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Secretion of foreign proteins from *Saccharomyces cerevisiae* directed by α-factor gene fusions

(yeast MFα precursor/synthetic DNA/β-endorphin/consensus α-interferon/hybrid protein processing)

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ABSTRACT **Fusions between the cloned yeast α-factor structural gene and chemically synthesized DNA segments encoding human protein analogs have been constructed. The gene fusions encode hybrid proteins that include the first 89 amino acids of the native α-factor precursor fused to either a small (β-endorphin, 31 amino acids) or large (α-interferon, 166 amino acids) foreign protein. Proteolytic cleavage sites involved in α-factor maturation from the native precursor immediately precede the foreign peptide in the hybrid protein. The α-factor promoter was utilized to express the gene fusions in *Saccharomyces cerevisiae* and resulted in the efficient secretion of the foreign proteins into the culture medium. The processing of the hybrid proteins has been characterized by amino acid sequence analysis of the secreted proteins. The proteolytic cleavages involved in the maturation of α-factor peptides from the native precursor also occur accurately in the hybrid protein. In addition, cleavages occurred on the carboxyl side of two lysines within the β-endorphin peptide. Internal cleavages in the interferon protein were also detected. However, in this case, the cleavages occurred at a very low frequency such that >95% of the secreted interferon remained intact.**

The yeast mating pheromone α-factor is a 13-amino acid peptide that is secreted into the culture medium by *MATα* cells. α-Factor arrests *MAT***a** cells in G_1 phase and induces specific biochemical and morphological changes as a prerequisite to mating of the **a** cell to an α haploid. On a molar basis, α-factor is produced as efficiently as the highly expressed yeast glycolytic enzymes (1). It seems likely, therefore, that the components responsible for the efficient secretion of α-factor might be utilized to direct secretion of foreign proteins into the culture medium.

Kurjan and Herskowitz (2) cloned the *Saccharomyces cerevisiae* α-factor structural gene by using a bioassay to screen for yeast clones that overproduce the pheromone due to the presence of the α-factor gene on a multicopy plasmid. From the DNA sequence of the cloned gene these authors deduced that the 13-amino acid α-factor peptide is synthesized as a 165-amino acid prepropolyprotein precursor containing four copies of the α-factor peptide. A probable processing pathway in α-factor maturation was proposed (2), which has, in large part, been experimentally substantiated (3–5). The precursor contains a hydrophobic amino-terminal 22-residue segment that presumably initiates translocation into the endoplasmic reticulum. The function of the next 61 amino acids (pro-segment) is not known, but it does contain three glycosylation sites and may be involved in directing the precursor into the correct secretory pathway. The reiterated α-factor peptides are separated by spacer peptides and are cleaved from the precursor and processed by three enzymatic activities: trypsin-like or cathepsin B-like cleavage at Lys-Arg; carboxypeptidase B-like cleavage of basic residues from the excised peptides; and removal of the Glu-Ala or Asp-Ala dipeptides by dipeptidyl aminopeptidase A.

Subsequently, Singh *et al.* (6) reported cloning two α-factor structural genes. One gene (MFα1) appears to be the same as the original Kurjan and Herskowitz isolate, whereas the other clone (MFα2) encodes a precursor containing only two α-factor peptides. We used a synthetic oligonucleotide as a hybridization probe to clone a segment of the yeast genome with complementarity to the α-factor peptide coding region (7). The DNA sequence of our clone, at least in the coding region, is identical to the MFα1 gene of Kurjan and Herskowitz (data not shown). We have constructed fusions between the α-factor structural gene and chemically synthesized DNA segments encoding foreign proteins. Utilizing the endogenous α-factor promoter on the cloned segment to express the gene fusions in *S. cerevisiae*, we have obtained efficient secretion into the culture medium of a number of foreign proteins. Recently, Emr *et al.* (8) reported an α-factor gene fusion that directed secretion of yeast invertase to the periplasmic space. In the present report, we describe the processing events associated with secretion into the medium of β-endorphin and an analog of human α-interferon, IFN-αCon_1.

