The Role of an Activating Peptide in Protease-mediated Suicide of Escherichia coli K12∗

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Activation of latent proteinases ensures that the timing of proteolysis is regulated precisely, a process that generally involves proteolytic excision of a pro-region or a tightly bound inhibitor. Here we define the activation mechanism for Lit, a dormant suicide proteinase in Escherichia coli K-12. Previous work has shown that Gol, a short sequence within the major capsid protein gp23, activates Lit during the latter stages of T4 phage infection. This results in cell death and exclusion of the phage from the culture. The Lit site specifically cleaves the host translation factor EF-Tu (elongation factor Tu) after it has formed a weak complex with Gol, which can be supplied as a 29-residue peptide. Gol is absolutely required for Lit activation, but its role in proteolysis is unknown. Using a purified three-component system and kinetic analysis, we demonstrate that under physiological conditions Lit hydrolyzes its substrate very slowly (kcat of ~1 s⁻¹). Given the abundance of EF-Tu in the cell, this finding is consistent with a cell-killing mechanism in which a few cleaved EF-Tu proteins are able to block translating ribosomes from functioning. We also demonstrate that less than half of the 29 Gol residues are needed for Lit activation and that the role of the peptide is not to provide catalytic groups but to influence catalysis indirectly through stabilization of the ternary Lit-Gol-EF-Tu complex. Hence, phage-elicited suicide of E. coli K-12 by Lit is a variant form of “cofactor-induced activation,” a mechanism of protease activation that has only been documented previously in pathogen subversion of mammalian hemostasis cascades.

Bacteriophages are believed to be the most abundant organisms in the biosphere, outnumbering bacteria by an order of magnitude in many environments (1). As many as 10²⁸ bacteriophage infections occur per second, giving rise to a huge number of opportunities for DNA recombination between host and pathogen that have been occurring for ~3 billion years (2, 3). Temperate bacteriophages are important mediators of horizontal gene transfer and may contribute up to 20% of a bacterial genome (4), often providing genes that confer a selective advantage to the host cell. These advantages include the antigenic variation of lipopolysaccharide, the production of extracellular enzymes or outer membrane proteins, or the encoding of exotoxins implicated in bacterial pathogenicity (5, 6). An example is the conversion of non-pathogenic commensal Escherichia coli to Shiga toxin-producing strains by bacteriophage during infection by E. coli O157:H7 (7).

Because of the significant number of bacteriophage infections, bacteria that have developed or acquired mechanisms of resistance to phage or the means for preventing production of phage progeny have a distinct advantage in such competitive environments. Some temperate phages encode toxins that prevent infection by competing bacteriophages in a process called bacteriophage exclusion. This is achieved through the activation of a prophage-encoded toxin that mediates bacterial cell death during viral infection, thereby preventing viral propagation (8, 9). Three such mechanisms have been identified in E. coli: (i) Rex, a pore-forming toxin encoded by λ phage; (ii) PrrC, a RNase that targets the anticodon loop of aminoacyl-tRNA⁹⁰⁰⁰⁰; and (iii) Lit, a peptidase that cleaves the host translation factor EF-Tu¹¹ (10).

The molecular basis of bacteriophage exclusion is poorly understood. Rex is a two-component system with the cytoplasmic sensor RexB proposed to activate the channel-forming toxin RexA, thus de-energizing the cell in the early or middle phase of T4 infection (11). Rex toxicity induces a stationary phase-like state from which some cells recover after viral infection (12, 13). PrrC (or anticodon nuclease) is an RNase that is activated by a viral gene product called Stp. In the absence of viral infection the anticodon nuclease is inactive because of the formation of a latent complex with the EcoprrI DNA restriction modification system, giving the EcoprrI-PrrC complex or latent holoenzyme (14, 15). Stp is a small nuclease inhibitor that activates the anticodon nuclease activity through its interactions with the DNA restriction-modification endonuclease, EcoPrrI. This interaction modulates the conformation of the latent holoenzyme, inducing the anticodon nuclease activity of PrrC that cleaves amino acid-tRNA⁹⁰⁰⁰⁰, although the molecular basis of this activation mechanism is not known (10, 14, 15).

