119 of the heavy chain was subcloned in a 3′ to 5′ orientation in M13mp11. Various mutations in this fragment were then prepared by the two-primer oligonucleotide-directed mutagenesis technique (Zoller & Smith, 1984). All mutants were sequenced by the dideoxy chain termination technique to verify each mutation and the fidelity of the remaining sequence. RF DNA was prepared from mutant plaques, and the mutant *Sst*I fragments were used to replace the corresponding fragment of the wild-type protein C cDNA in the expression vector.

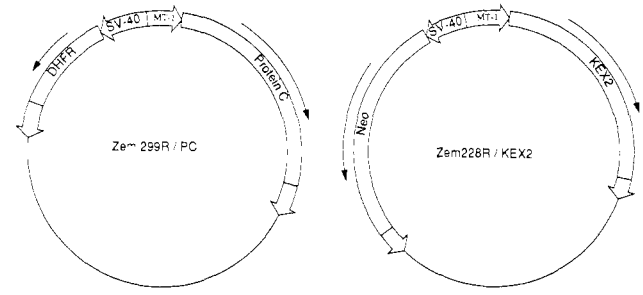

FIGURE 2: Mammalian expression vectors used for coexpression of the protein C and KEX2 genes. A full-length protein C cDNA was ligated into a unique *Eco*RI cloning site downstream of the metallothionein promoter in the vector Zem229R. Similarly, the intact KEX2 gene, with its own secretory leader signal sequence, was ligated as a *Bam*HI fragment into a unique *Bam*HI site in the vector 228R.

Vectors. The mutant protein C cDNAs were ligated into a mammalian cell expression vector called Zem229R (Figure 2). This vector permits insertion of *Eco*RI fragments or *Bam*HI fragments into a cloning site downstream of the mouse MT-1 promoter and upstream of an SV-40 polyadenylation sequence and also carries an expression unit for the dihydrofolate reductase (DHFR) gene under control of the SV-40 early promoter. The yeast *KEX2* gene (obtained from G. Ammerer, ZymoGenetics) was ligated into the *Bam*HI site of a similar vector called Zem228R. This vector is identical with Zem229R but carries the Neo gene as a selectable marker (Figure 2).

Transfection/Selection. BHK cells were grown in Dulbecco's-modified Eagle's medium containing 10% fetal calf serum and vitamin K (5 μg/mL). Cells were transfected with the protein C expression plasmids by the calcium phosphate procedure (Graham & van der Eb, 1973). For selection of stable colonies, the cells were divided 48 h after transfection into medium containing 250 nM methotrexate (MTX). Colonies were screened for protein C production by an immunofilter assay as previously described (Busby et al., 1988) and grown individually for protein analyses. For coexpression of the *KEX2* gene, stable clones expressing protein C were transfected a second time with the *KEX2* expression plasmid, and transfectants were selected in medium containing both 250 nM MTX and 500 μg/mL G418. Resistant colonies were selected at random and cultured individually for analysis of cleavage of the protein C product.

Pulse-Labeling. For metabolic labeling, confluent 10-cm plates of cells were washed with PBS and then incubated overnight with 2 mL of cysteine-free Dulbecco's MEM supplemented with 25 μCi/mL [^{35}S]cysteine, 5 μg/mL vitamin K, and 1% fetal calf serum. Following the labeling period, the medium was harvested; the cell layer was washed with PBS and then lysed in 2 mL of lysis buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 300 mM sucrose, and 0.5% Triton X-100). Labeled media and cell lysates were stored at −20 °C until use. Radioimmunoprecipitations of labeled samples were performed with a monoclonal antibody (PCH-1, unpublished results), which is directed against an epitope in the heavy chain of human protein C. Immunoprecipitated samples were boiled in reducing gel sample buffer prior to electrophoresis on 10% SDS–PAGE gels.

Immunopurification of Protein C. Recombinant wild-type and mutant protein C samples were purified by affinity chromatography on a Sepharose-bound anti-protein C monoclonal antibody column (PCL-2). This antibody has been shown to be specific for the calcium-induced conformation of protein C but does not selectively fractionate one-chain from two-chain protein C (unpublished results). Culture medium

containing protein C was made 10 mM in $CaCl_2$, filtered through a 0.45-μm filter, and applied to the antibody column which was previously equilibrated with 50 mM Tris-HCl, pH 7.4, buffer containing 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3. The unbound protein was washed off with 1.0 M NaCl in the equilibration buffer, and bound protein C was eluted with 15 mM EDTA in 50 mM Tris-HCl, pH 7.4.

