Roles of the signal peptide and mature domains in the secretion and maturation of the neutral metalloprotease from *Streptomyces cacaoi*

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The neutral metalloprotease (Npr) of *Streptomyces cacaoi* is synthesized as a prepro-Npr precursor form consisting of a secretory signal peptide, a propeptide and the mature metalloprotease. The maturation of Npr occurs extracellularly via an autoproteolytic processing of the secreted pro-Npr. The integrity of the propeptide is essential for the formation of mature active Npr but not for its secretion [Chang, Chang and Lee (1994) J. Biol. Chem. **269**, 3548–3554]. In this study we investigated whether the secretion and maturation of Npr require the integrity of its signal peptide region and mature protease domain. Five signal peptide mutants were generated, including the substitution mutations at the positively charged region (mutant IR6LE), the central hydrophobic region (mutants GI19EL and G19N), the boundary of the hydrophobic core-cleavage region (mutant P30L) and at the residues adjacent to the signal peptidase cleavage site (mutant YA33SM). All these lesions delayed the export of Npr to the growth medium and also resulted in a 2–10-fold decrease in Npr export. The most severe effect was noted in mutants GI19EL and P30L. When these signal peptide mutations

were fused separately with the propeptide lacking the Npr mature domain, the secretory defect on the propeptide was also observed, and this impairment was again more severely expressed in mutants GI19EL and P30L. Thus the Npr signal peptide seems to have more constraints on the hydrophobic core region and at the proline residue within the boundary of the hydrophobic core-cleavage site. Deletion mutations within the C-terminal mature protease domain that left its active site intact still blocked the proteolytic processing of mutant precursor forms of pro-Npr, although their secretions were unaffected. These results, together with our previous findings, strongly suggest that the signal peptide of Npr plays a pivotal role in the secretion of both Npr and the propeptide, but not in the maturation of Npr. On the contrary, the integrity of mature domain and propeptide is not critical for secretion of the Npr derivative but is essential for the formation of a functional Npr. Therefore the secretion and maturation of Npr are dependent on the integrity of the signal peptide, propeptide and mature protease domains, and the roles of these domains in this regard are functionally distinct.

INTRODUCTION

Virtually all prokaryotic secretory proteins destined for extracellular or membrane compartments are characterized by the presence of a signal peptide or leader sequence located at the N-terminus, although some internal signal peptides exist [1,2]. Analyses of a large number of the signal peptides reveal certain common structural features [2]: (1) all possess from one to three basic residues at the N-terminus followed by a hydrophobic segment of 10–18 residues; (2) residues capable of forming β -turns (e.g. proline and glycine) are often near the C-terminal end of this hydrophobic sequence; (3) specific residues with small side-chains (e.g. serine, alanine and glycine) immediately preceding the cleavage site constitute a recognition site for the signal peptidase. The relative importance of these structural features in secretion and their interactions with the components of the secretory apparatus of the cell have been the subject of extensive studies, largely in *Escherichia coli* (reviewed in [1–4]). In comparison, however, the secretory mechanisms of Gram-positive bacteria such as *Bacillus* or *Streptomyces* have received insufficient characterization. Few signal peptide sequence mutants in these two genera have been reported [5–8].

Our laboratory has been interested in the secretory system of *Streptomyces*. We have chosen MelC1 in the melanin gene (*melC*) operon and neutral metalloprotease (Npr) as model

systems for the study of *Streptomyces* secretory proteins. MelC1 has an N-terminal signal peptide sequence and acts as the molecular chaperone for apotyrasinase (MelC2); it has a dual role in secretion and *trans*-activation of apo-tyrosinase [8–11]. Npr is a neutral metalloprotease of *Streptomyces cacaoi* [12] and is synthesized as a precursor protein (prepro-Npr) consisting of an N-terminal signal peptide (34 residues) followed by a propeptide (171 residues) and a mature protease domain (345 residues) [12,13]. This protease has zinc-binding ligands located at His-202, His-206 and Glu-240 residues of the mature protease domain that are indispensable to the protease activity and proteolytic processing of the pro-Npr derivative [13]. On the basis of our previous studies [13,14] we propose a model (Figure 1) for the extracellular secretion and maturation of Npr. Our assumption is that the signal peptide guides the prepro-Npr precursor to pass through the membrane via the regular co- or post-translational transport route (step 1). During translocation the signal peptide of the prepro-Npr is removed by signal peptidase, resulting in the release of pro-Npr into the medium (step 2). Soon after arrival in the medium the propeptide promotes the correct folding of the Npr mature domain to acquire the active conformation, which then subsequently catalyses the autoproteolytic cleavage of pro-Npr propeptide (step 3). The released propeptide is further degraded, to form the mature protease (step 4). This maturation mechanism is sup-

Abbreviations used: *melC*, melanin gene locus; Npr, neutral metalloprotease; *npr*, gene encoding Npr; prepro-Npr (p60), preproenzyme of Npr; pro-Npr (p57), proenzyme of Npr; Pro, propeptide of Npr; TSB, tryptic soy broth.

