# Intracellular Targeting and Structural Conservation of a Prohormone-Processing Endoprotease

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**The prohormone-processing endoprotease (*KEX2* gene product) of the yeast *Saccharomyces cerevisiae* is a membrane-bound, 135,000-dalton glycoprotein, which contains both asparagine-linked and serine- and threonine-linked oligosaccharide and resides in a secretory compartment. Analysis of mutant *kex2* genes truncated at their 3′ end indicates that carboxyl terminal domains of the enzyme are required for its proper localization within the cell. A human gene product, "furin," shares 50% identity with the catalytic domain of Kex2 protease and is, therefore, a candidate for a human prohormone-processing enzyme.**

BIOACTIVE PEPTIDES ARE GENERATED by both endo- and exoproteolytic cleavage of larger precursors during their secretory transport (*1*). The only prohormone-processing endoprotease definitively identified in eukaryotic organisms is the *KEX2* gene product of *Saccharomyces cerevisiae* (*2, 3*). The Kex2 enzyme is a $Ca^{2+}$-dependent, neutral serine protease (*4, 5*) that cleaves pro–α factor and pro–killer toxin at Lys-Arg and Arg-Arg sites during transit of these precursors through the secretory pathway. We now describe structural features of mature Kex2 and demonstrate that COOH-terminal sequences of the protein are important in delivery of the enzyme to (or retention of the enzyme in) its normal intracellular compartment. We also identify a putative human homolog of Kex2.

We determined the sequence of 4118 nucleotides from a genomic clone containing *KEX2* (*4*) and deposited these data in GenBank (accession number M24201). Within this sequence is a single, long open reading frame that encodes an 814-residue polypeptide (calculated molecular mass of 90,013 daltons). "Hydropathy" analysis (*6*) of the protein sequence reveals two markedly hydrophobic segments. The first is located at the $NH_2$-terminus and has the hallmarks of a signal sequence (*7*), with likely cleavage sites at $Ala^{19}$, $Ser^{18}$, or $Ser^{22}$. The second hydrophobic segment ($Tyr^{679}$ to $Met^{699}$) occurs near the COOH-terminus and has the characteristics of a transmembrane domain (TMD) (*8*). Correspondingly, we have shown that Kex2 behaves as an intrinsic membrane protein during fractionation (*3–5*). The putative TMD separates the $NH_2$-terminal domain (678 residues) from an acidic (net charge −15) COOH-terminal "tail" (115 residues). We have shown previously that the first 614 $NH_2$-terminal residues are sufficient for $Ca^{2+}$-dependent enzymic activity (*5*). Furthermore, we have found (*2, 5, 9*), and another group has confirmed on obtaining the nucleotide sequence of *KEX2* (*10*), that the amino acid sequence of this $NH_2$-terminal region contains a domain (residues 142 to 447) with significant homology (24 to 33% identity) to members of the subtilisin family of serine proteases. Thus, the enzyme is probably oriented with the $NH_2$-terminal catalytic domain in the lumen of a secretory compartment and the COOH-terminal tail exposed to the cytosol.

Polyclonal antibodies were raised in rabbits to a hybrid protein generated after fusing the COOH-terminal 101 codons of *KEX2* to the COOH-terminus of *Escherichia coli lacZ* (*11*). This antiserum immunoprecipitated a polypeptide from *S. cerevisiae* that migrated as a broad band with an apparent molecular mass of ~135,000 daltons (Fig. 1A). Several observations demonstrated that this species represents the authentic *KEX2* gene product. The protein was not detected by preimmune serum, and addition of excess partially purified fusion protein prevented detection. Furthermore, when cell extracts were examined by immunoblotting, the antiserum recognized a polypeptide of the same apparent molecular mass, and the amount of this polypeptide was proportional to the degree of overproduction of Kex2 activity in every strain examined. Finally, this protein was undetectable in strains in which the chromosomal copy of *KEX2* was deleted (*12*).