Amino Acid Sequence Determination. Automated Edman degradation was performed on a gas-phase sequencer equipped with an on-line Applied Biosystems 120A PTH analyzer. PTH-amino acids were separated and identified on a microbore PTH C-18 column employing solvents and gradient suggested by the manufacturer.

Results

The apparent analogy of mammalian cell proteolytic maturation of plasma proteins to the more clearly elucidated process of α-factor maturation in yeast (Figure 1) led us to speculate that these processes are functional analogues, but with an apparent difference between the mammalian and yeast system being the extended substrate specificity of the mammalian enzyme beyond the −1 and −2 positions. If functional analogy exists between these processes, and if the yeast *KEX2* gene product is functional in mammalian cells (Thomas et al., 1988), then one would expect that the *KEX2* gene product should recognize and cleave the wild-type protein C precursor when coexpressed in the same cells. This speculation led to construction of a mammalian expression vector carrying the wild-type *KEX2* gene (Figure 2) from *Saccharomyces cerevisiae* with its own secretory leader sequence directing synthesis and targeting to post-Golgi secretory vesicles.

A stable BHK cell line was chosen which had been transfected with the wild-type protein C expression vector (Figure 2) using DHFR as a selectable marker. This cell line was chosen because BHK cells cleave the Lys-Arg sequence in the protein C precursor poorly, and thus serve as a useful model for studying factors which enhance the cleavage. These cells were retransfected with the Zem228R/*KEX2* vector and then subjected to selection using both MTX *and* G418 to select the subpopulation which had acquired the second expression cassette and would express both the protein C and *KEX2* gene products. Colonies which are resistant to both drugs would be expected to be identical in their level of production of protein C, since they are all derived from a clonal population arising from a first transfection with the protein C expression vector. However, colonies would be expected to vary in their level of synthesis of Kex2 product, since they would represent unique events of uptake of the second expression vector.

In order to determine whether introduction of the *KEX2* gene product would affect the degree of processing, these colonies were cultured individually, and the degree of endoproteolytic processing was analyzed by SDS–PAGE fractionation of the protein C immunoprecipitated from culture media of cells that had been pulse-labeled with [^{35}S]Cys. The gels were run under reducing conditions to separate two-chain molecules and then dried and autoradiographed for evaluation of the relative amounts of one-chain and two-chain forms of the protein.

Figure 3 shows the results of this analysis both for the parent cell line (lane 1) and for a representative sample of doubly transfected colonies expressing both protein C and Kex2 (lanes 2–4). As expected, the quantity of protein C synthesized by all the clones appears to be identical. Since the distribution of cysteines is nonuniform, the light chain shows a higher relative specific activity than the heavy chain and appears darker on the autoradiograms. The heavy chain shows molecular weight heterogeneity similar to that observed in plasma protein C which appears to be due to differential glycosylation (Miletich & Broze, 1990). In the parental cell line (lane 1), the secreted protein C is cleaved to two-chain form poorly, with most of the radioactivity appearing in the band associated with intact, single-chain protein. In the doubly transfected progeny cell lines (lanes 2–4), the degree of cleavage to two-chain form varies from somewhat better than the parental line (lane 3) to nearly 100% cleaved (lanes 2 and 4). A high degree of cleavage in the progeny clones can only be attributed to introduction of the *KEX2* gene, since progeny derived from subcultures of the original parent exhibit no variability in the cleavage rate (data not shown). The variation in degree of cleavage is a function of the level of Kex2 product expressed in the progeny, since colonies exhibiting higher cleavage rates show higher levels of *KEX2* mRNA on Northern blots than colonies with lower cleavage rates or than the parent BHK/protein C cell line (data not shown).

