Appl Microbiol Biotechnol (2000) 54: 277–286

MINI-REVIEW

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Yeast secretory expression of insulin precursors

Received: 8 February 2000 / Received revision: 2 May 2000 / Accepted: 2 May 2000

Abstract Since the 1980s, recombinant human insulin for the treatment of diabetes mellitus has been produced using either the yeast *Saccharomyces cerevisiae* or the prokaryote *Escherichia coli*. Here, development of the insulin secretory expression system in *S. cerevisiae* and its subsequent optimisation is described. Expression of proinsulin in *S. cerevisiae* does not result in efficient secretion of proinsulin or insulin. However, expression of a cDNA encoding a proinsulin-like molecule with deletion of threonine^{B30} as a fusion protein with the *S. cerevisiae* α-factor prepro-peptide (leader), followed either by replacement of the human proinsulin C-peptide with a small C-peptide (e.g. AAK), or by direct fusion of lysine^{B29} to glycine^{A1}, results in the efficient secretion of folded single-chain proinsulin-like molecules to the culture supernatant. The secreted single-chain insulin precursor can then be purified and subsequently converted to human insulin by tryptic transpeptidation in organic–aqueous medium in the presence of a threonine ester. The leader confers secretory competence to the insulin precursor, and constructed (synthetic) leaders have been developed for efficient secretory expression of the insulin precursor in the yeasts *S. cerevisiae* and *Pichia pastories*. The Kex2 endoprotease, specific for dibasic sites, cleaves the leader-insulin precursor fusion protein in the late secretory pathway and the folded insulin precursor is secreted to the culture supernatant. However, the Kex2 endoprotease processing of the pro-peptide-insulin precursor fusion protein is incomplete and a significant part of the pro-peptide-insulin precursor fusion protein is secreted to the culture supernatant in a hyperglycosylated form. A spacer peptide localised between the leader and the insulin precursor has been developed to optimise Kex2 endoprotease processing and insulin precursor fermentation yield.

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Insulin and diabetes

The pancreatic peptide hormone insulin was discovered in 1921–1922 by Banting and Best (Best 1922, 1923). In the β-cells of the pancreatic islets of Langerhans insulin is synthesised as prepro-insulin, then folded and processed into the globular two-chain 51-amino-acid-residue insulin molecule in the secretory pathway (Steiner et al. 1967, 1986; Docherty and Steiner 1982). The complex process of intracellular transport and secretion of insulin from the β-cells is highly regulated. In the Golgi apparatus proinsulin is targeted to secretory granules. Enzymatic conversion of proinsulin to insulin occurs within acidifying secretory granules where the pro-peptide (C-peptide) is removed. Two mammalian functional equivalents of the *S. cerevisiae* Kex2 endoprotease PC1 (or PC3) and PC2 cleave proinsulin at dibasic processing sites, removing the C-peptide, and carboxypeptidase H is presumed to remove the two basic amino acid residues from the B-chain's C-terminus.

Insulin is essential for maintaining glucose homeostasis and normal metabolic regulation. When the blood glucose level increases, the β-cells release insulin. Insulin binds to specific receptors in various target tissues such as muscle, adipose tissue and liver. Insulin lowers the blood glucose level by increasing glucose uptake and subsequent metabolism by tissues such as muscle and fat, and by suppressing hepatic glucose output. Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia, in which glucose is not taken up and metabolised normally by the cells because of insufficient insulin concentration. This may be due to β-cell destruction (in type 1 diabetes) or to a combination of β-cell failure and resistance of the target tissues to insulin action (in type 2

diabetes). Without effective insulin treatment, type 1 diabetes leads to coma and death.

Since the early 1920s it has been possible to treat diabetes mellitus with insulin, and for decades this was purified from porcine or bovine pancreas. The average daily dose for patients treated with insulin is approximately 40–60 U, equal to 1.4–2.1 mg of insulin. In the 'industrialised' part of the world more than 130,000 mega U (approximately 4600 kg) of insulin are used yearly for the treatment of patients with diabetes mellitus, and the requirement for insulin is increasing by 3–4% (in volume) yearly. Approximately 150 mg of insulin can be purified from one porcine pancreas and the quantity of insulin needed to satisfy current requirements would be difficult to produce by pancreatic extraction. Developments in molecular biology and biotechnology opened up new possibilities, and in 1987 genetically engineered human insulin was produced in the yeast *S. cerevisiae*. This simple eukaryote is remarkably efficient at expressing and secreting insulin, and it offers high productivity.

The incidence of diabetes is increasing rapidly and it is estimated that, by the year 2025, the number of diabetic patients worldwide will increase from 120 million to approximately 300 million. Only a minor fraction (approximately 10%) of these will have type 1 diabetes, dependent on insulin for survival. The vast majority will have type 2 diabetes. Although only relatively insulin-deficient and therefore not dependent on insulin for survival, many of these patients will progress to a state that requires insulin treatment for metabolic control. Consequently, the pharmaceutical requirement for insulin is increasing steadily, and even the productivity of current insulin expression systems is beginning to impose limitations. Efficient insulin production is also required if the development of novel routes for administering insulin (such as the intrapulmonary route where bio-availability is lower than with subcutaneous administration) is to be both realistic and economically feasible. One of the challenges within applied biotechnology is to provide expression systems that satisfy the increasing insulin requirements for the treatment of diabetes mellitus. Here expression and optimisation of insulin secretory expression in the yeast *S. cerevisiae* is described.