The *KEX2* sequence predicts five consensus sites [Asn-X-Ser/Thr] (*13*) (where X represents any amino acid) for attachment of Asn-linked (N-linked) oligosaccharide in the $NH_2$-terminal domain (an additional site is present in the cytoplasmic tail) (*14*). Immunoprecipitated Kex2 was digested thoroughly with either endoglucosaminidase H (endo H) (*15*) or peptide: *N*-glycosidase F (PNGase) (*16*). Treatment with either enzyme reduced the apparent molecular mass by ~3000 to 5000 daltons (Fig. 1B), indicating that mature Kex2 contains a maximum of three N-linked oligosaccharides [if we assume that the N-linked carbohydrate represents processed cores, (*N*-acetylglucosamine)$_2$-(mannose)$_8$ (*17*)]. This amount of N-linked carbohydrate could not account for the large difference between the observed molecular mass of the mature protein (135,000 daltons) and either the calculated molecular mass from the primary sequence

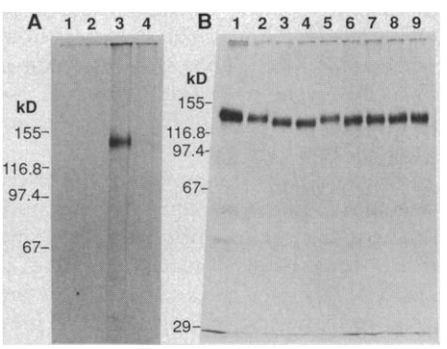


**Fig. 1.** (**A**) Immunoprecipitation of Kex2 protein. *Saccharomyces cerevisiae* strain AB110 containing plasmid pAB23-KX211 (*5*) was grown at 30°C in low sulfate medium (LSM) (*23*) containing 100 μ*M* $(NH_4)_2SO_4$ and lacking uracil to a density of ~$10^7$ cells per milliliter and then concentrated twice into 12.5 ml of the same medium [except containing 20 μ*M* $(NH_4)_2SO_4$]. After 20 min at 30°C (to allow $SO_4^{2-}$ depletion), the cells were labeled with $^{35}SO_4^{2-}$ (100 μCi/ml) for 30 min, harvested at 0°C in the presence of 1 m*M* $NaN_3$, frozen on dry ice, and lysed in the presence of 1% SDS by glass bead breakage (*23*). $^{35}$S-labeled Kex2 was immunoprecipitated (*23*) from the lysate derived from 1 ml of cells with 5 μl of preimmune (lanes 1 and 2) or anti-Kex2 (lanes 3 and 4) serum (*11*) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 14 μg of β-galactosidase–Kex2 fusion protein. Protein A–Sepharose (Pharmacia) was used as the immunoadsorbent. Immunoprecipitates were solubilized in gel loading buffer [50 m*M* tris-HCl (*p*H 6.8) 1% SDS, and 5% 2-mercaptoethanol], heated to 95°C for 5 min, and subjected to SDS-PAGE (8% gel). The gel was soaked in ~200 ml of 1*M* sodium salicylate for 30 min, dried, and fluorographed on Kodak XAR-5 film. (**B**) Asn-linked carbohydrate content of Kex2. To analyze N-linked carbohydrate, we solubilized immunoprecipitates prepared as in (A) with gel loading buffer (*p*H 7.6) in this case. After diluting tenfold with immunoprecipitation buffer (*23*), Kex2 was reprecipitated with antiserum. The second precipitates were solubilized in gel loading buffer and either subjected to SDS-PAGE (lane 1), or diluted tenfold into buffer for endo H digestion [250 m*M* sodium citrate (*p*H 5.5), 0.05% SDS, and 10 m*M* $NaN_3$] (lanes 2 to 4) or buffer for PNGase digestion [100 m*M* tris-HCl (*p*H 9.0), 10 m*M* EDTA 0.5% Triton X-100, and 10 m*M* $NaN_3$] (lanes 5 to 9) and digested with the respective enzyme [(lanes 2 to 4) 0, 1, and 5 milliunits of endo H per milliliter, respectively; (lanes 5 to 9) 0, 0.4, 1.9, 7.4, and 26 units of PNGase per milliliter, respectively] for 15 hours at 30°C (PNGase) or 37°C (endo H). Digestion products were recovered by precipitation with 10% trichloroacetic acid, and subjected to electrophoresis as in (A).