Lit is a metallopeptidase encoded by e14, a cryptic prophage element in the E. coli K-12 genome that excludes T-even bacteriophages (16–18). It was identified as an agent that inhibited T4 late gene expression, giving rise to its name late inhibition of T4 (19, 20). Lit is constitutively expressed in E. coli but lies dormant in the absence of its activator, the Gol sequence of the T4 major capsid protein gp23 (21). Following activation, Lit cleaves after glycine in the RGITI motif of EF-Tu, part of the conserved switch 1 region of the GTPase domain. This single cleavage inhibits protein synthesis and blocks viral replication (22).

The Gol region of gp23 was identified by mutations that enable T4 replication in the presence of Lit or grow on Litt (Gol)
(20, 23, 24), although how these mutations decrease Lit activity remained unclear. Subsequently, a peptide mimic of the gol region was shown to activate Lit cleavage of EF-Tu in vitro (21), first forming a weak binary complex with the substrate (25). In this way, the Gol region may be contributing to catalysis through the provision of catalytic groups, the allosteric activation of Lit, or simply through the completion of the molecular surface required for the recognition of EF-Tu.

In the present study, we address the role of the activating Gol sequence in Lit-mediated cleavage of EF-Tu through alanine-scanning mutagenesis and kinetics. We determine how Gol activates Lit and provide explanations as to how some of the original gp23 gol mutants exert their effect. Using a more active preparation of Lit than hitherto reported, we also determine the EF-Tu substrate dependence for the enzyme. These data are discussed in the biological context of Lit-mediated phage exclusion in E. coli and the more general problem of why proteins and peptides bind to translation factors, particularly during viral maturation. Finally, we address the similarities and differences between Gol activation of Lit and other cases of cofactor-induced activation.

EXPERIMENTAL PROCEDURES

Synthetic Gol peptide was prepared chemically by Affiniti Research Products Limited (Manheud, UK). Purification of EF-Tu was performed essentially as described (26).

**Purification of Lit**—Lit was purified from inclusion bodies by a modified procedure of that originally described (21). BL21(DE3) + pJL2901 (21) were grown at 37 °C to an A600 of ∼0.6 and induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 4 h. Cells were harvested by centrifugation at 8000 × g for 20 min, washed twice by resuspension in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 5 mM 2-amiinoethylbenzenesulphonyl fluoride and sonicated. The Lit-containing insoluble fraction was collected by centrifugation at 12,000 × g for 20 min, washed twice by resuspension in 50 mM Tris-HCl, pH 8, 1 mM DTT, and 4 mM 2-aminoethylbenzenesulphonyl fluoride and dialyzed against 50 mM Tris-HCl, pH 9, 1 mM DTT, and 1 mM urea, followed by 50 mM triethanolamine, pH 7.5, 1 mM DTT, and 1 mM urea. The final dialysis step was 50 mM triethanolamine pH 7.5, 1 mM DTT, and 5 mM ZnCl2, and further purification by size exclusion chromatography in the same buffer. Active fractions were determined by an in vitro cleavage assay, pooled, concentrated by ultrafiltration, and stored until use in 50% (v/v) glycerol at −20 °C.

**Purification of Im9Gol**—The construction of the imm9Gol gene will be described in detail elsewhere. The Im9Gol fusion protein and its mutants were purified by overexpression in BL21 (DE3) transformed with pLGmGol (pET11c containing the imm9Gol gene). The cells were grown at 37 °C until induction by 1 mM isopropyl-1-thio-β-D-galactopyranoside at an A600 of ∼0.6 and incubated for a further 3–4 h at 30 °C before harvesting (8000 × g for 10 min). Cells were resuspended in 50 mM Tris-HCl, pH 7, 10 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, and 1 mM DTT and disrupted by sonication. The supernatant was subsequently clarified by centrifugation at 40,000 × g for 30 min before the addition of ammonium sulfate to 35% saturation. The precipitate was removed by centrifugation for 10 min at 12,000 × g and resuspended in 50 mM Tris-HCl pH 7, 1 mM DTT, and 10 mM EDTA. The resuspended protein was subsequently dialyzed into 50 mM Tris-HCl, pH 7, 100 mM NaCl, 10 mM EDTA, and 1 mM DTT. A 15-ml Q-Sepharose fast flow column (Amersham Biosciences) was pre-equilibrated in the same buffer, and Im9Gol was eluted using a gradient of 50–600 mM NaCl. Pooled fractions after analysis by SDS-PAGE were concentrated and applied onto a HR 20/60 preparative grade Superdex 75 column (Amersham Biosciences) and equilibrated in 50 mM Tris-HCl, pH 7, 150 mM NaCl, and 1 mM DTT. After size exclusion chromatography, the fusion protein was >95% pure as judged by SDS-PAGE. Protein concentration was determined by a Bradford assay. Mutagenesis of the gol region was performed using the Strategene QuikChange method with suitable primers synthesized by MWG Biotech.