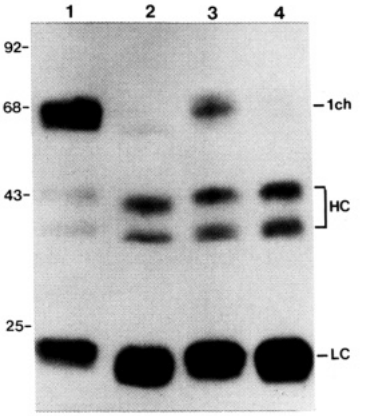

FIGURE 3: Immunoprecipitation of wild-type protein C secreted from cells with or without coexpression of the KEX2 gene. A clonal cell line expressing wild-type protein C under DHFR selection was transfected with the Zem228R/KEX2 vector (neo selection). Several doubly transfected clones were picked at random and cultured individually. Both the parent cell line (lane 1) and the doubly transfected clones (lanes 2–4) were labeled with [^{35}S]Cys, and the protein C products were immunoprecipitated as described under Materials and Methods.

In all colonies expressing both gene products, we observed Kex2-dependent cleavage enhancement of the protein C precursor. Furthermore, the resulting cleavage products comigrated with the predicted positions of the natural heavy- and light-chain bands of two-chain protein C from plasma. The size correlation suggested that cleavage by Kex2 occurred following the Lys156-Arg157 pair which separates the light and heavy chains in the precursor. However, since the protein C precursor contains eight dibasic pairs which all represent potential sites of cleavage for the Kex2 endopeptidase, it was important to determine the precise site(s) of cleavage in the protein secreted from these cells. In order to address this structural question, one cell line (PC594/KEX2-1) which shows essentially complete cleavage (lane 2, Figure 3) was cultured in larger scale, the protein C product was purified, and the N-terminal sequence was determined. As shown in Table I, two amino acid residues were determined at each cycle of sequence analysis. These corresponded to the two N-termini expected for the mature light and heavy chains of two-chain protein C. Thus, it was demonstrated that the *KEX2* gene product recognized and cleaved the protein C precursor at the correct Lys-Arg pair which connects the two chains in the precursor structure. However, it was not proven either from the electrophoretic patterns or from the sequence analysis that this represented the *only* site of cleavage by the Kex2 protease.

Table I: Amino-Terminal Sequence of Wild-Type Protein C Secreted from Cells Coexpressing KEX2[a]

	light chain		heavy chain	
cycle	expected	observed	expected	observed
1	A	A (353)[b]	D	D (293)
2	N		T	T (137)
3	S	S (107)	E	E (210)
4	F	F (289)	D	D (184)
5	L	L (309)	Q	Q (239)
6	E[c]		E	E (118)
7	E[c]		D	D (156)
8	L	L (127)	Q	Q (194)
9	R	R (76)	V	V (262)
10	H	H (36)	D	D (161)
11	S	S (59)	P	P (117)
12	S	S (73)	R	R (60)

[a] Protein C was purified from a clonal cell line which coexpressed the wild-type protein C and the KEX2 gene (lane 2, Figure 3). The protein was immunopurified as described under Materials and Methods on a calcium-dependent monoclonal antibody column. Approximately 500 pmol of purified protein was subjected to N-terminal sequence analysis as described under Materials and Methods. Numbers in parentheses represent the yield in picomoles at each cycle. [b] Numbers in parentheses represent the yield in picomoles at each cycle. [c] γ-Carboxyglutamic acid residues.

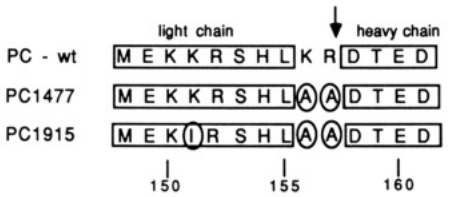

FIGURE 4: Processing site sequences of human protein C cleavage site mutants created by site-directed mutagenesis. Amino acids representing the mature C-terminus of the light chain and the mature N-terminus of the heavy chain are boxed. Amino acids substituted by point mutation are circled. The endoproteolytic cleavage site is indicated by an arrow. Amino acid positions relative to amino acid 1 of the light chain are indicated by numbering at the bottom of the figure.

Other potential sites exist in close proximity to the expected cleavage site which would not significantly alter electrophoretic mobility. Such cleavage fragments might not remain associated with the protein during subsequent purification and therefore would not be detected in sequence analysis.