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(90,013 daltons) or the apparent molecular mass of the in vitro translation product (~110,000 daltons) (*18*). However, just proximal to the TMD in Kex2, there is a segment (residues 617 to 672) of high Ser and Thr content (28 of 56 amino acids). In other transmembrane proteins (*19*), such Ser- and Thr-rich domains are modified by O-linked glycosylation. When Kex2 was detergent-solubilized from membrane fractions of cells treated with tunicamycin [to block N-linked glycosylation (*20*)], and then applied to a concanavalin A (Con A)–Sepharose column, 98% of Kex2 activity was retained. A significant fraction (35%) of the bound activity was eluted by sequential washes with buffer containing 0.2*M* and 0.5*M* α-methylmannoside, showing that retention on the column was due to specific binding by the lectin. These results suggest that the Ser- and Thr-rich domain is modified by O-linked oligosaccharides [which, in yeast, consist of linear chains of up to four α-linked mannosides (*17*)] and that this decoration contributes about 20,000 daltons to the apparent molecular mass of the mature protein. We reached a similar conclusion by comparing the electrophoretic mobilities of mutant proteins retaining or lacking the Ser- and Thr-rich domain (*5*). A substantial fraction of Kex2 activity remained on the Con A–Sepharose even after elution with 0.5*M* α-methylmannoside. This avidity of binding suggests that a large number of O-linked chains may be present on the protein. On the basis of the correlation between anomalous electrophoretic migration and analytically determined carbohydrate content for well-characterized *O*-glycosylated proteins, including the low density lipoprotein (LDL) receptor (*21*) and yeast MATa agglutinin (*22*), mature Kex2 may carry O-linked sugars at 10 to 12 sites within the Ser- and Thr-rich domain.

Our previous analysis of pro–α factor maturation in temperature-sensitive secretion-defective mutants (*23*) suggested that cleavage by Kex2 occurs in the Golgi body, and, therefore, that Kex2 may be a component of this organelle. Also, membrane fractionation by density gradient centrifugation separates most Kex2 from a marker enzyme in secretory vesicles (acid phosphatase) (*24*), but only partially resolves Kex2 activity from another marker enzyme (α-1,3-mannosyl transferase) thought to reside in some Golgi cisterna (*25*). Both the N- and O-linked oligosaccharides present on mature Kex2 carry α-1,3-linked mannose residues (*18*), providing further support for the conclusion that Kex2 resides in a late compartment of the Golgi body.

In other membrane proteins with a single COOH-terminal TMD, such as the 215-kD mannose-6-phosphate receptor (M6PR) (*26*), the LDL receptor (*27*), and influenza virus hemagglutinin (*28*), COOH-terminal sequences, especially the cytoplasmic tail, have a marked influence on subcellular distribution. We produced mutant forms of Kex2 (*5*) by deleting sequences in *KEX2* that encode the COOH-terminal domains (Fig. 2A), and their subcellular distribution was compared to that of the wild-type enzyme (Table 1). The truncated polypeptides are all glycosylated and retain $Ca^{2+}$-dependent proteolytic activity (*5*). In yeast producing the normal enzyme, only a small fraction (~2%) of total Kex2 activity was detected on intact cells, even though the protein was overproduced by a factor of 200 from a plasmid expression vector (*5*) (Table 1). This result contrasts with the shunting of yeast vacuolar enzymes to the cell surface that occurs upon even modest (three- to fivefold) overproduction (*29*). The amount of extracellular Kex2 activity detected was comparable to the fraction of cells (2 to 3%) that were permeable to methylene blue, and thus represents activity released by, or accessible within, dead cells. Therefore, we conclude that little or no Kex2 is normally present at the cell surface.

In contrast, the truncated molecules all showed a readily detectable change in their cellular distribution (Table 1). The enzyme produced by the Δ4 deletion (which removes 99 amino acids from the 115-residue cytoplasmic tail) displayed a nearly sevenfold increase in activity at the cell surface, without a concomitant increase in the fraction of dead cells. For deletion Δ5 (which