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Figure 1 Proposed model for maturation and secretion of Npr

The signal peptide directs prepro-Npr across the membrane via the regular co- or post-translational transport route (step 1), where the signal peptide is removed, resulting in the release of pro-Npr (step 2). In the medium, folding of the mature domain of the protease occurs, including the metal ion (Zn)-binding site guided by the propeptide (step 3). A propeptide and mature protease are released from the precursor by autoproteolytic cleavage between the propeptide and the protease region. The propeptide is further degraded, leaving the mature Npr (step 4).

ported by the detection of pro-Npr in the medium of cells harbouring a mutant *npr* gene defective in the zinc-binding site [13]. However, in the cells harbouring a wild-type *npr* gene only the mature Npr can be detected in the medium [12–14], suggesting that the processes of propeptide-mediated folding and auto-processing of pro-Npr occur spontaneously.

An important feature of this model (Figure 1) is the involvement of the propeptide region in the maturation and secretory pathway of Npr. Earlier work conducted in our laboratory on the role of propeptide in Npr has shown that its complete removal inhibits the production of functional Npr [14]. Partial deletion of the propeptide region while leaving an intact mature Npr sequence resulted in the secretion of inactive, apparently unstable, truncated forms of pro-Npr [14]. Thus the integrity of the propeptide is essential for the maturation and correct processing of Npr but not for the secretion of Npr or its derivatives. This feature distinguishes the mechanism of maturation and secretion of Npr from those of subtilisin and the thermolysin-like neutral protease of *Bacillus*. In subtilisin the folding and the secretion functions of the propeptide sequences are not separable, because only properly folded and processed forms of subtilisin are secreted efficiently [15,16]. In the neutral protease of *Bacillus cereus*, the intact propeptide region is not necessary for the processing of *B. cereus* neutral protease precursor [17]. Therefore the protein export and maturation of these three proteases of Gram-positive bacteria seem to be considerably different with regard to the role of the propeptide.

Apart from the role of the propeptide, the relative importance of the mature domain and the signal peptide in Npr maturation and secretion remains unknown. It is also unclear to what extent transport of the precursor of Npr shares mechanistic features with the transport of the precursor protein to the periplasm of

Gram-negative bacteria, which in general is signal-peptide-dependent and requires that the nascent protein be in an unfolded state [18,19]. It is also important to know whether the signal peptide sequences in these two streptomycete proteins, MelC1 and Npr, have the same features in regard to their export function. Previously we reported the effects of mutations in the MelC1 signal peptide on its secretion and *trans*-activation functions [8].

In this study we examined the effects on the maturation and secretion of Npr by various mutations within the signal peptide sequence and mature region. Our results indicate that the integrity of the Npr signal peptide is crucial for the secretion of Npr and the propeptide. However, the integrity of the mature domain is not required for secretion of the Npr derivative, but is essential for the maturation of a functional Npr.

EXPERIMENTAL

Bacterial strains, plasmids, culturing conditions and recombinant DNA techniques

Streptomyces lividans TK64 (*pro-2*, *str-6*) [20] was the host for harbouring and expressing the cloned *npr* gene. The *Streptomyces* plasmid pSP105 harbouring the cloned *npr* gene has been described previously [13]. Plasmids pMUL1 and pMUS1 were constructed by subcloning the 5.4 and 0.93 kb *Pst*I–*Sac*I fragments of pSP105 respectively into the corresponding sites on vector pUC19 [14]. pSelect-BS carrying the 0.38 kb *Bam*HI–*Sac*I fragment containing the signal peptide and propeptide regions of *npr* gene was constructed by inserting this fragment into the corresponding sites on pSelect®-1 (Promega). Plasmids pVU1200 and pVU5100 were generated by inserting the 1.2 or 5.1 kb *Pvu*II fragments of pSP105 into the *Pvu*II site of the vector pUC19

	Charged region	Hydrophobic region	Cleavage site
Wild type	<u>MPMFRI</u> RLPKPAALIAAGGIGACIATVAVPSAYAAAP	*****	↑
IR6LE	MPMFRL <u>EL</u> PKPAALIAAGGIGACIATVAVPSAYAAAP		
GI19EL	MPMFRI <u>RL</u> PKPAALIAAG <u>EL</u> GACIATVAVPSAYAAAP		
G19N	MPMFRI <u>RL</u> PKPAALIAAG <u>NG</u> GACIATVAVPSAYAAAP		
P30L	MPMFRI <u>RL</u> PKPAALIAAGGIGACIATVAV <u>L</u> SAYAAAP		
YA33SM	MPMFRI <u>RL</u> PKPAALIAAGGIGACIATVAVPSA <u>SM</u> AAP		

Figure 2 Sequence of the wild-type and mutant signal peptides of Npr

The amino acid sequence of the wild-type Npr signal peptide is shown as standard single-letter abbreviations. The N-terminal positively charged region is boxed. The asterisks indicate the hydrophobic core region. The arrow marks the cleavage site by signal peptidase [13]. The residues substituted in each mutant are underlined.

respectively [13]. Culturing and gene manipulations of *E. coli* and *Streptomyces* were by the methods of Sambrook et al. [21] and Hopwood et al. [22].

