Vol. 183, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1992 Pages 212-219

A NOVEL KEX2 ENZYME CAN PROCESS THE PROREGION OF THE YEAST ALPHA-FACTOR LEADER IN THE ENDOPLASMIC RETICULUM INSTEAD OF IN THE GOLGI

Bhabatosh Chaudhuri,* Sarah E. Latham, Stephen B. Helliwell and Peter Seeboth

Department of Biotechnology, K-681.106, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Received January 15, 1992

The prepro sequence of the yeast prepro-α-factor, usually referred to as the α-factor leader, has often been used for the efficient secretion of heterologous proteins from the yeast Saccharomyces cerevisiae. The α-factor leader consists of a 19-amino acid N-terminal pre or signal sequence followed by a 66-amino acid proregion. After removal of the signal sequence during membrane translocation, the proregion is cleaved from the precursor protein by the Kex2 endoprotease only in a late Golgi compartment. Here we report that a modified Kex2 enzyme, containing at the C-terminus the HDEL tetrapeptide, cleaves the proregion from the α-factor leader - human insulin like growth factor-1 fusion protein in the endoplasmic reticulum. The processing of pro-proteins earlier in the secretion pathway could be helpful in defining the cellular function of the proregions present naturally in various eucaryotic precursor proteins.

For proteins destined for secretion a signal sequence, consisting of a central hydrophobic core [1], mediates interaction with the membrane of the endoplasmic reticulum (ER) [2]. Usually, the signal sequence is cleaved during translocation into the ER [3] and the resultant protein is identical to the mature secreted form. However, there are a variety of secretory proteins, hormones [4-6], growth factors [7] and proteases [8] which initially form intracellular intermediates after cleavage of the signal sequence. These precursor molecules contain extra amino acid residues, termed propeptides, which are absent in the mature secreted protein. The proregion of a protein is processed from the intermediate precursor, either in the late Golgi or in the secretory vesicles, by enzymes which cleave after pairs of dibasic amino acids [9]. In S.cerevisiae, the maturation of the α-factor pheromone [10], or heterologous proteins [11] fused to the α-factor leader (αFL) occurs when the Ca^{++} dependent serine protease Kex2p removes the proregion from precursor molecules by cleaving at a Lys-Arg residue [10,12].

* To whom correspondence should be addressed.

Abbreviations. A_{600}, absorbance at 600nm; αFL, alpha-factor leader; D, aspartate; DTT, dithiothreitol; E, glutamate; ER, endoplasmic reticulum; EDTA, ethylene diamine tetraacetic acid; H, histidine; HPLC, high performance liquid chromatography; IGF1, human insulin-like growth factor-1; L, leucine; Mr, molecular mass; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminomethane; WT, wild type.

0006-291X/92 $1.50

In contrast to the signal peptides involved in the targeting of proteins to different cellular organelles [13], the function of the proregions is not well understood. It is unlikely that there is one uniform role for all known proregions, which vary not only in length and structure but also in their location in precursor molecules [14]. There have been attempts to define the possible cellular function of the proregions. In the case of subtilisin and α-lytic protease the proregions have been shown to assist in the folding of these prokaryotic proteases by acting as molecular chaperones [15,16]. It has also been suggested that the proregion could be a sorting signal which would guide a precursor protein through the secretory pathway from the ER to the Golgi [17].

In order to investigate the role of the proregion in a nascent polypeptide after signal sequence mediated translocation, it would be advantageous if the proregion was removed from the precursor protein in the lumen of the ER rather than in the Golgi. In this communication we provide evidence that the yeast Golgi membrane-bound Kex2p can indeed function in the ER if the ER-retention signal HDEL [18] is attached to the C-terminus of a soluble form of the Kex2 protein (sKex2p) [19-21]. The novel sKex2pHDEL has been distinguished from sKex2p and Kex2p by using, as a model system, expression cassettes containing αFL sequences fused to a reporter gene human insulin-like growth factor-1 (IGF1). The fact that an HDEL sequence permits ER retention of the sKex2p reveals the possibility to study, in an eucaryotic cell, the role of the proregions in protein folding and intracellular transport.