Structure of the insulin molecule

The primary structure of insulin was determined by Sanger et al. (1955). The mature human insulin molecule consists of an A-chain and a B-chain, joined by two disulfide bonds. In addition, the A-chain has an intrachain disulfide bond (A6–A11). Vertebrate insulins have a high frequency of invariant and conserved amino acid residues. The six cysteines and the disulfide bond location are invariant in all vertebrate insulins. The three-dimensional structure of insulin hexamers has been determined by X-ray crystallography (Adams et al. 1969; Blundell et al. 1971; Smith et al. 1984; Baker et al. 1988; Derewenda et al. 1989), and the three-dimensional structure of some soluble monomeric insulin analogues has been derived by applying nuclear magnetic resonance spectroscopy (Ludvigsen et al. 1994; Olsen et al. 1996, 1998). The insulin A-chain (21 amino acids) features two α-helices (A2–A8 and A13–A19) joined by a loop (A9–A12). The A-chain is joined to the 30-amino-acid B-chain by two disulfide bonds (A7–B7 and A20–B19). The B-chain features a single α-helix from B9 to B19 followed by a turn and a β-strand from B21 to B30 (termed the T-state; Fig. 1). In the presence of phenol the B-chain's α-helix can extend to B1 (termed the R-state; Derewenda et al. 1989; Kaarsholm et al. 1989). Insulin's tertiary structure is stabilised by a hydrophobic core composed of highly conserved amino acid residues, including leucine and isoleucine residues (leucine^{B6}, leucine^{B11}, leucine^{B15}, isoleucine^{A2} and leucine^{A16}), the A6 and A11 cysteines, alanine^{B14} and tyrosine^{A19}. Amino acid residues in the hydrophobic core have numerous non-covalent interactions with peripheral amino acid residues, and the overall structure is stabilised by the disulfide bonds and interchain hydrogen bonds.

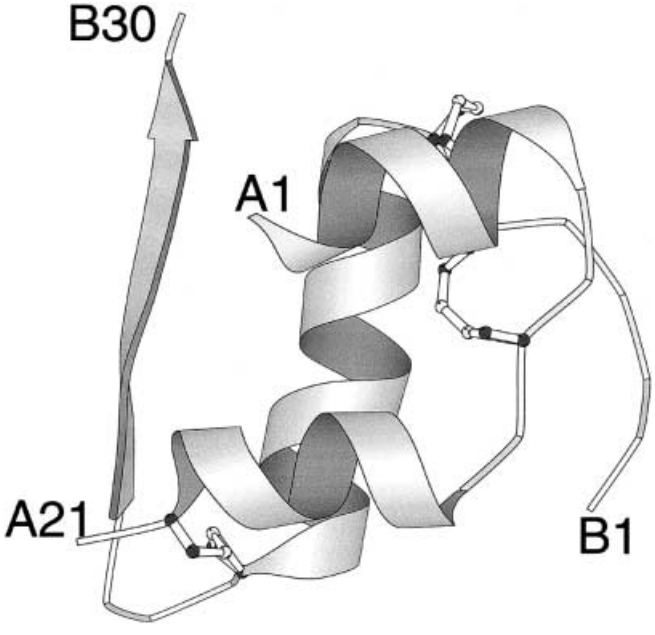

Fig. 1 Model of insulin structure (courtesy of Svend Ludvigsen, Insulin Research, Novo Nordisk). The model is based on the histidine^{B16}insulin analogue structure determined by nuclear magnetic resonance spectroscopy (Ludvigsen et al. 1994). The structure of the histidine^{B16}insulin analogue resembles the majority of structures determined by X-ray crystallography (Smith et al. 1984) and by nuclear magnetic resonance spectroscopy (Ludvigsen et al. 1994). The purpose of the figure is to orient the reader rather than to suggest a specific structure for insulin. The figure was produced using the MOLSCRIPT program (Kraulis 1991)

Secretory expression of heterologous proteins in *S. cerevisiae*

Yeasts are unicellular eukaryotes which have a life cycle with certain stages where they divide by budding (Phaff 1990). Yeasts belong to the fungi, lack photosynthetic abilities and are saprophytic or parasitic, being dependent on organic carbon sources provided by other organisms (Phaff 1990). The yeast *S. cerevisiae*, also known as baker's or brewer's yeast, has played a considerable role in food production for several thousand years. This is due to its highly specialised metabolism for alcoholic fermentation, converting mono- and some disaccharides into carbon dioxide and alcohol by means of enzymes. The extensive use of *S. cerevisiae* by mankind for the preparation of wine, beer and bread has lead it to be regarded as a GRAS organism (generally regarded as safe).

Over the past 50 years, *S. cerevisiae* has also become a powerful model system for biological research, involving numerous scientific groups in a wide range of fields of study. *S. cerevisiae* is an unicellular eukaryote but shares many genes, organelles and functions with higher eukaryotes, including humans. It is now one of the best characterised eukaryotes. The entire *S. cerevisiae* genome has been sequenced and more than a thousand genes have been characterised (Goffeau et al. 1996). The secretory pathway of *S. cerevisiae* exhibits much of the structure and function of the mammalian secretory system, with the capacity for folding, proteolytic processing, glycosylating and secreting proteins.