MATERIALS AND METHODS

Vector Construction, Cell Transformation, and Culture Conditions. Plasmid pαF consists of a 2.1-kilobase yeast *Eco*RI DNA fragment containing an α-factor structural gene cloned in pBR322 (7). A 1.7-kilobase *Xba* I–*Eco*RI fragment was subcloned by using *Bam*HI linkers into pBRΔH (pBR322 derivative in which *Hin*dIII site has been deleted) or pBRΔHS (pBR322 derivative in which *Hin*dIII and *Sal* I sites have been deleted; constructed by D. Hare) to generate pαC1 and pαC2, respectively, which were used for construction of the gene fusions. The *Xba* I site is 930 base pairs (bp) 5′ to the ATG translation initiation codon and the *Eco*RI site is 330 bp 3′ to the termination codon of the α-factor structural gene. The β-endorphin gene was cloned between the *Hin*dIII sites at the alanine codon of the first and fourth spacer peptide. The IFN-αCon_1 gene was cloned between the *Hin*dIII site of the first spacer peptide and the *Sal* I site 35 bp beyond the termination codon of the α-factor structural gene. Details of vector construction and DNA sequences of the synthetic genes will be published elsewhere.

After construction of the gene fusion and confirmation of the DNA sequence, the hybrid gene was cloned as a *Bam*HI fragment into a yeast–*Escherichia coli* shuttle vector. pGT41 contains a *LEU2* selectable marker and has been described (9). pYE was constructed from pGT41 by replacing the *Bam*HI–*Sal* I 2.5-kilobase *LEU2* gene segment with the 275-bp *Bam*HI–*Sal* I segment of pBR322 and subsequently cloning the yeast *TRP1* gene (*Eco*RI–*Bgl* II 852-bp fragment; ref. 10) into the *Sal* I site by blunt-end ligation. pYα-E and pYE/α-E contain an α-factor/β-endorphin gene fusion

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Abbreviation: bp, base pair(s).

cloned into pGT41 and pYE, respectively. pYE/αF-Con$_1$ contains an α-factor/IFN-αCon$_1$ gene fusion cloned into pYE. *S. cerevisiae* was transformed according to the method of Hinnen *et al.* (11). Transformants of strain GM3C-2 (*MATα leu2-3 leu2-112 trp1-1 his4-519 cyc1-1 cyp3-1*) were selected by leucine prototrophy in SD medium (0.67% yeast nitrogen base without amino acids/2% glucose) supplemented with 0.01% histidine and 0.01% tryptophan, whereas 20B-12 (*MATα trp1 pep4-3*) transformants were selected in SD medium containing 0.5% Casamino acids.

DNA Sequencing. DNA sequence determinations were by the method of Maxam and Gilbert (12) or by the dideoxy chain-termination method (13) on fragments subcloned into M13.

Chemical Synthesis of DNA. Oligonucleotides were synthesized essentially according to the chemistry outlined by Tanaka and Letsinger (14). The oligonucleotide segments of the β-endorphin gene were designed to allow only one ligation pathway. All purified oligonucleotides except the 5′-end segments were phosphorylated, mixed in equimolar ratios, and ligated and the full-length synthetic β-endorphin gene was purified by polyacrylamide gel electrophoresis. The 5′ termini of the synthetic gene were phosphorylated prior to cloning into M13mp9 as a *Hin*dIII–*Bam*HI segment. After confirming the DNA sequence of the synthetic gene, which utilized optimal yeast codons (ref. 15; unpublished data), it was excised from the phage RF as a *Hin*dIII fragment for subsequent vector construction. The chemical synthesis and cloning of the IFN-αCon$_1$ gene has been described (ref. 16; unpublished data).

Amino Acid Sequence Determinations. Automated sequence analyses (17, 18) of peptide fragments isolated by HPLC were performed with a gas-phase sequenator (Applied Biosystems, Foster City, CA). Phenylthiohydantoin-amino acids obtained from each sequenator cycle were identified by reverse-phase HPLC (19).

