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PURIFICATION OF A PROPHAGE ENCODED EF-TU-SPECIFIC PROTEASE AND STUDIES OF ITS MECHANISM OF ACTIVATION

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ABSTRACT

PURIFICATION OF A PROPHAGE ENCODED EF-TU- SPECIFIC PROTEASE AND STUDIES OF ITS MECHANISM OF ACTIVATION

By

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Bacteria use a number of mechanisms to defend themselves against bacteriophage. The death of the infected cells to halt the spread of infection to healthy cells is one such survival mechanism. This survival mechanism is called phage exclusion. One of the best understood phage exclusion systems is *e14* exclusion of T4. In this system, a 34 kDa *Escherichia coli (E. coli)* protease (Lit) encoded by the cryptic DNA element, *e14* plays a crucial role. Upon infection of *E. coli* by phage T4, this protease cleaves the 43 kDa translation elongation factor Tu (EF-Tu), disrupting the translation machinery needed for phage growth and propagation. This proteolysis is activated by a 29 amino acid long phage polypeptide determinant (Gol) internal to the major head protein.

One of the structural genes for EF-Tu, *tuf*A, was cloned into an expression vector in such a way that the gene was over-expressed and the protein was fused to an affinity tag to facilitate its purification. Similarly, the PCR engineered gene for Lit protease was cloned into a vector that has a different affinity tag, over-expressed, and the fusion protein was affinity

purified. The 29 amino acid long phage polypeptide determinant (Gol) was chemically synthesized. When these three components were mixed together, the 43 kDa EF-Tu was cleaved to yield 37 kDa and 6 kDa fragments. Therefore, only three components are required for the cleavage reaction: Lit protein, EF-Tu and Gol peptide.

There are three possible mechanisms of Gol peptide activation of the proteolysis of EF-Tu by Lit protein: (a) Gol peptide may bind to EF-Tu and Lit protein recognizes this Gol-bound EF-Tu as a substrate for proteolysis; (b) Gol peptide may bind to Lit protein to make Lit protein active so that it can proteolyze EF-Tu; or (c) Gol peptide may bind both Lit protein and EF-Tu to bring about the proteolysis of EF-Tu. Results from this research work appear to favor (a) above.

Another question is what type of protease is Lit protein. Some evidence suggests that it may belong to the zinc metalloprotease superfamily. First, it has the signature sequence of zinc metalloproteases, HEXXH. Second, it is sensitive to inhibitors of metal enzymes and not to inhibitors of other types of proteases. Plasma emmission spectroscopy also shows the presence of some zinc in Lit protein. Finally, site-directed mutagenesis shows that the residues H and E in the HEXXH sequence could play a role in the conformation and or activity of Lit protein.

To The Blessed One At Whose Feet We Lay Our Burden

and

My Parents On Whose Shoulders I Have Stood

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INTRODUCTION

The study of the biology of bacteriophages, especially T4 and λ, has led to the discovery of numerous basic principles concerning replication, transcription, regulation and recombination. To give some examples, there is the classic work that provided the evidence that deoxyribonucleic acid is genetic material Hershey and Chase (1952); recombination within genes was demonstrated in the *rII* genes of phage T4 Benzer (1955); bacteriophages and their host bacteria were used to show that the genetic code works as redundant unpunctuated triplets Crick *et al.* (1961). The discovery of many phage enzymes in the early 1960s, which included such enzymes as polynucleotide kinase, DNA ligases, polymerases, phosphatases, and endonucleases, led to the rapid development of new technologies in molecular genetics and molecular biology.

Bacteriophages are viruses that infect bacteria. Although they are able to persist on their own, they are incapable of replication except within a bacterial cell. They are usually composed of DNA or RNA surrounded by a coat of protein. Their presence is usually demonstrated by the plaques they form on a lawn of bacteria which represent regions where bacteria have been killed and lysed by the infecting phage.

Bacteria have mechanisms in place for protecting themselves against bacteriophage attack. These mechanisms include phage exclusion systems in which the host cell is killed to prevent multiplication of the phage.

Although phage exclusion mechanisms have been extensively studied over the past fifty years, most are not well understood. This dissertation deals with one of the best understood phage exclusion mechanisms, the *e14* exclusion of phage T4.

While searching for *Escherichia coli* mutants that restrict the growth of T4 mutants that are deficient in the production of polynucleotide 5'-kinase, 3'- phosphatase, Cooley *et al.* (1979) isolated *E. coli* K12 mutants that were unable to support the late gene expression of T4. They called these mutants *lit* mutants (for late inhibitor of T4 development). However, certain rare T4 mutants are able to grow and form plaques on these *E. coli* K12 *lit* mutants Champness and Snyder (1982). These rare T4 mutants were named *gol* mut-ants (for grow on lit). The inhibition of late gene expression in wild type T4 was found to be due to the interaction between the Lit protein from the *E. coli* K12 *lit* mutant and either the RNA or peptide or both encoded by the *gol* region internal to the major head protein gene of T4, gene 23 Bergsland *et al.* (1990). The molecular basis for the inhibition of gene expression became apparent when Yu and Snyder (1993) found that extracts of cells in which Gol peptide had been induced in the presence of Lit protein were defective in translation and showed that this defect was due to the cleavage of EF-Tu. The cleavage site is between ^{59}Glycine and ^{60}Isoleucine in the sequence: Arg - Gly - Ile - Thr - Ile a 5 amino acid sequence conserved in translation elongation factors from prokaryotes to eukaryotes.

Delineation of the participants involved in a reaction in which crude cell extracts were used is not possible. Therefore, to be able to determine the components involved in the *in vitro* cleavage experiments of Yu, it is necessary to use purified proteins. The goal of my project was to purify the

proteins in the *in vitro* cleavage experiments and use these to determine the components involved in the Gol-induced Lit cleavage of EF-Tu. Experiments were also conducted to determine how Gol peptide activates the proteolysis of EF-Tu. Since Lit protein has the HEXXH sequence characteristic of zinc metalloproteases, experiments were done to answer the question: Is Lit protein a zinc metalloprotease? Experiments to determine what residues may be required for activity of Lit protein were conducted. From these experiments, a model is proposed for how the cleavage of EF-Tu by Lit protein is activated.

CHAPTER 1

LITERATURE REVIEW

Overview of Bacteriophage T4

Bacteriophage T4, a representative of the T-even phages, has been extensively studied. Entire books have been published on what is known about T4. Therefore this overview is merely a highlight of some of the more basic information about this phage.

Although the structure of T4 is more complex than those of most other bacteriophages, T4 is still basically the same as other viruses. T4 has an icosehedral head in which the large T4 DNA molecule is housed. This head is attached to a tubular tail that has fibers at its free end. T4 contains one of the largest known phage chromosomes. Its genome is a large, linear, duplex DNA molecule (165,000 base pairs). This large genome consists of about 200 genes, organized into extensive functional groups, some of which are referred to as "essential genes" and others, "non-essential genes". As the name suggests, essential genes are required for phage multiplication in laboratory strains of *E. coli*. Therefore, a mutation in an essential gene is usually deleterious to the phage. The large DNA molecule is packaged into the protective icosehedral protein head by the headful mechanism whereby enormously long DNA molecules, concatemers, resulting from multiple rounds of DNA replication and recombination are packaged into the head until the head is completely filled. The concatemer is then cut. The cutting and packaging of the concatemers is mediated by the products of terminase genes 16 and 17 Franklin and Mosig (1996). For these large DNA molecules to be made quickly, as required by the phage, actively metabolizing bacteria

are required. This leads to the question of how T4 infection of *E. coli* is achieved.

Since bacteriophages are not capable of independent multiplication, they require a bacterial host for growth. *E. coli* is the host for T4. In an overview of the T4 developmental program, Mathews (1994) lucidly lays out the events, factors and known mechanisms of phage infection. The first step in the infection process is phage attachment to the host cell, *E. coli.* T4 adsorbs to host bacteria very rapidly and efficiently by utilizing its tail fibers, the distal tips of which are made up of gp37 protein. Together with gp38, gp37 is a "host-range cassette" functional unit Snyder and Wood (1989). T4 tail fibers bind to specific receptor molecules in the bacterial outer membrane. In *E. coli* B, this receptor molecule is lipopolysaccharide (containing diglucosyl residues), whereas in *E. coli* K12, it is the OmpC protein. According to Bayer (1968a), the short tail fibers, made up of gp12, extending from the base plate "pin" the phage to the cell outer membrane. The large DNA molecule of phage T4 is then ejected from the phage head into the interior of the host cell. The mechanism of this ejection is not well understood, but it is believed to be brought about by the interaction of several proteins: gp18, gp19, gp5 and perhaps gp25. Within the cytoplasm of host *E. coli*, phage DNA has to be protected from endonuclease activity of *E. coli* enzymes. T4 protects the ends of the injected DNA from exonucleolytic degradation by binding gp2. T4 DNA is further protected from degradation by host cell enzymes by using a double modification of its DNA nucleotide, cytidine. First, cytidine is modified to hydroxymethyl-dCMP, and then it is glucosylated to β-D-glucosyl-hydroxymethyl-dCMP. Nucleases that degrade cytosine-containing DNA will not degrade this modified phage DNA.

The T4 developmental program is broadly divided into an early period and a late period.

Early Period

Immediately after infection of the *E. coli* host cell by phage T4, some *E. coli* genes are transcribed. The others are shut down, and phage T4 begins to use the host RNA polymerase (RNAP) to transcribe its own early genes. Phage T4 is able to use host RNAP in this manner because it modifies the host RNA polymerase core enzyme by ribosylating the alpha units of RNAP with the phage proteins, gpalt, gpmod, and an RNA polymerase-binding phage protein called RpbA. Some of the early phage genes to be transcribed are the nuclear disruption, *ndd*, genes. The product of these genes disrupts the chromosome of the host cell. Along with other T4 encoded nucleases, the product of the *ndd* gene causes *E. coli* DNA to be degraded to mononucleotides. These mononucleotides serve as precursor molecules for *de novo* synthesis of phage DNA during the replication stage which starts about six minutes after infection. The phage proteins that participate in the replication of phage DNA include DNA polymerase (gp43), proteins that enhance the processivity of DNA polymerase (gp45 and gp44/62), single-strand DNA binding protein (gp32), topoisomerase (gp39, gp52 and gp60), DNA ligase (gp30), RNaseH, and a host of helicases (Nossal and Alberts, 1983; Nossal, 1994). Phage DNA replication usually increases in rate for several minutes in preparation for the late stage.

Late Period

This period ushers in the transcription of T4 late genes whose translation products are required for making the structural components of the phage: the head, the tail and its fibers. The work of Epstein *et al.* (1963) and of Edgar and Wood (1966) showed that the late genes were not sequentially expressed but rather simultaneously expressed. However, the interaction of the gene products was sequential. The transcription of phage T4 late genes differs from that of *E. coli* genes in several ways. First, it occurs from late promoters that have only the -10 sequence, TATAAATA, upstream of the transcriptional start site as opposed to the usual -35 to -10 sequence of the σ^{70} or *sig*A promoters of *E. coli* Kassavetis and Geiduschek (1982), Christensen and Young (1982) and Elliott and Geiduschek (1984). Another difference is that transcription of T4 late genes requires replicating DNA because it is enhanced by components of the T4 DNA replication apparatus Herendeen *et al.* (1989). Finally, it is different because it requires a modified host RNAP Herendeen *et al.* (1990, 1992). So how are T4 late genes turned on?

The gp44 and gp62 proteins are clamp-loaders that load the gp45 sliding clamp on the DNA. The gp45 clamp binds to DNA polymerase and tracks with it along the DNA where it increases its processsivity. If the gp45 encounters RNAP bound to a late promoter, it activates transcription. Thus, the replication apparatus serves as a sort of "mobile enhancer" activating transcription from late promoters as it moves along the DNA, and making late transcription replication dependent.

Late transcription also requires that host RNAP be modified by binding two phage proteins, gp33 and gp55. The gp33 protein serves as a bridge between the gp45 sliding clamp and RNAP. The gp55 is a sigma factor, specific for the late promoters, that confers promoter specificity on the modified RNAP by out-competing host σ^{70}. Therefore, the modified RNAP recognizes only T4 late promoters Williams *et al.* (1987) and Malik and Goldfarb (1988).

The products of many of the late genes of the phage are involved in making the T4 phage particle. About 20 proteins are involved in making the head and another 30 in making the tail and associated structures. In all, more than 25% of the entire genome is devoted to assembly of the phage particle. Of the 11 proteins that make up the phage head, gp23 is made in larger amounts than any of the other late proteins with about 1000 copies of the protein in each head. The interaction of a region of this protein with a protease encoded by a defective prophage is the subject of this thesis.

e14 DNA Element

DNA elements are foreign DNAs integrated into the chromosome. They can be transposons, integrated plasmids or prophages. A prophage is a stable, inherited, noninfectious provirus form of temperate phage in which the phage DNA has become incorporated into and replicates with the host bacterial DNA. Some examples of prophages are: λ prophage, *E. coli prr*, and the *e14* element. Some of these prophages can be defective in that they lack some essential genes to make a phage. The *e14* DNA element of *E. coli* K12 is an example of a defective prophage. This element was discovered when Greener and Hill (1980) observed that the induction of the SOS repair

process in *E. coli* cells that have been exposed to damaging ultraviolet radiation caused a 14.4 kb fragment of covalently closed circular DNA to be excised from the *E. coli* chromosome. They named this DNA *e14* because it contains 14 kilobases of DNA. Hill *et al.* (1989) found that this *e14* DNA element was integrated into the *E. coli* K12 isocitrate dehydrogenase structural gene *(icd)* between *pur*B and *umu*C at 25 min in the *E. coli* chromosome. This integration is site-specific like the integration of λ and P4 phage. All of the prokaryotic DNA elements that integrate by site-specific recombination use enzymes that belong to the integrase family Campbell (1993). DNA element *e14* is not found in either *E. coli* B or C.

Many gene products are now known to be encoded by the *e14* element including a restriction modification system directed against methylated DNA, a DNA invertase similar to the invertase responsible for phase shift in *Salmonella,* and tail fiber genes related to those of phage λ. It is the presence of these tail fiber genes that has led to the conclusion that *e14* is a defective prophage, which has lost some of the genes required to make phage particles. Another of the genes carried by *e14* is *lit* which encodes the protease involved in phage exclusion and which is the subject of this dissertation.