In order to address the question of potential additional sites, we constructed a protein C mutant (PC1477, Figure 4) in which the Lys156-Arg157 pair was changed to Ala-Ala. This mutant cDNA was ligated into the expression vector (Zem229R) and transfected into BHK cells with MTX selection. A stable clone was chosen and retransfected with the Zem228R/*KEX2* vector, and colonies resistant to both MTX and G418 were isolated. Both the parent PC1477 cell line and several doubly transfected progeny were pulse-labeled with [^{35}S]Cys, and the protein C products were analyzed for the degree of processing as described earlier for the wild-type protein C. The results (Figure 5A) clearly show that in the absence of Kex2 (lane 1) this mutant precursor is not detectably cleaved by the mammalian endopeptidase(s) present in BHK cells. However, in the presence of Kex2 (lane 2), some colonies show relatively high rates of processing. This result indicates that there are other sites within the protein C precursor which are not suitable substrates for the more restrictive mammalian processing enzymes (Foster et al., 1990) but which are subject to cleavage by Kex2.

In order to determine the location of this alternative cleavage site, the PC1477/*KEX2* protein C product was affinity-purified and subjected to N-terminal sequence analysis. The sequence

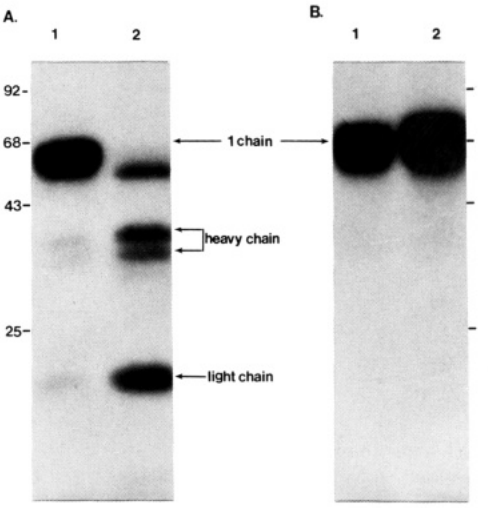

FIGURE 5: Immunoprecipitation of mutants PC1477 and PC1915 secreted from cells with or without coexpression of the KEX2 gene. A clonal cell line expressing either PC1477 or PC1915 (see Figure 4) under DHFR selection was transfected with the Zem228R/KEX2 vector (neo selection). In each case, several doubly transfected clones were picked at random and cultured individually. Both the parent cell line (lane 1: either PC1477, panel A, or PC1915, panel B) and the doubly transfected clones (lane 2, both panels) were labeled with [^{35}S]Cys, and the protein C products were immunoprecipitated as described under Materials and Methods.

Table II: Amino-Terminal Sequence of PC1477 from Cells Coexpressing Kex2 Endopeptidase[a]

	light chain		heavy chain	
cycle	expected	observed	expected	observed
1	A	A (469)[b]	S	S (270)
2	N	N (187)	H	H (27)
3	S	S (148)	L	L (170)
4	F	F (175)	A	A (155)
5	L	L (168)	A	A (209)
6	E[c]		D	D (88)
7	E[c]		T	T (77)
8	L	L (79)	E	E (58)
9	R	R (34)	D	D (50)
10	H	H (14)	Q	Q (62)
11	S	S (49)	E	E (38)
12	S	S (83)	D	D (26)

[a] Protein C was purified from a clonal cell line which coexpressed the mutant protein C (PC1477) and the KEX2 gene. The protein was immunopurified as described under Materials and Methods on a calcium-dependent monoclonal antibody. Approximately 500 pmol of purified protein was subjected to N-terminal sequence analysis as described under Materials and Methods. Numbers in parentheses represent the yield in picomoles at each cycle. [b] Numbers in parentheses represent the yield in picomoles at each cycle. [c] γ-Carboxyglutamic acid residues.

(Table II) shows two amino acids identified at each cycle—one corresponding to the mature N-terminus of the light chain and another beginning with Ser153, indicating a cleavage in the precursor following the Lys150-Lys151-Arg152 sequence near the C-terminal end of the light chain. Presumably, the wild-type protein C precursor was also cleaved at this site, and the resulting small peptide corresponding to Ser153-Leu155 was released. It is not surprising that cleavage at this second site produced products which were not detectably different than the native heavy and light chain on SDS–PAGE.