In more recent years, *S. cerevisiae* has also been developed as an efficient eukaryotic expression system for biotechnology (Smith et al. 1985; Brake 1989; Buckholz and Gleeson 1991; Romanos et al. 1992; Hadfield et al. 1993; Heinisch and Hollenberg 1993). *S. cerevisiae* is well suited for expressing heterologous proteins of scientific and commercial interest. It does not produce toxic compounds, and the wealth of experience from its extensive use in food production has made it an attractive host for producting pharmaceutical proteins, including human insulin and insulin analogues. Development of plasmids combining efficiently regulated or constitutive transcriptional promoters has allowed the expression of numerous foreign proteins in *S. cerevisiae* (Romanos et al. 1992; Hadfield et al. 1993; Heinisch and Hollenberg 1993). The production by *S. cerevisiae* of a substantial part of the insulin used worldwide for the treatment of diabetes is but one example of the importance of yeast in biotechnology.

The generation of secretory expression systems in yeasts and other eukaryotic hosts involves processes that reach well beyond classical molecular biology. Efficient secretion of heterologous proteins from eukaryotic cells requires more than just signal peptide-mediated passage through the endoplasmic reticular membrane. Secretory expression is a complex multi-step process and many factors influence the export of heterologous proteins. Heterologous proteins need to be folded, transported through the secretory pathway and exported to the culture supernatant.

The secretion process is multifunctional, consisting of translocation across the endoplasmatic reticulum membrane, attachment of N-linked carbohydrate chains and folding in the endoplasmatic reticulum lumen, transport from the endoplasmatic reticulum to the Golgi apparatus, post-translational modification in the Golgi apparatus, transport by secretory granules to the cell membrane and finally exit to the extracellular space. Proteins destined for secretion typically feature a signal peptide at the N-terminus. In the endoplasmic reticulum, the signal peptide is removed by the signal peptidase, and primary oligosaccharides ($GlnNAc_2Man_9Glc_3$) are covalently attached by the oligosaccharyltransferase to glycoproteins at consensus sites for N-linked glycosylation (NXT/S), creating a core glycosylated protein (Kornfeld and Kornfeld 1985; Tanner and Lehle 1987; Lehle and Tanner 1995). Disulfide bonds and the tertiary structure of membrane and secretory proteins are formed in the endoplasmic reticulum before being transported to their site of action. The correct folding of proteins in the endoplasmatic reticulum may depend on the function of auxiliary proteins (such as chaperones and protein disulfide isomerase) which mediate folding and disulfide bond formation (Freedman 1989; Freedman et al. 1994; Hartl 1996; Ferrari and Söling 1999). A vesicle system mediates transport of the protein to the Golgi compartment in a highly regulated fashion (Nothwehr and Stevens 1994; Warren and Malhotra 1998). In the Golgi apparatus, core carbohydrate chains of glycoproteins are further modified by outer-chain glycosylation. From the Golgi compartment, the protein is loaded into vesicles and transported to their destination. Genetic analyses suggest that vacuolar and secretory proteins share a common pathway to the Golgi apparatus where sorting occur (Schekman 1982).

S. cerevisiae cells of mating type α secrete a 13-residue peptide pheromone (α-factor) essential for mating with cells of mating type **a** (Thorner 1981). The α-factor is the product of the *MF*α1 gene which encodes a 165-residue polypeptide (prepro-α-factor) featuring a leader which consists of the 19-amino-acid signal (pre-) sequence, followed by the 66-amino-acid pro-sequence with three consensus sites for N-linked glycosylation and a dibasic Kex2 endoprotease processing site at the C-terminus, together with four repeats of the mature α-factor during secretion (Thorner 1981; Kurjan and Herskowitz 1982). In the prepro-α-factor, each 13-residue α-factor is preceded by a spacer peptide of 4–6 amino-acid residues $[(E/D)A]_{2-3}$, preceded by a dibasic processing site, lysine-arginine (KR; Thorner 1981; Kurjan and Herskowitz 1982). Maturation of the α-factor requires processing of the prepro-α-factor by four different proteolytic enzymes (Emter et al. 1983; Julius et al. 1983, 1984a, b; Fuller et al. 1988; Brake 1989). The signal peptidase cleaves the prepro-α-factor between amino

acid residues 19 and 20 (Waters et al. 1988). The Kex2 endoprotease encoded by the *KEX2* gene cleaves the glycosylated pro-α-factor after the pro-peptide and between the α-factor repeats (Julius et al. 1984a, b; Achstetter and Wolf 1985). Cleavage by the Kex2 endoprotease occurs on the C-terminal side of the dibasic sequence KR in the pro-peptide (Julius et al. 1984a; Achstetter and Wolf 1985; Brake 1989). Maturation of the excised α-factor requires exoproteolytic processing of both C- and N-termini (Brake 1989). The dipeptidyl aminopeptidase A encoded by the *STE13* gene removes EA or DA dipeptides from the N-terminus (Julius et al. 1983; Fuller et al. 1988). In addition, the carboxypeptidase serine protease encoded by the *KEX1* gene removes arginine (R) and lysine (K) residues from the C-terminus (Dmochowska et al. 1987).