Phage Exclusion

Usually, extra-chromosomal elements like prophages and plasmids confer on their host cells some advantage. It may be antibiotic resistance, increased ability to compete for scarce nutrients or simply improved survival rate in a hostile environment. Resistance to phage is one such advantage often conferred on the bacterial host that harbours a DNA element.

Bacteria use a number of mechanisms to defend themselves against bacteriophage. One such survival mechanism is called phage exclusion. All phage exclusions are superficially similar. A gene product made by the prophage or other DNA element kills the cell upon infection by another type of phage, thereby preventing the multiplication of the infecting phage and its spread to other cells. In all the known exclusions, the infection begins normally, but then a catastrophe occurs, gene expression stops and the cell dies. In most cases it is not known what causes the cessation of gene expression and what aspect of phage infection triggers it. Many phage exclusion systems have been studied over the past forty years, and they include:

(a) P2 *old* gene exclusion of phage λ

(b) F plasmid *pif* exclusion of wild type T7 and mutant T3

(c) λ *rex* exclusion of T4 rII mutants and many other phages

(d) *prr* element exclusion of T4 *rlī* and *pnk*⁻ mutants

(e) *e14* element exclusion of T4

Most phage exclusion systems are not well understood. For example, λ phage is excluded in *E. coli* carrying P2 phage because of the interaction between the products of *gam* and *red* genes of λ and the product of P2 *old* gene although the reason the cell dies is not known. Similarly, wild type T7 and mutant T3 are known to be excluded by the product of the *pif* gene of the conjugative plasmid, F, but again, the reason the cell dies is not known Molineux (1991).

T4 *rII* mutant exclusion by λ *rex*

One of the most extensively studied exclusion mechanisms is that due to the *rex* genes of λ prophage. Although λ *rex* exclusion of T4 *rII* mutants was the first to be described Benzer (1955), the mechanism of exclusion still remains unclear. The products of two genes, *rex*A and *rex*B genes of λ phage are required for the exclusion of T4 *rII* mutants Matz *et al.* (1982), Landesmann *et al.* (1982). After λ lysogens are infected by T4 *rII* mutants, the infection proceeds normally until about the time T4 DNA replication starts. At this point, a severe loss of membrane potential occurs, accompanied by a drop in cellular ATP levels Sekiguchi (1966), Snyder and McWilliams (1989), Parma *et al.* (1992). The loss of cellular energy is most severe if there are monovalent cations in the medium and if the pH is below 7 Garen (1961), Sekiguchi (1966), Brock (1965), Ames and Ames (1965). RexB protein has been proposed to be a membrane ion channel that allows the passage of monovalent cations. Its activation by the already activated RexA protein would reverse the membrane ATPase leading to a loss of cellular ATP Parma *et al.* (1992). While T4 *rII* mutants would be excluded under such conditions, wild type T4 would not be so affected. However, Shinedling *et al.* (1987) observed that the overproduction of *rex* gene products will cause even wild type T4 to be excluded.

T4 Exclusion by the *prr* element

One of the best understood phage exclusion systems is the *prr* system. The *prr* element is not found in most laboratory strains of *E. coli.* It was found in a clinical isolate of *E. coli*, CT196, where it is integrated at 29 min in the genetic map of the *E. coli* chromosome Depew and Cozzarelli

(1974), Abdul-Jabbar and Snyder (1984), Levitz *et al.* (1990). It was subsequently transduced into laboratory strains of *E. coli* Abdul-Jabbar and Snyder (1984). Wild type T4 can multiply in *E. coli* carrying the *prr* element because it has polynucleotide kinase and RNA ligase. T4 mutants lacking these enzymes cannot multiply in *E. coli* carrying the *prr* element. This was how the element was found Depew and Cozzarelli (1974), Sirotkin *et al.* (1978), Runnels *et al.* (1982), Abdul-Jabbar and Snyder (1984). Work done by the Kaufmann group and their collaborators led to the discovery of the molecular basis of the *prr* exclusion of T4. The *prr* element carries a gene, *prr*C, which encodes a ribonuclease specific for host lysine tRNA, $tRNA^{Lys}$. This ribonuclease cleaves $tRNA^{Lys}$ in the anticodon loop after T4 infection. Amitsur et al. (1987) have shown that the cleavage occurs immediately 5' of the anticodon in the anticodon loop, thereby leaving 5' hydroxyl and cyclic 2':3' PO_4 ends. This cleavage of $tRNA^{Lys}$ blocks translation and stops phage development. T4 that has polynucleotide kinase and RNA ligase can repair this cut and multiply in the cells possessing the *prr* element, while mutant T4 that lack these repair enzymes would not survive because translation has been blocked and so phage development is halted. If such mutants possess a second mutation that suppresses the original mutation, then the mutant T4 can multiply in cells that possess the *prr* element. Such mutations, termed suppressor of three prime phosphatase, *stp*, lie in a gene that encodes a small peptide that is 26 amino acids long. The Stp peptide is not essential for normal growth of the phage. So why does the phage make the peptide? Levitz *et al.* (1990) discovered that *prr*C gene which encodes the ribonuclease, is associated with a restriction-modification system. It was found that the *prr*C gene was sandwiched between the *hsd*R and *hsd*S genes

of the restriction-modification system. The *prr*C gene product is physically associated with the products of the *hsd*M, *hsd*R and *hsd*S genes. Amitsur *et al.* (1992) showed that in the presence of the restriction proteins, the activity of the ribonuclease appears to be masked. If exogenous Stp was added to this mix of restriction proteins and ribonuclease, the activity of the ribonuclease was restored. Synthesis of the ribonuclease from a clone containing only the *prr*C gene yielded an active but very unstable ribonuclease Morad *et al.* (1993). The Stp peptide also inactivates this type of restriction *in vivo*. Based upon these findings, Penner *et al.* (1995) proposed that the Hsd proteins mask the activity of the PrrC ribonuclease by binding to it, and in so doing, increases the stability of the protein so that any free PrrC ribonuclease is rapidly inactivated and is not available to cleave any $tRNA^{Lys}$. In Type I restriction-modification systems, both restriction and modification activities are contained in the same multisubunit complex. The Stp peptide may inactivate this complex by causing dissociation. Therefore when Stp peptide binds the Hsd proteins complex, it may dissociate the complex thereby removing the masking of PrrC by the restriction-modification proteins. PrrC is activated and can then cleave $tRNA^{Lys}$ thereby blocking translation and halting phage propagation.

Work in Snyder's laboratory has also focused on an exclusion system similar to the *prr* element exclusion system. It is the T4 exclusion by *e14* DNA element of *E. coli* K12 which is the subject of this thesis.

T4 Exclusion by e14 element

As mentioned, *e14* is integrated at 25 min in the genetic map of *E. coli* K12 chromosome Greener and Hill (1980). *E. coli* K12 mutants that were

deficient in their ability to support the late gene expression of T4 were isolated. The mutations were named *lit* (for late inhibition of T4) mutants Cooley *et al.* (1979). Kao *et al.* (1987) cloned and characterized the *E. coli* K12 *lit* gene and showed that the inability to plate T4 was due to the constitutive expression of this gene, named the Lit(Con) phenotype. Small fragments of the DNA element, *e14*, were found to confer the Lit(Con) phenotype on cells. These clones were sequenced and found to contain an open reading frame (ORF) that encoded a 34 kDa protein, the Lit protein. The inhibition of late gene expression in T4 was found to be due to the interaction between the Lit protein (the product of the *lit* gene of the *e14* element) and the Gol peptide, a small portion of the major head protein of T4 gene 23 Bergsland *et al.* (1990). The *gol* region was found through isolation of rare T4 mutations that overcame this defect in late gene expression. These rare mutations allowed T4 to form plaques in the presence of Lit protein, hence they were named *gol* (for grow on lit), Champness and Snyder (1982).

To determine the reason for the block in late gene expression, Bergsland *et al.* (1990) measured the rate of DNA, RNA and protein synthesis after induction of synthesis of the Gol peptide in the presence of Lit protein. They found that protein synthesis was specifically inhibited. DNA replication and RNA synthesis continued unabated. The question remained: what is happening to the translation apparatus when the Gol peptide is synthesized in the presence of Lit protein?

Yu and Snyder (1993) answered this question when they prepared extracts of cells in which translation had been inhibited and discovered that elongation factor Tu (EF-Tu) was cleaved. They could also show that cleavage of EF-Tu was probably solely responsible for the inhibition of

translation. The cleavage of EF-Tu occurs between Gly^{59} and Ile^{60} giving rise to 37 kDa and 6 kDa fragments.

Zinc Metalloproteases

Four major classes of proteases are known and are designated by the principal functional group in their active site. They are:

(a) Serine proteases: which have a serine residue in their active sites.

(b) Thiol proteases: which have a cysteine thiol group in their active sites.

(c) Carboxyl (acid) proteases: which have acidic residues in their active sites.

(d) Metalloproteases: which have a Group II metal in their active sites.

Since some evidence suggests that Lit protein might be a member of the zinc metalloprotease superfamily, I shall review the properties of such proteases in more detail. Metalloproteases that have Zn^{+2} in their active sites are called zinc metalloproteases. Although metalloproteases that have Cu^{+2} and Co^{+2} in their active sites exist, those that have Zn^{+2} in their active sites occur more frequently in nature. Zinc has been found to be an essential and integral component in over 300 enzymes. Zinc plays a role in both enzyme catalysis and structure. It plays a structural role when it stabilizes the structure of proteins and nucleic acids.

In zinc metalloproteases, zinc plays a catalytic role in proteolysis. One of the identifying characteristics of zinc metalloproteases is that they possess the **HEXXH** signature sequence. The number of zinc metalloproteases/peptidases identified in recent years has increased greatly Hooper (1994), Jongeneel *et al.* (1989). Using a scheme based on the comparison of the sequences around the **HEXXH** motif of the zinc-binding site, this super-

family has been classified into five distinct sub-families: (1) Thermolysin (2) Astacin (3) Serratia (4) Matrixin and (5) Reprolysin (snake venom) metalloproteinases. Two histidines and a glutamate are the zinc ligands in the thermolysin sub-family while three histidines and one tyrosine are the zinc ligands in the other four sub-families Jiang and Bond (1992). The non-thermolysin sub-families differ from one another in unique ways. For example, members of the astacin sub-family possess a glutamate following the third histidine which is used to form a salt bridge with the N-terminus of the mature enzyme. There is also a glycine residue that is important for secondary structure Bode *et al.* (1992). The serratia sub-family possesses a proline instead of the second glutamate of the astacin sub-family, while the reprolysin sub-family has an aspartic acid residue at position 12 away from the first histidine of the HEXXH motif. This sequence has been found in all clostridial neurotoxins whose sequences are available Schiavo *et al.* (1992), elastase of *Pseudomonas aeroginosa* Kawamoto *et al.* (1993), lethal factor of anthrax toxin of *Bacillus anthracis* Klimpel *et al.* (1994), insulin degrading enzyme in which the sequence is HXXEH, a mirror image of the standard HEXXH motif Perlman and Rosner (1994) and deformylase, encoded by *E. coli fms* gene Meinnel *et al.* (1995). In all these enzymes, this motif is required for the binding of the metal ion that is needed for the activity of the enzyme. The role zinc plays in catalysis has been studied in many zinc metalloproteases. Vallee and Auld (1990) reviewed the results of such studies. They suggest the following as a possible general mechanism for the catalytic activity of metalloproteases. In all zinc metallopeptidases for which crystal structures are known, the catalytic zinc atom is coordinated to three amino acid residues of the protein and an "activated" water molecule. A

combination of any three residues of His, Glu, Asp, or Cys creates a tridentate active zinc site although histidine is the most frequent ligand. An "activated" water molecule fills and completes the coordination sphere of the active zinc site. From the work of Vallee *et al.* (1983) and Auld and Vallee (1987) on carboxypeptidase A, Quiocho and Lipscomb (1971) and Schmid and Herriott (1976) on the bovine carboxypeptidase A and B enzymes, and Tan *et al.* (1980) on carboxypeptidase M, it is now believed that 2 His and a Glu are ligands to the zinc atom at the active site.

Activation of Proteases

Some proteases can be activated from inactive precursor proteins by processes that are not related to metal incorporation. Vallee and Auld (1990) proposed a model for how zinc metalloproteases could be activated from these longer precursors called the "Velcro model". In this model, the inactive precursor peptide is proteolyzed to remove a peptide from one end, resulting in the conversion of the zinc coordinated to four amino acid residues (tetradentate) to zinc that is coordinated to only three residues (tridentate) through the removal of a cysteine ligand, which is then replaced by water. Other models for the activation of proteases involve an exchange reaction or a conformational change. An example of an exchange reaction activating a protease is demonstrated by the adenovirus protease, Ad2, a thiol protease that is required for virus maturation. For this protease to be active, it requires an 11 amino acid long peptide determinant, GVQSLKRRRCF, derived from the C-terminus of the structural protein, pVI. Using its cysteine residue, this peptide participates in a disulphide exchange reaction with Ad2 to activate Ad2 by exposing the Ad2 active site cysteine Webster *et al.* (1993). The

cleavage of the largest subunit, p220, of eukaryotic translation initiation factor 4F (eIF-4F) in the presence of a functional poliovirus protease $2A^{pro}$ is another example of a thiol protease being involved in proteolysis. This proteolytic event requires the presence of eukaryotic translation initiation factor 3 (eIF-3). Wyckoff *et al.* (1990) proposed that eIF-3 - eIF-4F complex presents p220 in a proper conformation to be a substrate for cleavage by protease $2A^{pro}$. An example of conformational change as a regulator of protease activity is dimerization. Human immunodeficiency virus (HIV) protease, an aspartyl protease, is activated by dimerization Navia *et al.* (1989) and Krausslich (1991). The active HIV protease is composed of two copies of the HIV protease monomer linked to each other. The active protease proteolyses HIV polyprotein substrates. If dimerization occurs before the mature virus particle is formed, the processing of viral polyproteins will prevent particle formation and infectivity.