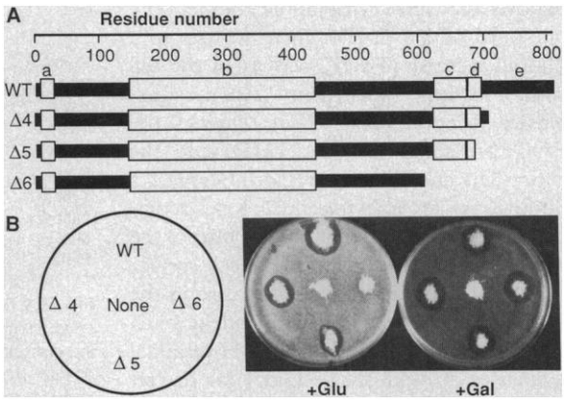


**Fig. 2.** Mislocalization of Kex2 causes inefficient processing of pro–α factor. (**A**) Structures of Kex2 and COOH-terminal truncation mutants. Boxes indicate elements of the predicted primary sequence: a, signal sequence; b, domain with homology to subtilisin; c, Ser- and Thr-rich region; d, TMD; and e, cytoplasmic tail. Construction of the deletions has been described (*5*). (**B**) Bioassays of α factor production. The wild-type (WT) and deletion mutant genes were expressed under the control of the *GAL1* promoter on high copy number vector pLG1-4Δ60 as described (*5*). The plasmids were introduced into *S. cerevisiae* strain BFY101-32C (*MATα kex2Δ1::URA3-S*) in which the chromosomal copy of *KEX2* was deleted and replaced with *URA3* (*12*). The transformants were grown as patches on minimal medium lacking leucine, then replica-plated onto plates [containing 1% yeast extract, 2% peptone, 2% agar, and either 2% glucose (+Glu) or 2% galactose (+Gal)] onto which lawns had been spread of a *MAT***a** strain, RC634, that is supersensitive to α factor because of a mutation in *SST1* (*4*).

**Table 1.** Mislocalization of mutant Kex2 proteins truncated at the COOH-terminus. Transformants of *S. cerevisiae* strain BFY101-32C [*MATα kex2Δ1::URA3-S* (*5*)] containing plasmids pLG-KX212, pLG-KX212Δ4, pLG-KX212Δ5 or pLG-KX212Δ6 (*5*) were grown at 30°C in galactose (2%) minimal medium (*42*) lacking leucine, harvested when $A_{600}$ reached 1.0 ($2 \times 10^7$ cells per milliliter), washed in 0.15*M* NaCl, and then resuspended in 1/40 volume of buffer A containing protease inhibitors (*5*). To assay cell surface Kex2 activity, we added intact cells directly to 50 μl of reaction mix under the conditions described (*5*), except that Triton X-100 was omitted. To assay total activity, we lysed cells by three cycles of freezing on dry ice and thawing at 0°C in buffer A containing 2% (w/v) sodium deoxycholate, followed by incubation at 0°C for 30 min. Enzyme activity was measured by following hydrolysis of *t*-butoxycarbonyl-Gln-Arg-Arg-4-methylcoumarin-7-amide, which releases the fluorescent compound 4-methyl-7-amino-coumarin (AMC) (*5*). Activity per $5 \times 10^8$ cells is expressed in units, where 1 unit represents 1 pmol of AMC released per minute. N.D., not determined.

| *KEX2* | Experiment | Kex2 activity (units) | | Fraction of Kex2 activity at cell surface (%) | Dead cells (%) |
|---|---|---|---|---|---|
| | | Cell surface | Total | | |
| Wild type | 1 | 670 | 25,700 | 2.6 | 2.6 |
| | 2 | 920 | 52,600 | 1.7 | N.D. |
| Δ4 | 1 | 5,625 | 26,700 | 21 | 2.6 |
| | 2 | 4,560 | 45,200 | 10 | N.D. |
| Δ5 | 1 | 6,690 | 15,700 | 43 | 3.8 |
| | 2 | 4,280 | 37,900 | 11 | N.D. |
| Δ6 | 1 | 2,280 | 3,360 | 68 | 3.1 |
| | 2 | 5,280 | 8,960 | 59 | N.D. |

removes the entire cytoplasmic tail, but only one-third of the TMD), there was up to a 20-fold increase in Kex2 activity at the cell surface in some experiments. The most extensive deletion, Δ6 (which removes the COOH-terminal tail, the TMD, and the entire Ser- and Thr-rich region), showed a 30-fold increase in extracellular Kex2 activity, such that the majority (60 to 70%) of the total activity was present at the cell surface (Table 1), again without an increase in the fraction of cells stained by methylene blue. These results indicate that COOH-terminal sequences of Kex2 are necessary for correct localization of the enzyme.