Following the same rationale as outlined above, it was important to establish whether there were yet other nearby potential cleavage sites which were recognized by the Kex2 peptidase. Accordingly, we constructed another mutant protein C in which the Lys156-Arg157 pair was changed to Ala-Ala *and* also the Lys151 was changed to Ile (PC1915, Figure 4)

so that both identified sites of Kex2 cleavage were eliminated. Following the same strategy of sequential expression of the double mutant and coexpression of Kex2, both the parent cell expressing PC1915 alone and a number of progeny cell lines coexpressing Kex2 were examined by pulse-labeling and immunoprecipitation. Representative results are shown in Figure 5B. Neither the parent (lane 1) nor coselected progeny cell lines (lane 2) showed significant cleavage of the single-chain protein C precursor to two-chain form, suggesting that all exposed cleavage sites susceptible to either the mammalian cell processing enzyme or the *KEX2* gene product have been eliminated from this mutant.

Discussion

Previous studies from this laboratory (Foster et al., 1990) have demonstrated that many mammalian cells have an endoproteolytic activity in their secretory pathway which recognizes and cleaves exposed clusters of basic amino acids in the precursors of human protein C and human factor X, converting the single-chain precursors to the mature, two-chain forms which circulate in plasma. Human factor X circulates in plasma completely in the two-chain form and is also secreted from most transfected mammalian cell lines completely in the two-chain form. However, human protein C circulates in plasma as a mixture of approximately 80% two-chain form and about 20% unprocessed single-chain form. Human protein C is also poorly cleaved to the two-chain form in a variety of mammalian cell recombinant systems (Foster et al., 1987; Grinnell et al., 1987; Oppenheimer & Wydro, 1988) and in human HepG2 cells (J. Miletich, personal communication). Our studies on mutation of the sequence surrounding the Lys-Arg pair in the protein C precursor demonstrated that the reason for this poor cleavage of the protein C precursor relative to that of factor X appears to be the substrate specificity of the mammalian cell endopeptidase for sequences which contain basic amino acids at the −1, −2, and −4 positions relative to the cleavage site.

This type of cleavage after Lys-Arg sequences to yield mature coagulation factors bears striking similarity both to the processing of polyhormone precursors in mammalian cells and also to processing of the mating-type α-factor precursor in yeast. The obvious similarities in these processes led us to speculate that such endoproteolytic maturation systems are also functionally analogous and that the cleavage of precursors to blood coagulation factors (and apparently also removal of their propeptides) may be carried out by the same enzymes which normally also participate in the maturation of polyhormone precursors. None of these enzymes has yet been firmly identified in mammalian tissues; however, both the protein and the gene for the Kex2 endopeptidase have been isolated from the yeast *Saccharomyces cerevisiae*. In order to test this hypothesis in the current studies, we have chosen a mammalian cell expression system which cleaves the protein C precursor poorly and then introduced the gene for the yeast Kex2 endopeptidase and looked for the functional effect on cleavage of the protein C.

In the present study, we have shown that the Kex2 endopeptidase does indeed recognize and cleave the protein C precursor in the "natural" position, following Lys156-Arg157. Mutagenesis of this site to nonbasic amino acids revealed the presence of at least one additional Kex2-sensitive cleavage site in the precursor, and N-terminal sequence analysis of the cleaved mutant showed the position of the second site to be after the Lys150-Lys151-Arg152 sequence. This upstream alternative site was not sensitive to cleavage by the mammalian cell endopeptidase, since it was not detectably cleaved in the absence of Kex2. Subsequent experiments with a double mutant, in which both identified cleavage sites had been mutated, showed that no other sites within the protein C precursor were sensitive to cleavage by the Kex2 endopeptidase.