Role of Elongation Factor Tu in Translation

Because Lit protein cleaves EF-Tu I shall briefly review what is known about EF-Tu. The information contained in messenger RNA, mRNA, is converted or "translated" into protein. EF-Tu is one of the components of the protein translation machinery. Although translation may be divided into three steps: initiation, elongation and termination, I shall focus only on the elongation step. This step is brought about by the elongation factors, primarily, EF-Tu, EF-Ts and EF-G. There are two forms of EF-Tu: the active GTP bound form and the inactive GDP bound form. In its active GTP bound state, EF-Tu recognizes, transports and positions the codon-specified aminoacyl-tRNA onto the acceptor (A) site of the 70S ribosome that is

tracking along the mRNA Miller and Weissbach (1977). After the tRNA has bound and the GTP bound to EF-Tu is cleaved to GDP, the EF-Tu is released. EF-Tu is recycled when EF-Ts dissociates the EF-Tu-GDP complex and GTP binds again to EF-Tu. EF-G translocates the tRNA to the peptidyl (P) site to make room for another charged tRNA at the A site. Two tRNAs are, at this stage, attached to the ribosome, with the one at the P site having a growing polypeptide chain attached to it. The enzyme peptidyl transferase catalyzes the formation of a peptide bond between the amino acid attached to the A site tRNA and that at the P site. The P site tRNA then releases the polypeptide chain which is now attached to the A site. EF-G then translocates the tRNA and the polypeptide chain which is now one amino acid longer to the P site so that the A site is once again empty. The ribosome moves along the mRNA in the 3' direction to the next three nucleotides, a charged tRNA is once again brought to the A site by GTP bound EF-Tu and the process is repeated. In eukaryotes, the counterparts of EF-Tu and EF-Ts are eEF-1α and eEF-1β, respectively and the analog of EF-G is EF-2.

Structure and Function of Elongation Factor Tu

Elongation factor Tu (EF-Tu) has been the subject of extensive investigation since it was first discovered Lucas-Lenard and Lipman (1966). It is one of the most abundant proteins in the bacterial cell and accounts for 5-10% of the protein mass Van der Meide *et al.* (1980) and Jacobson and Rosenbusch (1976). *E. coli* EF -Tu is encoded by two almost identical structural genes *tuf*A and *tuf*B Jaskunas *et al.* (1975). The amino acid sequences encoded by the two genes differ only by one amino acid: TufA has a glycine at its carboxyl terminus while TufB has a serine Arai *et al.* (1980)

and Jones *et al.* (1980). The *tuf*A gene is the distal gene in the *str* operon which also contains the genes for ribosomal proteins S7 and S12 and EF-G Zengel and Lindahl (1990). The *tuf*B gene is in an operon for tRNAs. It is not known why there are two genes for EF-Tu, but the two genes may be regulated differently. The *tuf*A gene is expressed 50% more than the *tuf*B gene under normal growth conditions. The *tuf*B gene is regulated by ppGpp.

EF-Tu is a 43 kDa monomeric protein that contains 393 amino acid residues arranged in three domains: I, II, and III. Domain I is the nucleotide binding domain of EF-Tu. The crystal structure of EF-Tu revealed that domain I of EF-Tu is similar to that of other nucleotide binding proteins, especially those that have GTPase activity such the *ras* oncogene proteins, p21 Jurnak (1985), Wittinghofer (1993), Weijland *et al.* (1992) and Sprinzl (1994). It is made up of five β-sheets and six α-helices. Within this domain, there is a region that connects the first α-helix to the second β-sheet. This region was named the "effector region", L2, since its structure changes between GDP-bound and GTP-bound forms of EF-Tu. Domains II and III are made up of anti-parallel β-sheets exclusively, forming two β-barrels. In EF-Tu-GTP, the active form of EF-Tu, all three domains are tightly packed, whereas in the inactive form, EF-Tu-GDP, domain II is separated from domain I Sprinzl (1994), apparently to release the bound tRNA.

The cleavage of EF-Tu by the *e14*-encoded Lit protein occurs between Gly^{59} and Ile^{60} in the highly conserved sequence Arg-Gly-Ile-Thr-Ile found in elongation factors of both prokaryotes and eukaryotes. This sequence is in the carboxyl terminal part of the L2 region within the nucleotide binding domain. In EF-Tu, the arginine residue of this sequence is a trypsin-hypersensitive site while the threonine residue coordinates the Mg^{2+} ion and the γ-phosphate of

the EF-Tu bound GTP Berchtold *et al.* (1993). In all regulatory GTPases, this region is involved in binding the GTPase activating protein. In the case of EF-Tu, the ribosome functions as the GTPase activating protein Georgiou *et al.* (1998) and Zeidler *et al.* (1996). Ziedler et al. (1996), in studies on the effector region of EF-Tu of *Thermus thermophilus,* showed that the structural integrity of the L2 region of EF-Tu around the arginine in the sequence Arg-Gly-Ile-Thr-Ile, is important for the control of the GTPase activity by ribosomes. This structural integrity is not maintained when EF-Tu is cleaved by the Lit protein. Thus the cleavage may abolish the ability of ribosomes to stimulate GTPase activity.

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CHAPTER 2

PURIFICATION AND PRELIMINARY CHARACTERIZATION OF e14 ENCODED LIT PROTEIN: PRELIMINARY RESULTS SUGGESTING THAT LIT PROTEIN MAY BE A ZINC METALLOPROTEASE.

ABSTRACT Some strains of *Escherichia coli* harbour genes that trigger cell death upon infection by bacteriophage. The death of the infected cells halts the spread of infection to healthy cells ensuring their survival. This survival mechanism is called phage exclusion. One of the best understood phage exclusion systems is the *e14* exclusion of T4 caused by the interaction of the *e14* encoded protein, Lit, and a short polypeptide sequence encoded by *gol* from within the major head protein gene of T4. This interaction caused severe inhibition of all translation. Using purified proteins, we have shown here that translation inhibition is due to the activation of the proteolysis of the 43 kDa translation elongation factor Tu (EF-Tu) by the 34 kDa *E. coli* protease (Lit) encoded by the cryptic DNA element *e14*. The proteolysis is activated by the 29 amino acid long phage polypeptide determinant (Gol) internal to the major head protein of bacteriophage T4. This Lit-specific Gol peptide-activated proteolysis of EF-Tu leads to the disruption of the protein biosynthetic machinery needed for phage growth and propagation. A comparison of the sequence of Lit protein to known sequences shows that Lit protein has a sequence similar to the consensus sequence of zinc binding region of zinc metalloproteases. Here we present some evidence suggesting that Lit protein may be a member of the zinc metalloprotease superfamily, and that the consensus sequence His-Glu-Xaa-Xaa-His might play a role in the activity, stability or conformation of Lit protein.

Introduction

The phenomenon of phage exclusion has been known and studied for over 40 years and several phage exclusion systems are now well understood and characterized. Among the best understood of these systems is the *e14* exclusion of bacteriophage T4. The defective prophage *e14* is a DNA element integrated into the the isocitrate dehydrogenase gene in the chromosome of many *E. coli* K-12 strains (1). It encodes a 34 kDa protein, Lit (late inhibitor of T4) which proteolyses translation elongation factor Tu (EF-Tu) after infection by bacteriophage T4. Using purified proteins, we were able to show the cleavage of EF-Tu in a system involving Lit protein, chemically synthesized Gol peptide and EF-Tu (2). Also using purified proteins, we present evidence here that Lit protein may belong to the zinc metalloprotease superfamily, and that the characteristic zinc metalloprotease sequence His-Glu-Xaa-Xaa-His it possesses could play a role in its activity, stability or its conformation. In addition, we show that Lit protein is active even after being pre-incubated over a range of temperatures, and is inhibited by inhibitors of metalloproteases.

MATERIALS AND METHODS

Overproduction and Purification of EF-Tu. *E. coli* DH5α harbouring pGEX2T-*tuf*A (3), an EF-Tu over-producing clone in which the *tuf*A gene of EF-Tu was cloned downstream of the glutathione-S-transferase gene in the pGEX-2T vector (a gift from the Parmeggianni laboratory), was grown in 500 ml Luria Bertani (LB) broth supplemented with ampicillin (50 μg/ml) at

30°C with shaking. EF-Tu was overproduced by inducing the culture with isopropyl β-D-thiogalactoside (IPTG) (0.2 mM final conc.) for 3 hrs at mid-log phase during growth. Cells were harvested by centrifugation, re-suspended in buffer A: 50 mM Tris.Cl, pH 7.5/ 150 mM KCl/ 5 mM $MgCl_2$/ 1 mM DTT and disrupted by sonication. The suspension was then centrifuged at 12,000 x g for 1 hr at 4°C. The supernatant was loaded onto pre-washed Redipack glutathione column (Pharmacia) and allowed to stand at 4°C for 30 min. The column with the bound GST-EF-Tu fusion protein was washed several times with ice cold buffer A. The EF-Tu was eluted by developing the column with re-suspended thrombin according to manufacturer's instructions (Pharmacia). The glutathione-S-transferase remaining on the column was eluted with reduced glutathione. The protein was stored in 15% glycerol at -70°C. The EF-Tu was >90% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 1). Protein concentration was determined by the Bradford method (4), using bovine serum albumin as the standard. The yield was typically 25-50 mg/liter of culture.

Overproduction and Purification of Lit. To facilitate purification of Lit protein, the *lit* gene was cloned into the pET-30b vector (Novagen). This vector expresses cloned genes from the powerful promoter and the highly efficient ribosome binding site (rbs) from the phage T7 major capsid protein gene and also fuses the protein to a string of six histidines (His-tag) and an S-tag to allow its detection. To clone the *lit* gene in frame, flanking *Bam*HI sites were engineered by PCR onto the ends of a 998 bp *lit* gene fragment lacking its own promoter and rbs. The amplicon was cloned first into the *Bam*HI site

of pUC8 vector (Fig. 2). Colorless colonies were selected. The *lit* gene was then sub-cloned into the *Eco*RI/*Sal*I sites of pET-30b immediately downstream of the cleavable N-terminal His-tag/S-protein tag. The resulting plasmid was called pEKS (Fig. 3). The His-tag allows for a one-step protein purification using the nickel affinity column (Novagen) and the S-tag allows its detection. The pEKS plasmid was used to transform competent JM109DE3lit^0pLysS *E. coli* strain. Transformants were tested for over-production of Lit protein. *E. coli* strain JM109DE3lit^0pLysS containing the pEKS plasmid was grown in 120 ml of LB broth supplemented with kanamycin (50 μg/ml) at 37°C in a shaker water bath to mid-log phase. The culture was shifted to 23°C for 30 min and then induced with isopropyl-β-D-thiogalactoside (IPTG) (0.1 mM final conc.) to over-produce the fusion Lit protein. After 3 hrs., cells were harvested by centrifugation, re-suspended in 1X binding buffer: 20 mM Tris.HCl, pH 7.9/0.5 M NaCl/5 mM imidazole, and disrupted by sonication. The suspension was centrifuged at 12,000 x g for 40 min at 4°C. The supernatant was filtered through a 0.45 μm filter and then layered on a pre-equilibrated 2.5 ml nickel affinity column and allowed to stand for 20 min. The column with the bound fusion protein was washed with 1X wash buffer: 20 mM Tris-HCl, pH 7.9/0.5 M NaCl/60 mM imidazole. The fusion protein was eluted from the column by developing the column with 3 ml of 1X Wash buffer for 8 hrs. The fusion protein was stored in 15% glycerol in -70°C. The Lit protein was >90% pure as judged by SDS/PAGE (Fig. 4). Protein concentrations were determined as above. Typically, protein yield was 10-25 mg from 1 liter of cell culture.

Lit Activity Assays. *Standard EF-Tu in-vitro cleavage assay*. Lit protein, EF-Tu and chemically synthesized Gol peptide were combined in a total volume of 45µl at final concentrations of 0.466 µg, 2.55 µg and 0.2 mM respectively. This reaction mixture was incubated at 30°C for 30 min. after which it was stopped by addition of sodium dodecyl sulfate (SDS) gel loading buffer followed by boiling. The reaction products were analyzed on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) containing 13% acrylamide. The gel was electrophoresed at 60 volts for 3 hrs, fixed in 12% trichloroacetic acid and stained with 0.1% Coomassie blue. The depletion of the 43 kDa EF-Tu band and the appearance of a 36 kDa EF-Tu* band was indicative of Lit protease activity. The bands were quantified in the AMBISS laser densitometer.

RESULTS.

Effect of Temperature on Lit Stability. We have previously reported the cleavage of EF-Tu in a purified three-component system containing Lit protein, chemically synthesized Gol peptide and EF-Tu (2). Those studies also had shown that Lit protein functions enzymatically. One of the factors that affect enzyme function is temperature. We studied the effect of temperature on the stability of Lit protein by first incubating the Lit protein to be used for the *in vitro* cleavage assay at different temperatures for 15 min, transferring to 30°C for 2 min before performing the cleavage assay at 30°C for 30 min as previously described. The results were expressed as the percentage of EF-Tu cleaved. From these results shown in Figure 5, it is clearly evident that while temperature has an effect on the stability of Lit

protein, the protein is thermostable. Lane 4 shows that with incubation at 30°C, virtually all EF-Tu in the reaction was cleaved under these conditions. Although activity of Lit protein was reduced as the temperature of incubation was increased, as shown in Lanes 5 - 7, the reduction only became substantial from 65°C and higher. This suggests that Lit protein is thermostable.

Effect of Inhibitors on Activity of Lit Protein. Lit protein has the consensus zinc metalloprotease motif: His-Glu-Xaa-Xaa-His shown in Figure 6, that may suggest membership within zinc metalloprotease superfamily (5-10). If Lit protein is a zinc metalloprotease, it should be sensitive to inhibitors of metalloproteases such as ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline, while phenylmethylsulfonyl-fluoride (PMSF) a serine protease inhibitor, Leupeptin, a serine/thiol protease inhibitor, and Pepstatin A, an acid protease inhibitor, ought not inhibit its activity. However, these compounds do not inhibit all enzymes within the indicated classes and alternate inhibition are known. The effect of each of these different protease inhibitors on the activity of Lit protein was studied. Lit protein was first incubated at room temperature for 10 min with the following protease inhibitors, separately : EDTA (5 mM); 1,10-phenanthroline (0.1 mM); PMSF (0.5 mM); Leupeptin (50 μM); and Pepstatin A (50 μM). These samples were then used in the standard *in vitro* EF-Tu cleavage assay as previously described. Results shown in Figure 7 demonstrate inhibition of activity of Lit protein by the metal chelator, EDTA and 1,10-phenanthroline. Surprisingly, Lit protein activity was also inhibited by PMSF, an inhibitor of serine proteases. This would seem to suggest that Lit protein is a serine protease. It

is not clear why PMSF would have this effect on Lit protein. It seems unlikely that Lit protein could be a serine protease because it is not known to possess a catalytic triad. However, there is a case reported of a different metalloprotease being inhibited by PMSF. The urease isolated from *Staphylococcus xylosus* is a metalloprotease that contains threonine instead of cysteine, an amino acid located within a highly conserved domain of 17 amino acids, that is supposed to be a part of the enzyme active site (11). Neither Leupeptin nor Pepstatin A, inhibitors of thiol and acid proteases respectively, significantly inhibited Lit protein, suggesting that Lit protein is neither a thiol nor acid protease.

