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Improved processing of secretory proteins in *Hansenula polymorpha* by sequence variation near the processing site of the alpha mating factor prepro sequence

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ABSTRACT

The literature as well as databases are ambiguous about the exact start of human interleukin-6 (IL-6) – three possibilities for the initiation of the mature protein are described. These three variants of IL-6, different in the exact initiation of the mature protein (A28, P29, or V30), were expressed in *Hansenula polymorpha* using the *Saccharomyces cerevisiae* MFα prepro sequence instead of the homologous pre sequence. All three IL-6 variants were secreted but the processing by the Kex2 protease showed significant differences. V30-IL-6 showed correctly processed material but also a molecule species of higher molecular weight indicating incomplete processing of the MFα pro peptide. P29-IL-6 did not yield any correctly processed IL-6, instead only the unprocessed pro form was found in the culture supernatant. Only A28-IL-6 led to 100% correctly processed material. N-terminal sequencing of this material revealed a start at V30 – obviously the first two amino acids (Ala28-Pro29) have been removed by a so far unknown protease. Thus expression of both A28-IL-6 and V30-IL-6 as MFα prepro fusion proteins resulted in the very same mature V30-IL-6, however, the ratio of correctly processed molecules was significantly higher in the case of A28-IL-6.

The expression of an MFα prepro-interferon α-2a (IFNα-2a) fusion protein in *H. polymorpha* leads to about 50% correctly processed molecules and 50% misprocessed forms which contain part of the pro peptide at the N-termini. The insertion of A28 and P29 of IL-6 between the pro peptide and the start of the mature IFNα-2a led to correct processing and elimination of all high molecular weight isoforms observed in earlier experiments

1. Introduction

The MFα prepro sequence derived from *Saccharomyces cerevisiae* has been very successfully used for secretory expression of a wide variety of heterologous target proteins in various yeasts. It is derived from the α-mating factor precursor and is essential for the correct processing and secretion of pheromone. During translocation the pre sequence (residues 1–19) is cleaved off by the signal peptidase. During the passage through the ER and Golgi the pro sequence is glycosylated at three N-glycosylation sites near its C-terminus. In the late Golgi the Kex2 protease cleaves off the pro sequence (66 amino acids) and releases 4 α-factor precursor units (Julius et al., 1984). The C-terminal KR-residues are removed by the Kex1 carboxypeptidase (Wagner and Wolf, 1987) followed by the removal of the N-terminal Glu-Ala-Glu-Ala spacer by the action of the Ste13 dipeptidyl aminopeptidase A (Julius et al., 1983). With this last step the processing is completed, and mature α-factor becomes secreted. The MFα prepro sequence has been successfully used in heterologous gene expression amongst others in the following yeast derived expression systems: *S. cerevisiae* (Brake et al., 1984), *Pichia pastoris* (Clare et al., 1991), *Kluyveromyces lactis* (Chen et al., 1992), *Zygosaccharomyces bailii* (Porro et al., 2005), *Ogataea minuta* (Akeboshi et al., 2007) and *Hansenula polymorpha* (Weydemann et al., 1995). The finding that these yeasts are able to correctly process the MFα prepro sequence via the dibasic motif supports that Kex2p homologs exist in all these organisms.

Interleukin-6 (IL-6) is a multifunctional human protein which belongs to the group of cytokines. It has a pre sequence which in the natural host is cleaved off during ER uptake of the protein. However the exact transition between signal sequence and sequence of the mature IL-6 is unclear (Fig. 1A). Parekh et al. (1992) described the

Abbreviations: IL-6, interleukin-6; IFNα-2a, interferon-α 2a; IFNα-2b, interferon-α 2b; MFα, mating factor α.

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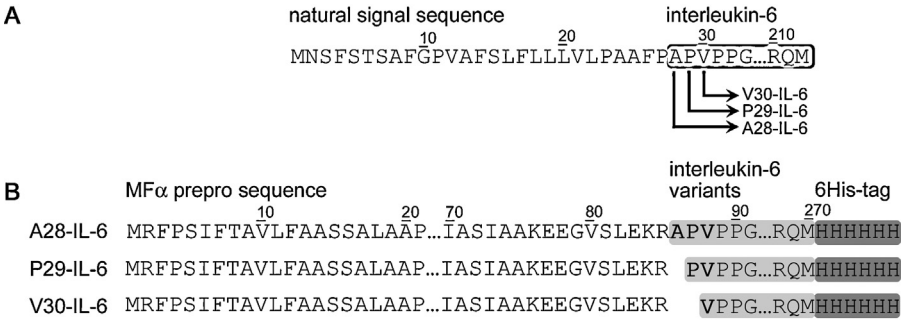

Fig. 1. (A) Transition from signal peptide to mature protein in the case of interleukin-6. The arrows indicate the three possible initiation amino acids described in the literature and the resulting nomenclature. (B) Due to the three potential N-terminal ends, three IL-6 variants were fused to the MFα prepro sequence for expression in *H. polymorpha*.

N-terminal sequence of human IL-6 to commence with the amino acids A28-P29-V30, but additionally they found two other sequence initiations in which one (A28) or two amino acids (A28-P29) were removed. Since the exact start also in the databases is ambiguous, three possibilities for the initiation of the mature protein need to be considered, which are referred to as V30-, P29-, or A28-IL-6 (mature IL-6 starting at the indicated amino acid residue, respectively).