Site-specific mutagenesis of the signal peptide region of *npr*

The oligonucleotide-directed site-specific mutagenesis of the *npr* signal peptide sequence was performed on plasmid pSelect-BS, using the Altered Site *In Vitro* Mutagenesis System (Promega Co.) in accordance with the specifications of the manufacturer. Oligonucleotides (designated by the position of the mutated amino acid and the amino acid before and after the mutation) are listed below. Mutated bases are shown in bold, and new restriction sites created after mutation are also indicated.

IR6LE	5'-TTTCGGCAACT TCGAG TCGGAACAT-3' <i>XhoI</i>
GI19EL	5'-ATGCAGGCGCC GAGCT CGCCCGCCGC-3'
G19N	5'-AGGCGCCTATG TTG CCCGCCGC-3'
P30L	5'-AGGCGGACAGTACGGCG-3'
YA33SM	5'-CGGGGGCGGCC ATCGAT GCGGACGGTAC-3' <i>ClaI</i>

The *Bam*HI–*Sac*I fragment (0.38 kb) encoding the mutated residues in pSelect-BS plasmid derivatives was excised and used to replace the corresponding segment on pMUS1 to generate a series of mutant derivatives of pMUS1 (pMUS-IR6LE, pMUS-GI19EL, pMUS-G19N, pMUS-P30L and pMUS-YA33SM). Each 0.93 kb *Pst*I–*Sac*I fragment on these pMUS derivatives was isolated and ligated with the 5.4 kb *Pst*I–*Sac*I fragment of pMUL1 to generate the mutant derivatives of pSP105 (pSP105-IR6LE, pSP105-GI19EL, pSP105-G19N, pSP105-P30L and pSP105-YA33SM) carrying the designed mutations in the signal peptide of Npr (Figure 2).

Deletion mutagenesis of the mature region of *npr*

To construct pSP105ΔC, the 81 bp *Sca*I fragment at the 3' end of the *npr* gene was deleted, resulting in an excision of the 27 residues at the C-terminus of the Npr mature domain (Figure 3). To construct pSP105ΔM, an *Xba*I amber linker (BioLab) was introduced into the unique *Sma*I restriction site of plasmid pVU1200 [13], and the 1.2 kb *Pvu*II fragment of the resulting plasmid pVU1200ΔM was recovered and ligated with the 5.1 kb *Pvu*II DNA fragment of pVU5100 [13]. In pSP105ΔM the expected Npr derivative contained 284 residues of mature domain in addition to the signal sequence and propeptide region, followed

by an additional four residues (Pro-Ser-Leu-Asp) from the linker region at the C-terminus of the protein (Figure 3). To construct pSP105ΔD, plasmid pVU1200 was treated with restriction enzyme *Dra*III and repaired by T4 DNA polymerase. The 1.02 kb *Pvu*II fragment of the resulting plasmid pVU1200ΔD was excised and re-ligated with the 5.1 kb *Pvu*II fragment of plasmid pVU5100 to produce plasmid pSP105ΔD. This construct was expected to produce the fusion protein containing the Npr mature domain's N-terminal 42 residues, followed by an out-of-frame polypeptide sequence of 162 residues (Figure 3).

Construction of mutated secretory expression plasmids for propeptide

To construct the plasmids for directing the propeptide secretion by the mutated signal peptides, a similar strategy as described previously for the construction of secretory plasmid for the propeptide region was used [14]. Briefly, the 0.93 kb *Sac*I–*Pst*I fragment of each corresponding signal peptide mutant derivative of pMUS (pMUS-IR6LE, pMUS-GI19EL, pMUS-G19N and pMUS-P30) was ligated to the 5.4 kb *Sac*I–*Pst*I fragment of pSP105-Pro [14], resulting in pSP6LE-Pro, pSP19EL-Pro, pSP19N-Pro and pSP30L-Pro.

Preparation of the antiserum against pro-Npr

To overexpress the propeptide in *E. coli* for use in the antigenic generation of antiserum, the expression plasmid pET25b-Pro was constructed. The DNA segment that encodes for the pro-Npr region was amplified by PCR with the primers PS (5'-CGTACCGTCCGCCTCCATGGCCGCCCGCCCCG-3') and END (5'-CTCGGTACCCGGGCTAGCGTACTGGATG-GTGTGG-3'). The amplified fragment encompassed the complete region of pro-Npr and also contained *Nco*I and *Nhe*I sites at the 5' and 3' ends of the fragment respectively. This 1.5 kb PCR fragment was cloned into the *Sma*I site of pUC19 and re-excised as an *Nco*I–*Nhe*I fragment and inserted into the corresponding sites of 5.5 kb *E. coli* expression vector pET25b(+) (Novagen Co.) to generate the plasmid pET25b(+)-proNpr (His). Then the *Sac*I–*Kpn*I (0.74 kb) fragment of pMUL-Pro [14] was used to replace the corresponding fragment in pET25b(+)-proNpr (His) to produce plasmid pET25b-Pro. In this construct the translation stop codon was introduced into the last codon of the propeptide; therefore an *E. coli* strain harbouring this expression construct produces the propeptide directed by the pelB leader sequence after induction with isopropyl β-D-thiogalactoside [23]. *E. coli* strain BL21 harbouring this expression construct was cultured overnight in Luria–Bertani medium and induced for 3 h at 37 °C with 1 mM isopropyl β-D-thiogalactoside before harvest. The expected pelB–propeptide fusion protein (molecular mass 24 kDa) was separated on SDS/PAGE, and the protein band was sliced, eluted and used as antigen for the immunization of rabbits.