MATERIALS AND METHODS

Strains and transformations: All newly constructed plasmids were transformed in E.coli HB101. For site-directed mutagenesis [22] E.coli strains JM101 and BMH71-18 MutS [22] were used. Yeast transformations [23] were performed in S.cerevisiae strains AB110 (his4-580, leu2, ura3-52, pep4-3, [cir^0]) [24] and AB110 kex2$^-$. The chromosomal copy of the KEX2 gene in AB110 was disrupted by transformation with the linearised plasmid pUC19/kex2::LEU2 where a 2.9kb BglII fragment containing the functional copy of the LEU2 gene has been inserted at the BglII site of the KEX2 gene [25]. LEU2 prototrophs were confirmed by Southern blot analysis of the genomic DNA.

DNA construction of Kex2 variants: The expression cassette for sKex2p [21] was obtained by deleting from the KEX2 gene [25] the DNA encoding the C-terminal 200-amino acids. The gene construct for sKEX2pHDEL was made from p18kexp [21] which consists of the DNA sequence for sKEX2p inserted into the polylinker region of pUC18. The 3' end of the DNA sequence was cut with Asp718 and EcoRI. The cut plasmid was isolated and ligated in the presence of the two annealed oligonucleotides,

5'-GTACCGTTCGAACACGACGAATTATAATAG-3' and
3'-GCAAGCTTGTGCTGCTTAATATTATCTTAA-5'

resulting in the ligation product p18kexpHDEL. The 3' end of the DNA insert encoding the His-Asp-Glu-Leu peptide sequence and two stop codons was sequenced [26]. The plasmid was cut with SalI, PvuII and ScaI. For further subcloning in yeast expression plasmids BamHI linkers (Boehringer) were added to the isolated 2572 bp SalI-PvuII fragment.

Construction of mutated αFL sequences: Mutations in the αFL sequence were introduced by the Zoller-Smith two-primer protocol of site-directed mutagenesis [22] using mutagenic primers,

5'-GCGGAGGATGCgttGAATAAAACTGC-3'
and 5'-ATCTAAGTAgttGATGACAGC-3' (for Mut1),
5'-GTAGTGTTGACTaGAtCtGCTAATGCGGAGG-3' (for Mut2),
5'-AGCTTCAGCCGGAGCAGCTAATGC-3' (for Del1).

The lower case letters in the primers indicate the mutations which were introduced in the M13 mp18 DNA template containing the positive DNA strand of the IGF1 expression cassette [24]. Site-specific mutations and deletions in the DNA were confirmed by sequencing [26]. The altered amino acids in the two mutated sequences (Mut1 and Mut2) and the deleted amino acids in another (Del1) are shown in Table 1.

Construction of plasmids: A unique BglII site was created at the SacI site of the E.coli-S.cerevisiae shuttle vector pDP34A [24] using BglII linkers (Boehringer), and the resulting vector was named pDP34B. The BamHI fragments containing the genes encoding Kex2p [25, 21], sKex2p [21] and sKex2pHDEL were subcloned in the BglII site of pDP34B to yield plasmids pBC8, pBC9 and pBC10. The BamHI fragments containing the IGF1 expression cassettes with the wild type αFL (αFLWT) [24] and the three mutated sequences of the αFL (Mut1, Mut2 and Del1) were ligated to BamHI digested pDP34B. The resulting plasmids were named pBC1 [24], and pBC11, pBC12 and pBC13 (Table 2). In another set of subcloning experiments the IGF1 expression cassettes bearing the mutated sequences of the αFL were subcloned in the unique BamHI sites of pBC8, pBC9 and pBC10. Clones which had on the plasmid the KEX2 and IGF1 expression cassettes in the same orientation were chosen for further expression of IGF1 (Table 2).

Expression of IGF1: pBC1 has been transformed in both the yeast strains, AB110 and AB110 kex2$^-$. Yeast strain AB110 alone has been used for the transformation of plasmids pBC11, pBC12 and pBC13. All other plasmids listed in Table 2 have been transformed in AB110 kex2$^-$. Three transformants from each of the transformations were grown for 72h in a uracil-selective medium for expression of IGF1 exactly in the way reported previously [24].