It was rapidly established that the *S. cerevisiae* α-factor leader was able to confer secretory competence on proteins expressed in *S. cerevisiae* (Emr et al. 1983; Bitter et al. 1984; Brake et al. 1984; Singh et al. 1984). Subsequently, the α-factor leader has become the classical leader for secretory expression in *S. cerevisiae*, is generally applicable for secretory expression and has been used to facilitate secretion of numerous heterologous proteins, including insulin (Bitter et al. 1984; Brake et al. 1984; Zsebo et al. 1986; Markussen et al. 1987; Thim et al. 1987, 1989; Brake 1989; Romanos et al. 1992; Jonassen et al. 1994; Kjeldsen et al. 1996).

Expression of insulin in *S. cerevisiae*

Expression of proinsulin fused to the α-factor leader in *S. cerevisiae* did not result in efficient secretion of proinsulin or insulin to the culture supernatant (Thim et al. 1986a). However, expression of a cDNA encoding a proinsulin-like molecule with deletion of threonine^{B30}, followed either by replacement of the human proinsulin C-peptide with a small C-peptide (SK or AAK), or by direct fusion of lysine^{B29} to glycine^{A1}, and fusion to the α-factor leader, resulted in efficient expression of these single-chain proinsulin-like molecules (Table 1, Fig. 2; Markussen et al. 1987, 1990). Likewise, Jonassen et al. (1994) developed an efficient *S. cerevisiae* secretory expression system for insulin, based on the insulin B-chain residues 1–29 linked to the A-chain by short connecting peptides and characterised by having a dibasic processing site prior to glycine^{A1}, e.g. EKR. Furthermore, expression of a full-length B-chain connected to the A-chain by various mini C-peptides (e.g. RRLQKR) led to the secretion of a mixture of insulin and des(B30) insulin to the culture supernatant (Thim et al. 1986a, b, 1987, 1989). This type of insulin precursor can be converted into insulin by digestion with trypsin (EC 3.4.21.4) and carboxypeptidase B after purification (Thim et al. 1987, 1989). Taken together these data indicate that although the *S. cerevisiae* secretory pathway does not have the ability to express human proinsulin (efficient expression being impaired by the C-peptide), it does have the ability to fold and export single-chain proinsulin-like precursors. During transport through the secretory pathway, proteolytic enzymes involved in maturing the prepro-α-factor also mature the fusion protein, and the single-chain proinsulin-like precursor is secreted to the culture supernatant (Fig. 3). The fusion proteins are processed by the signal peptidase in the endoplasmic reticulum and by the Kex2 endoprotease in the late secretory pathway (Fig. 3). The single-chain insulin precursor could then be purified and subsequently converted to human insulin by tryptic transpeptidation in organic–aqueous medium in the presence of a threonine ester (Markussen 1987; Markussen et al. 1987).

Table 1 Fermentation yield of insulin precursor expressed in *Saccharomyces cerevisiae* by different leaders. Insulin precursors comprise the first 29 amino acids of the human insulin B-chain, joined to the 21 amino acids of the human insulin A-chain by a "mini C-peptide", AAK. The insulin precursor was expressed in the *S. cerevisiae* strain MT663[d] (a gift from Mogens Trier Hansen, Novo Nordisk; *MATa*/*MATα pep4*-3/*pep4*-3 *HIS4*/*his4 tpi*::*LEU2*/*tpi*::*LEU2* Cir$^+$) using the *POT* expression plasmid (Fig. 4). Each leader consists of a pre-peptide (the α-factor leader signal peptide or the Yap3 endoprotease signal peptide) and a pro-peptide, but only the pro-peptide and spacer peptides are shown here, see Fig. 2. Fermentation was at 30 °C for 72 h in YPD. Yield was determined by RP-HPLC of the culture supernatant, and is expressed as mean ± standard deviation (SD) of the values obtained in four individual fermentations. Where leaders lack the Kex2 endoprotease site, fermentation yield was determined as des(B30) insulin after maturation by *A. lyticus* lysyl specific endoprotease (EC 3.4.21.50). Consensus N-linked glycosylation sites [NX(T/S)] as well as mutated non-functional glycosylation sites (QTT) are underlined

S. cerevisiae strain	Pro-peptide	Pro-peptide amino acid sequence	Spacer peptide	Yield (mg/l)
MT748[d]	α	APVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFI NTTIASIAAKEEGVSLDKR	–	16.5 ± 0.2
yAK708	α[a]	APVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFI NTTIASIAAKEEGVSMAKR	EEAEAEAEPK	41.1 ± 0.9
yAK721	LA19	QPIDDTESNTTSVNLMADDTESRFATNTTLALDVVNLISMAKR	EEAEAEAEPK	50.3 ± 3.8
yAK775	LA34	––––––––QTT––––––––––––––––QTT––––––––––––– ΔΔ	EEAEAEAEPK	25.6[c] ± 3.9
yAK817	TA39	––––––––NTT––––––––––––––––NTT–GGLDVVNLISMAKR	EEGEPK	59.5 ± 6.7
yAK855	TA57	––––––––QTT––––––––––––A–––QTNSGGLDVVGLISMAKR	EEGEPK	79.2 ± 3.7

[a] An α-factor leader in which the C-terminus has been modified from "SLDKR" to "SMAKR". The LA19 pro-peptide amino acid sequence is shown; for the next three pro-peptides the sequence is given only at positions where it differs from the LA19 pro-peptide amino acid sequence