RESULTS

We constructed gene fusions between the α-factor leader region and two chemically synthesized genes. The gene fusions encode a hybrid protein (Fig. 1*A*), which includes the first 89 amino acids of the α-factor precursor: signal peptide, pro-segment containing three glycosylation sites, and the first spacer peptide. The first amino acid of the foreign protein occupies the same position in the hybrid protein as does the first amino acid (tryptophan) of the first α-factor peptide in the native precursor. One fusion contains a human β-endorphin analog (Leu5) as the last 31 amino acids of the hybrid. The second gene fusion encodes a hybrid of the α-factor leader and a 166-amino acid α-interferon analog (IFN-αCon$_1$). IFN-αCon$_1$ is an average amino acid sequence of all the known naturally occurring leukocyte interferons and expression of this gene in *E. coli* results in a protein with a 10-fold higher antiviral activity than any of the known naturally occurring α-interferons (16). The gene fusions include the 930 bp of yeast DNA upstream from the ATG translation initiation codon of the α-factor structural gene (*Materials and Methods*). Thus, we expected this fragment to include all sequences necessary for α-factor promoter function *in vivo* in addition to including 300 bp of 3′-untranslated yeast DNA.

The gene fusions were cloned into various yeast–*E. coli* shuttle vectors (Fig. 1*B*) and reintroduced into *S. cerevisiae*. Transformants were cultured in liquid medium, the cells were removed by centrifugation, and the conditioned medium was tested for the presence of the foreign protein. β-Endorphin was quantitated by competitive RIA and IFN-αCon$_1$ antiviral activity was determined by an end-point cytopathic effect assay as described (16). Strain GM3C-2 containing

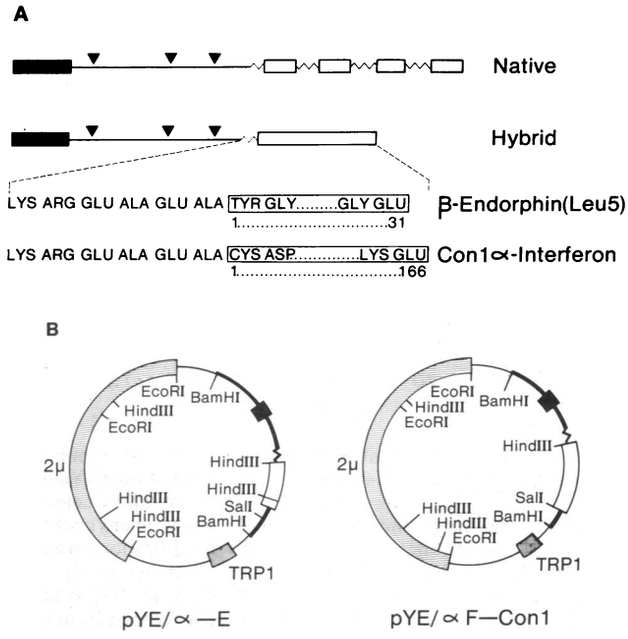

FIG. 1. (*A*) Native and hybrid precursor proteins. The α-factor precursor signal peptide (closed box), pro-segment (straight line), *N*-linked glycosylation sites (inverted triangles), and spacer peptides (squiggle) are indicated. The α-factor peptides and foreign protein segments are represented by open boxes. The amino acid sequence at the junction between the α-factor and foreign protein segments of the precursors is detailed. (*B*) Vectors for expressing α-factor gene fusions in yeast. pBR322 DNA is represented by the thin line and coding regions of the gene fusions are indicated as above. pYα-E is identical to pYE/α-E, with the exception that the yeast *LEU2* gene is incorporated instead of the *TRP1* gene. The α-factor/β-endorphin gene fusion includes the coding region for the last α-factor peptide, but this is not expressed since a termination codon was included in the synthetic gene.

pYα-E and cultured to an OD_{600} of 1 secreted 150–200 μg of β-endorphin immunoreactive material into the medium per liter of culture. No β-endorphin was detected in conditioned medium from cells containing a gene fusion in which the β-endorphin segment was in the incorrect orientation relative to the α-factor gene. Strain 20B-12 transformed with pYE/αF-Con$_1$ secreted 2×10^8 units of IFN-αCon$_1$ into the medium per liter of culture at an OD_{600} of 1. These results demonstrate that the expression system results in the efficient secretion of both small (31 amino acids) and large (166 amino acids) proteins into the culture medium.

