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THE LEADER SEQUENCE OF STREPTOKINASE IS RESPONSIBLE FOR ITS POST-TRANSLATIONAL CARBOXYL-TERMINAL CLEAVAGE

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SUMMARY: When the expression of streptokinase from two tac promoter-controlled expression vectors, one of these deleted a putative leader sequence of streptokinase and the other not, was compared, both normal and degraded streptokinase were detected in proteins expressed from the leader-contained vector, but only normal streptokinase was detected from the leader-deleted vector. These findings indicate that the characteristic carboxyl-terminal cleavage of streptokinase is correlated with its leader sequence and occurs during the defective secretion. The homogeneous preparation of streptokinase was facilitated by expressing from this leader-deleted vector.

Streptokinase (SK) is an extracellular secretory protein produced by many strains of hemolytic streptococci, which lyses blood clots by activating the plasma zymogen, plasminogen, to the fibrinolytic enzyme, plasmin. Unlike other plasminogen activators, it does not possess the inherent ability to catalyze the proteolytic cleavages necessary to convert plasminogen to plasmin (1). By the interaction of SK and plasminogen, the proteolytic active site is generated in the plasminogen moiety (2), which then activates the free plasminogen to plasmin. We have cloned the SK coding gene (skc) from Streptococcus equisimilis ATCC9542 (3), which was almost identical to the skc gene cloned by Malke and Ferretti (4), and prepared three single amino acid-substituted SKs to understand the structure-function relationship between SK and plasminogen (5). However, when the cloned skc gene was expressed in Streptococcus sanguis, the

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Abbreviations: bp, base pairs; IPTG, isopropyl β-D-thiogalacto-pyranoside; kD, kilodalton; OD, optical density; SD, Shine-Dalgarno; SDS, sodium dodecyl sulfate; SK, streptokinase.

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expressed SK had a molecular weight of about 44 kD by the posttranslational proteolysis of carboxyl-terminal residues (6, 7), whereas the molecular weight of the native SK is 47.4 kD. And it was also found that the expressed SK in Escherichia coli, Bacillus subtilis or S. equisimilis appeared as mixed forms of 44 and 47.4 kD (8). In this paper, we report the finding that the characteristic posttranslational cleavage of SK is closely connected with its putative leader sequence.

MATERIALS AND METHODS

Materials: Enzymes used in DNA manipulation were purchased from New England Biolabs (Beverly, U.S.A.); isopropyl β-D-thiogalactopyranoside (IPTG) from Promega (Madison, U.S.A.). The preparation of human plasminogen, standard SK and anti-SK polyclonal serum were described before (5). Oligonucleotide adapter: (EcoRI) 5'-AATTCCATGATTGCTG-3'
3'-GGTACTAACGACCTG-5' (AvaII)
was synthesized by a DNA synthesizer.

Bacterial Strains and Plasmids: E. coli JM107 was used for plasmid construction and gene expression. Plasmid pSK2.5 is the plasmid pBR322 carrying skc gene which was cloned previously in the PstI site (3), and the expression plasmid pKK223-3 was obtained from Pharmacia (Uppsala, Sweden). Recombinant DNA procedures were conducted as described by other (9).

Expression System: E. coli JM107 was used as the host for the expression vector. Colonies were picked, grown for 12 hr at 37°C in 3 ml of LB media containing ampicillin (50 μg/ml) and transferred in 50 ml of LB media containing ampicillin for 2.5% inoculation. When OD_{600} of the culture was about 1.0, the cultures were induced by addition of final 1 mM IPTG and then incubated for 3 hr at 37°C. The cells were pelleted, suspended directly in Laemmli sample buffer (10) and heated for 10 min in the boiling water bath. Following centrifugation, the clear supernatant was subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (10). Western blotting of the transferred proteins onto a nitrocellulose paper was carried out by the method of Burnette (11) with slight modification.

RESULTS AND DISCUSSION

To construct a expression vector for leader sequence-deleted SK, the 1370 bp skc AvaII fragment was isolated from cloned skc gene and ligated into EcoRI digested pKK223-3 with a short synthetic oligonucleotide adapter (Fig. 1A). The 1370 bp skc fragment contains almost all of the SK structural gene and its transcription terminator, but lacks its putative promoter, SD sequence, leader sequence and first 2 codons of processed SK (Fig. 1B). A initiation codon and first 2 codons of processed SK were recovered by the insertion of a oligonucleotide adapter (see Materials and Methods). When pKS601 was expressed in E.