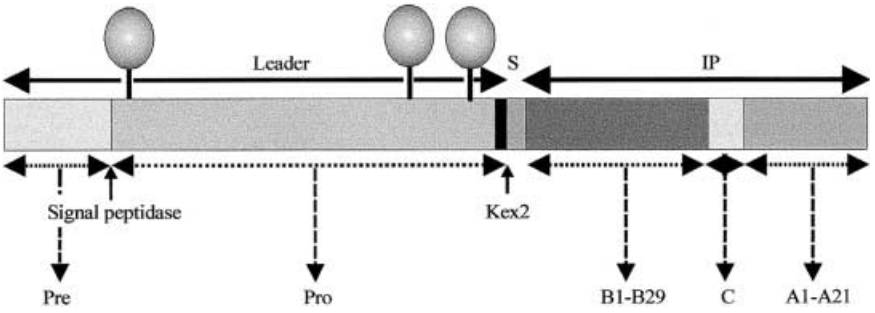

Fig. 2 Schematic representation of the leader-insulin precursor fusion protein expressed in *Saccharomyces cerevisiae*. A leader consists of a pre-peptide and a pro-peptide. Here the *S. cerevisiae* α-factor leader is indicated (*leader*). *Pre* indicates the pre-peptide (signal peptide) and *Pro* indicates the pro-peptide. The dibasic Kex2 endoprotease processing site, localised at the pro-peptides C-terminus, is shown in *black*. The single-chain insulin precursor (*IP*), comprising the first 29 amino acids of the human insulin B-chain (*B1–B29*) is joined to the 21 amino acids of the human insulin A-chain (*A1–A21*) by the mini C-peptide AAK (*C*) connecting lysine^{B29} and glycine^{A1}. Certain fusion proteins also feature a spacer peptide (*S*) with the amino acid sequence (EEAEAEAEPK, or derivatives of this sequence). Sites for attaching N-linked carbohydrate chains, for processing by the signal peptidase and for processing by the Kex2 endoprotease are indicated

The *S. cerevisiae* α-factor leader has three consensus sites for the attachment of N-linked carbohydrate chains. N-linked glycosylation is important, but not essential, for the ability of the α-factor leader to secrete α-factor (Julius et al. 1984b; Caplan et al. 1991). The α-factor pro-peptide's three N-linked carbohydrate chains are also important for the secretion of the insulin precursor. Mutation of all three sites by changing the asparagine to a glutamine decreased the quantity of secreted insulin precursor to 13% of that obtained using the α-factor pro-peptide with three functional N-linked glycosylation sites. However, there was a clear difference in the relative importance of the three sites with regard to their influence on insulin precursor secretion. The two N-linked carbohydrate chains closest to the insulin precursor were significantly more important than the third site for the ability to facilitate the secretion of the insulin precursor, and there was no synergistic effect of the N-linked oligosaccharide chains with respect to secretion (Kjeldsen et al. 1998a).

Insulin was expressed in *S. cerevisiae* utilising a synthetic gene encoding the fusion protein α-factor-leader-insulin precursor inserted into a *S. cerevisiae-Escherichia coli* shuttle plasmid, the *POT* plasmid (Fig. 4), which is based on the *S. cerevisiae* 2 μm plasmid (Markussen et al. 1987; MacKay et al. 1990; Kawasaki and Bell 1999). Transcription of the synthetic fusion protein gene was controlled by the *S. cerevisiae* triose phosphate isomerase gene (*TPI1*) promoter and terminator (Fig. 4; Alber and Kawasaki 1982; Thim et al. 1986a; Markussen et al. 1987; MacKay et al. 1990; Kawasaki and Bell 1999). Auto-selection of the *POT* expression plasmid in *S. cerevisiae* was based on the *Schizosaccharomyces pombe* TPI encoded by the *POT* gene. *S. cerevisiae* strains [like MT663 (*MATa*/*MATα pep4*-3/*pep4*-3 *HIS4*/*his4*