Analysis of Metal content of Lit Protein. If Lit protein is a zinc metalloprotease, it should contain zinc. The result of metal analysis using inductively coupled plasma emission spectroscopy is shown in Table 1. Less than 1% of the Lit protein contain a zinc atom. It is still possible that Lit protein is a zinc metalloprotease but , if so, it may have lost zinc during the purification based on this result. We have not made careful measurements of the percentage of Lit protein which is active when purified by this method.

Site-directed Mutagenesis of the ^{160}His-^{161}Glu-Xaa-Xaa-^{164}His **motif**. If Lit protein is a zinc metalloprotease, then changing the zinc metalloprotease motif would be expected to affect its activity. Using the PCR-based QuikChange site-directed mutagenesis method of Stratagene, the mutations: H160A (cat160gct), E161A (gaa161gca), were made. The template DNA used in both mutations is DNA of the over-expressing clone of *lit*, pEKS. The PCR products were used to transform the competent *E. coli* strain,

JM109DE3*lit*^{0}pLysS. 40% of all transformants tested *lit*^{0} (do not make Lit protein). These were selected. From these, "mutant" Lit protein was produced, purified and tested for activity in the standard *in vitro* EF-Tu cleavage assay as previously described. Figure 8 shows the result of the SDS-PAGE analysis. Lane 3 shows cleavage of EF-Tu as expected of the "wild type" Lit protein from the non-mutated over-expressing clone. In Lane 6, "mutant" Lit protein from E161A does not cleave EF-Tu. The mutation giving rise to E161A Lit inactivates the protease, consistent with the interpretation that Lit protein is a zinc metalloprotease. The mutation that yields H160A protein appears to significantly reduce the yield of Lit protein. Figure 8 shows dot blot of wild type Lit protein and mutant Lit proteins from H160A and E161A. From this figure, it is clear that more of the mutant Lit protein from H160A mutation is in the pellet, suggesting that it has gone into inclusion bodies. This suggests that the H160A mutation changes the conformation of Lit protein and makes it difficult to determine whether the ^{160}His is required for its protease activity. Also from this figure, it is clear that more of the wild type Lit protein and the mutant Lit protein from E161A mutation are in the soluble fraction. More changes e.g. (H160R, H160K, E161D, E161Q, H164R, and H164K) would be needed to fully study the effects of mutating this motif before a definite role for these residues can be ascribed. However, based on what is known of the chemistry of active sites of proteins that possess this motif, ^{160}H and ^{164}H may serve as metal ligands, while ^{161}E may function as a catalytic base (7-10).

DISCUSSION

We have purified both the translation elongation factor Tu (EF-Tu) and Lit protein (product of *e14* element of *E. coli* K12) from clones that over-express these genes. The purification of these proteins was facilitated by cloning their genes into cloning vectors that introduce affinity tags. This allows the resulting fusion proteins to bind preferentially to affinity columns and so permit a one-step purification.

Using the purified proteins in an *in vitro* cleavage assay reaction, we were able to show that Lit protein remained active as the temperature of pre-incubation was raised from 30°C to 55°C. The reduction in activity became substantial from 65°C and higher. This suggests that Lit protein is quite thermostable.

We were also able to show that Lit protein is inhibited by EDTA and 1,10-phenanthroline. EDTA is a strong metal chelator as is 1,10-phenanthroline which has a strong affinity for zinc and has been used in many cases to remove zinc from an enzyme to produce the apoenzyme (12-13). We were unable to restore activity to Lit protein by adding back zinc. Although the activity of Lit protein was inhibited by PMSF, a serine protease inhibitor, we have no compelling evidence to indicate that Lit protein is a serine protease (Lit protein does not have the catalytic triad characteristic of serine proteases). It is not clear why PMSF has this effect on Lit protein. However, there is a case reported in which the activity of a known metalloprotein, urease, isolated from *Staphylococcus xylossus* was inhibited by PMSF (11). The activity of Lit protein was not inhibited by inhibitors of both acid and thiol proteases. Although less than 1% of Lit protein contains

zinc as shown in the results from metal analysis of Lit protein, it may still be a zinc metalloprotein. It could have lost most of its zinc during purification. If the metal analysis result is taken in conjunction with the inhibitor studies, the site-directed mutagenesis of E161A and the presence of the His-Glu-Xaa-Xaa-His in what is presumably the active site of the protein, it could suggest that Lit protein may be a member of the zinc metalloprotease superfamily. Structural studies would be required to identify the active site of Lit protein and to definitely classify Lit protein as a zinc metalloprotease.

The His-Glu-Xaa-Xaa-His motif has been used to identify zinc-binding sites in metalloendopeptidases (15). If this conserved consensus sequence which is present in the Lit protein plays a similar role as it does in zinc metalloproteases, then mutating the invariant residues could shed some light on the role of those residues in the activity of the Lit protein. We successfully mutated H160A and E161A and tested the resulting "mutant" Lit proteins. Mutation H160A appears to have changed the conformation of Lit protein because most of the protein is found in the pellet, suggesting that the protein went into inclusion bodies. This makes it difficult to determine whether this residue plays a similar role in Lit protein as it does in the canonical His-Glu-Xaa-Xaa-His sequence of zinc enzymes where it is required to ligand zinc (7, 14-16). Figure 4a shows that the mutant Lit protein from E161A is made and more of it is in the soluble fraction. However, this mutant Lit protein is not active. In a majority of metalloproteases studied so far, the glutamic acid residue serves as a catalytic base (15). So the loss of function due to the E161A mutation in which alanine is unable to fulfill the catalytic base function of glutamic acid may be responsible for the lack of activity observed with mutant Lit protein from E161A and not zinc binding since the glutamic

acid residue is generally not used for zinc binding. There are cases where the activity of the protein is only slightly or not affected by such a change. In these proteins, glutamic acid in that conserved sequence may be replaced by aspartic acid with little or no loss of activity (10). Therefore from these preliminary results, ^{160}His and ^{161}Glu may play some role in the activity of Lit protein or at least its stability or conformation. More detailed structural studies will be needed to assign definite roles in the activity of Lit protein to these residues.

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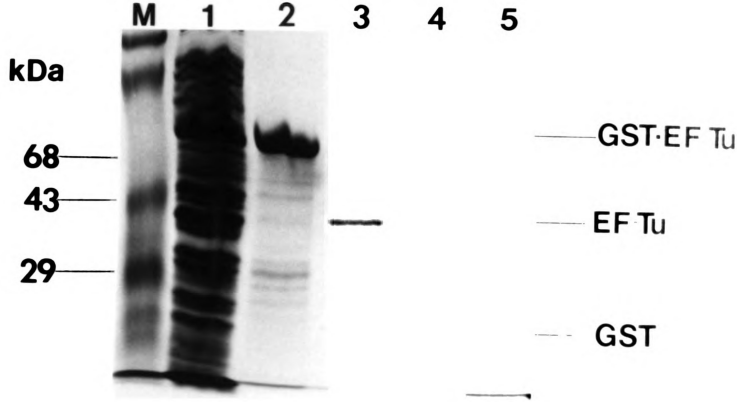
Figure Captions

Figure 1: Purification of EF-Tu. This figure shows the purification of EF-Tu from the overexpression clone, pGEX2T-*tuf*A. Lane 1 has cell extracts of the overexpressed cell culture; Lane 2 shows GST-EF-Tu fusion protein eluted from the glutathione affinity column; Lanes 3 and 5 represent EF-Tu cleaved off GST-EF-Tu fusion protein with thrombin and Lane 4 is GST alone.



of EF-
mass of
protein
EF-Tu
is GST

Figure 2: Cloning of *lit* gene. PCR*lit*3 is a PCR-amplified 998 bp *lit* gene fragment that was designed such that it lacked its own promoter and ribosome binding site, but possessed *Bam*HI sites engineered onto its ends to facilitate its cloning into the *Bam*HI site of pUC8 cloning vector.

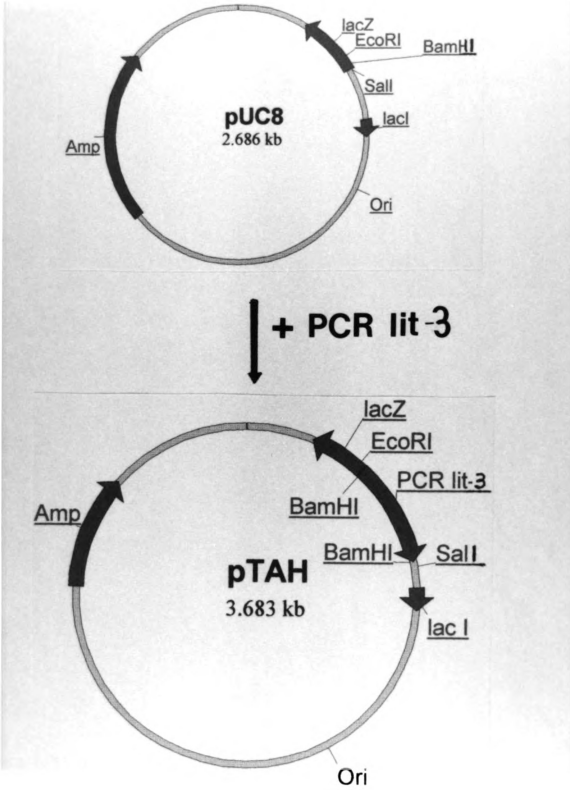

lacZ
EcoRI
BamHI
SalI
lacI
pUC8
2.686 kb
Amp
Ori
+ PCR lit-3
lacZ
EcoRI
PCR lit-3
Amp
BamHI
BamHI
SalI
pTAH
3.683 kb
lac I
Ori

Figure 3: Cloning of *lit* gene (continued). The *lit* gene fragment from pTAH was subcloned between the *Eco*RI and *Sal*I sites of pET30b cloning vector. The resulting plasmid, called pEKS, overexpresses the *lit* gene.

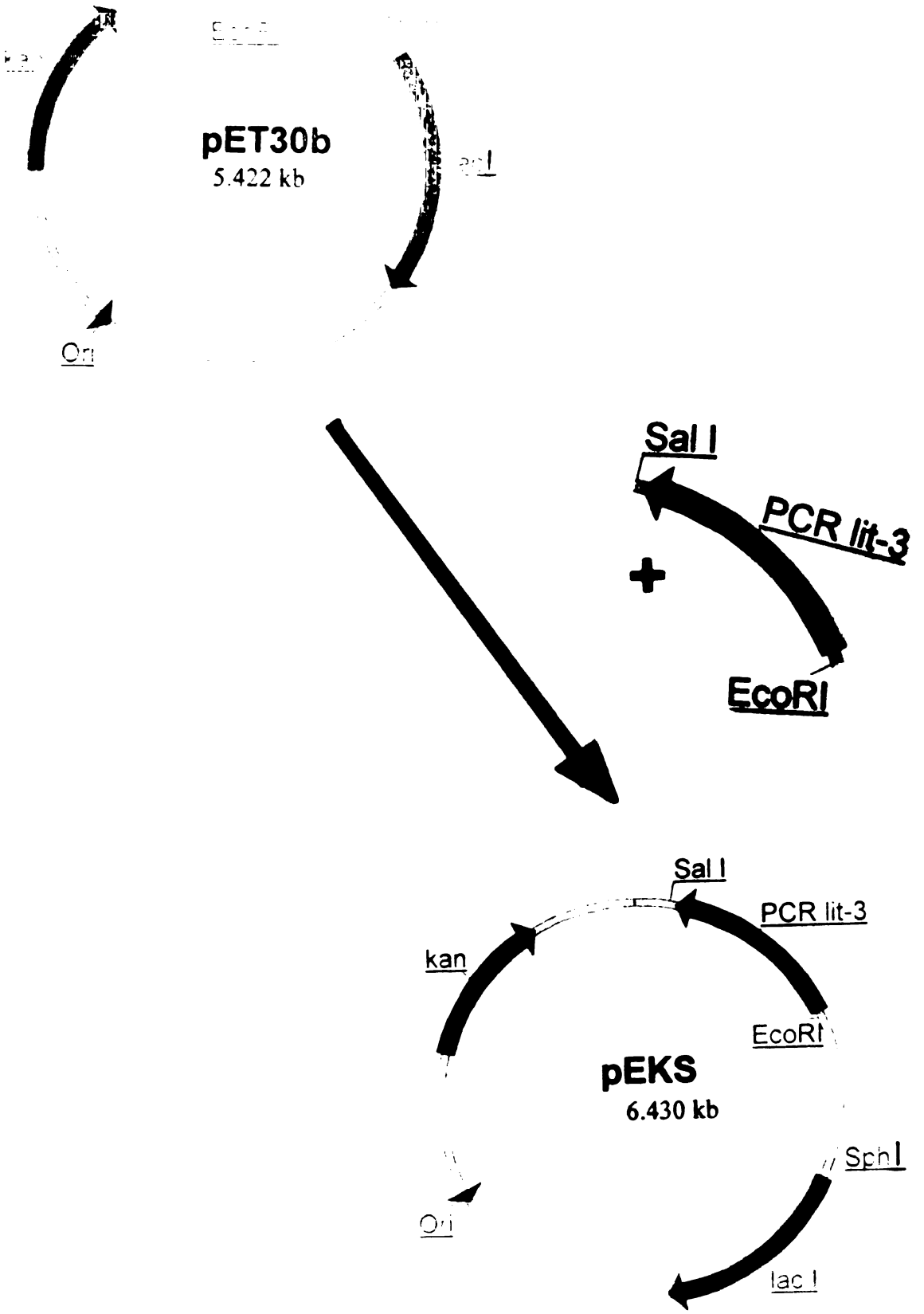

pET30b
5.422 kb
Ori
Sal I
PCR lit-3
EcoRI
Sal I
PCR lit-3
kan
EcoRI
pEKS
6.430 kb
Sph I
Ori
lac I

Figure 4: Purification of Lit protein/Activity assay. Lane 1 was loaded with cell extracts of uninduced cell culture from *E. coli* strain JM109DE3*lit*^{0}pLysS containing the pEKS plasmid. Lane 2 has cell extracts of induced cell culture from the same *E. coli* strain as in Lane 1. Lane 3 contained column flow-through fraction after induced cell extracts were loaded on the Ni-affinity column. Lane 4 was loaded with purified His-tag/S-tag Lit fusion protein. **A**: Lane 5 represents purified EF-Tu. Lanes 6 and 7 represent Lit protein activity assay. In Lane 6, 4.66 μg Lit protein and 2.55 μg EF-Tu were combined in a total volume of 45 μl. Lane 7 is the same as Lane 6 except that 2 mM chemically synthesized Gol peptide was added. Contents of Lanes 5-7 were incubated at 30°C for 30 minutes. Activity of Lit protein is shown by the cleavage of EF-Tu. This is seen in Lane 7 where the band corresponding to EF-Tu is missing and a band, EF-Tu*, representing the cleavage fragment appears.