Immunoblot analysis: 100 A_{600} cells were mechanically lysed using 0.3g glass beads (0.5mm diameter) in 200µl of 0.1M sodium phosphate buffer (pH 7). After denaturation of 10µl of cell lysate with 1µl of 10% SDS at 95°C for 3min, the mixture was subjected to endoglycosidase F (Boehringer) digestion in 50µl of incubation buffer (50mM potassium phosphate pH6.5, 50mM EDTA, 2% Triton X-100) for 14h at 30°C and 1h at 37°C. All samples for SDS-PAGE were prepared by adding 0.5V of Laemmli buffer (4% SDS, 0.1M Tris chloride pH6.8, 4mM EDTA, 15% glycerol, 1.5% DTT). Immunoblot analyses were performed according to previously published protocols [24]. Rabbit polyclonal IGF1 anti-serum [24]] was generated using purified IGF1 monomer. The polyclonal antibodies raised against prepro-α-factor was kindly provided by J.Rothblatt [27].

Determination of N-terminal amino acid sequences: The intracellular IGF1-like protein, from cells grown from a yeast transformant bearing pBC11, was immune-precipitated using an anti-IGF1 polyclonal antibody [24]. After dissociation of the immune-complex, the proteins were fractionated by 15% SDS/PAGE. The purified protein [28] was subjected to N-terminal amino acid sequence analysis using an Applied Biosystems 477A protein sequencer.

RESULTS AND DISCUSSION

The prepro sequence of the yeast prepro-α–factor has been used to study the intracellular location of the novel protein sKex2pHDEL. The proregion of the prepro sequence has three sites for N-linked glycan formation [29]. The different stages of glycosylation and oligosaccharide processing [30,31], which the propeptide encounters in the secretion pathway [32], permit identification of products which are in the ER and also those which have gone through the Golgi apparatus [33]. To facilitate this study we have chosen the heterologous IGF1 gene as a convenient tag for the

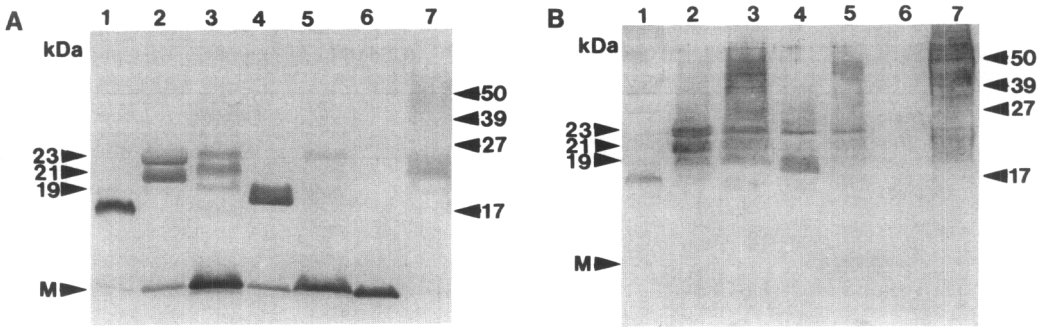

Fig.1. Western blot analysis of intracellular IGF1-like proteins from AB110 transformants harbouring the IGF1 expression plasmids pBC1 and pBC11, pBC12, pBC13 (Table 2) bearing the WT [24] and the mutated αFL sequences (Table 1). All cell lysates were from cultures grown for 72h, except for the WT sequence where cells were also harvested at 22h. Proteins from 2µl cell lysate in Laemmli buffer were separated on a SDS-15% polyacrylamide gel. After blotting on to PVDF membrane the transferred proteins were detected with **(A)** anti-IGF1 antiserum, and **(B)** antibodies raised against the prepro-α-factor. Lane 1: Mut1; lane 2: Mut2; lane 3: WT, cells from a 22h culture; lane 4: Del1; lane 5: WT,cells from a 72h culture; lane 6: 150ng of HPLC purified IGF1 monomer [24]; lane 7: WT, in AB110 kex2⁻, for visualising Golgi-associated outer-chain glycosylation. The 17 ,27, 39 and 50kDa bands used as markers belong to the pre-stained low-range standard proteins (Bio-Rad); M, purified IGF1 monomer.

proregion of the αFL. After the initial expression of the αFL-IGF1 fusion protein, the steps toward maturation of IGF1 can then be monitored via Western blot analysis.