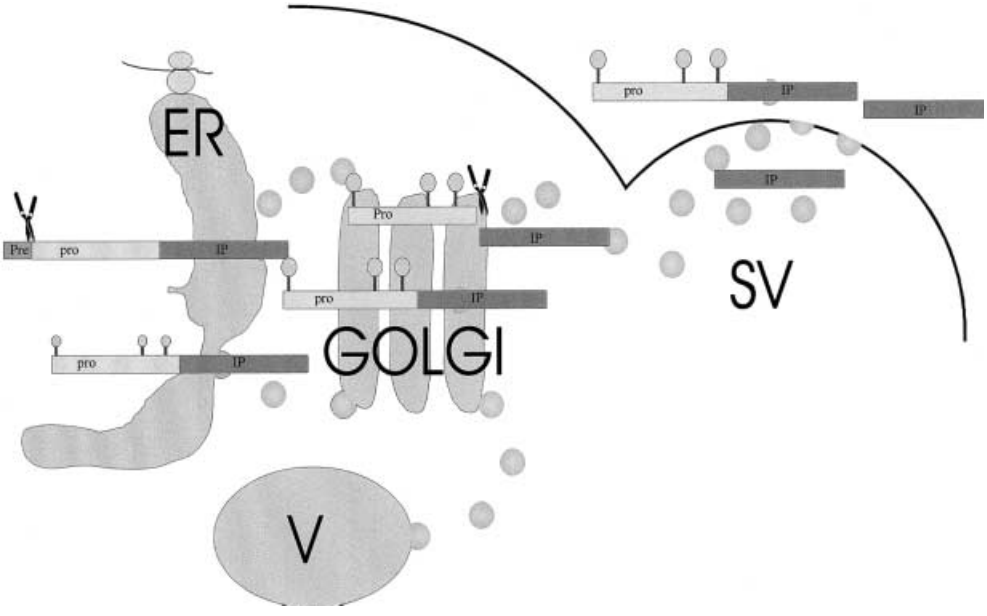

Fig. 3 Schematic representation of the *S. cerevisiae* secretory pathway, showing transport, processing and export of the α-factor leader insulin precursor fusion protein. Endoplasmic reticulum (*ER*), Golgi apparatus (*GOLGI*), vacuole (*V*), secretory vesicles (*SV*), α-factor pre-peptide (*pre*), α-factor pro-peptide (*pro*) and human insulin precursor (*IP*) are indicated. Attachment and modification of the α-factor pro-peptide's three N-linked carbohydrate chains, as well as processing, both by the signal peptidase and by the Kex2 endoprotease, are also shown

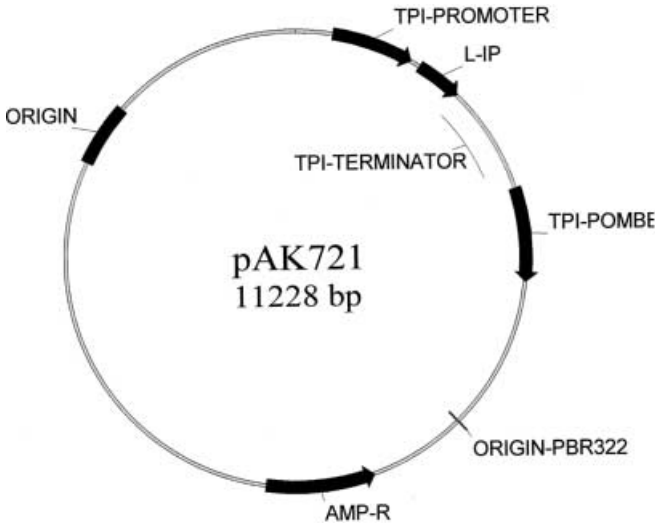

Fig. 4 The pAK721 *S. cerevisiae* expression plasmid expressing the LA19 leader-EEAEAEAEPK-insulin precursor fusion protein. The pAK721 expression plasmid was based on the *S. cerevisiae–Escherichia coli* shuttle *POT* plasmid (MacKay et al. 1990; Kawasaki and Bell 1999). *L-IP* indicates the fusion protein expression cassette, *TPI-PROMOTER* the *S. cerevisiae TPI1* promoter and *TPI-TERMINATOR* the *S. cerevisiae TPI1* terminator. *TPI-POMBE* indicates the *S. pombe POT* gene used for selection in *S. cerevisiae*; *ORIGIN* indicates a *S. cerevisiae* origin of replication derived from the 2-μm plasmid. *AMP-R* indicates the *β*-lactamase gene conferring resistance toward ampicillin and facilitating selection in *E. coli*. *ORIGIN-PBR322* indicates an *E. coli* origin of replication

tpi::*LEU2*/*tpi*::*LEU2* Cir^+)] with a non-reverting deletion/disruption in the *TPI1* chromosomal gene encoding TPI grow poorly when glucose is the only carbon source (MacKay et al. 1990; Kawasaki and Bell 1999). Transformation of *Δtpi1 S. cerevisiae* strains (like the MT663 strain) with the *POT* expression plasmid allows selection by the ability to grow on glucose in rich medium (MacKay et al. 1990; Kawasaki and Bell 1999). The *Sch. pombe POT* gene is poorly expressed in *S. cerevisiae* and multiple copies of the *POT* plasmid are required to generate sufficient gene product to allow growth on glucose as the sole carbon source (MacKay et al. 1990; Kawasaki and Bell 1999). However, the *TPI1* promoter is constitutive and powerful, and transcription of synthetic genes on high-copy plasmids by this promoter may induce down-regulation of the plasmid copy number. Such plasmid down-regulation may influence the quantity of secreted heterologous protein. The *POT* plasmid expression system has been successfully scaled up (Diers et al. 1991) and used for stable long-term continuous cultures with high production levels. The combination of a powerful constitutive glycolytic promoter, autoselection to stabilise the expression plasmid and growth in rich medium, an insulin molecule specifically modified for secretion, and long-term large-scale continuous fermentation has established the ability of *S. cerevisiae* to produce pharmaceutically important polypeptides, with the required quality, authenticity and quantity.