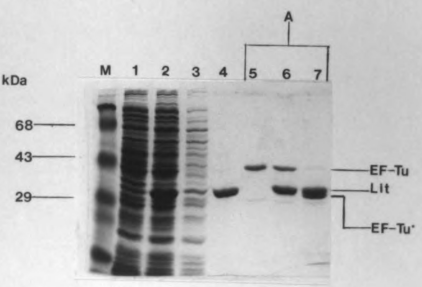
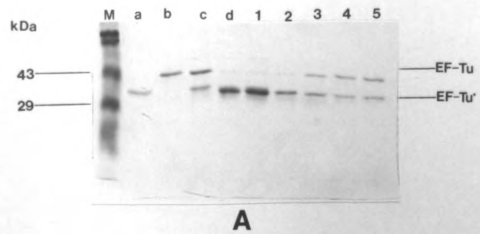
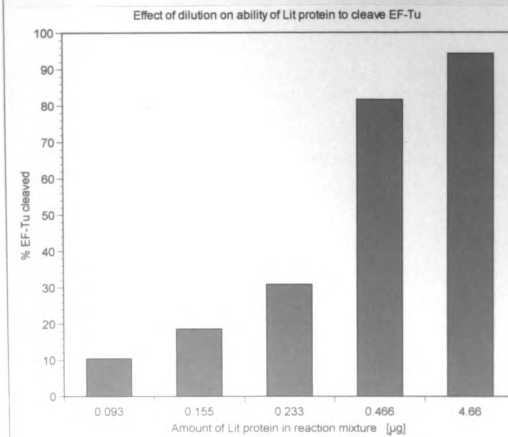

A
kDa
M
1
2
3
4
5
6
7
68
43
29
EF-Tu
Lit
EF-Tu*

Figure 4-1: Effect of dilution on ability of Lit protein to cleave EF-Tu. Panel A: Lanes a, b and c were loaded with 0.466 μg of purified Lit protein, 1.3 μg of purified EF-Tu and a mixture of purified Lit protein and EF-Tu at concentrations of 0.466 μg and 1.3 μg respectively. Lane d was the same as Lane c except for the addition of 0.2 mM Gol peptide. The reaction volume of each set up was 45 μl. The reaction was done at 30°C for 30 minutes. Lane d shows the cleavage of EF-Tu with the resulting cleavage fragment, EF-Tu*. Lanes 1-5 contained the same reactants as Lane d except that the concentrations of Lit protein were 4.66 μg, 0.466 μg, 0.233 μg, 0.155 μg and 0.093 μg respectively. These results show that as the concentration of Lit protein is reduced, less EF-Tu is cleaved.
Panel B: This is a plot of results of Lanes 1-5 showing the amount of EF-Tu cleaved in percent versus the concentration of Lit protein.

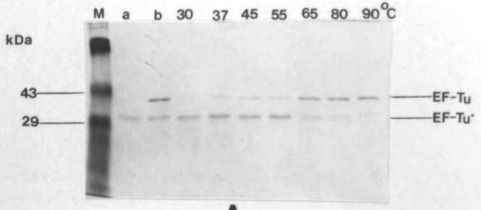

kDa
M
a
b
c
d
1
2
3
4
5
43
29
EF-Tu
EF-Tu*

A

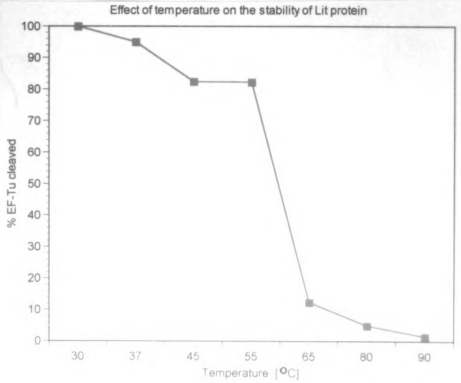

Effect of dilution on ability of Lit protein to cleave EF-Tu
% EF-Tu cleaved
100
90
80
70
60
50
40
30
20
10
0
0.093
0.155
0.233
0.466
4.66
Amount of Lit protein in reaction mixture [µg]

B

Figure 5: Effect of temperature on Lit protein stability.
Panel A: shows SDS-PAGE. The standard Lit activity assay was modified by using Lit protein that had been incubated at different temperatures before assay was conducted. In all cases, the Lit protein was incubated at the chosen temperature for 15 minutes. Lane a contained 0.466 μg Lit protein; Lane b was loaded with 0.466 μg Lit protein and 1.3 μg EF-Tu. These two lanes were controls. All other lanes were loaded as in Lane b except that 0.2mM Gol peptide was added to each one. The incubation temperatures of the Lit protein used were 30, 37,45, 55, 65, 80 and 90°C. Lit protein showed stability up to 55°C as evidenced by the amount of EF-Tu cleaved.
Panel B: is a graphical representation of Panel A.


M a b 30 37 45 55 65 80 90°C
kDa
43
29
EF-Tu
EF-Tu*

A


Effect of temperature on the stability of Lit protein
% EF-Tu cleaved
100
90
80
70
60
50
40
30
20
10
0
30
37
45
55
65
80
90
Temperature [°C]

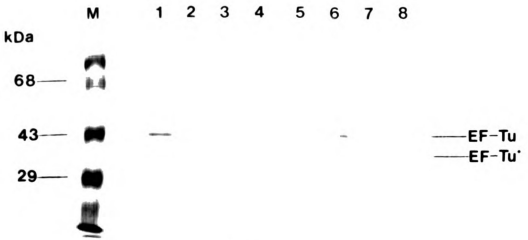
B

Figure 6: Consensus sequence of zinc-binding region. Comparison of Lit protein sequence with the signature motif of zinc peptidases to show region of similarity.

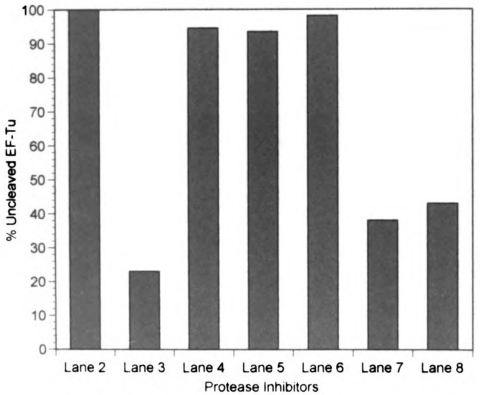
Lit protein: -^{157}Ile Leu His His* Glu Ile Ser His*

Zn peptidase: --Ile X X His Glu Ile Ser His
Ser Val Thr
Ala Met Ala
Val Leu
Thr Phe
Trp

Figure 7: Effect of typical protease inhibitors on activity of Lit protein. Panel A: Standard assay: 0.002 mM Lit + 0.04 mM EF-Tu + 0.2 mM Gol peptide in a total volume of 45µl. Incubated at 30°C for 30 minutes. Stopped by adding SDS loading buffer followed by boiling. Lane 1 is EF-Tu alone. Lane 2 is EF-Tu + Lit protein, while Lane 3 is EF-Tu + Lit protein + Gol peptide. Lanes 4-8 are the same as Lane 3 except that the Lit protein has been incubated with a different protease inhibitor for 10 minutes before being assayed. The protease inhibitors used at the final concentrations shown were EDTA (5 mM), 1,10-phenanthroline (0.1 mM), PMSF (0.5 mM), Leupeptin (50 µM) and Pepstatin A (50 µM) respectively. PMSF was dissolved in isopropanol. In Lanes 4-6, Lit protein was inhibited by the typical protease inhibitors in those lanes, whereas inhibition was only partial in Lanes 7 and 8.
Panel B: graphical representation of Panel A.


M
1
2
3
4
5
6
7
8
kDa
68
43
29
EF-Tu
EF-Tu*

A


100
90
80
70
60
50
40
30
20
10
0
% Uncleaved EF-Tu
Lane 2
Lane 3
Lane 4
Lane 5
Lane 6
Lane 7
Lane 8
Protease Inhibitors

B

Table

Table 1: Metal analysis of Lit protein using inductively coupled plasma emission spectroscopy.

The values shown in the table are (ppm). The concentration of Lit protein is 4 mg/ml. The blank is 1X binding buffer: 20 mM Tris-HCl, pH 7.9/0.5 M NaCl/5 mM imidazole. 1 mM metal solutions were made by dissolving the metal salts in 1X binding buffer. The samples were analyzed in the Chemical Analysis Laboratory of University of Georgia, Athens, GA.

Note: Lit protein was purified on a Ni-affinity column.

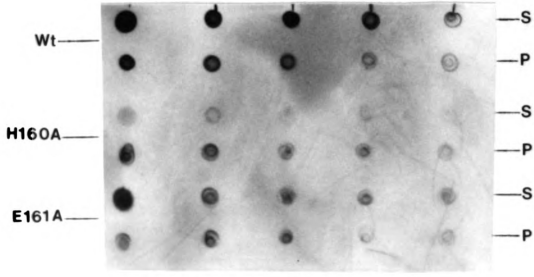
Element	BLANK	Co	Cu	Fe	Zn	Ni	Lit
Ag	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000
Al	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
As	2.4810	0.048250	<0.000000	<0.000000	<0.000000	<0.000000	2.4588
B	1.1404	0.14480	0.069652	0.095008	0.093756	0.080956	0.63970
Ba	<0.000000	<0.000000	<0.000000	0.005081	<0.000000	<0.000000	<0.000000
Be	<0.000000	0.001145	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000
Bi	0.016386	0.048726	0.59980	<0.000000	<0.000000	0.10823	<0.000000
Ca	0.69430	<0.000000	0.025907	0.20984	0.023316	0.090674	0.57513
Cd	0.008493	0.018850	0.007457	0.021543	0.024443	0.011807	0.016365
Co	<0.000000	36.7406	0.021628	0.012734	0.013745	0.020011	<0.000000
Cr	<0.000000	<0.000000	0.041014	0.048471	<0.000000	<0.000000	<0.000000
Cu	0.011861	0.088403	39.689	0.45101	0.042448	0.037454	<0.000000
Fe	0.14198	<0.000000	<0.000000	44.322	0.021812	<0.000000	0.11514
K	<0.000000	3.2563	2.6339	2.6784	2.7673	2.8895	<0.000000
Mg	0.050563	<0.000000	0.011798	0.031180	<0.000000	0.032866	0.085114
Mn	0.013442	0.043551	0.009140	0.073660	0.031184	0.039787	0.037636
Mo	0.002281	0.001996	<0.000000	0.023955	0.047339	0.002566	0.007129
Na	8596.8	1.1613	0.65039	<0.000000	0.045137	<0.000000	8980.9
Ni	<0.000000	0.072970	<0.000000	0.096164	<0.000000	35.776	0.47769
P	0.11045	<0.000000	0.85005	<0.000000	<0.000000	<0.000000	0.17228
Pb	<0.000000	0.11854	0.086011	0.094478	0.022951	0.069299	<0.000000
Sb	<0.000000	<0.000000	<0.000000	0.036312	0.19833	<0.000000	<0.000000
Se	0.16898	0.016093	<0.000000	0.022351	0.054537	0.001788	0.24676
Si	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000	<0.000000
Sn	3.4115	<0.000000	<0.000000	0.018664	<0.000000	<0.000000	3.1163
Sr	0.017803	<0.000000	<0.000000	0.000812	0.001178	0.000812	0.015202
Ti	<0.000000	<0.000000	0.000465	<0.000000	<0.000000	<0.000000	<0.000000
U	<0.000000	0.45795	0.51709	0.67215	0.64763	0.62166	<0.000000
V	<0.000000	0.007678	<0.000000	0.017050	0.024722	0.007885	<0.000000
W	<0.000000	<0.000000	<0.000000	<0.000000	0.86983	<0.000000	<0.000000
Zn	0.044744	<0.000000	0.062895	0.000325	43.558	0.21982	0.12047

Table 2: The characteristics and references of bacterial strains, plasmid constructs and phage mutants used in this article.

STRAIN		
DH5α	supE44ΔlacU169(ϕ80lacZΔM15)hsdR17recA1 endA1gyrA96thi-1relA1	Hanahan 1983; Bethesda Research Laboratories 1986.
609	JM101*lit*(Con), overproduces Lit protein.	Kao and Snyder, 1987.
610	JM101*lit*^{o}, cured of e14 by UV	Kao and Snyder, 1987.
JM109λ(DE3) *lit*^{o}pLysS	JM109, E. coli K cured of e14, carrying λ DE3 with T7 RNA polymerase gene under LacUV5 promoter and the T7 lysozyme plasmid.	This work.
PLASMID		
pGEX2T-*tuf*A	*tuf*A gene cloned into pGEX2T cloning vector	Parmeggiani laboratory.
pUC 8	high copy number plasmid that displays α-complementation in appropriate host.	Messing 1983; Norrander et al., 1983; Yanisch-Perron et al., 1985.
pET 30b	cloning vector carrying an N-terminal His-tag/ Thrombin/S-tag/enterokinase sequence. Contains inducible T7 promoter.	Novagen.
pTAH	PCR *lit*3 gene fragment cloned into *Bam*HI site of pUC 8.	This work.
pEKS	PCR *lit*3 gene fragment ex. pTAH subcloned between *Eco*RI and *Sal*I sites of pET 30b; overproduces Lit protein.	This work.
PHAGE		
T4*gol*6B	spontaneous mutant whose mutation allows phage Growth in *lit*(Con) strains.	Champness, W. and Snyder, L. 1982.