Mature IGF1 is the predominant protein expressed in strains which use the αFLWT as a secretion signal (lane 5, Fig.1A). However, the same strains grown only up to 22h, instead of 72h, show formation of intermediate precursors besides the mature molecule (lane 3, Fig.1) [24]. Strains which also use the αFLWT for the secretion of IGF1 but lack the genomic copy of the KEX2 gene show diffused bands on Western blots (lane 7, Fig.1) and no mature IGF1 at all. This suggests the formation of precursors, where Golgi-associated outer-chain glycosylation has occurred [31]. The precursor molecules remain unprocessed because of the absence of the KEX2 gene product. It can be inferred from these observations that the secretion of IGF1 with the αFLWT sequence involves precursor intermediates which have traversed through the classical secretion pathway [32,34].

The three mutated αFL secretion signals (Table 1) yield appreciably less mature IGF1 than the wild type sequence, both intracellularly (Fig.1A) and in the supernatant of the yeast expression medium (data not shown). Western blot analysis of proteins from cell lysates show accumulation of unprocessed precursor forms of IGF1 even after 72h of cell growth (Fig.1A). An antibody raised against the prepro-α-factor which recognizes only the prepro sequence [27], but not the mature IGF1 molecule, has been used to confirm that a part or whole of the αFL polypeptide is still fused to IGF1 in these IGF1-like proteins (Fig.1B).

The intracellular proteins obtained from constructs bearing αFLMut2 (19-23kDa bands, lane 2, Fig.1) and αFLDel1 (17-19kDa bands, lane4, Fig.1) show succinct bands and the near absence of high mannose oligosaccharides. The apparent molecular mass (Mr) of the intracellular entities is

Table 1. Specific amino acid changes (Mut1 and Mut2) and a 13-amino acid deletion involving the first glycosylation site (Del1), in the αFL

Mutated αFL sequence	Mutation	Deletion
Mut1	Ala13 to Asn13 and Gly40 to Asn40	
Mut2	Ala20 to Asp20 and Pro21 to Leu21	
Del1		Pro21 to Ile33

indicative of proteins which have undergone only core-sugar attachment [30,31] to the sequons for N-glycosylation (Asn-X-Ser/Thr) in the αFL sequence [29]. Endoglycosidase F digestion (Fig.2) should remove oligosaccharides with a Mr of about 2kDa for each N-linked core-glycosylation present in the precursor protein. Removal of sugars yield proteins corresponding to a Mr of about 17kDa (Mut2) and 15.5kDa (Del1) suggesting that the molecules were indeed core-glycosylated (Mut2 has three and Del1 has two potential N-glycosylation sites). IGF1-like protein from strains bearing Mut1, however, is unglycosylated (Fig.2). N-terminal amino acid sequencing of this molecule proves that the pre sequence is still uncleaved. These results imply that, although precursor molecules from strains bearing Mut2 and Del1 have undergone translocation, they have not traversed beyond the ER, and that the precursor intermediate from Mut1 is still in the cytosol.