These results suggest that the protein C precursor contains an exposed loop on its surface which presents both the Lys150-Lys151-Arg152 sequence and also the Lys156-Arg157 pair in a conformation accessible to cleavage by proteases. The mammalian cell endopeptidase recognizes and cleaves these sites poorly since neither has an appropriate basic amino acid in the −4 position. However, the Lys156-Arg157 site is cleaved partially in most cell lines and also during physiologic biosynthesis in the liver. It is possible that the His154-Leu155 sequence in positions −4 and −3 confers some recognition of the Lys156-Arg157 pair, whereas the Glu149 in position −4 for the upstream site precludes cleavage, consistent with our earlier observations on the effect of negative charge in the −4 position (Foster et al., 1990). The yeast Kex2 endopeptidase has no obvious extended substrate specificity beyond the Lys-Arg pair and consequently recognizes and cleaves both of these exposed sites. The fact that no other pairs of basic amino acids in the precursor are cleaved by Kex2 suggests that either they are not in exposed surface positions or alternatively they lack some other unidentified substate requirement of the Kex2 endopeptidase.

This demonstration of functional analogy between Kex2 and mammalian cell endoproteolytic maturation of coagulation factor precursors suggests that very similar proteases may be involved in both processes. The Kex2 peptidase has already been shown to be capable of correctly cleaving the POMC polyhormone precursor in mammalian cells (Thomas et al., 1988) and also to be active in removing the proalbumin propeptide in vitro (Bathurst et al., 1987). Two groups have recently shown (Fuller et al., 1989; van den Ouweland et al., 1990) that the *KEX2* gene sequence shows remarkable homology with a previously unidentified human sequence which was named *furin* because of its physical proximity to the c-fes protooncogene. The furin sequence predicts a protein domain structure highly similar to that of Kex2, consisting of an approximately 700 amino acid lumenal subtilisin-type catalytic domain, a transmembrane region presumably spanning the membrane of secretory vesicles, and a short cytoplasmic tail. It is highly likely that the furin gene product represents at least one of the mammalian endopeptidases which perform endoproteolytic maturation at basic sites within precursors. If so, it may have the extended substrate specificity observed in most mammalian cells and in the liver. Experiments are currently in progress in this laboratory to examine the substrate specificity of the furin gene product.

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Registry No. Endopeptidase, 9001-92-7.

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Metabolism of Unusual Membrane Phospholipids in the Marine Sponge *Microciona prolifera*†

Wai-kwan Lam, Mark F. Beatty, Soonkap Hahn, and Carl Djerassi*
Department of Chemistry, Stanford University, Stanford, California 94305
Received July 24, 1990; Revised Manuscript Received September 10, 1990

ABSTRACT: Sponges are unique in regard to membrane phospholipid composition. Features virtually without parallel in other organisms are the predominance of the C_{26}–C_{30} polyenoic acids (demospongic acids) in the phosphatidylethanolamines (PE) and the attachment of identical acyl groups to the glycerol moiety. The biosynthesis and disposition of these unusual phospholipids were followed in the marine sponge *Microciona prolifera* where PE($\Delta^{5,9}$-26:2,$\Delta^{5,9}$-26:2) is a major molecular species. Incorporation experiments with radiolabeled fatty acids, bases, and intact phospholipids revealed the de novo biosynthesis of the two major phosphatides, phosphatidylethanolamines (PE) and phosphatidylcholines (PC), via the cytidine pathway as in higher animals, with ethanolamine selectively incorporated into PE($\Delta^{5,9}$-26:2,$\Delta^{5,9}$-26:2). Methylation of PE and random acyl chain migration across different phospholipid classes were marginal, but the exchange of PC for PE, apparently mediated by the action of phospholipase, was indicated after uptake of the unnatural PC(Δ^{9}-26:1,Δ^{9}-26:1). The present study demonstrates in the most primitive multicellular animals a phospholipid metabolic pattern similar to that in higher organisms, with unique acyl and phosphoethanolamine transferases apparently involved in the biosynthesis of the (demospongic) di-C_{26}-acyl-PE molecular species.

Phospholipids are molecules of crucial importance in the regulation of cell membrane properties and in biological signal transmission (Hirata & Axelrod, 1980). Although the major pathways of phospholipid biosynthesis are well established in both prokaryotes and eukaryotes (Vance & Vance, 1985; Hawthorne & Ansell, 1982; Mudd, 1980), extensive efforts are still being directed to the study of phospholipid class interconversion, for instance the methylation of PE to PC.[1]

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* Address correspondence to this author.

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