Purification of Npr from signal peptide mutants, and assay of Npr activity

The mature Npr was purified from the culture medium of *S. lividans* TK64 harbouring various pSP105 derivatives essentially as previously described [12]. Briefly, *S. lividans* were cultured in R2YE/starch liquid medium for 24–36 h [13]. The acetone-concentrated pellets of crude supernatants of media were dissolved in 50 mM sodium citrate buffer, pH 5.2 (buffer A), and then applied to a Fractogel CM TSK650(s) ion-exchange column. After washing with 130 mM NaCl in buffer A, the bound Npr was eluted by 0.15 M NaCl in buffer A.

Figure 3 Schematic representation of deletions in the mature domain of Npr

The solid line at the top represents the coding sequence of the wild-type mature Npr. The asterisks indicate the regions coding for the active sites of Npr [13]. In the three deletion mutants shown, the dotted line represents the deleted portion of Npr mature region and the chequered bar indicates the frame-shifted region after position 42. The open bar in ΔM indicates the commercial amber linker region. The amino acid numbering is relative to the N-terminus (+1) of mature Npr. The sizes of the truncated Npr forms are indicated at the right (aa, amino acids). The nature and position of the residues at the junction of deletion or frameshift in each mutant are also indicated.

The phenotype of Npr was examined by the clear zones on R2YE agar [24] containing 2% (w/v) casein [13]. The protease level in liquid tryptic soy broth (TSB, Difco) culture supernatant was measured by using Azocoll (Sigma) as substrate [12,25]. One unit of protease activity was defined as previously reported [25].

Western blotting

Rabbit antibodies against Npr (35 kDa) and pro-Npr (57 kDa) were prepared as described elsewhere [13]. Antiserum against the propeptide of Npr (Pro) was prepared as described above. Proteins separated by SDS/PAGE were electroblotted on a nitrocellulose filter (Schleicher and Schuell) with a Bio-Rad mini trans-blot apparatus. Antigens were detected by anti-Npr, anti-pro-Npr [13] or anti-Pro antibodies. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) was used as the secondary antibody, and chromagenic development was performed as previously described [14].

RESULTS

Altered signal peptide sequence affects the secretion of Npr

The N-terminal 34 residues of prepro-Npr exhibited a typical prokaryotic signal peptide: three positive charged residues (Arg-5, Arg-7 and Lys-10) followed by an 11-residue hydrophobic stretch (Ala-12 to Ala-22) and a small side-chain amino acid at the putative signal peptidase cleavage site (Ala-34 and Ala-35) [12,26] (see also Figure 2). To elucidate whether mutations in the signal sequence would affect the secretion and maturation of Npr, five individual mutations were generated in the signal peptide (Figure 2; see the Experimental section for construction of mutants). In IR6LE the net charge on the N-terminal basic region was decreased from +3 to +1. In GI19EL and G19N a negatively charged (Glu) or a neutral polar (Asn) residue was incorporated into the central portion of the hydrophobic region to shorten the hydrophobic length. In P30L the proline residue that supposedly breaks the hydrophobic core α -helix structure [26] was replaced by leucine. In YA33SM the Tyr-Ala dipeptide upstream from the processing site was altered to Ser-Met.

When examined for the Npr activity on casein plates, all mutants displayed positive results (results not shown). However, the use of Azocoll as the assay substrate for secreted Npr in liquid broth revealed different profiles of protease activity among these mutants and the wild-type (Figure 4). Although the onset of extracellular protease production in all strains examined

exhibited a strong correlation with cell growth, the wild-type strain secreted Npr much earlier (12–18 h culture). The wild-type strain reached the peak of extracellular Npr activity at 24 h of

culturing time and declined thereafter. All mutants except GI19EL reached their peaks of Npr activity after 36–48 h of culturing and the duration of the expression was sustained from 18 to 24 h, which was similar to the wild-type strain. All mutants exhibited a decrease in secretory Npr activity. In mutants IR6LE and YA33SM the secreted Npr activity was 54–55% of the wild-type; in mutants G19N and P30L the secreted Npr activity was 33% and 18% of the wild-type respectively. In mutant GI19EL the Npr activity was less than 10% of the wild-type.

When analysed by immunoblot with anti-pro-Npr antiserum, the amounts of secreted Npr protein (P35) from the mutants correlated well with their activities (see Figures 4 and 5). A decrease in the secreted Npr to between one-third and one-half was found in IR6LE, YA33SM and G19N, and a decrease in P30L and GI19EL to between one-fifth and one-tenth, compared with the wild-type strain. Interestingly, a distinct band estimated at about 57 kDa, which accounted for 30–50% of the Npr species, was detected in mutants GI19EL and P30L at 12–54 h (mutant GI19EL) or 36–54 h (mutant P30L) of culturing (Figure 4). A similar protein species with weaker intensity was also found in 12–24 h cultures of the wild-type strain and much later in mutants IR6LE (36–48 h), G19N (24–30 h) and YA33SM (30–54 h). This protein species presumably represented the unprocessed pro-Npr species (p57) [13]. Several smaller protein species of 13–21 kDa, which probably represented the degradative forms of the Npr, were also detected in the immunoblots of the wild-type and mutant strains.