Fig. 1. (A) Schematic representation of streptokinase expression vector pKS601. The closed and open boxes represent the coding regions of streptokinase (skc) and β-lactamase (bla), respectively. The cross-hatched arrows correspond to the tac promoter and rrnB T_1T_2 transcription terminator, respectively. Ec, EcoRI; Hd, HindIII; PvI, PvuI; PvII, PvuII; Sl, SalI; and Hd...Ec, multicloning site of pKK223-3 (HindIII-PstI-SalI-BamHI-SmaI-EcoRI). (B) Comparison of the translation initiation and N-terminal region of streptokinase carried on pSK2.5 and pKS601. The leader sequence of streptokinase is represented by italic letters. The bent arrow represents the N-terminal of processed streptokinase. The asterisks and the thick underline represent the proposed SD sequence and translation initiation codon, respectively.

coli JM107 by IPTG induction, a new protein of about 47.4 kD was accumulated (Fig. 2A). By the Western blotting with rabbit anti-SK antibody, the accumulated proteins were identified as the newly induced SK and its molecular size coincided with that of standard SK (Fig. 2B). Surprisingly, in contrast with SK expressed from original skc gene in E. coli, B. subtilis or S. sanguis, SK expressed from this leader sequence-deleted vector

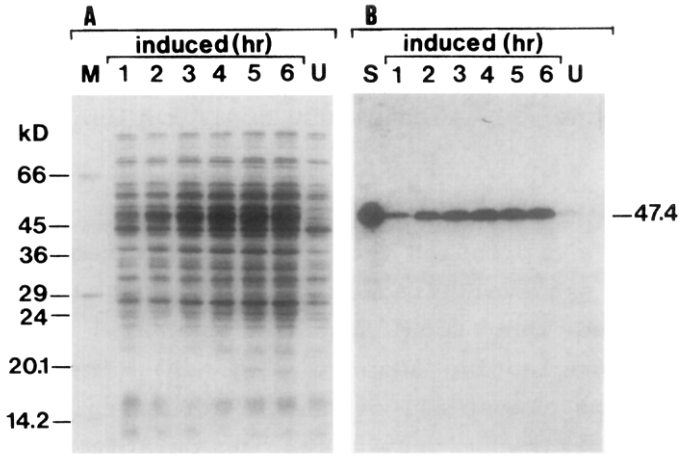

Fig. 2. SDS-polyacrylamide gel electrophoresis (A) and Western blotting (B) of the total cellular proteins of E. coli JM107 carrying pKS601 after IPTG induction. Lane 1-6, 1 mM IPTG-induced cell lysates according to the induction time (hr); lane U, uninduced cell lysate; lane M, protein molecular weight size markers; lane S, standard streptokinase.

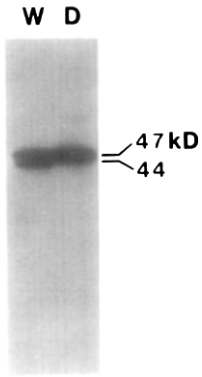

Fig. 3. Western blotting of the total intracellular proteins of E. coli JM107 carrying pOS421 (lane W), the leader sequence-contained vector, and pKS601 (lane D), the leader sequence-deleted vector, after IPTG induction.

showed only a single 47.4 kD protein band and no degraded 44 kD band.

To confirm the effect of putative SK leader sequence to the characteristic posttranslational cleavage, we also constructed a leader sequence-contained SK expression vector, pOS421, in where only the putative promoter was replaced with a *tac* promoter and its putative SD and leader sequence were remained. The SK expression level from leader sequence-contained pOS421 vector was very low and the culture was lysed immediately after IPTG induction because of the pronounced deleterious effects of wild-type *skc* leader sequence (8). As shown in Fig. 3, SK expressed from pOS421 (lane W) showed both normal 47.4 kD and degraded 44 kD SK, but SK expressed from pKS601 (lane D) showed normal 47.4 kD SK only. This suggests that the characteristic cleavage of SK is leader sequence specific. The possibility of proteolytic resistance of expressed products by forming inclusion bodies was excluded by the following reasons; 1) no inclusion bodies were found by microscopic observations, 2) almost all fraction of expressed SK was soluble in cytoplasm, and 3) the SK activity was increased according to the increase of expressed proteins. Recently, Muller *et al*. (8) found that *skc* and *prlA* (*secY*) gene, which regarded as a receptor for the signal sequence (12), are incompatible and this incompatibility is related to deleterious effects of the SK signal sequence. We concluded that the characteristic carboxyl-terminal cleavage of SK is correlated with its putative signal sequence and happened during the process of defective secretion. By expressing from

this leader-deleted vector, the homogeneous preparation of SK was facilitated.

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