The hybrid proteins encoded by pYα-E and pYE/αF-Con$_1$ contain proteolytic processing sites involved in the maturation of α-factor peptides from the native precursor (Fig. 1*A*). The gene fusions in our constructs were designed with the anticipation that these sites might also be recognized in the hybrid proteins and thus generate secreted proteins with the native amino termini. We have characterized the processing of the hybrid protein by determining the complete amino acid sequence of the secreted β-endorphin immunoreactive peptides. When conditioned medium is subjected to HPLC, three β-endorphin immunoreactive species are resolved (Fig. 2). Each peptide was sequenced by the automated Edman degradation procedure and the results are included in Table 1. Peak I is the carboxyl 12-amino acid fragment of β-endorphin that was generated by cleavage on the carboxyl side of lysine at position 19 of the β-endorphin peptide. Peak II is the amino-terminal 19-amino acid fragment of β-endorphin and thus represents the cleavage complement of peak I. Since peak II has the authentic β-endorphin amino-terminal sequence, this result demonstrates that the proteolytic cleav-

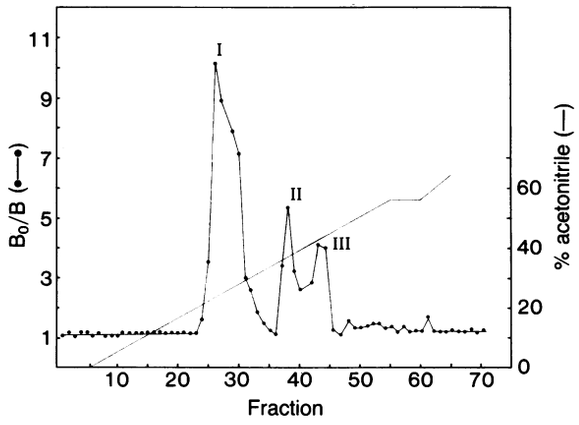

FIG. 2. HPLC fractionation of secreted β-endorphin immunoreactive peptides. *S. cerevisiae* GM3C-2 transformed with pYα-E was cultured to an OD_{600} of 1 and the cells were removed by centrifugation. The conditioned medium was made 1 mM phenylmethylsulfonyl fluoride and concentrated 20-fold by ultrafiltration through an Amicon YM2 membrane. The concentrated medium was adjusted to pH 2.1 and applied to a C_{18} column (0.39 × 30 cm, 10-μm particle size, Waters Associates) and a gradient was developed from 0–56% acetonitrile in 0.1% trifluoroacetic acid. Individual 1-min fractions were assayed by competitive RIA (New England Nuclear) and the results are presented as B_0 (cpm in reaction lacking unlabeled β-endorphin) divided by B (cpm in reaction containing sample). No β-endorphin immunoreactive material was detected in the column flow-through.

ages involved in α-factor biogenesis also occur accurately in the α-factor/β-endorphin hybrid protein. Peak III of Fig. 2, consists of the peptide IIIc, which has also been accurately processed on the amino terminus but cleaved on the carboxyl side of lysine at position 9 in the β-endorphin peptide. Other peptides resulting from cleavage within the β-endorphin peptide (e.g., the fragment corresponding to residues 10–19) would escape detection in our analysis if they are not reactive in the RIA.