Taking these results together, we concluded that the integrity of the signal peptide is important for the export of Npr and its derivatives. Alterations within the four major regions of the signal peptide attenuated the export of Npr protein. In some mutants (GI19EL and P30L) the lesions seemed to delay the proteolytic processing of the secreted Npr precursor, resulting in the accumulation of unprocessed intermediate species.

A certain degree of variability in cell growth pattern (as reflected by the measurement of the total cellular proteins) was present in these signal peptide mutants compared with the wild-type strain (Figure 4). This suggests that the export defect in Npr might cause perturbations in cellular physiology. A similar observation was found in a signal peptide mutant of the major

Figure 4 Analysis of protease activity in the strains harbouring the signal peptide mutations

TK64 harbouring wild-type or the *npr* signal peptide mutants were cultured in TSB medium for 8–60 h. The secreted Npr activity was determined from the culture supernatant as described in the Experimental section. The total protein in 50 ml of culture was also determined [67]. The results shown in the Figure are means $\pm$ S.E.M. for three independent experiments performed in duplicate. Abbreviations: Host, TK64; WT, TK64[pSP105]; IR6LE, TK64[pSP105-IR6LE]; GI19EL, TK64[pSP105-GI19EL]; G19N, TK64[pSP105-G19N]; P30L, TK64[pSP105-P30L]; YA33SM, TK64[pSP105-YA33SM].

outer membrane lipoprotein of *E. coli* [27], where a deletion mutation in the lipoprotein signal peptide can cause severe effects on the viability of the host cell, resulting in cessation of growth and rapid cell death. Interestingly, in Npr this degree of interference with cell growth was not correlated with the severity of the export defect exerted by the respective signal peptide mutation introduced. The exact reason for this phenomenon is not clear, especially when one considers that mutant IR6LE showed a significant decrease in cell growth in spite of its exhibiting only a moderate inhibition of Npr production, whereas mutants GI19EL and P30L displayed a strong inhibition of Npr production but their cell growth showed a slight enhancement (Figure 4). Presumably this indicates that, in addition to the differences noted in the secretion of Npr, the pleiotropic effect of the Npr signal peptide mutation on the host cell might differ between mutants.

Npr derived from the signal peptide mutants has the same specific activity as that from the wild-type

To investigate whether mutations introduced into the signal peptide sequence might affect the folding of prepro-Npr and thus decrease the protease activities, the specific activities of Npr from the mutants were determined (Table 1). The results indicated that with the possible exception of G19N the specific activities of the

Npr from the other mutants (IR6LE and YA33SM) were essentially the same as that of the wild-type (Table 1). Results were not available for GI19EL and P30L, owing to the difficulty encountered in purifying the low quantities of Npr produced by these two mutants. Nevertheless the fact that similar levels of decrease in Npr export were found by Western blot and by enzymic assay strongly suggests that the same conclusion might be applicable to these two mutants.

Defects in the signal peptide sequence affect the secretion of propeptide

The propeptide of Npr can be secreted when fused directly to the signal peptide in the absence of the Npr mature domain [14]. To establish whether the mutations in signal sequence affect the secretion of propeptide as it did the secretion of Npr, the propeptide sequence was fused to the four mutant signal peptide sequences (IR6LE, GI19EL, G19N and P30L). Western blot analysis with anti-Pro antiserum demonstrated that all except P30L directed the secretion of the full-length (p22) propeptide into the medium at 16–20 h of culturing (Figure 6). However, the amounts of propeptide secreted by these mutants were much smaller than that secreted by the wild-type. The amounts of secreted propeptide directed by IR6LE, G19N, GI19EL and P30L at 16 or 20 h of culturing were approx. 88 %, 68 %, 23 %

Figure 5 Western blot analysis of Npr production in Npr signal peptide mutant strains

The strains were cultured in TSB medium for the indicated times (6–60 h), and the culture supernatant (300 μ l) was precipitated with 10% (w/v) trichloroacetic acid at 4 °C. The recovered pellets were dissolved in sample buffer and analysed by SDS/PAGE [12.5% (w/v) gel] [68], followed by Western blot analysis using anti-pro-Npr (P57) antiserum as described in the Experimental section. Arrows indicate the positions of pro-Npr (P57) or mature Npr (P35). The designations of the host or *npr* strains are the same as those in Figure 4.

Table 1 Specific activity of purified Npr proteases from wild-type and various signal peptide mutants

The Npr proteases from wild-type and mutant strains were purified by column chromatography as described in the Experimental section. The protease activity was assayed by the method of Chavira et al. [25]. The results are means $\pm$ S.E.M. for three independent experiments.

Enzyme	Specific activity (units/mg of protein)
Wild-type	623 $\pm$ 121
IR6LE	573 $\pm$ 21
GI19N	470 $\pm$ 18
YA33SM	583 $\pm$ 77

and 0% of that of the wild-type. Smaller protein species, which presumably represented the degraded propeptide, were also found. Thus it seems that those mutants with strong defects in the secretion of Npr (e.g. mutants GI19EL and P30L) were also defective in propeptide secretion (compare Figures 5 and 6).