The three mutated αFL sequences have been used to identify that sKex2pHDEL is distinct from sKex2p. The sKex2p molecule obtained after removal of the transmembrane domain and C-

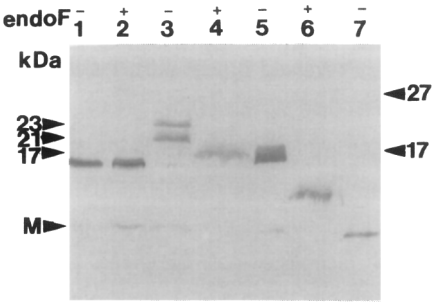

Fig.2. Endoglycosidase F digested products (lanes 2, 4, 6) of intracellular IGF1-like proteins from AB110 transformants harbouring IGF1 expression plasmids pBC11, pBC12 and pBC13 (Table 2) bearing the mutated αFL sequences (Table 1). After completion of digest, 0.5V of Laemmli buffer was added. Western blot analysis (15% SDS-PAGE) was performed using anti-IGF1 antiserum as in Fig.1. Lanes 1, 2: Mut1; lanes3, 4: Mut2; lanes 5, 6: Del1; lane 7: 150ng of HPLC purified IGF1 monomer. The markers used were the same as in Fig.1.

Table 2. Plasmids where the pDP34B vector either bears only the IGF1 expression cassette [24] containing one of the αFL secretion signals, or both the IGF1 expression cassette and one of the three KEX2 variants. Expression of IGF1 is under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter [24]

KEX2 variant	αFL sequence	Plasmid
-	WT	pBC1
-	Mut1	pBC11
-	Mut2	pBC12
-	Del1	pBC13
KEX2	Mut1	pBC14
sol KEX2	Mut1	pBC15
sol KEX2HDEL	Mut1	pBC16
KEX2	Mut2	pBC17
sol KEX2	Mut2	pBC18
sol KEX2HDEL	Mut2	pBC19
KEX2	Del1	pBC20
sol KEX2	Del1	pBC21
sol KEX2HDEL	Del1	pBC22

terminal tail from Kex2p is an active protease and is secreted in considerable amounts [21]. sKex2pHDEL, too, secretes into the culture medium (unpublished results). If mere attachment of the tetrapeptide HDEL allows an ER retention, then only sKex2pHDEL should release mature IGF1 from precursor molecules which have accumulated in the ER.

The yeast strain AB110 kex2$^-$ has been transformed with various multi-copy yeast plasmids (Table 2) which allow co-expression of any of the three Kex2p variants together with IGF1 fused to one of the mutated αFL sequences. Only over-expression of sKex2pHDEL causes maturation of IGF1 from precursor molecules which have accumulated in the ER (Fig.3A&3B). Western blot analyses clearly show that Kex2p and sKex2p do not have a similar effect. These observations are in agreement with the idea that some molecules of sKex2pHDEL in the yeast secretion pathway [32] may be recycled from an intermediate compartment between the ER and the Golgi through the ERD2 receptor-mediated pathway [35]. It is probable that over-expression of sKex2pHDEL causes its secretion into the culture medium because the ERD2 receptor molecules are saturated. If these assumptions are correct then sKex2pHDEL, which is functional in the ER, should not act on precursor molecules which have accumulated in the cytosol and should behave identically to Kex2p and sKex2p. The processing products of the three forms of the Kex2 protein are indeed indistinguishable (Fig.3C) using unprocessed cytosolic proteins from the Mut1 construct as in vivo substrates for the expressed proteases.

The work described here suggests that an endoprotease which is normally resident in the late Golgi can be mislocated to a cellular compartment which is earlier in the classical secretion pathway [32,34]. We anticipate that the site-specific cleavage of the proregion in the ER by an enzyme which recognizes dibasic amino acids would enable the assessment of the role of proregions in the folding [36,37] and intracellular transport of a variety of proteins.