The proteolytic cleavages within the β-endorphin peptide do not correspond to cleavage sites in the native α-factor precursor. These cleavages could result from exposure of the hybrid precursor to vacuolar proteases during the secretory process. Alternatively, the cleavages could occur by proteases in the extracellular medium, perhaps released by cell lysis. To test this possibility, we subcloned the α-factor/β-endorphin gene fusion into another shuttle vector and transformed the new plasmid (pYE/α-E) into *S. cerevisiae* 20B-12. This strain lacks 95% of the vacuolar protease activity of wild-type strains (20) due to the presence of the *pep4-3* mutation. For as yet uncharacterized reasons, these transformants secrete more β-endorphin immunoreactive material (up to 450 μg/liter of culture at an OD_{600} of 1). However, when conditioned medium from cultures of these cells was fractionated by HPLC, the three β-endorphin immunoreactive peaks noted above were observed in the same ratios (data not shown). These results suggest that the cleavages within the β-endorphin peptide occur during passage of the hybrid precursor through the normal secretory pathway. HPLC peak III from strain 20B-12 transformed with pYE/α-E was also subjected to amino acid sequence analysis. In this case, multiple phenylthiohydantoin-amino acids were identified in each sequencing cycle. Thus, it did not consist of a single peptide, despite the fact it chromatographed as a single immunoreactive peak. From the sequencing data, we deduced that this peak contained, in addition to peptide IIIc (Table 1), peptides IIIb and IIIa, which contain Glu-Ala and Glu-Ala-Glu-Ala extensions, respectively, on the amino terminus of peptide IIIc.

The processing of the α-factor/IFN-αCon_1 hybrid precursor was also investigated. Strain 20B-12 transformed with pYE/αF-Con_1 was cultured in liquid medium, the cells were removed by centrifugation, and the conditioned medium was concentrated by ultrafiltration. Denaturing polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) of the secreted proteins (Fig. 3*A*) reveals a 20,000-dalton polypeptide (lane 2) that is not present in medium proteins from a negative control strain (lane 3). The IFN-αCon_1 secreted from yeast migrates somewhat slower than IFN-αCon_1 purified from *E. coli* (lane 1). From the sequencing results of the secreted β-endorphin immunoreactive peptides (Table 1), we infer that the retarded electrophoretic migration of IFN-αCon_1 may be due to incomplete processing of the amino-terminal Glu-Ala dipeptides. Fig. 3*B* depicts an electrophoretic transfer immunoblot of the same protein samples electrophoresed in the gel shown in Fig. 3*A*. There is some nonspecific background, as evidenced by reaction with proteins from the negative control yeast strain (lane 3). However, IFN-αCon_1 specific reactivity is clear (lanes 1 and 2). In addition to the intact 20,000-dalton polypeptide, secreted yeast proteins include subfragments of IFN-αCon_1. A major subfragment of 14,500 daltons and minor species with masses of 13,500, 12,500, and 11,000 daltons are observed. Thus, internal cleavages also occur in IFN-αCon_1 during α-factor-directed secretion from yeast. The autoradiograph of the immunoblot was purposely overexposed to detect low-abundance subfragments. However, inspection of the stained gel (Fig. 3*A*) reveals that these

Table 1. Amino acid sequence of secreted β-endorphin immunoreactive peptides

Peptide	Amino acid sequence
IIIa	Glu-Ala-Glu-Ala-Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys
IIIb	Glu-Ala-Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys
IIIc	Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys (1–9)
II	Tyr-Gly-Gly-Phe-Leu-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys (19)
I	Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu (20–31)

Purified β-endorphin immunoreactive peptides (*Results*) were subjected to automatic amino acid sequence determination. Numbers refer to residue positions in authentic β-endorphin.

cleavages occur at a low frequency such that >95% of the secreted IFN-αCon$_1$ remains intact.