Development of spacer peptides for optimisation of Kex2 endoprotease processing of the pro-peptide-insulin precursor fusion protein and concomitant increase of the yield of the insulin precursor

In some early attempts to use the α-factor leader for the secretion of heterologous proteins in *S. cerevisiae* (e.g. interferon-α1, *β*-endorphin and human epidermal growth factor) the α-factor spacer peptide $[(E/D)A]_{2-3}$ was retained between the dibasic Kex2 endoprotease site and the heterologous protein (Bitter et al. 1984; Brake et al. 1984; Singh et al. 1984; Zsebo et al. 1986; Piggott et al. 1987). However, removal of the spacer peptide from the heterologous proteins by the dipeptidyl aminopeptidase A was found to be inefficient. Also expression of an α-factor leader EAEA-insulin precursor fusion protein led to the secretion of a molecule N-terminally extended by either EAEA or EA (Thim et al. 1986a). In general, removal of EA dipeptides from the heterologous protein by the dipeptidyl aminopeptidase A encoded by the *STE13* gene has proved to be incomplete. Moreover, expression of the *MF*α1 gene on a high-copy plasmid resulted in the secretion of incompletely processed α-factor similar to that produced by *ste13 S. cerevisiae* mutants (Julius et al. 1983). These data suggested that the quantity of dipeptidyl aminopeptidase A present in the secretory pathway was insufficient to process the amount of synthesised heterologous protein expressed by synthetic genes on high-copy plasmids. Consequently, spacer peptides were generally avoided and heterologous proteins, including insulin, were fused directly to the leader (Markussen et al. 1987; Brake 1989; Markussen et al. 1990). However, expression of fusion protein without a spacer peptide might result in the secretion of unprocessed and partially processed fusion protein, indicating that the spacer peptide may facilitate Kex2 endoprotease activity (Zsebo et al. 1986; Piggott et al. 1987; Brake 1989). Pulse-chase analysis of an α-factor leader-insulin precursor fusion protein without a spacer peptide expressed in *S. cerevisiae* showed secretion of hyperglycosylated fusion protein to the culture supernatant (Kjeldsen et al. 1996). Thus, Kex2 endoprotease processing of the pro-peptide-insulin precursor fusion protein was incomplete and a significant part of the pro-peptide-insulin precursor fusion protein was secreted to the culture supernatant in a hyperglycosylated form (Fig. 3; Kjeldsen et al. 1996).

To improve Kex2 endoprotease processing of the fusion protein, a charged spacer peptide was introduced between the pro-peptide and the insulin precursor (Kjeldsen et al. 1996). However, this resulted in the secretion of an insulin precursor molecule which was N-terminally extended. To facilitate in vitro removal of the spacer peptide from the insulin precursor, a lysine (K) residue was introduced in the spacer peptide C-terminus before phenylalanine^{B1}. Transpeptidation (Markussen 1987) of the N-terminally extended insulin precursor,

either by trypsin (EC 3.4.21.4) or by *Achromobacter lyticus* lysyl-specific endoprotease (EC 3.4.21.50), removed both the spacer peptide and the "connecting-peptide" as well as adding a threonine residue to lysine^{B29}, to generate human insulin.

Introduction of a spacer peptide (e.g. EEAEAEAPK), based on the prepro-α-factor $[(E/D)A]_{2-3}$ spacer peptide after the dibasic Kex2 endoprotease site and before the phenylalanine^{B1} of the insulin precursor, significantly increased the fermentation yield of the insulin precursor expressed in the *S. cerevisiae* (Table 1; Kjeldsen et al. 1996). Concomitantly, the quantity of hyperglycosylated pro-peptide-insulin precursor fusion protein secreted to the culture supernatant decreased.

Development and characterisation of leaders (prepro-peptide) for secretory expression of the insulin precursor in *S. cerevisiae*

The pro-peptide was essential for export of the insulin precursor expressed in *S. cerevisiae* (Markussen et al. 1987, 1990; Kjeldsen et al. 1999b). Consequently, development of leaders and especially pro-peptides has been a key target for optimising the fermentation yield of the insulin precursor expressed in yeast. Semi-random mutation has been used to develop a number of constructed (synthetic) leaders for secretory expression of the insulin precursor in *S. cerevisiae* (Kjeldsen et al. 1997) and some of these are listed in Table 1. The constructed leaders were equal to or better than the *S. cerevisiae* α-factor leader at facilitating export of the insulin precursor to the culture supernatant (Table 1). Surprisingly, the constructed leaders did not depend on N-linked glycosylation for their ability to facilitate secretion of the insulin precursor (Table 1; Kjeldsen et al. 1998a). The secretory capacity of constructed leaders which lack N-linked glycosylation sites is illustrated by the TA57 leader which, combined with the spacer peptide EEGEPK, increased the fermentation yield of insulin precursor to 480% relative to the α-factor leader insulin precursor fusion protein (Table 1).

Secreted fusion protein based on a constructed leader which lack both a dibasic processing site and N-linked glycosylation was readily processed into des(B30) insulin by the addition of *A. lyticus* lysyl specific endoprotease (EC 3.4.21.50) to the culture supernatant (Table 1; Kjeldsen et al. 1998b). The Kex2 endoprotease catalytic efficiency toward the fusion protein can be a limiting factor in yeast secretory expression. In vitro maturation of a purified fusion protein by an alternative enzyme would eliminate limitations on the fermentation yield imposed by the Kex2 endoprotease and would also prevent N-terminal processing of heterologous proteins by dipeptidyl aminopeptidase A. Constructed leaders without hyperglycosylation provide an opportunity for the purification of secreted fusion protein and subsequent in vitro maturation with a suitable enzyme (Kjeldsen et al. 1998b).