To determine if mislocalization affects the biological function of Kex2, we measured the efficiency of pro–α factor processing in *MAT*α cells expressing either wild-type Kex2 or one of the three truncated proteins (Fig. 2B). A zone of growth inhibition (halo) is produced in a lawn of tester *MAT***a** cells only if mature α factor is secreted by *MAT*α cells. The *MAT*α strain we used was deleted of its chromosomal copy of *KEX2* and, as expected, failed to make such a halo (Fig. 2B). Introduction of a high copy number plasmid with either wild-type *KEX2* or deletion Δ4 (each under control of the inducible *GAL1* promoter) resulted in halo formation regardless of whether the plasmid-borne genes were expressed at a moderate level [growth on glucose results in five to ten times the expression seen with the wild-type promoter (*5*)] or at a high level [growth on galactose results in over 200 times the expression seen with the wild-type promoter (*5*)]. However, the halo size was detectably reduced when deletion Δ5 was expressed at the moderate level. No halo was observed when deletion Δ6 was expressed at a moderate level (Fig. 2B), even though direct assay showed that total Kex2 activity in these cells was threefold higher than that observed in wild-type cells. Thus, the mislocalization displayed by the Δ6 mutant (Table 1) prevents processing of pro–α factor. The fact that a significant fraction (30 to 40%) of the Δ6 enzyme does not appear at the cell surface (Table 1) may indicate that, in the absence of proper localization, a substantial portion of the mutant molecules are diverted to (or dwell longer in) all of the compartments of the secretory system. This situation could explain why pro–α factor processing was able to occur when the Δ6 enzyme was produced at a high level (Fig. 2B). Under this circumstance, pro–α factor molecules presumably encounter by chance a sufficient amount of Kex2 to generate some mature α factor.

Our results show that, in otherwise normal cells, mutant Kex2 enzymes lacking COOH-terminal sequences are partitioned to the cell surface. Conversely, Payne and Schekman (*30*) have found that wild-type Kex2 is shunted to the cell surface in yeast mutants lacking clathrin heavy chain. Taken together, these findings suggest that the COOH-terminal domains of Kex2 and clathrin are important recognition elements in the processes required to localize a constituent enzyme of the secretory pathway to the Golgi compartment. Several mechanisms could account for the role of COOH-terminal sequences in localizing Kex2 to its specific intracellular compartment. A portion of the Kex2 molecules might normally be transported to the cell surface in secretory vesicles, but then be rapidly cleared (or recycled) from the cell surface by endocytosis. In this model, alteration or removal of COOH-terminal sequences would block endocytosis, as found for the LDL receptor (*27*), and, therefore, more Kex2 would remain at the cell surface. Alternatively, the enzyme might be stably tethered within an intracellular compartment or cycled between two intracellular compartments. Deletion of COOH-terminal sequences might in this case interfere with the mechanism of retention or intracellular recycling, thereby causing more Kex2 to be diverted to the cell surface.

The Δ4 form of Kex2 still retains an 18–amino acid tail [16 authentic Kex2 residues and 2 residues from the terminator linker (*5*)]. Tails as short as ten amino acids are competent to promote endocytosis through coated pits in animal cells (*28*). Although the Δ4 enzyme was detectably mislocalized (Table 1), its biological function did not seem to be impaired (Fig. 2B). However, a more profound mislocalization (Table 1) and pronounced reduction in biological function (Fig. 2B) occurred for the Δ5 and Δ6 enzymes, which both lack the entire cytoplasmic tail. The difference in behavior between Δ4 and the other two mutants may reflect the retention of some partial targeting function by the 18-residue remnant of the cytoplasmic tail remaining in the Δ4 enzyme. This short segment carries one of only two Tyr residues present in the entire 115–amino acid cytoplasmic domain. This feature is noteworthy because Tyr residues are critical recognition elements for a set of proteins, called "adaptins," that are required in animal cells to link clathrin to the cytoplasmic tails of membrane proteins (*28, 31*). The region of the Kex2 tail that contains both its Tyr residues occurs at about the same relative position and bears significant similarity (30% identity) (*32*) to the segment of the cytoplasmic tail of M6PR (*26*) that is responsible for its recognition by adaptins (*31*). The TMD (and perhaps the Ser- and Thr-rich domain) of Kex2 also appears important for targeting and retention of the protease within the cell because the Δ6 mutant displayed a more severe mislocalization than Δ5, as judged both by appearance of enzyme at the cell surface (Table 1) and by inability to reside in the appropriate secretory compartment at a level sufficient to achieve pro–α factor processing (Fig. 2B).