Deletions of the mature domain abolish the maturation but not the secretion of Npr derivatives

To investigate whether the integrity of the mature region is essential for the maturation and secretion of Npr, three deletion mutations (Δ C, Δ M and Δ D) were created in the Npr mature region (see Figure 3). In Δ C the deletions spanned the C-terminal 26 residues (320–345). In Δ M the C-terminal 62 residues (284–345) were deleted and the C-terminus of the resulting mutant protein was replaced by four residues (Pro-Ser-Leu-Asp) encoded by the *Xba*I amber linker. In Δ D the N-terminal 42-residue fragment of the mature domain was fused with a different

Figure 6 Western blot analysis of the propeptide of Npr directed by mutant signal peptides

Culture supernatants (600 μ l; 16 or 20 h) from host or different propeptide expression variants was precipitated with 10% (w/v) trichloroacetic acid at 4 °C. The recovered pellet was dissolved in sample buffer and analysed by SDS/PAGE [68], followed by Western blot analysis with anti-Pro antiserum. The arrow indicates the propeptide-specific bands. The band intensity was quantified by a densitometer and expressed as the relative amount to that of wild-type. Abbreviations: Host, TK64; WT-Pro, TK64[pSP105-Pro]; 6LE-Pro, TK64[pSP6LE-Pro]; 19N-Pro, TK64[pSP19N-Pro]; 19EL-Pro, TK64[pSP19EL-Pro]; 30L-Pro, TK64[pSP30L-Pro].

Figure 7 Analysis of Npr activity in Npr mature-region deletion mutants

Npr activity in the signal peptide mutants was revealed on the casein plate as described in the Experimental section. Abbreviations: TK64, host; WT, TK64[pSP105]; Δ D, TK64[pSP105 Δ D]; Δ C, TK64[pSP105 Δ C]; Δ M, TK64[pSP105 Δ M].

Table 2 Analysis of Npr activity in Npr mature-region mutants

The Npr proteases from wild-type and Npr mature-region mutant strains were assayed as described in the Experimental section. Protease activity is expressed as units/ml of culture supernatant. The designations for the host or *npr* strains are the same as in Figure 7. The data are means $\pm$ S.E.M. for three independent experiments.

Culturing time (h)	Protease activity (units/ml of culture supernatant)				
	TK64	WT	Δ C	Δ D	Δ M
16	0.6 $\pm$ 0.0	8.0 $\pm$ 0.1	0.2 $\pm$ 0.0	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0
21	0.7 $\pm$ 0.0	13.0 $\pm$ 2.0	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1
26	0.6 $\pm$ 0.0	13.4 $\pm$ 1.0	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1
31	0.2 $\pm$ 0.0	4.9 $\pm$ 1.1	0.4 $\pm$ 0.0	0.4 $\pm$ 0.1	0.2 $\pm$ 0.0

reading frame polypeptide sequence with 162 residues resulting from a frame shift beyond Val-42 of the mature region. The expected molecular masses of the truncated Npr protein secreted from Δ C, Δ M and Δ D were 34, 32 and 22 kDa respectively, if the mutant propeptides were processed as in the wild-type.

As shown in Figure 7, all three mutants displayed an Npr[−] phenotype on casein plates, although the deletions in mutants Δ C and Δ M did not encompass the residues essential for the Npr activity (i.e. His-202, Glu-203, His-206 and Glu-240) [13]. No detectable protease activities were found in the liquid broth of these mutant strains either (Table 2). These results suggest that deletions in the mature domain of Npr might affect the matur-

Figure 8 Western blot analysis of the production of Npr derivatives from the culture supernatants of *npr* mature-region mutants

The host and *npr* mature-region deletion mutants were cultured in TSB medium for 16–31 h, and the culture supernatant (600 μ l for host strain and 100 μ l for the wild-type or mutant strains) was precipitated with 10% trichloroacetic acid and the recovered pellets were dissolved in sample buffer and analysed by SDS/PAGE [68], followed by Western blot analysis with anti-pro-Npr antiserum. The immunoreactive bands specific to anti-pro-Npr antisera are indicated with arrows. The abbreviations for the wild-type or mutant strains are as in Figure 7.

ation and/or processing of Npr. Immunoblot analysis with the anti-pro-Npr antibody detected 56 and 54 kDa proteins secreted by ΔC and ΔM (Figure 8). The size range of these protein species corresponded to the expected truncated pro-Npr derivatives if the pro-Npr derivatives were not processed. In the frame-shift mutant, ΔD , several protein species (55, 34 and 24 kDa) exceeding the expected size (22 kDa) for the processed Npr fusion protein were detected by anti-pro-Npr antiserum but not by anti-Npr antiserum (Figure 8). These protein species were presumably the unprocessed Npr fusion protein derivatives. All these results indicate that a truncation as small as 26 residues of the C-terminus of the mature domain completely abolishes the auto-processing activity of Npr. Interestingly, these truncations did not block the secretion of these Npr derivatives.