DISCUSSION

We have demonstrated that the α-factor leader region directs secretion of foreign proteins from *S. cerevisiae*. Fusions have been constructed between the yeast α-factor leader region (signal peptide, pro-segment, and first spacer peptide) and chemically synthesized DNA segments encoding human protein analogs. Utilizing the α-factor promoter to express the gene fusions results in the efficient secretion of both small (e.g., β-endorphin, 31 amino acids) and large (e.g., IFN-αCon$_1$, 166 amino acids) foreign proteins into the culture medium. It should be noted that we have not yet determined the intracellular levels of the foreign polypeptides nor the amount secreted but associated with the cell wall. The present report documents the processing events associated with secretion of the foreign protein segment of the hybrid precursors into the culture medium.

All secreted β-endorphin immunoreactive peptides contain only β-endorphin sequences, with the exception of peptides IIIa and IIIb, which contain amino-terminal Glu-Ala-Glu-Ala and Glu-Ala extensions, respectively, derived from the α-factor portion of the hybrid protein precursor. These results indicate that processing of the precursor at the trypsin-like Lys-Arg cleavage site is not a rate-limiting step in processing, even when the hybrid protein is overproduced from multicopy vectors. Alternatively, cleavage of the precursor at Lys-Arg may be a prerequisite of secretion of carboxyl peptide segments. Since we have examined only peptides secreted through the cell wall into the extracellular medium, there may, in actual fact, be an accumulation of the immediate polypeptide precursor to the trypsin-like cleavage within the cell. Whether the cleavage at Lys-Arg is absolutely required for secretion may be determined by expressing precursors in which the cleavage site is altered or deleted. The amino-terminal Glu-Ala-Glu-Ala and Glu-Ala extensions on peptides IIIa and IIIb, respectively, indicate that production of the precursor from multicopy vectors results in incomplete processing by dipeptidyl aminopeptidase A. These results are consistent with those of Julius *et al.* (3), which indicated that this protease is rate-limiting in the processing of α-factor peptides from the native precursor. In our analysis, we have determined the molar ratio of peptides IIIa/IIIb/IIIc to be 2.4:1.0:1.5 based on phenylthiohydantoin-amino acid recovery during sequencing. The accumulation of peptide IIIb with only one Glu-Ala dipeptide indicates that dipeptidyl aminopeptidase A is not a processive enzyme. It should be possible to secrete peptides with homogeneous, completely processed amino termini by overproducing dipeptidyl aminopeptidase A, a product of the cloned *STE13* gene (3). Alternatively, hybrid genes may be constructed that encode precursors containing the Lys-Arg cleavage site but lacking the Glu-Ala codons. The processing of such precursors would thus be independent of the rate-limiting dipeptidyl aminopeptidase A.