The function of Kex2 and its true mammalian counterpart appears to have been highly conserved. Kex2 accurately processes human proinsulin at its Lys-Arg and Arg-Arg sites when this precursor is expressed in yeast (*33*). Conversely, expression of *KEX2* in processing-deficient mammalian cells results in specific cleavage of mouse pro-opiomelanocortin at Lys-Arg sites (*34*). Finally, membrane preparations enriched in Kex2 properly cleave human proalbumin at its normal Arg-Arg processing site (*35*). Therefore, we anticipated that the structural features of an authentic mammalian prohormone-processing endoprotease would close-

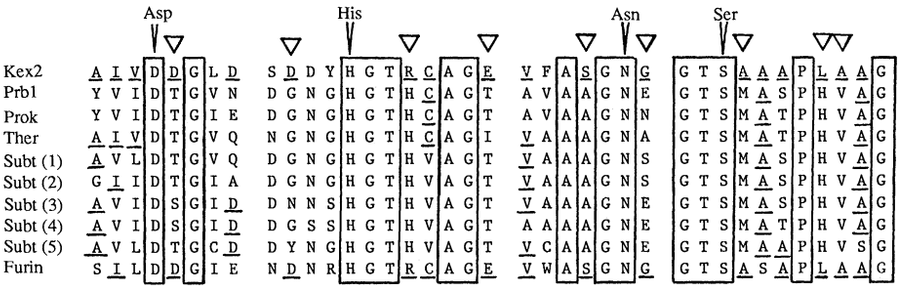


**Fig. 3.** Conservation of active site residues between Kex2 and furin. Active site Asp, His, Asn, and Ser residues found in the subtilisin family of serine proteases are indicated by arrows (*41*). Boxed residues are conserved in all sequences. Triangles indicate positions at which Kex2 and furin are identical and deviate from all the other subtilisin family members. Underlined residues are conserved between Kex2 and at least one other sequence. Prb1, *S. cerevisiae* vacuolar proteinase B; Prok, *Tritirachium album Limber* proteinase K; Ther, *Thermoactinomyces vulgaris* thermitase; Subt(1), *Bacillus subtilis* subtilisin Carlsberg; Subt(2), *B. subtilis* subtilisin DY; Subt(3), *B. amyloliquefaciens* subtilisin BPN′; Subt(4), *B. subtilis var. amylosaccharitícus* subtilisin S; and Subt(5), intracellular subtilisin from *B. subtilis* A-50 [see (*40*) for a compilation of these sequences).

ly resemble those of yeast Kex2.

A search of databases (*36*) revealed a partial sequence for a human gene product homologous to Kex2. The corresponding human gene (*fur*) lies immediately upstream of the *fps/fes* oncogene (*37*). The sequence of 513 amino acids of the *fur* gene product ("furin") can be deduced from a partial cDNA clone (3.1 kb) and from the immediately proximal genomic sequence (*37*). On the basis of the length of the *fur* mRNA (4.5 kb) (*37*), the complete protein could be significantly larger. Within the first 150 amino acids of the 513 residues, there are 75 matches (50% identity) to the Kex2 sequence, with only a single two-residue gap. This region of the Kex2 sequence corresponds to the COOH-terminal one-third of the catalytic domain and includes both the active site Ser and an Asn residue important for enzymic activity of (and substrate binding by) the subtilisin family of serine proteases (*38*). Additional cDNA sequence for the *fur* gene has recently been obtained (*39*) and permits comparison of the segments that correspond to the active site Asp and His residues of Kex2 and the subtilisin family.

In all four of these regions (Fig. 3), Kex2 and furin are significantly more homologous to one another than either is to the other subtilisin family members. Most importantly, Kex2 and furin share those residues that distinguish Kex2 from all the other subtilisin family members.

Further, distal to the catalytic domain, the next 150 residues of furin show significant relatedness (24% identity) to the corresponding segment of Kex2. Beyond this point, the sequences of Kex2 and furin diverge; however, the COOH-terminal portions of both proteins are almost identical in length and contain a single potential TMD followed by a hydrophilic and highly charged tail. The putative cytoplasmic tail of furin contains just two Tyr residues, which are situated at a distance (11 residues) from the TMD, and with a spacing between them (8 residues), nearly identical to those observed in Kex2. The similarity between the putative catalytic domains and other regions of furin and Kex2 suggests that furin is a human prohormone-processing protease.