**Kinetic Analysis**—Rates of EF-Tu cleavage were determined under pseudo-first order conditions as described previously (18, 21, 25), except that 10 μM Gol peptide and 5 μM Im9Gol were used to activate Lit. To elucidate the kinetics of Lit activation, the concentration of Gol peptide or Im9Gol was varied between 0.05 and 50 μM while maintaining the concentration of Lit and EF-Tu at 0.2 and 2 μM, respectively. At each concentration of Gol, the observed cleavage rates were determined and plotted against the concentration of activator, and the data were fitted to Equation 1,

\[

E + G_{\text{act}} = k_{\text{cat}}/K_m \text{Gol} + [\text{Gol}]
\]

where \( V_{\text{cat}} = \frac{V_{\text{cat}}}{K_m} \text{Gol} + [\text{Gol}] \)

**RESULTS**

Lit Is a Poor Enzyme—EF-Tu is one of the most abundant proteins in a bacterial cell with a concentration of 0.1–0.2 mM (27). An important consideration in understanding T4 phage exclusion by Lit is how such a high concentration relates to the substrate dependence of this suicide proteinase. Past attempts at evaluating this issue have been hampered by the relatively poor solubility of Lit, a zinc metalloenzyme. We have devised a new protocol for the solubilization of Lit inclusion bodies using urea and zinc (see “Experimental Procedures”). As a result, the specific activity of the enzyme is an order of magnitude greater than that previously reported, allowing for the first time an evaluation of substrate dependence at low concentrations of enzyme (20 mM).

To determine the kinetic parameters for EF-Tu cleavage, its concentration was varied over a 250-fold range, spanning an order of magnitude above and below \( K_m \). The activating Gol peptide was kept at 10 μM, which gives maximal activation of Lit under these conditions (18). The initial rate data for each concentration of EF-Tu was fitted to the Michaelis-Menten equation, which yielded values for \( V_{\text{max}} \) and \( K_m \) of 5.6 μM and 1.3 s⁻¹, respectively, at 30 °C.

Similarly, to determine the kinetics of EF-Tu cleavage by Lit-Gol, the Lit concentration was reduced to 10-fold (0.02 μM), and the concentration of EF-Tu varied between 0.2 and 50 μM using the optimum concentrations of Lit-Gol peptide (21). For concentrations of EF-Tu <1 μM, acetone precipitation was required to visualize the reaction products by SDS-PAGE. The initial rate of EF-Tu cleavage was corrected for enzyme concentration, and the data were fitted to the Michaelis-Menten equation using Sigmaplot (SPSS, Inc. Chicago, IL).

**Fig. 1. Lit displays hyperbolic kinetics for EF-Tu cleavage.** Rates of EF-Tu cleavage were obtained (in duplicate) from the percentage of cleaved-to-uncleaved substrate, estimated by laser densitometry of Coomassie-stained SDS-polyacrylamide gels over a range of EF-Tu concentrations (0.2–50 μM) and where Lit and Gol concentrations were 0.02 and 10 μM, respectively (see “Experimental Procedures”). Data were fitted to the Michaelis-Menten equation, which yielded values for \( K_m \) and \( k_{\text{cat}} \) of 5.6 μM and 1.3 s⁻¹, respectively, at 30 °C.
bacteria, is a relatively poor enzyme, hydrolyzing just one molecule of EF-Tu per second. It is difficult to reconcile such slow kinetics on an abundant substrate with the ability of Lit to exclude T4 from infected cells in a process that takes place in minutes without invoking some other aspect to the cell death mechanism. We return to this issue under “Discussion.”