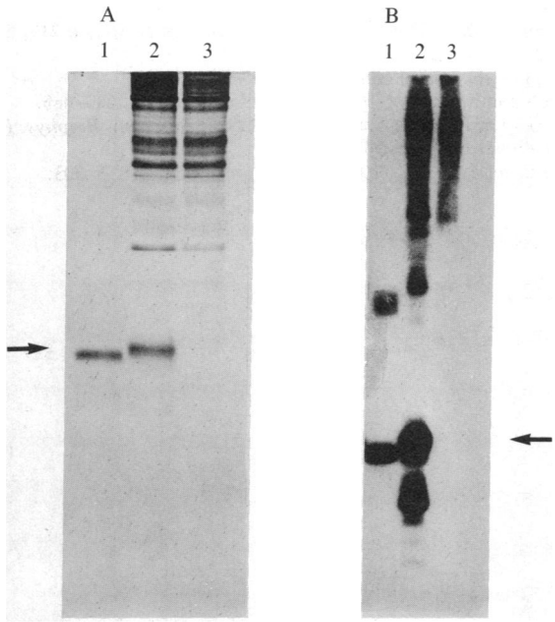

FIG. 3. NaDodSO$_4$/PAGE analysis of secreted yeast proteins. *S. cerevisiae* 20B-12 transformed with either pYE/αF-Con$_1$ or pYE/αF-calc was cultured to an OD$_{600}$ of 2 and the cells were removed by centrifugation. The strain containing pYE/αF-calc secretes human calcitonin (M_r 3500) directed by an α-factor gene fusion and will be described elsewhere. The conditioned medium was made 1 mM phenylmethylsulfonyl fluoride and concentrated 50-fold by ultrafiltration through an Amicon YM10 membrane (M_r cutoff, 10,000). Lanes 1, IFN-αCon$_1$ purified from *E. coli*; lanes 2, pYE/αF-Con$_1$ conditioned medium; lanes 3, pYE/αF-calc conditioned medium. The 20,000-dalton secreted IFN-αCon$_1$ is indicated by the arrow. Molecular sizes were determined by comparison to the migration of standards (ovalbumin, M_r 43,000; α-chymotrypsinogen, M_r 25,700; β-lactoglobulin, M_r 18,400; lysozyme, M_r 14,300; cytochrome *c*, M_r 12,300; bovine trypsin inhibitor, M_r 6200). (*A*) A 15% polyacrylamide gel was run as per Laemmli (21) and stained with Coomassie brilliant blue R250. (*B*) Proteins were electrophoresed in a 10–20% polyacrylamide gradient gel and transferred to nitrocellulose, and the immobilized proteins were incubated with rabbit antiserum (23) raised against IFN-αCon$_1$ produced in *E. coli*. Immune complexes were allowed to react with radioiodinated staphylococcal A protein and the nitrocellulose was exposed to Kodak XAR-2 film.

In addition to the processing events characteristic of the native α-factor precursor, two additional cleavage sites were mapped in the α-factor/β-endorphin hybrid precursor. These cleavages occurred on the carboxyl side of lysines in the β-endorphin portion of the protein at ^{8}Glu-^{9}Lys-^{10}Ser and ^{18}Phe-^{19}Lys-^{20}Asn. Peptides arising from cleavage at the lysines in position 24, 28, or 29 of the β-endorphin peptide were not observed. The cleavage at position 19 occurred in all peptides since no secreted peptides were observed that spanned this region. In contrast, the cleavage at Lys-9 of the β-endorphin peptide occurred in ≈50% of the secreted peptides. It is not clear whether these cleavages are effected by the same protease that cleaves at Lys-Arg in the native precursor or by another protease to which the hybrid precursor is exposed. The mature α-factor peptide contains a lysine (^{6}Leu-^{7}Lys-^{8}Pro) that has not been observed to be cleaved during maturation. However, it should be noted that Lys-Pro bonds in peptides are not cleaved by trypsin. The fact that different lysine-containing sequences in the β-endorphin peptide were cleaved quantitatively, partially or not at all, indicates that these internal cleavages may be dependent on the conformation of the β-endorphin peptide. Consistent with this hypothesis is the observation that the most susceptible bond in native β-endorphin to mild trypsin digestion is ^{19}Lys-^{20}Asn (22) and this bond was also the most susceptible to proteolysis during secretion from yeast.

The processing of the α-factor/IFN-αCon$_1$ hybrid protein was characterized by NaDodSO$_4$/PAGE. The electrophoretic mobility of the secreted IFN-αCon$_1$ suggests that this product may also contain amino-terminal Glu-Ala extensions. This possibility, which is consistent with the results of secreted β-endorphin, must be tested by direct amino acid sequencing. Although internal cleavages could be detected by electrophoretic transfer immunoblot analysis, >95% of

the secreted IFN-αCon_1 protein remained intact. The widely disparate degrees of internal cleavage, both between different lysines within β-endorphin and between β-endorphin and IFN-αCon_1, indicate that the extent to which internal cleavages occur will be determined by the conformation-dependent accessibility of susceptible bonds in the foreign protein.

These findings also have implications for the secretion of native yeast proteins. Thus, for proteins that follow the same secretory pathway as the α-factor precursor, it is likely that susceptible bonds (involving basic amino acids) are protected from proteases to which the precursor is exposed. This may be effected via conformation-determined inaccessibility or protection of the susceptible bond by glycosylation. In either case, this hypothesis implies a co-evolution of the secretory apparatus and secreted protein structure with selection for efficient processing and secretion of biologically active (intact) proteins.

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