Figure 8: Site-directed mutagenesis of the DNA encoding the ^{160}His-^{161}Glu-Xaa-Xaa-^{164}His motif. Panel A shows the standard Lit protein activity assay done with a mutant Lit protein. Lanes 1-3 show wild type Lit protein: Lane 1 is Lit protein alone, Lane 2 is Lit protein + EF-Tu and Lane 3 is Lit protein + EF-Tu + Gol peptide. Lanes 4-6 show E161A mutant Lit protein: Lane 4 is mutant Lit protein alone, Lane 5 is mutant Lit protein + EF-Tu and Lane 6 is mutant Lit protein + EF-Tu + Gol peptide. Panel B shows dot blots of soluble fractions and pellets of wild type Lit, mutant H160A and E161A protein. Going from left to right, the dots are undiluted, 1:5, 1:10,1:15 and 1:20 dilution respectively. The pellet was resuspended in 4 ml 1X Binding buffer. 3 µl of each boiled protein solution was spotted on nitrocellulose and the protein was detected using the S-tag detection Kit (Novagen).

A



B

CHAPTER 3

THE PEPTIDE ENCODED BY A SHORT REGULATORY REGION OF BACTERIOPHAGE T4 BINDS TO TRANSLATION ELONGATION FACTOR Tu.

To Be Submitted to Cell

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Summary

The defective prophage e14 in *E. coli* K12 excludes T4 and other T-even bacteriophages by encoding a protease, called Lit, that cleaves translation elongation factor Tu (EF-Tu) after phage infection, thereby blocking translation and preventing the multiplication of the infecting phage and its spread to other e14-containing cells. The proteolysis of EF-Tu by Lit protease is activated by the appearance in the cell of the short Gol peptide internal to the major head protein of the infecting phage, and the cleavage of EF-Tu occurs when the region of the major head protein gene encoding this peptide, the *gol* region, is transcribed and translated in the late stage of infection. The cleavage of EF-Tu has been demonstrated in a purified *in vitro* system containing only Lit protein, EF-Tu, and a chemically synthesized 29 amino acid peptide that is encoded by the *gol* region. No detectable cleavage of EF-Tu occurs unless the Gol peptide is added. The Gol peptide might activate the proteolysis of EF-Tu by Lit protein in one of two ways. Either the Gol peptide binds to the Lit protein and activates an otherwise dormant protease activity, or the Gol peptide binds to EF-Tu and somehow creates the unique substrate for an already active Lit protease. In this paper, we present a number of lines of evidence in support of the second possibility: the Gol peptide binds to EF-Tu and this binding is required to convert EF-Tu into a substrate for the Lit protease.

Introduction

Important insights and research tools have come from studies of how cells protect themselves against viruses. One method of protection is through programmed cell death, in which the infected cell kills itself to prevent multiplication of the virus and its spread to other cells. Even single-celled bacteria use this strategy in the form of phage exclusion systems (Snyder, 1995; Yarmolinsky, 1995). The two phage exclusion systems that are best understood (*e14* and *prr* exclusions of T4) are directed against T-even bacteriophage and use remarkably similar strategies although their molecular bases are very different. In both these exclusions, an enzyme encoded by the indigenous prophage is constitutively synthesized. After infection, due to the appearance in the cell of a peptide encoded by the infecting phage, the enzyme then specifically cleaves an evolutionarily highly conserved component of the translational apparatus, thereby blocking translation and multiplication of the infecting phage. In one such exclusion system, the defective prophage *e14* constitutively synthesizes a protease, Lit (for Late inhibitor of T4) which is highly specific for translation elongation factor Tu (EF-Tu) (Kao and Snyder, 1989; Yu and Snyder, 1994). The proteolysis of EF-Tu is activated by a short polypeptide determinant, the Gol peptide, internal to the major head protein of the infecting phage (Champness and Snyder, 1984; Bergsland *et al.*, 1990; Georgiou *et al.*, 1998). Cleavage of EF-Tu occurs site-specifically between glycine 59 and isoleucine 60 in the highly conserved effector region of EF-Tu responsible for triggering the cleavage of

GTP when EF-Tu with tRNA bound enters the A site of the ribosome. The cleavage of EF-Tu causes a *cis*-dominant inhibition of translation of mRNA sequences downstream of the *gol* region and a strong *cis*-polarity effect on downstream transcription (Bergsland *et al.*, 1990), thereby preventing the synthesis of the T4 major head protein and blocking the development of the infecting phage. Overproduction of the Lit protease in the presence of Gol peptide can cause the cleavage of all the cellular EF-Tu, totally blocking cellular translation (Bergsland *et al.*, 1990; Yu and Snyder, 1994). Note the similarity between the mechanism used by these phage exclusions and other types of viral defense mechanisms such as those induced by interferon in which infected cells are killed by specific enzymes that target evolutionarily highly conserved cellular constituents, often components of the translation apparatus.

The Gol peptide, so named because mutations in this region of the major head protein gene allow the phage to grow on Lit protein containing bacteria (*gol* mutations, for grow on Lit), is less than 29 amino acids long, and approximately one-fifth of the way in from the N terminus of the protein. The amino acid sequence of the Gol peptide, extending from amino acid 94 to amino acid 122 of the major head protein prior to processing, is almost identical in the four members of this diverse family of phages in which it has been sequenced (Monod *et al.*, 1997). Furthermore, a test of twenty wild type isolates of T-even phages revealed that all are vulnerable to the Lit exclusion (our unpublished observations). Therefore, the Lit exclusion system seems to play an important role in defending cells containing the e14 element against the large and ubiquitous family of T-even phages.

The activation of cleavage of EF-Tu has been demonstrated in a purified *in vitro* system by adding the chemically synthesized 29 amino acid Gol peptide to EF-Tu and Lit protein (Georgiou *et al.*, 1998). Therfore no other cellular components, either phage or host encoded, are required for the activation of cleavage of EF-Tu. Furthermore, the Gol peptide seems to be absolutely required for the activation of cleavage since no cleavage of EF-Tu has been detected if the Gol peptide is omitted from the reaction. Mutations in the *gol* region, which reduce the ability of the mutant Gol peptide to activate the cleavage of EF-Tu also alleviate the *cis*-dominant inhibition of downstream translation, indicating that even limited cleavage of EF-Tu can have a strong effect on translation in *cis*.

We imagine two general possibilities for how the Gol peptide might activate the proteolysis of EF-Tu by Lit. One possibility is that the Gol peptide binds to the Lit protein and somehow activates a dormant protease activity, for example by furnishing a needed amino acid for the active center. Another possibility is that the peptide does not activate Lit at all, but rather binds to EF-Tu and somehow converts EF-Tu into a unique substrate for the Lit protease. In this paper we present a number of lines of evidence in support of the latter possibility: that the peptide binds to EF-Tu, somehow creating the substrate for the protease.

Results

Equimolar amounts of Gol peptide and EF-Tu are required for complete cleavage of EF-Tu.

As mentioned in the Introduction, there are two general possibilities for how the Gol peptide activates the proteolysis of EF-Tu. Either the peptide binds to the Lit protease and somehow activates it, or the peptide binds to EF-Tu and somehow creates the unique substrate for the already active Lit protease. Clues to which general mechanism is correct may come from measurements of the molar ratios of peptide to Lit and EF-Tu required for complete cleavage of EF-Tu. Lower amounts of peptide may be required if the peptide binds to Lit and activates it since Lit acts enzymatically (Georgiou *et al.*, 1998), and each molecule of activated Lit protease should be able to cleave many molecules of EF-Tu. If, however, the peptide binds to EF-Tu and creates the substrate for Lit proteolysis, higher amounts of Gol peptide may be required because then one molecule of peptide might have to bind to each molecule of EF-Tu before it can be cleaved.

To determine the molar ratios of Gol peptide to EF-Tu and Lit protein required for complete cleavage of EF-Tu, we mixed the Lit protein and EF-Tu at a molar ratio of about 1 to 20 and added varying amounts of Gol peptide. In Figure 1 is shown the extent of cleavage of EF-Tu at each concentration of Gol peptide. Figure 1-1 shows percent EF-Tu cleaved at each concentration of Gol peptide and the molar ratio of peptide to EF-Tu

and peptide to Lit at that concentration. It can be seen that significant cleavage of EF-Tu occurred only when the molar ratio of Gol peptide to EF-Tu was 1:1 or higher, even though at these concentrations of Gol peptide there was still a great molar excess of peptide over Lit protein. The fact that such high concentrations of Gol peptide are required for efficient cleavage of EF-Tu suggests that one molecule of peptide must bind to each molecule of EF-Tu that is cleaved, rather than each molecule of peptide binds to and activates a molecule of Lit protein.

In interpreting the results of the above experiment, we have assumed that most of the peptide we added was active. The concentration of Gol peptide was determined solely by measuring the optical absorbance of the solution of the peptide (ex. Chiron Inc.) and it is conceivable that the mixture contained many inactive peptides, that were either, for example, too short or had been chemically damaged during synthesis or storage. If only a small fraction of the Gol peptide added was active, then the actual amount of active Gol peptide present when significant amounts of EF-Tu were cleaved might have been much less than estimated, and more commensurate with the lower amount of Lit protein in the reaction.

Lit protease is not activated by a preincubation with Gol Peptide.

Another way to test the hypothesis that Gol peptide must bind to EF-Tu to activate the proteolysis is to test a prediction of the alternative hypothesis: that the Gol peptide binds to Lit and thereby somehow activates its protease activity. If the Lit protease activity is activated by Gol peptide, then it should be possible to activate the Lit protease by incubating Lit with Gol peptide at high concentrations. Each molecule of activated Lit protease should then be

able to cleave many molecules of EF-Tu, since Lit protease acts catalytically. If, however, the Gol peptide must bind to EF-Tu to activate the proteolysis, the Lit protease will not have been activated during the preincubation and amounts of Gol peptide commensurate with the amount of EF-Tu may still have to be added to see significant cleavage of EF-Tu. Figure 2 shows the results of such an experiment. In Lane C, 0.2 μg Lit protein was preincubated with 0.2 mM Gol peptide. The mixture was diluted 1 to 20 and added to 1.5 μg EF-Tu in a total volume of 45 μl to initiate the cleavage reaction. Very little EF-Tu was cleaved, inconsistent with the interpretation that the protease had been activated during the preincubation. In Lane D is a control indicating that the Lit protease is not inactivated by the preincubation. In this control, the Lit protein was preincubated by itself, diluted 1 to 20 and then added to 0.2 mM Gol peptide and 1.5 μg EF-Tu in a total volume of 45 μl. Now, substantial amounts of EF-Tu was cleaved. The last lane (Lane E) contains an additional control that shows that preincubating Lit with Gol peptide is not deleterious to the activity of Lit and that the Gol peptide is not significantly inactivated by the preincubation. This lane was the same as Lane C except for the extra Gol peptide added after preincubation and dilution. More EF-Tu was cleaved. Lane B contains a control of undiluted components. Thus one prediction of the alternative hypothesis, that the Gol peptide can activate Lit during a preincubation, is not fulfilled, offering indirect support to the hypothesis that the Gol peptide does not activate the Lit protease but rather binds to EF-Tu and thereby creates the substrate for proteolysis.

Direct Evidence that Gol Peptide Binds to EF-Tu

If Gol binds to EF-Tu, it may be possible to detect this binding directly. One way to demonstrate binding between two protein molecules is to fix one protein on a column and then pass the second protein through the column, usually accompanied by other proteins (cf. Formosa *et al.*, 1991). If the second protein binds to the first protein affixed to the column, its passage through the column may be retarded relative to the other proteins. It may also be that the second protein fails to elute altogether, or only elutes under conditions in which the first protein elutes.

To determine whether the Gol peptide binds to EF-Tu, either EF-Tu or the Gol peptide could have been bound to a column and the other component passed through the column. However, EF-Tu often behaved anomalously on columns, eluting after most other proteins, even if no Gol peptide had been attached to the column. This anomalous behaviour might reflect the tendency of EF-Tu to polymerize under some conditions (cf. Beck *et al.*, 1978). Therefore, it proved more feasible to pass the Gol peptide through a column to which EF-Tu had been bound.

To determine if Gol peptide was retained on a column to which EF-Tu had been attached, we needed a way to bind EF-Tu to a column matrix as well as a way to detect the Gol peptide, since the latter is too small to visualize on SDS PAGE gels. To fix EF-Tu to a column, we expressed the EF-Tu protein from a plasmid pGEX*tufA* (Cetin *et al.*, 1998) in which the *tufA* gene encoding EF-Tu is translationally fused to the *gst* gene encoding

glutathione-S-transferase (GST). The GST portion of the resultant fusion protein binds tightly to a glutathione column, and can be eluted with an excess of reduced glutathione. To allow detection of the Gol peptide, we expressed the Gol peptide from a plasmid pET30PZ1 in which the *gol* coding sequence has been translationally fused to an S tag (see Experimental Methods). The Gol peptide expressed from this plasmid is also attached to a string of six consecutive histidines (His-tag) which allows its purification on nickel affinity columns, which will be important for later experiments.

To investigate the ability of Gol peptide to bind to EF-Tu, extracts were prepared separately from cells in which synthesis of the GST-EF-Tu fusion protein and S-tagged Gol peptide had been induced (see Experimental Procedures). The extracts were then mixed in a 1:1 ratio, incubated, and layered on a glutathione column as in Experimental Procedures. The columns were washed with an equal volume of the loading buffer followed by a buffer containing reduced glutathione to elute the GST-EF-Tu fusion protein. Fractions were collected during the loading as well as during the washes and after elution with reduced glutathione. Aliquots of these fractions were then analyzed by SDS-PAGE and protein staining to follow the elution of the GST-EF-Tu fusion protein as well as other proteins in the extract and analyzed by S-tag dot blots to detect elution of the Gol peptide (see Experimental Procedures). Figure 3A shows the results of such an experiment. Most of the GST-EF-Tu fusion protein, indicated by the arrow, was stripped from the extracts as expected, and did not elute until the column was washed with reduced glutathione. Most of the S-tagged Gol peptide flowed through the column unimpeded and could be detected in the flow through fractions (see dots below the lanes). However, some of the S-tagged

Gol peptide was retained on the column, and did not elute until the GST-EF-Tu fusion protein eluted.