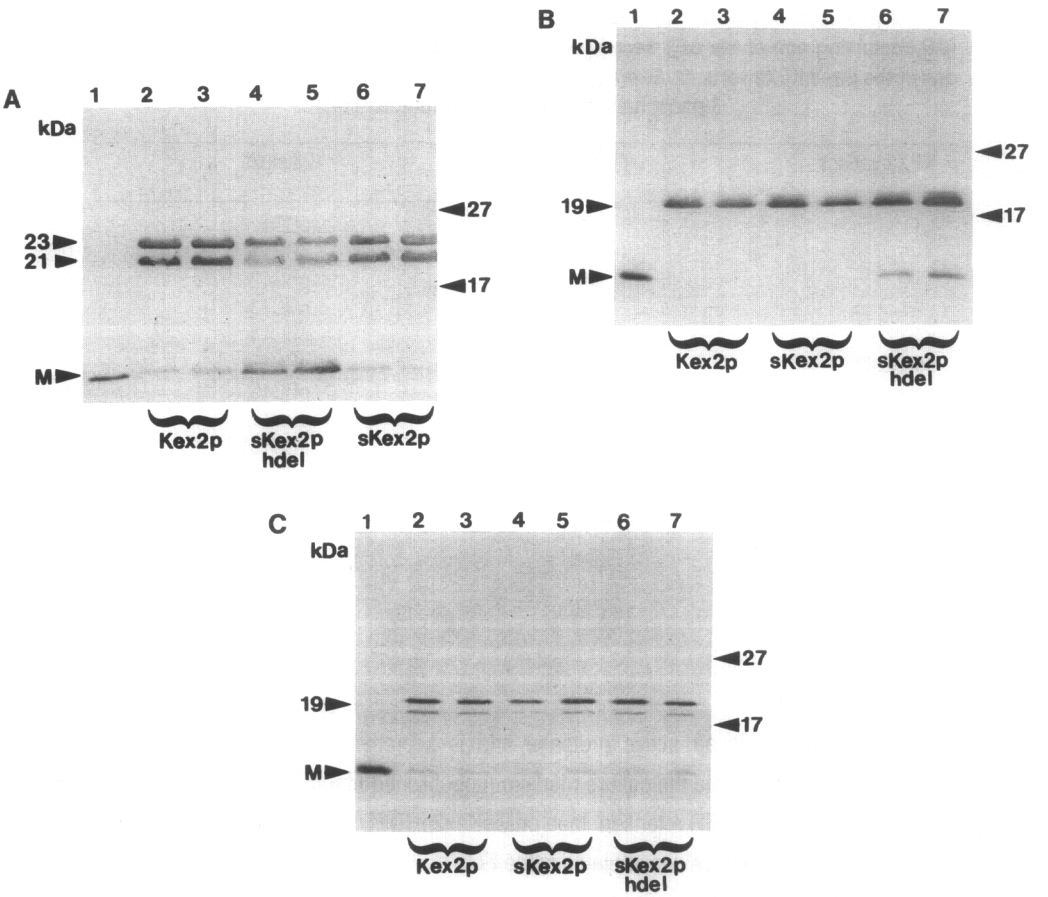

Fig.3. In vivo comparison of Kex2p, sKex2p and sKex2pHDEL using as substrates different intracellular entities accumulated in the ER and in the cytosol. IGF1-like proteins from cells grown from AB110 kex2$^-$ transformants bearing plasmids **(A)** pBC17, pBC18, pBC19; **(B)** pBC20, pBC21, pBC22; and **(C)** pBC14, pBC15, pBC16. All plasmids are listed in Table 2. Two transformants from each of the nine transformations were used for the Western blot analysis. Proteins from 2µl cell lysates in Laemmli buffer were separated by 15% SDS-PAGE, blotted on to PVDF membrane, and then detected with the anti-IGF1 antiserum. The markers used were the same as in Fig.1.

REFERENCES

1. von Heijne,G. (1985) J.Mol.Biol. 184, 99-105.
2. Walter,P., Gilmore,R., and Blobel,G. (1984) Cell 38, 5-8.
3. Nothwehr,S.F., Folz,R.J., and Gordon,J.I. (1989) J.Biol.Chem. 264, 4642-4647.
4. Douglas,J., Civelli,O., and Herbert,E. (1984) Ann.Rev.Biochem. 53, 665-715.
5. Fischer,J.M., and Scheller,R.H. (1988) J.Biol.Chem. 263, 16515-16518.
6. Cohen,P., Gomez,S., Gluschankof,P., Clamagirand,Ch., Plevrakis.I., Brakch,N., Créminon,Ch., Lepage,A., Rholam,M., and Camier,M. (1989) in The Second Forum on Peptides (Aubray,A., Marraud,M., and Vitoux,B. eds) Vol.174, pp3-9, Colloque INSERM/J.Libbey Eurotext.
7. Gentry,L.E., and Nash,B.W. (1990) Biochemistry 29, 6851-6857.
8. Fabre,E., Nicaud,J.-M., Lopez,M.C., and Gaillardin,C. (1991) J.Biol.Chem. 266, 3782-3790.
9. Barr,P.J. (1991) Cell 66, 1-3.
10. Fuller,R.S., Sterne,R.E.,and Thorner,J. (1988) Ann.Rev.Physiol. 50, 345-362.