DISCUSSION

In this report we have shown that the secretion of active Npr in *Streptomyces* is dependent on the presence of the intact N-terminal signal peptide. Substitution mutations in four major regions (basic region, hydrophobic core, β -turn region and signal-peptidase cleavage site) of the signal peptide sequence led to an attenuation of Npr export, both kinetically and quantitatively, although the degree of impairment varied (Figures 4 and 5). Similar results were obtained when these mutant signal peptides were used to direct the secretion of Npr propeptide in the absence

of the mature domain (Figure 6). Thus the integrity of the Npr signal peptide is important in the secretion of the Npr and propeptide. Compared with the signal peptides of other bacterial secretory proteins, several features of the Npr signal sequence are noted. For instance, although the translocation of a number of *E. coli* secretory proteins (e.g. lipoprotein, staphylokinase and maltose-binding protein) across the cytoplasmic membrane does not absolutely require a net positive charge at their N-terminus [28–30], we found that in mutant IR6LE even the retention of a net charge of +1 at the N-terminus still caused a negative effect on Npr export (see IR6LE in Figures 4 and 5). The requirement for charged residues for the secretion of Npr in *Streptomyces* may be due to the nature of the host membrane, or it could be unique to Npr.

The introduction of charged or polar residues to the hydrophobic core of the Npr signal peptide impaired the secretion of Npr and pro-Npr (see GI19EL and G19N in Figures 4–6). Similar results were obtained with *E. coli* secretory proteins, although the severity of impairment varied considerably in different systems [2]. For instance, Goldstein et al. [31] introduced an asparagine residue (mutants I8N and V10N) into the hydrophobic core region of OmpA protein, which resulted in the complete blocking of secretion. In the Gly-14 to Asp-14 mutation (mutant 14D) in the lipoprotein signal sequence, translocation of precursor protein across the cytoplasmic membrane was not affected [32]. In contrast, the introduction of an aspartate or glutamate residue at positions 14 and 15 respectively of the LamB signal sequence markedly decreased secretion [33]. In our study, replacement of central-core Gly-Ile residues at positions 19 and 20 by Glu-Leu residues in mutant GI19EL resulted in a severe impairment of Npr export and proteolytic processing (Figures 4 and 5). Less severe defects were conferred by the G19N mutation, in which the polar residue Asn was introduced (Figures 4 and 5). All these findings indicate that the N-terminus and hydrophobic region in the Npr signal peptide are sensitive to the lesions introduced.

It was also surprising to find that the alteration introduced in the residues preceding the signal peptidase cleavage site in the YA33SM mutant caused a moderate export defect in Npr (Figures 4 and 5), whereas a similar substitution at the corresponding region in MelC1 signal peptide yielded no detectable effects on export [8]. This contrast might reflect their respective secondary structures at the processing site. In addition we observed an almost completely defective signal peptide in mutant P30L, in which a mutation at the hydrophobic core–cleavage site junction supposedly disrupted the predicted β -turn (Figures 4 and 5). This finding agrees with the hypothesis that a β -turn structure four to six residues upstream of the cleavage site is required for precursor protein processing [26,34,35]. However, this result stands in contrast with the observation of Borchert and Nagarajan [7] that the export of *Bacillus amyloliquefaciens* levansucrase remained unaffected when its β -turn structure at the end of the hydrophobic core of the signal peptide was disrupted. It is likely that the sequence requirement in the Npr signal peptide is more constrained than other secretory proteins discussed here.

Several roles of the signal peptide in facilitating protein export have been proposed [1,36–38]. In the present study we cannot extrapolate the exact mechanisms by which these signal peptide mutants hinder or delay Npr export across the membrane. The mutations introduced into the signal peptide that deteriorate protein secretion might result from the alteration of the conformation or hydrophobicity of the signal peptide, which might affect its insertion into the membrane or its interactions with secretory components and/or alter the export competence of the secretory proteins attached to it [1,36–38]. Notably, when we

Table 3 The β -turn structure features of bacterial signal peptide sequences in secretory proteins predicted by the method of Chou and Fasman [39]

The window width for the analysis was four amino acid residues. The program used in this analysis was PC/GENE of Intelligentics.