Alanine-scanning Mutagenesis Defines the Role of the Gol Peptide in Lit Activation—The 29-residue Gol peptide forms a weak binary complex with EF-Tu (K_i of ~300 μM), interacting preferentially with the GDP-bound form and inhibiting the intrinsic GTPase activity of the translation factor (25). More recently, a study of the Gol concentration dependence of EF-Tu cleavage by Lit showed that whereas the peptide activates Lit at low concentrations (K_m of ~1 μM), at higher concentrations Gol inhibits Lit in a manner analogous to substrate inhibition (K_m = ~100 μM) (18). The activation K_m reflects the concentration of Gol that achieves 50% activated cleavage of EF-Tu at 2 μM EF-Tu. This value is significantly lower than the equilibrium dissociation constant for the complex of EF-Tu and Gol, suggesting that the affinity of the Gol peptide for the EF-Tu-Gol-Lit ternary complex is much higher than that of the binary substrate complex.

Having established a system whereby steady-state kinetics can be used to evaluate Lit activation by Gol (18), a combined mutagenic and kinetic strategy has been used to analyze the role of the Gol region in Lit activation. To facilitate the rapid purification of mutants, the Gol region of T4 gp23 was fused to the C terminus of the endonuclease inhibitor protein Im9, which provides protection to bacteria during colicin E9 production (28). Im9 is a small and highly soluble protein, making it an ideal fusion partner. The ability of the Im9Gol fusion to activate Lit-mediated EF-Tu cleavage was compared with that of the isolated Gol peptide. Im9Gol was slightly more effective in activating cleavage, as determined by the lower activation K_mGol (~0.3 μM) as compared with 1 μM for the isolated Gol peptide; Fig. 2). This result is caused by the additional six residues of gp23 present within the fusion protein (35 residues compared with 29 for the synthetic Gol peptide) and not due to the presence of the Im9 protein, because exogenous Im9 had no effect on the ability of the peptide to activate EF-Tu cleavage (data not shown).

Alanine scanning was performed on the 29 residues of the previously identified Gol region using the Im9Gol fusion protein to generate the mutants. The effect of each Gol alanine mutant (or Gly for Ala) on Lit activity was determined relative to the wild type sequence under standard EF-Tu cleavage conditions (18, 21, 25). Almost half of the mutations (13/29) reduced Lit activation significantly (~50% relative to wild type Im9Gol), and these were largely restricted to the central 20 residues of the Gol sequence (residues 98–117; Fig. 3A). The 13 mutation sites generally fall into two types, those that reduced Lit activation significantly (M98A, V99A, L100A, I101A, A108G, C112A, V114A, Q115A, and I117A). These included six of seven residues that had the largest effect on Lit action (P104A could not be purified in high enough yield because of degradation of the fusion protein during purification) and half of the remaining residues (R101A, C112A and Q115A). For comparison and to validate the kinetic analysis, F109A, a Gol mutant that displayed wild type levels of activity, was also analyzed (Fig. 3A).

The activation parameters V_maxGol and K_mGol for F109A were...
Activation of Lit-mediated Bacterial Suicide by Gol

The role of the activating Gol sequence in Lit-mediated cleavage of the nucleotide-binding domain of EF-Tu has been established through a combination of kinetics and mutagenesis. Using more active enzyme than was previously available, Lit shows no sign of cooperativity either in terms of its EF-Tu substrate or activating Gol peptide dependence. The substrate $K_m$ for EF-Tu in the presence of Gol is in the low micromolar range, ~2-orders of magnitude lower than the apparent dissociation constant for the binary substrate complex of EF-Tu bound to Gol. This finding implies that the Gol peptide engages in bridging interactions between Lit and its substrate, EF-Tu, resulting in a higher affinity ternary complex. The Gol peptide-binding site on EF-Tu has been shown by chemical cross-linking to span two of the three domains of the translation initiation factor.