IL-6 has been expressed in various yeast systems using the MFα prepro sequence. Guisez et al. (1991) expressed P29- and A28-IL-6 in *S. cerevisiae*. They observed different processing for each type. The P29-IL-6 showed incorrect processing of the pro peptide, whereas in the case of A28-IL-6 the first two amino acids A28 and P29 were removed. Steinborn et al. (2006) expressed V30-IL-6 in *S. cerevisiae* and observed N-terminal truncation of eight amino acids, however in *Arxula adeninivorans* they observed correct processing and the full length mature protein was secreted.

The methylotrophic yeast *H. polymorpha* has been established as a highly efficient expression system (Janowicz et al., 1991) amongst others due to the strong promoters derived from the methanol metabolism pathway and the possibility of mitotically stable high-copy integration. Degelmann et al. (2002) expressed the V30-IL-6 in *H. polymorpha*. Secretion was observed but no further sequence analysis was performed. Steinborn et al. (2006) also expressed the V30-IL-6 in a *H. polymorpha* system. These authors found an eight-amino-acid truncation of the N-terminus of the secreted molecules.

In this study we investigated the influence of three different start amino acids (A28, P29, and V30) on expression and processing of mature IL-6 using in all three cases the MFα prepro sequence as a signal and transport sequence. Furthermore, we applied the positive processing results from A28-IL-6 for the secretion of interferon α-2a.

2. Materials and methods

2.1. Strains and culture conditions

For construction and propagation of plasmid DNA *Escherichia coli* NEB10β (araD139 Δ(ara-leu)7697 fhuA lacX74 galK (ϕ80 Δ(lacZ)M15) mcrA galU recA1 endA1 nupG rpsL (Str^R) Δ(mrr-hsdRMS-mcrBC); New England Biolabs, Frankfurt a. M., Germany) was used. Cultivation took place in Luria Bertani media, when needed supplemented with 50 μg ampicillin per ml. For protein expression *H. polymorpha* strain RB11 deficient in orotidine-5′-phosphate decarboxylase (*ura3*) (Suckow and Gellissen, 2002) or KLA8-1 deficient in orotidine-5′-phosphate decarboxylase and β-isopropylmalate dehydrogenase (*ura3 leu2*) (Latchev et al., 2002) were used. Cultivation took place either in yeast extract-peptone-dextrose (YPD) medium or yeast nitrogen base (YNB) medium. All cultivations were performed at 37 °C.

2.2. Vector construction

To generate the expression plasmids pFPMT-MFaE-A28-IL-6, pFPMT-MFaE-P29-IL-6, and pFPMT-MFa-V30-IL-6, a synthetic open reading frame for each variant based on Universal Protein Resource Knowledgebase (UniProt ID: P05231) were ligated with the 6.8 kb *Hind*III/*Aat*II fragment of pFPMT-MFaE-IFNα-2a (Wendel, 2009). This plasmid contains all features of the vector pFPMT121 (Degelmann et al., 2002) and in addition the MFα prepro sequence with an amino acid exchange at position (Asp to Glu at position 83, D83E). A His-tag was added C-terminally to the IL-6 protein sequence. The cloned IL-6 sequences with additional flanking recognition sites (5′ *Hind*III and 3′*Aat*II) were synthesized by GeneArt (Regensburg, Germany).

To generate the expression plasmid pFPMT-MFaE-AP-IFNα-2a a PCR fragment was amplified with oligonucletide primers AP IFN hin (5′-AGGGGTAAGCTT GGAAAAGAGAGCTCCATGTGATTTGCCACAGACACACTCCCTGGGC) and AP IFN her (5′-GCGAGGGGGATCCTTATTACTCCTTCGATC) using plasmid pFPMT-MFaE-IFNalpha-2a as a template. The PCR product has been digested with *Hind*III/*Bam*HI resulting in a 0.523 kb fragment. This fragment has been ligated with the 7.217 kb *Hind*III/*Bam*HI fragment of pFPMT-MFaE-IFNalpha-2a. *H. polymorpha* was transformed by electroporation as described in Faber et al. (1994). The following strain generation was performed according to Guengerich et al. (2004). The passaging procedure lasted for four passages.

2.3. Protein expression and preparation of protein material

For heterologous protein expression recombinant strains were subjected to derepression/MeOH induction. Cells were cultivated in 3 ml scale in YNB/2% glycerol for 48 h and then for additional 24 h in YNB/1% methanol (37 °C). After cultivation the cells were harvested by centrifugation, and the supernatant was collected and stored at −20 °C.

2.4. Protein analysis

For molecular weight determination the samples were analyzed by SDS-PAGE according to Laemmli (1970). For the electrophoretic separation 4–12% Bis–Tris acrylamide XT gels (BioRad, Hercules, CA, USA) were used. The gels were stained either with the Coomassie stain SimplyBlue (Invitrogen, Karlsruhe, Germany) or the protein patterns were transferred to nitrocellulose-membranes. The protein bands were then visualized by immunoblotting. For immunological detection a His-tag-specific antibody (Micromol, Karlsruhe, Germany) or an IFNα-specific antibody (US Biologicals, Massachussetts, MA, USA) was applied. As secondary antibody a goat-anti-mouse-AP-conjugate (BioRad) was applied