The retention of some of the S-tagged Gol peptide on the column to which the GST-EF-Tu fusion protein was fixed and its subsequent elution with the GST-EF-Tu fusion protein suggested, but did not prove, that some Gol peptide bound to the EF-Tu attached to the column. For example, the Gol peptide may be binding to the GST portion of the fusion protein, or binding to some column component through either its S tag or His tag rather than its Gol peptide sequence. If the Gol peptide was retained by virtue of its binding to EF-Tu, it should not be retained if only the GST protein with no EF-Tu attached is fixed to the column, or if the peptide being passed through the column does not contain the Gol peptide sequence, but contains only the His and S tags. The same experiment was repeated with the S-tagged peptide made from the cloning vector pET30c without the *gol* sequence cloned into it, so the peptide did not contain the internal Gol peptide sequence although it was identical at both the N and C termini. The GST-EF-Tu fusion protein was retained on the column. Very little S-tagged peptide was retained and eluted with the GST-EF-Tu fusion protein indicating that the S-tagged peptide is being retained on the column because of its Gol peptide sequence rather than some other sequence on the peptide (Figure 3b). We conclude that at least some of the Gol peptide is being retained on the column through its binding to EF-Tu that is attached to the column. Note that the peptide that flows through the column unimpeded in the experiment in Figure 3A may have been bound to the chromosomally-encoded EF-Tu (represented by the strong band at 43 kDa in the flow through) which does not contain a GST tag so it is not retained on the glutathione affinity column. Based on the results of

the binding experiments, a model for the activation of the proteolytic cleavage of EF-Tu by Lit protease is proposed as shown in Figure 4.

Discussion

We have presented evidence that a small region of the major head protein of T4 phage, the Gol peptide, binds to translation EF-Tu during the normal course of phage infection. This binding was revealed because the peptide-bound EF-Tu is the target of the specific Lit protease, encoded by the defective prophage e14. Cleavage of EF-Tu by the protease blocks T4 and other T-even phage development, causing phage exclusion. We have presented separate lines of evidence that the Gol peptide binds to EF-Tu. First, the amount of Gol peptide required for complete cleavage of EF-Tu is more commensurate with the amount of EF-Tu in the cleavage reaction than it is with the amount of Lit protease. Second, the Lit protease is not activated by a preincubation with the Gol peptide, as might be expected if the Gol peptide activates the Lit protease by binding to the Lit protein. Finally, the binding has been demonstrated directly even in the presence of all other cellular proteins by showing that the Gol peptide is retained on an EF-Tu affinity column and elutes when the EF-Tu elutes.

The binding of the Gol peptide to EF-Tu is not likely to be an *in vitro* artifact since it is substantiated by both genetic and physiological evidence. For example, T4 mutations that prevent the cleavage of EF-Tu after T4 infection or allow transformation of cells containing the Lit protein by plasmids containing clones of the major head protein gene, T4 gene 23, all lie in the Gol peptide coding region of T4 gene 23 (Champness *et al.*, 1984;

Bergsland *et al.*, 1990). At least some of these same *gol* mutations also reduce the ability of the peptide to activate cleavage of EF-Tu in a purified *in vitro* system (Georgiou *et al.*, 1998). It will be interesting to determine if some of these same *gol* mutations prevent or weaken the binding of the peptide to EF-Tu, providing a test of this model for how they prevent the cleavage. Our previous work had also indicated that only the Gol peptide is required to activate cleavage of EF-Tu by Lit protease *in vivo* and *in vitro* (Yu and Snyder, 1994; Georgiou *et al.*, 1998). Finally, the experiments reported here indicate that the binding of the Gol peptide to EF-Tu is required to activate cleavage of EF-Tu by the Lit protease in a purified *in vitro* system, and it seems very likely that the mechanism of activation of cleavage of EF-Tu occurs by the same mechanism *in vivo*.

The results reported here and in earlier publications offer a satisfying explanation for how the defective prophage e14 excludes T-even phages. Late in infection by a T-even phage, when the synthesis of late proteins including the major head protein of the phage has begun, the Gol peptide internal to newly synthesized major head proteins binds to EF-Tu. This binding is part of the normal course of infection, even in cells that are not lysogenic for e14. However, if the cells are lysogenic for e14, as are most laboratory *E. coli* K12 strains, EF-Tu with Gol peptide bound provides a unique substrate for the e14-encoded Lit protease which is made constitutively but has no substrate in the uninfected cell. The Lit protease then cleaves EF-Tu, blocking the multiplication of the infecting phage and its spread to other neighboring *E. coli* cells which, because they are often siblings, also contain the e14 prophage. In this way, the e14 prophage

protects the population of host bacteria, and thereby itself, from phage contagion.

The normal situation is probably somewhat more complicated than this, however. Normally, not enough Lit protease is made from a wild type e14 prophage to cleave all the EF-Tu in the cell, and as much as 50% of the EF-Tu remains uncleaved after T4 infection of wild type *E. coli* K12 strains (our unpublished observations). These levels of uncleaved EF-Tu are sufficient to maintain almost normal levels of total protein synthesis so total translation is only partially blocked when T4 infects wild type *E. coli* K12 strains. Nevertheless, T4 phage production in wild type *E. coli* K12 strains is severely delayed, especially at lower temperatures, due to a severe delay in the synthesis of the major head protein. This inhibition of the translation of genes containing the *gol* sequence is apparently strongly *cis*-acting, as has been demonstrated in a number of ways. One demonstration of the *cis*-effect came from coinfecting Lit protease-containing cells with wild type T4 and T4 with a *gol* mutation (Champness and Snyder, 1984). The major head protein was almost exclusively synthesized from the phage genomes with the *gol* mutation. Another demonstration came from measuring the expression of reporter genes fused translationally downstream to the *gol* region (Bergsland *et al.*, 1990). Very little expression of the downstream reporter gene occurred, even though cell growth and expression of most of the other genes in *E. coli* were not significantly affected. Assuming this *cis*-effect is due to cleavage of EF-Tu, which seems likely considering that it is affected by the same *gol* mutations and, as far we know, has all the same requirements as the global inhibition of translation due to cleavage of all the cellular EF-Tu, it appears that those EF-Tu proteins involved in translating the *gol* region are

preferentially cleaved and then act to block translation from ribosomes on the same messenger RNA. But how could a cleaved EF-Tu due to a Gol peptide exiting a ribosome act retroactively to block translation by that ribosome? According to the textbook picture of the role EF-Tu plays in translation, EF-Tu binds to GTP and aminoacylated tRNA and the ternary complex enters the A site of the ribosome, promoting proper pairing between the codon in the mRNA and the anticodon in the tRNA. The GTP is then cleaved and EF-Tu exits the ribosome. Perhaps the newly synthesized Gol peptide activates cleavage of EF-Tu and then remains attached to the cleaved EF-Tu, tethering the cleaved EF-Tu to the ribosome from which the Gol peptide was translated, and limiting its diffusion to other ribosomes. The cleaved EF-Tu then preferentially enters the A site of the ribosome to which it is tethered and blocks translation, perhaps because the defective EF-Tu cannot cleave GTP and exit the ribosome (Georgiou *et al.*, 1998). However, there are recent indications that this textbook picture of the role of EF-Tu in translation may be too simple and it may not be necessary to invoke tethering to explain the *cis*-effect. Some evidence suggests that two EF-Tu molecules rather than only one may be involved in each amino acid addition on the ribosome (Weijland and Parmeggianni, 1993; Ehrenberg *et al.*, 1990). It seems possible that this second EF-Tu molecule could be making contact with the peptidyl tRNA at the P site. Furthermore, nonsense suppression by some mutant forms of EF-Tu is affected by the nature of the penultimate amino acid in the polypeptide chain, suggesting that EF-Tu may be in contact with the peptidyl tRNA at the P site of the ribosome (Mottagui-Tabar and Isaksson, 1996).

While it seems clear that the Gol region of the major head protein of T4 binds to EF-Tu during the normal course of infection, left unanswered is the

question of why a protein would bind to EF-Tu in the first place. The evidence suggests the Gol region of the major head protein probably binds to EF-Tu during its synthesis on the ribosome and not afterwards when the mature protein has been synthesized and folded into heads where it is probably not accessible for binding. From the available evidence, it is not clear whether the Gol region of the major head protein is exposed on the surface of heads where it could bind to EF-Tu (cf. Black *et al.*, 1994). However, the *cis*-inhibition of translation is much easier to explain if the Gol peptide activates cleavage of EF-Tu during the synthesis of the head protein. Moreover, inhibition of translation after T4 infection of Lit-containing cells is much more severe when the cells are infected by T4 with an amber mutation downstream of the *gol* region in gene 23 than when they are infected by wild type T4 (cf. Bergsland *et al.*, 1990). In retrospect, we interpret this to mean that there is more exposed Gol peptide to activate proteolysis of EF-Tu after infection by the amber mutants when the free amber fragments accumulate, than after wild type T4 infection, when the newly synthesized gp23 is immediately assembled into heads.

Possible reasons why the Gol region of the major head protein of T-even phages may bind to EF-Tu during synthesis can be divided into three categories. In the first category are those hypotheses in which the binding has nothing to do with the normal function of EF-Tu in translation, for example by playing a scaffolding role in the assembly of phage heads or a chaperone role in the folding of the major head protein. There are precedents for proteins being used as scaffoldings. For example, the RNA ligase of T4 bacteriophage is also used as a scaffolding in tail fiber assembly and the two roles for this protein seem unrelated (Snopeck *et al.*, 1977). A chaperone role

for EF-Tu in head protein folding is also not far-fetched. The head protein of T4 certainly needs chaperones to assist in its folding and T4 even encodes a special co-chaperonin dedicated to the folding of this protein alone. Moreover, EF-Tu has recently been shown to play a chaperone role in the folding of some proteins (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998). The Gol peptide might be expected to bind to the GTPase activating region, since this is where the cleavage by Lit occurs and the GTPase cleavage and recycling functions of EF-Tu may be important for its chaperon activity on some proteins, perhaps by promoting cycling on and off the protein during folding (Kudlicki *et al.*, 1997).

In the second category are those hypotheses based on the translational role of EF-Tu. One attractive hypothesis within this category has the binding of the Gol region to EF-Tu temporarily halting the translation of the major head protein until it can be fed into the growing phage head, in analogy to the stoppage of translation until the signal sequences of membrane-destined proteins enter the rough endoplasmic reticulum in eukaryotes. It has been known for some time that the uncontrolled synthesis of major head protein, in the absence of head assembly, leads to the formation of largely insoluble structures. Perhaps EF-Tu with the peptide bound can enter the A site on the ribosome, thereby blocking translation, invoking the same arguments used to explain the *cis*-blocking of translation when EF-Tu is cleaved by the Lit protease. Perhaps the hypothesized blockage of translation due to Gol peptide binding to EF-Tu is only temporary and normally reversed by other cellular components, unlike the blockage due to the cleaved form of EF-Tu which may be irreversible but otherwise similar. A pause in translation could be difficult to detect if it is too transient.

The third general category of reasons for why the peptide binds to EF-Tu proposes that the binding of the Gol peptide to EF-Tu affects some other, heretofore unknown, function of EF-Tu. However, this must await the discovery of another function for EF-Tu other than in translation and protein folding.

Experimental Procedures

Purification of Lit protein

Experiments to determine the mechanism of activation of the Lit protease require a source of purified Lit protein. Earlier studies used Lit protein purified by solubilization from inclusion bodies after overexpression from a T7-based expression vector (Georgiou *et al.*, 1998). The Lit protein obtained by this method was quite pure but the purification procedure was laborious and most of the Lit protein obtained by this method was in the form of inactive aggregates from which active monomers had to be separated. To facilitate the purification of Lit protein, we PCR amplified a 998 bp fragment extending from nucleotide 262 to 1260 in the *lit* gene sequence. This fragment had neither a promoter sequence nor a ribosome binding site. Flanking *Bam*HI sites were introduced at its ends by PCR so that it could be cloned into the vector pET-30b (Novagen) in such a way that the AUG of the Lit protein is fused in frame to a His tag of six consecutive histidines. The fusion protein could then be purified to near homogeneity on nickel affinity columns (Novagen). The fusion protein with the His tag attached was still active in the proteolysis of EF-Tu in the presence of Gol peptide making it

adequate for studying the mechanism of activation of proteolysis by the peptide.

Assay of Lit Protease

In the standard assay, the following final concentrations of 0.466 μg Lit protein, 2.55 μg EF-Tu and 0.2 mM Gol peptide were combined in a total volume of 45μl. This reaction mixture was incubated for 30 min at 30°C. The reaction was stopped by addition of sodium dodecyl sulfate (SDS) gel loading buffer followed by boiling. The reaction products were analyzed on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) containing 13% acrylamide. The gel was run at 60 volts for 3 hrs, fixed in 12% trichloroacetic acid and then stained with 0.1% Coomassie blue. The depletion of the 43 kDa EF-Tu band and the appearance of a 36 kDa EF-Tu* band was indicative of Lit protease activity. The bands were quantified in the AMBISS laser densitometer.

Synthesis and isolation of Gol peptide

The chemically-synthesized 29 amino acid Gol peptide was purchased from Chiron Inc., California. To synthesize the Gol peptide *in vivo* the *gol* coding sequence from the *Pst*I site in gene 23 to the *Hin*dIII site at the Δ1 deletion (Bergsland, *et al.*, 1990) was cloned between the *Pst*I and *Hin*dIII sites of the plasmid pET-30b (Novagen). In the presence of T7 RNA polymerase, the resultant plasmid, pET-30PZ1, directed the synthesis of an approximately 12 kDa peptide containing a 53 amino acid sequence from the major head protein gene of T4, including the 29 amino acid Gol peptide sequence and an S-Tag and His-Tag on its N terminus. That this plasmid

made active Gol peptide was confirmed by the cleavage of EF-Tu in crude extracts of cells containing the induced plasmid to which purified Lit protein was added.