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11. Production of antiserum to Kex2 (anti-Kex2): Plasmid pLZ-KXR was constructed by inserting a 1500-bp Eco RI fragment of plasmid pKX2 (*5*) encoding the COOH-terminal 101 residues and translational terminator of *KEX2* into the Eco RI site of plasmid pUR292 [U. Rüther and B. Müller-Hill, *EMBO J.* **2**, 1791 (1983)]. When introduced into *E. coli* DG99 (*thi1 endA hsdR lacZM15Δ lacI$^Q$ pro::Tn10 supE*), pLZ-KXR directed synthesis of a β-galactosidase fusion protein of the expected size (~130 kD). The fusion protein was purifed from 1 g of cells [grown at 37°C in 1.5 liters of LB medium (per liter: 10 g of Bacto tryptone, 5 g of Bacto yeast extract, 10 g of NaCl) (*p*H 7.5) containing ampicillin (100 μg/ml) until the absorbance at 600 nm ($A_{600}$) was 0.3, induced for 90 min by addition of 0.5 m*M* isopropylthiogalactoside, and harvested when $A_{600}$ was 0.77]. Cells were lysed by passage three times at 10,000 psi through a French pressure cell in 25 ml of lysis buffer [50 m*M* tris-HCl (*p*H 7.5), 5% saturated $(NH_4)_2SO_4$, 10% glycerol, 25 m*M* EDTA-NaOH, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* benzamidine-HCl, 0.1 m*M* tosylphenylalanylchloromethylketone, and 5 μ*M* pepstatin A]. The fusion protein was recovered in the insoluble debris (inclusion bodies) after centrifugation at 100,000*g* for 60 min. The pellet was washed by resuspension in 25 ml of lysis buffer containing 1% Triton X-100 and 0.5*M* NaCl followed by centrifugation. The pellet was dissolved in 5 ml of resuspension buffer [4*M* urea, 2% (w/v) SDS, 50 m*M* tris-HCl (*p*H 7.5), 1 m*M* EDTA-NaOH, and 5% (v/v) 2-mercaptoethanol] by incubation at 65°C for 15 min, extracted four times with an equal volume of $CHCl_3$:isoamyl alcohol (IAA) (24:1), three times with an equal volume of phenol:$CHCl_3$ (1:1), twice with an equal volume of $CHCl_3$:IAA (24:1), three times with 100% ethanol, and once with sterile $H_2O$. The insoluble pellet was resuspended in 5 ml of resuspension buffer, heated at 65°C for 10 min, and then subjected to preparative SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (8% gel) in buffer containing 0.1 m*M* sodium thioglycolate and 0.12% SDS. The fusion protein band was visualized with 4*M* sodium acetate, the band was sliced out, and the protein was eluted [M. W. Hunkapillar, E. Lujan, F. Ostrander, L. E. Hood, *Methods Enzymol.* **91**, 227 (1983)]. The purified fusion protein was lyophilized, extracted with triethylamine:acetic acid:acetone (5:5:85) to remove SDS [W. H. Konigsberg and L. Henderson, *Methods Enzymol.* **91**, 254 (1983)], resuspended in sterile saline solution, and used as immunogen for two New Zealand White rabbits. Primary injections were made at multiple subcutaneous locations with 100 μg of purified fusion protein in complete Freund's adjuvant, and boosts with multiple injection of 50 μg in incomplete Freund's adjuvant at 2-week intervals. After the first two boosts, subsequent boosts were made with protein that had been purified by chromatography on Sepharose CL-6B (Pharmacia). The animals were bled at the same time as boosters injections were applied. Substantial Kex2-specific immunoreactivity was observed against purified fusion protein after the fifth bleed as measured by enzyme-linked immunosorbent assay (ELISA) (antibodies to LacZ were blocked by incubating the serum with an excess of an unrelated β-galactosidase fusion protein). Antiserum used in immunoprecipitation came from the sixth bleed; the titer was estimated at about 10 to 20 μg of Kex2-specific immunoglobulin per milliliter of serum by ELISA, standardized with yeast 3-phosphoglycerate kinase (PGK) and affinity-purified rabbit antibodies to yeast PGK [P. Baum, J. Thorner, L. Honig, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4962 (1978)].
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Kex2- RRSRAETYEFDI IDTDSEYD-
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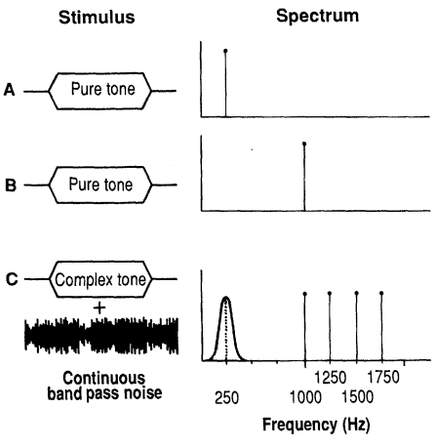


**Fig. 1.** Scheme of the stimulus conditions in (left) time- and (right) frequency-domain representation. (**A**) Pure-tone burst of 250 Hz (spectral pitch stimulus); (**B**) pure-tone burst of 1000 Hz (spectral pitch stimulus); (**C**) complex-tone burst (virtual pitch stimulus) consisting of the fourth through the seventh harmonic of 250 Hz, presented simultaneously with narrow-band noise centered at 250 Hz.