**Table 1**

| Gol mutant | $K_m^{Gol}$ | $V_{max}^{Gol}$ | $V_{max}^{Gol}/K_m^{Gol}$ | Effect |
|------------|------------|----------------|--------------------------|--------|
| WT         | 0.32 ± 0.1 | 1.18 ± 0.08    | 1                        | –      |
| M98A       | 1.9 ± 0.2  | 0.06 ± 0       | 0.008                    | –      |
| V99A       | 4.9 ± 0.6  | 0.3 ± 0.01     | 0.016                    | –      |
| R101A      | 2.2 ± 0.5  | 0.5 ± 0.05     | 0.067                    | –      |
| I103A      | 10.2 ± 2.2 | 0.03 ± 0.01    | 0.001                    | –      |
| F109A      | 0.55 ± 0.2 | 0.8 ± 0.08     | 0.4                      | –      |
| D110A      | 5.9 ± 0.95 | 0.08 ± 0       | 0.004                    | –      |
| I111A      | 8.1 ± 1.1  | 0.16 ± 0.01    | 0.005                    | –      |
| C112A      | 16.5 ± 1.1 | 0.33 ± 0       | 0.005                    | –      |
| Q115A      | 7.1 ± 1.2  | 0.53 ± 0.02    | 0.02                     | –      |
| M117A      | 8.1 ± 1.9  | 0.57           | 0.019                    | –      |

**Conclusion**

The present alanine scan and the previous gol mutation data illustrate that the peptide mediates specific interactions within the Lit-Gol-EF-Tu ternary complex involving polar and charged interactions as well as several hydrophobic contacts. These combine to stabilize ternary complex formation and reduce the activation energy for hydrolysis of the Gly59–Ile60 bond in the EF-Tu GTPase domain by Lit. The specific nature of Gol peptide contacts within the ternary complex are further validated by the differential effects of the three alanine-to-glycine substitutions, two of which (A94G and A102G) have no effect on Lit activation, whereas one (A108G) reduces Lit activation, whereas another (A107G) reduces Lit activation.

**Discussion**

The role of the activating Gol sequence in Lit-mediated cleavage of the nucleotide-binding domain of EF-Tu has been established through a combination of kinetics and mutagenesis. Using more active enzyme than was previously available, Lit shows no sign of cooperativity either in terms of its EF-Tu substrate or activating Gol peptide dependence. The substrate $K_m$ for EF-Tu in the presence of Gol is in the low micromolar range, ~2-orders of magnitude lower than the apparent dissociation constant for the binary substrate complex of EF-Tu bound to Gol. This finding implies that the Gol peptide engages in bridging interactions between Lit and its substrate, EF-Tu, resulting in a higher affinity ternary complex. The Gol peptide-binding site on EF-Tu has been shown by chemical cross-linking to span two of the three domains of the translation initiation factor.
factor, but not the GTPase domain (25). Moreover, Lit preferentially cleaves the GDP-bound form of EF-Tu in which both the Gol binding site and the scissile bond are exposed and adjacent to each other (25).

Lit is a zinc-metallopeptidase requiring the characteristic HEXXH motif for catalytic activity (18). However, a key issue has been whether Lit has a full complement of catalytic residues or whether the additional interactions provided by the Gol peptide include the contribution of catalytic groups. Peptide bond hydrolysis by zinc-metallopeptidases generally involves general base catalysis, the metal ion functioning as a Lewis acid, with additional active site residues stabilizing the transition state. Consequently, the substitution of any such residue by alanine should abolish enzymatic activity; this is indeed the case when any of the conserved HEXXH motif residues within Lit are mutated (18). It is striking then that no residues within the activating Gol sequence have such an effect (Table I). Most elevate the activation $K_m$ suggesting the stability of the Lit-Gol/EF-Tu ternary complex has been compromised. Some mutations affect $V_{max}$ but by no more than 100-fold. Hence, the Gol sequence of gp23 does not furnish catalytic groups to the Lit enzyme.

The Gol polypeptide is not predicted to be helical as deduced by the program AGADIR (30), and, consistent with this prediction, it has no apparent regular structure in solution (21). In addition, the pattern of mutations from the alanine scan does not suggest a helical structure for the bound peptide. If the bound peptide has an extended or $\beta$-structure, then the 20 residues that encompass the 13 important mutation sites (highlighted by asterisks in Fig. 4) would span >60 Å, significantly greater than the distance required to span the ~40 Å Gol peptide binding site on EF-Tu (25). This suggests that the peptide may fold back on itself possibly in a $\beta$-turn structure. In this regard, it is interesting to note that of the 29 Gol residues, six are glycine or proline and all are conserved.