11. Hirsch,H.H., Suarez-Rendueles,P., and Wolf,D. (1989) in Molecular and Cell Biology of Yeast (Walton,E.F., and Yarranton,G.T.,eds) pp.135-200, Blackie, Glasgow, London.
12. Hiramatsu,R., Horinouchi,S., Uchida,E., Hayakawa,T., and Beppu,T. (1991) Appl. Environ.Microbiol. 57, 2052-2056.
13. Pugsley,A. (1989) in Protein Targeting, pp. 20-53, Academic Press Inc., San Diego, CA.
14. Wiren,K.M., Potts,Jr.,J.T., and Kronenberg,H.M. (1988) J.Biol.Chem. 263, 19771-19777.
15. Zhu,X., Ohta,Y., Jordan,F., and Inouye,M. (1989) Nature 339, 483-484.
16. Silen,J.L., and Agard,D.A. (1989) Nature 341, 462-464.
17. Guan,J.L., and Rose,J.K. (1984) Cell 37, 779-787.
18. Pelham,H.R.B. (1988) EMBO J. 7, 913-918.
19. Fuller,R.S., Brake,A.J., and Thorner,J. (1989) Science 246, 482-485.
20. Fuller,R.S., Brake,A.J., and Thorner,J. (1989) Proc.Natl.Acad.Sci. 86, 1434-1438.
21. Seeboth,P.G., and Heim,J. (1991) Appl.Microbiol.Biotechnol. 35, 771-776.
22. Zoller,M.J., and Smith,M. (1987) Meth.in Enzymol. 154, 329-350.
23. Klebe,R.J., Harriss,J.V., Sharp,Z.D., and Douglas,M.G. (1983) Gene 25, 333-341.
24. Steube,K., Chaudhuri,B., Märki,W., Merryweather,J.P., and Heim,J. (1991) Eur.J.Biochem. 198, 651-657.
25. Mizuno,K., Nakamura,T., Ohshima,T. Tanaka, S., and Matsuo,H. (1988) Biochem.Biophys.Res.Commun. 156, 246-254.
26. Sanger,F., Nicklen,S., and Coulson,A.R. (1977) Proc.Natl.Acad. Sci. 74, 5463-5467.
27. Rothblatt,J.A., Webb,J.R., Ammerer,G., and Meyer,D.I. (1987) EMBO J. 6, 3455-3463.
28. Harrington,M.G. (1990) Meth.in Enzymol. 182, 488-495.
29. Kurjan,J., and Herskowitz,I. (1982) Cell 30, 933-943.
30. Kornfeld,R., and Kornfeld,S. (1985) Ann.Rev.Biochem. 54, 631-664.
31. Kukuruzinska,M.A., Bergh, M.L.E., and Jackson,B.J. (1987) Ann.Rev.Biochem. 56, 915-944.
32. Scheckman,R. (1982) Trends Biochem.Sci. 7, 243-246.
33. Graham,T.R., and Emr,S.D. (1991) J.Cell Biol. 114, 207-218.
34. Pfeffer,S., and Rothman,J. (1987) Ann.Rev. Biochem. 56, 829-852.
35. Semenza,J.C., Hardwick,K.G., Dean,N., and Pelham,H.R.B. (1990) Cell 61, 1349-1357.
36. Freedman,R.B. (1989) Cell 57, 1069-1072.
37. Nilsson,B., and Anderson,S. (1991) Ann.Rev.Biochem. 45, 607-635.