Strain	Protein	Signal peptide sequence	β -turn potential (residue number)	Reference
<i>S. ceolicolor</i> A3(2)	Agarase	MVNRDLIKWSAVALGAGAGLAPAPAAAHA	3 (31)	[50]
<i>S. griseus</i>	Amylase	MARRLATASLAVLAAAATALTAPTAAA	1 (28)	[51]
<i>S. hygroscopicus</i>	Amylase	MQQRSRVLGGLAGTVAATAATVAPWPSQA	4 (30)	[52]
<i>S. venezulae</i>	Amylase	MARKTVAAALALVAGAAVAVTGNAPQA	1 (28)	[53]
<i>S. limosus</i>	Amylase	MARRLATASLAVLAAAATALTAPTAAA	1 (28)	[54]
<i>S. cacaoi</i>	Npr	MPMFRIPLPKPAALIAAGGIGACIATVAVPSAYA	3 (34)	[13]
<i>S. albus</i>	β -Lactamase	VHPSTSRPSRRTLLTATAGAAALATLVPGTAHASSGGR	6 (39)	[55]
<i>S. sp36</i>	Xylanase	MNQDGKRYESEQNPFPFSGLSRRGLVAGAGAAAAGSGLLPGTAHA	11 (50)	[56]
<i>S. antibioticus</i>	MelC1	MPELTRRRALGAAAVVAGVPLVALPAARA	0 (30)	[57]
<i>B. amyloliquefaciens</i>	Barnase	MKKRLSWISVCLLVLSAAGMLFSTA	0 (26)	[47]
<i>Bacillus</i>	Alkaline protease	MRGKKVWISLLFALALIFTMAFGSTSSAQ	1 (30)	[47]
<i>Bacillus</i>	Neutral protease	MGLGKKLSVAVAAAFMSLTISLPGVQA	1 (27)	[47]
<i>B. amyloliquefaciens</i>	Amylase	MIQKRKRTVSFRLVLMCTLLFVSLPITKTS	0 (31)	[58]
<i>B. subtilis</i>	Amylase	MFAKRFKTSLLPLFAGFLLFHLVLAGPAA	0 (31)	[59]
<i>B. amyloliquefaciens</i>	Levansucrase	MNIKKIVKQATVLTFTALLAGGATQAF	1 (28)	[7]
<i>E. coli</i>	LamB	MMITLRKLPLAVAVAGVMSAQAMA	0 (25)	[60]
<i>E. coli</i>	Alkaline phosphatase	MKQSTIALALLPLFTPTKA	0 (21)	[61]
<i>E. coli</i>	OmpA	MKKTAAIAVALAGFATVQA	0 (25)	[62]
<i>E. coli</i>	Lipoprotein	MKATKLVLGAVILGSTLLAG	0 (20)	[63]
<i>E. coli</i>	Maltose-binding protein	MKIKTGARILALSALTMMMSASALA	0 (25)	[64]
<i>E. coli</i>	Staphylokinase	MLKRSLLFTVLLLFSSSITNQVSA	0 (27)	[65]
<i>E. coli</i>	β -Lactamase	MSIQHFRVALIPFAAFCLPVFA	1 (23)	[66]

used the Chou–Fasman [39] rule to predict the propensity of Npr signal peptide for β -turns, there are potentials for β -turns located at residues Leu-8, Ala-16 and Val-29. Moreover, for those mutants (GI19EL and P30L) yielding severely decreased Npr secretion, their predicted β -turn structures within the Npr signal peptide had altered. Interestingly, a similar observation can be made on the mutations of neutral protease signal peptide from *B. amyloliquefaciens* when fused with human parathyroid hormone, where changes in the predicted β -turn structure near the signal peptidase cleavage site substantially affect the secretory efficiency of the signal peptide [40]. Unexpectedly, an analysis of several secretory proteins from *Streptomyces* also showed a higher propensity for β -turn structure in their signal peptide regions than those from *Bacillus* or *E. coli* (Table 3). However, no such feature was observed in the signal peptide of MelC1 protein. This may account for the differential sensitivity to the similar mutation introduced into the cleavage site of the MelC1 or Npr signal peptide, as described earlier. The β -turn structure might be one of the requirements for proper function of the Npr signal peptide. However, the generalization of the β -turn requirement for secretion of other *Streptomyces* secretory proteins must await additional information obtained from other *Streptomyces* signal peptide mutants.

The mature region has been shown to have a profound impact on the export function of several secretory proteins [41–47]. Altman et al. [44], for example, have demonstrated that both the signal peptide and mature regions of LamB protein are required for the interaction with the export factor SecB in *E. coli*. In addition, the charge distribution as well as the secondary structure of the N-terminal mature region adjacent to the signal peptide have been recognized as important determinants for the compatibility of protein secretion [35,41–43,45]. In the present study, neither the N-terminal frame-shift nor the C-terminal deletion of Npr mature domain affected the secretion of Npr derivative (Figure 8). It had previously been observed that, although the integrity of propeptide is not essential for the export of the pro-

Npr derivatives, removal of propeptide while preserving intact mature region completely abolished secretion [14]. Thus, in contrast with the propeptide, if the mature region is directly fused with the signal peptide it remains incompetent for secretion. This dependence on the propeptide sequence for secretion might be due to structural incompatibility between the C-terminus of the signal peptide and the N-terminus of the mature domain, or possibly to the fact that signal peptide alone, without the help of the propeptide, cannot maintain the Npr mature region in an export-competent folded state. The presence of two positively charged residues at the N-terminus of the Npr mature sequence (positions 4 and 5), which has been suggested to be detrimental to protein secretion [35,42–45], is consistent with the first hypothesis. Nevertheless the second view is also very likely, because one of the major functions of the propeptide in protease is to act as an intramolecular chaperone for the modulation of protein conformation [48,49].

In summary, on the basis of our previous work [13,14] and results presented here it is clearer that in the processes of Npr secretion and maturation, the three distinct domains of Npr are essential and confer different functions. Although a functional signal peptide is essential for secretion, the propeptide guides the precursor domains to achieve a conformation that is competent for the export and maturation of Npr. Finally, the properly folded mature region yields the Npr activity and removes the propeptide sequence. Therefore the secretory mechanism of Npr precursor in *Streptomyces* is fundamentally analogous to the transport of precursor protein across the cytoplasmic membrane of Gram-negative bacteria in that both are signal-peptide-dependent and require a transport-competent conformation of precursor protein.

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