EF-Tu has a propensity to associate with peptides (31), an activity that has also been reported for the mammalian homologue EF-1$\alpha$, where the translation factor can bind nascent chains emerging from the ribosome (32). EF-Tu has also been reported to have chaperone activity, which helps to refold denatured proteins in vitro (33). These properties point to additional functions for translation factors, possibly as chaperones in protein assembly. Consistent with this possibility, human EF-1$\alpha$ is required for the maturation of the HIV virus through an association with the gag polyprotein (34), whereas EF-1$\beta$ has been shown to interact with herpes simplex virus 1-infected cell protein 0 (35), both associations causing the arrest of translation. Bingham et al. (25) have postulated that complexation between EF-Tu and Gol may be required for the maturation of the phage capsid. This is supported by recent work (36) in which EF-Tu was found to associate with the Gol region of T4 during protein synthesis, its cleavage by Lit blocking the translation of downstream reporters. Snyder et al. (36) proposed a model whereby the binding of EF-Tu to the Gol sequence arrests translation and allows the remainder of the gp23 polypeptide to bind the GroEL chaperone, which is required for capsid assembly before translation continues. This model has some similarities with the translational arrest mechanisms observed for the human immunodeficiency virus and the herpes simplex virus and points to a more general association between viral proteins and elongation translation factors that appears to be required for virus maturation. The Snyder mechanism (36) begins to explain how Lit is able to exclude T4 phage even though it is a slow enzyme hydrolyzing one of the most abundant proteins in the cell; Lit would only target EF-Tu molecules bound to Gol sequences that have emerged from the ribosome polypeptide exit tunnel, although how such cleavage events then poison the cell remains to be established.

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The Gol sequence is conserved across a broad spectrum of bacteriophages that target Gram-negative bacteria, including T4, KVP20, and Aehl, suggesting that Lit could exclude these phages. Gp23 proteins in bacteriophage are highly mosaic in structure, with blocks of conserved and variable sequence indicative of genetic recombination (37, 38). The Gol sequence is more conserved than the entire gp23 protein across this broad spectrum of phages (e.g. RB49 has 57% identity to T4 gp23 and 90% identity within the Gol region), hinting that some important functional or structural constraint has limited sequence variation in this region of the capsid protein. This possibility is further borne out by studies showing that certain classes of petite and giant (ptg) morphological mutants of T4 map within the Gol region (39). Protein-protein interactions are known to play an important role in the evolution of sequence variation at interfaces (40), and it is reasonable to assume then that the high sequence conservation of the Gol site may be due to its association with EF-Tu. This would explain why nearly all Gol

![Fig. 4. Correlation between sequence conservation of the Gol region and residues that affect Lit activation. Alignment of 10 gp23 sequences highlights how the Gol sequence is highly conserved among the bacteriophages that infect Gram-negative bacteria. Conserved sequences cluster into four groups, with Gol residues that are important for Lit activation found in three (indicated by asterisks). Five residues that have been identified previously as gol sites follow the same basic pattern. Almost all of the gp23 Gol residues that are important for Lit activation are completely conserved in T4-like bacteriophage.](http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/)
residues that are important for Lit activation correspond to highly conserved residues (Fig. 4).

Because the activation of Lit is dependent on the appearance of a peptide cofactor, suicide is prevented unless the cell becomes infected with a bacteriophage. This contrasts with the activation mechanism of most proteinases, where activation occurs through the proteolytic removal of a pro-region or a tightly bound inhibitor. In recent years a third form of proteinase activation has been elucidated, termed “cofactor-inducedzymogen activation.” Cofactor-induced zymogen activation resembles Lit activation by Gol in that a protein-protein interaction occurs between the inactive enzyme and a protein cofactor activates proteinase activity. This form of enzyme activation has, to our knowledge, only been documented in blood coagulation and fibrinolysis systems in which proteins secreted by bacterial pathogens subvert the normal proteolytic cascade mechanisms (41). The fibrinolytic activity of plasminogen, for example, is activated by the protein activators staphylokinase and streptokinase, whereas the coagulation cascade is initiated by protein activators staphylocoagulase and streptokinase, which activate prothrombin (41–43).

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