Secretion kinetics of the insulin precursor expressed in *S. cerevisiae*

Secretion kinetics of the leader-insulin precursor fusion protein was investigated by pulse-chase labelling (Kjeldsen et al. 1999b). Secretion was rapid, with the first insulin precursor appearing in the culture supernatant within 2–4 min of the chase. The overall kinetic profiles for insulin precursor secretion mediated by different leaders were comparable, indicating a similar intracellular transport and processing of these fusion proteins in the *S. cerevisiae* secretory pathway. The majority of secreted insulin precursor appeared in the culture supernatant within the first 15 min (Kjeldsen et al. 1999b). Furthermore, the rapid secretion of an insulin precursor with disulfide bonds identical to that of human insulin indicated a rapid disulfide bond formation and folding in the *S. cerevisiae* endoplasmic reticulum.

After 2.5 min of metabolic labelling of a leader insulin precursor fusion protein, the majority was core-glycosylated on one or both of the pro-peptide N-linked glycosylation sites, indicating that the majority of the fusion protein was present either in the endoplasmic reticulum or on its way to the Golgi apparatus (Kjeldsen et al. 1999b). This indicated translocation and stabilisation of the fusion protein in the endoplasmic reticulum mediated by the leader. Some extended carbohydrate chains appeared to be present on the pro-peptide, and enzymatic processing of the fusion protein by the Kex2 endoprotease located in a late Golgi compartment (Cunningham and Wickner 1989; Wilcox et al. 1992; Redding et al. 1996a, b) had already occurred to a minor degree. Therefore, after 2.5 min a minor fraction of the fusion protein had already reached a late Golgi apparatus compartment. After 35 min of the chase, only 2–4% of the insulin precursor was still present intracellularly as fusion protein. However, after 35 min approximately 30% of the metabolically labelled insulin precursor was still present as intracellular insulin precursor (Kjeldsen et al. 1999b). The fusion protein was almost completely proteolytically processed to insulin precursor by the Kex2 endoprotease. Therefore, the majority of this insulin precursor fraction was located in a Golgi or post-Golgi compartment. The intracellularly retained insulin precursor might have been sorted to the vacuole. Moreover, the selective intracellular retention of the insulin precursor relative to the fusion protein indicates that retention was associated with the biophysical/biochemical properties of the insulin precursor. Intracellular retention of a substantial amount of the synthesised insulin precursor indicated that the insulin precursor followed two different intracellular routes and that secretion to the culture supernatant reflected either the saturation of a Golgi or post-Golgi compartment due to overexpression, or secretion occurring in competition with intracellular retention.

Prevention of folding of the insulin precursor, whether by mutation or by preventing disulfide bond formation (using dithiothreitol), resulted in degradation and intracellular retention (Kjeldsen et al. 1999b). Misfolding or partial folding of the insulin precursor and consequent exposure of the insulin's hydrophobic core increased the hydrophobic surfaces of the molecule, and this might provide the signal for intracellular retention.

The insulin precursor has also been expressed in the methylotrophic yeast *Pichia pastoris* (Kjeldsen et al. 1999a). Proinsulin and the insulin precursor were expressed in *P. pastoris* using different leaders to confer secretory competence. Expression of an α-factor leader proinsulin fusion protein in *P. pastoris* did not result in the secretion of proinsulin. However, shortening the C-peptide, deleting threonine^{B30} and associating with the *S. cerevisiae* α-factor leader, as described for secretory expression in *S. cerevisiae* (Markussen et al. 1987, 1990), resulted in the efficient secretion of the insulin precursor in *P. pastoris*. Presumably, a *P. pastoris* endopeptidase functionally equivalent to the *S. cerevisiae* Kex2 endoprotease cleaved the fusion protein. Constructed leaders developed for secretory expression in *S. cerevisiae* also facilitated secretion of the insulin precursor in *P. pastoris*. As in *S. cerevisiae*, constructed leaders without N-linked glycosylation sites facilitated secretion of the insulin precursor expressed in *P. pastoris*. Furthermore, N-linked glycosylation was important for the ability of the α-factor pro-peptide to facilitate efficient transport and secretion of the insulin precursor. These data indicate that *P. pastoris* and *S. cerevisiae* have similar overall characteristics regarding the expression and secretion of insulin.

Summary and outlook

Steadily increasing requirements for insulin for treating the growing numbers of patients with diabetes mellitus have encouraged the search for efficient biotechnological expression systems for the production of human insulin. Since 1987, genetically engineered human insulin has been produced in the yeast *S. cerevisiae*. This simple eukaryote is remarkably efficient at expressing and secreting insulin, and it offers high productivity. However, it was necessary to adapt the insulin molecule to secretory expression in yeast by deleting threonine^{B30} and replacing human proinsulin's C-peptide with a small C-peptide, together with fusion to a leader. The leader appears to mediate the introduction of the insulin precursor into the endoplasmic reticulum and its stabilisation in the endoplasmic reticulum, emphasising the importance of the leader for secretory expression. Autoselection plasmid systems, as described here, allow for selection in rich medium and large-scale continuous fermentation and illustrate the capacity of the yeast *S. cerevisiae* for producing pharmaceutically important proteins. This points toward further development of autoselection plasmid systems and chromosomal integration of expression plasmids to ameliorate the secretory expression of recombinant proteins in *S. cerevisiae*, as regards both quantity and authenticity.

Acknowledgements The author is grateful to Annette Frost Pettersson for excellent technical assistance.

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