# Tonotopic Organization of the Auditory Cortex: Pitch Versus Frequency Representation

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**According to the place principles of the classical hearing theory, the physical entity frequency is encoded in the auditory periphery as place information (tonotopic representation), which is decoded in more central parts of the auditory system to form the subjective entity pitch. However, this relation is true only for pure-tone signals (spectral pitch); it can be quite different in the case of complex auditory stimuli (virtual pitch), thus requiring a multistage process for pitch formation. Neuromagnetic measurements showed that the tonotopic organization of the primary auditory cortex reflects the pitch rather than the frequency of the stimulus; that is, the pitch formation process must take place in subcortical regions.**

THE HISTORY OF HEARING THEORY is determined by two contradictory hypotheses: Helmholtz (*1*) proposed a systematic spatial representation of pure tones in the auditory system according to their frequency (tonotopic organization), which was subsequently confirmed with invasive physiological methods for any level of the auditory system. Frequency information was assumed to be encoded as place information, and the perceived pitch (place or spectral pitch) was assumed to be related to the place of cortical excitation. However, experiments carried out even before Helmholtz formulated his hearing theory (*2*) demonstrated that the pitch of complex tones composed of higher harmonics (that is, integral multiples of the fundamental frequency) corresponds to that of a pure tone whose frequency equals that of the fundamental frequency, and that the pitch does not change even when the fundamental frequency (missing fundamental) is removed. The perceived pitch was assumed to be related to the temporal structure of the auditory stimulus (periodicity or virtual pitch). A unification of the two hypotheses—place versus periodicity pitch—was originally attempted by Licklider (*3*), who proposed that pure tones are mapped by the auditory system to essentially the same brain structure as are complex tones with similar temporal periods and perceived pitch.

With the advent of biomagnetic measurements, the chance arose to directly test Licklider's unified hypothesis in humans. Auditory-evoked magnetic fields (AEF), because of their great spatial resolution, have already been used to reveal the tonotopic organization of the human primary auditory cortex; that is, neurons are arranged in space according to that frequency to which they respond best (*4–6*): the depth of the equivalent current dipole (ECD) calculated for wave M100 (termed according to its typical latency of approximately 100 ms) of the AEF increases with the logarithm of stimulus frequency. Using pure-tone and complex-tone stimuli, which are currently known to be the most effective for the perception of periodicity pitch (*7, 8*), we tested whether the depth of the ECD is the same for a pure-tone stimulus and a complex-tone stimulus producing the same pitch (spectral and virtual, respectively), or whether it moves in the latter case to the place characteristic of the higher harmonics composing the complex-tone stimulus. If the former proves true, then it can be deduced that the "pitch processor," which was postulated by Goldstein (*9*) and Terhardt (*10*), is located peripheral to the primary auditory cortex.

Three different stimulus signals were used (Fig. 1): bursts (duration, 500 ms) of pure tones, with carrier frequencies of 250 Hz and 1000 Hz, respectively, to elicit a spectral pitch sensation, and bursts of a complex tone composed of the fourth to the seventh harmonic of 250 Hz, which produces a strong virtual pitch. The virtual pitch of the complex-tone signal was tested in 15 individuals and was found to match very well with the spectral pitch of a 250-Hz pure-tone stimulus (250 ± 5 Hz). To ensure that the perceived pitch was not due to combination tones resulting from nonlinear interaction in the auditory periphery, we masked the 250-Hz region of the cochlea by simultaneously presenting a continuous band-pass noise centered at 250 Hz. The intensity of both the stimulus and the masker was 60 decibels (dB) relative to the normal hearing threshold. Three right-handed and three left-handed normal hearing individuals participated in the study. To obtain maximal AEF amplitudes, the measurements were done over the hemisphere contralateral to the side of handedness (*11*) with contralateral stimulation (*5*). The magnetic field normal to the skull was measured at 70 positions over the auditory cortex (*12*) by means of a second-derivative gradiometer, magnetically coupled to a DC-SQUID (superconducting quantum interference device, Biomagnetic Technology). The diameter of the pick-up coil was 20 mm, and the length of the baseline was 50 mm. The output signal of the SQUID control unit was comb filtered, to reduce the noise at the harmonics

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