Binding Experiments on Affinity Columns

Extracts containing the S-tagged Gol peptide or the GST-EF-Tu fusion protein were prepared from *E. coli* JM109DE3/pET30PZ1 or DH5/pGEX2T-*tuf*A, respectively. 500 ml of cells were grown to early log phase at 37°C and then shifted to 23°C for one hour before isopropyl-β-D-thiogalactoside (IPTG) was added to 0.1 mM final concentration. After 3 hrs., the cells were harvested, resuspended in 10 ml Buffer A (50 mM Tris pH 7.5, 150 mM KCl, 5 mM $MgCl_2$) and sonicated to lyse. They were centrifuged for 30 min at 25,000 g and the supernatants were stored at -70°C. Almost all the Gol peptide and approximately 30% of the GST-EF-Tu fusion protein remained soluble in the supernatant.

To perform the binding experiments, the extracts were mixed and incubated at 30°C for 30 min. They were then filtered through 0.45 μm Gelman filters and layered on a glutathione Redipack column (Pharmacia). The column was washed with 7.5 ml of Buffer A and the GST-EF-Tu fusion protein was eluted with reduced glutathione according to the manufacturer's instructions. Individual 1.5 ml fractions were assayed for the S-tag on the Gol peptide by spotting 3 μl on nitrocellulose and developing the filter with the S-tag kit (Novagen). Aliquots of the fractions were boiled in SDS loading buffer (final conc. 1% SDS, 10% glycerol plus a color indicator dye) and applied to 10% acrylamide gels (Laemmli, 1970).

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Figure Captions

Figure 1: The Gol peptide must be present at equimolar or higher concentrations with EF-Tu for efficient cleavage of EF-Tu. In this experiment, increasing dilutions of Gol peptide were added to purified Lit + EF-Tu, at a molar ratio of 1 Lit to 20 EF-Tu. The extent of cleavage of EF-Tu after a 30 min incubation at 30°C was determined by SDS PAGE. A Coomasie blue-stained gel is shown. EF-Tu* is the large cleavage fragment of EF-Tu, extending from ^{60}Ile to the carboxy terminus. The other cleavage fragment from the N terminus to ^{59}Gly is only about 6 kDa and ran off the gel. Lane A: EF-Tu + Lit protease molar ratio of 20 to 1, no Gol peptide. Lane B: same amounts of EF-Tu and Lit protease but with Gol peptide added to a molar ratio of Gol peptide to EF-Tu of 5:1. The following lanes have progressively lower ratios of Gol peptide to EF-Tu. Lane C: 1:1. Lane D: 1:2. Lane E: 1:4. Lane F: 1:6. Lane G 1:10. Lane H: 1:20. Note that there is very little cleavage of EF-Tu in the reaction in Lane H even though the molar ratio of Gol peptide to Lit protease is still 1:1.

EF-Tu
EF-Tu*
A B C D E F G H
kDa
68
43
29

Figure 1-1: Concentration of Gol peptide needed for cleavage of EF-Tu. This figure is a graphical representation of the experiment in Figure 1. It shows that at as low a concentration of Gol peptide as 0.02 mM, EF-Tu is cleaved. As the concentration of Gol peptide increases, more and more EF-Tu is cleaved. At a concentration of 2 mM of Gol peptide, over 90% of EF-Tu is cleaved. At this concentration, the molar ratio of Gol:EF-Tu is 5:1, and of Gol:Lit protease is 100:1 which suggests a very tight binding between Gol peptide and EF-Tu.

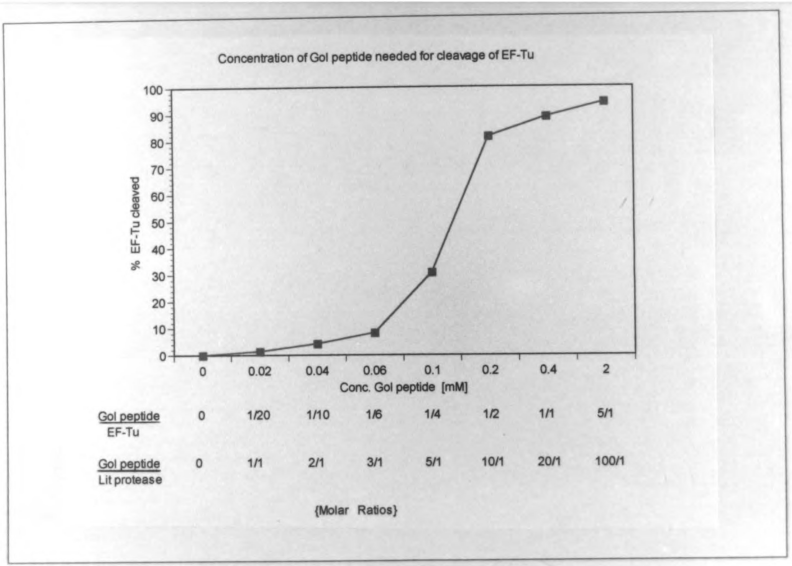

Concentration of GoI peptide needed for cleavage of EF-Tu
% EF-Tu cleaved
100
90
80
70
60
50
40
30
20
10
0
0
0.02
0.04
0.06
0.1
0.2
0.4
2
Conc. GoI peptide [mM]
GoI peptide / EF-Tu
0
1/20
1/10
1/6
1/4
1/2
1/1
5/1
GoI peptide / Lit protease
0
1/1
2/1
3/1
5/1
10/1
20/1
100/1
{Molar Ratios}

Figure 2: Can the Gol peptide activate Lit protease during a preincubation? In this experiment, the standard assay was done with these modifications. Gol peptide (2 mM) was incubated with 0.23 μg Lit protein at 30°C for 30 minutes. The mixture was then diluted 1:20, added to 1.3 μg EF-Tu and re-incubated at 30°C for 30 minutes. Lane C shows such an experiment. Very little EF-Tu was cleaved. In contrast, substantial EF-Tu cleavage occurred in the control experiment shown in Lane D in which 0.23 μg Lit protein was incubated alone, at 30°C for 30 minutes, diluted 1:20, added to 1.3 μg EF-Tu + Gol peptide (2 mM) and re-incubated at 30°C for 30 minutes. Apparently, the Gol peptide did not activate the Lit protease during preincubation, at least not irreversibly, and a molar excess of Gol peptide to EF-Tu must still be present for efficient cleavage. Lane E was the same as Lane C + extra Gol peptide (2 mM). More EF-Tu was cleaved in this lane than in Lane C, presumably because of the extra Gol peptide. Lanes A and B are standard assay controls. Lane A contains 1.3 μg EF-Tu + 0.23 μg Lit protein, while Lane B is the same as Lane A + 2 mM Gol peptide.

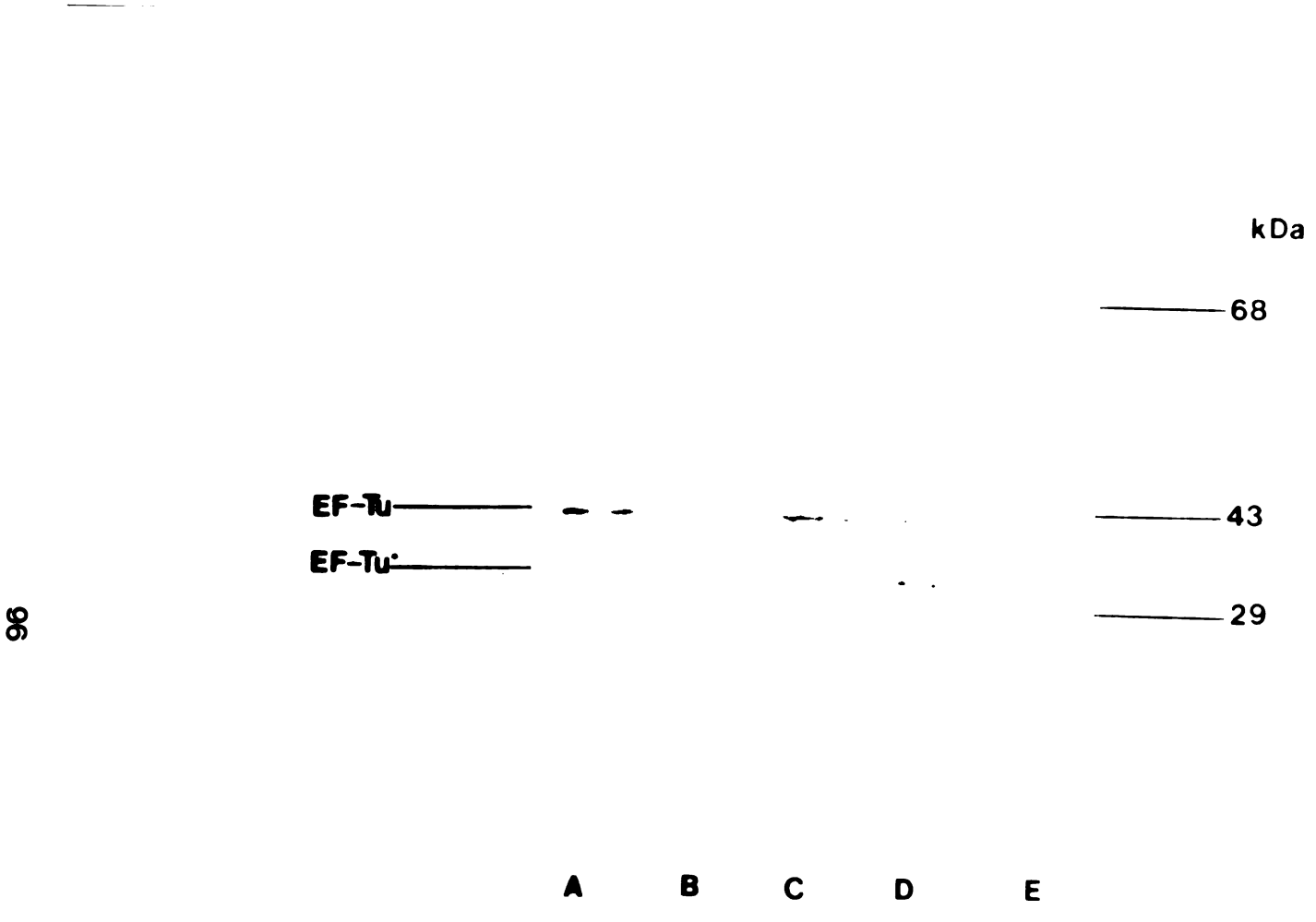

kDa
68
43
29
EF-Tu
EF-Tu
A
B
C
D
E

Figure 3A: Binding of Gol peptide to EF-Tu. Two extracts, one from cells in which synthesis of the Gol peptide had been induced fused to an S-tag (S-tag Gol- peptide) and the other in which EF-Tu had been induced fused to glutathione-S-transferase (GST-EF-Tu) were mixed and applied to a glutathione column (Pharmacia). After washing, the GST-EF-Tu fusion protein was eluted with glutathione and the fractions applied to an SDS-PAGE gel. The fractions were also spotted on nitrocellulose paper and the presence of the S-tag detected using an S protein alkaline phosphatase conjugate (Novagen). Some Gol peptide passed through the column but substantial amounts were retained and eluted with the GST-EF-Tu fusion protein. Lane A: Mixed extracts. Lane B; A flowthrough fraction. Lanes C, D, E and F: First 4 fractions eluted with reduced glutathione. The expression vector fusing GST to EF-Tu was the kind gift of Andrea Parmeggiani.

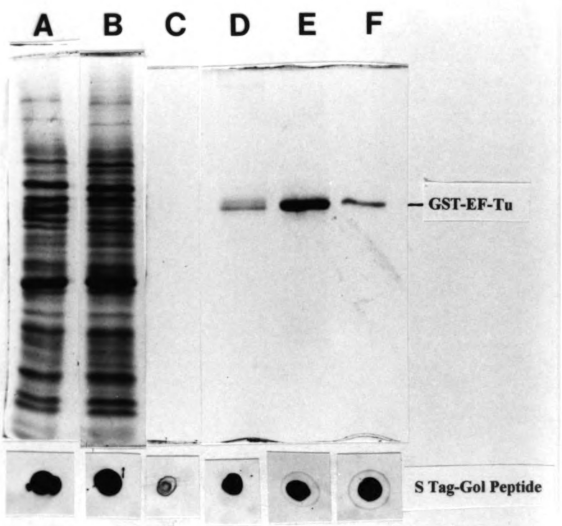

A
B
C
D
E
F
GST-EF-Tu
S Tag-Gol Peptide

Figure 3b: The peptide binds to EF-Tu through its Gol Sequence. Same as experiment in Figure 3A except that cells contain only the cloning vector so they make the S-tag peptide without the Gol portion. Much less S-tagged peptide is retained than in Figure 3A, even though more GST-EF-Tu fusion protein was retained in this experiment than in the one in Figure 3A. Apparently, the peptide is binding to GST-EF-Tu through its Gol peptide sequence. In a similar experiment, much less S-tagged Gol peptide was retained by a column on which only the GST portion of the fusion protein had been retained, indicating that the Gol peptide is binding to EF-Tu portion of the GST-EF-Tu fusion protein (result not shown).

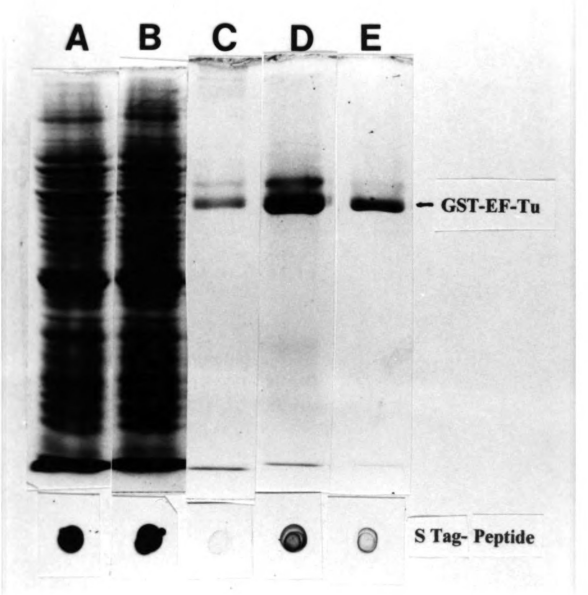
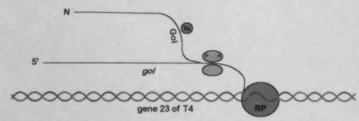

A
B
C
D
E
GST-EF-Tu
S Tag- Peptide

Figure 4: Model for the *cis*-inhibition of translation by Lit protease. RP stands for RNA polymerase; Tu is translation elongation factor, EF-Tu; A is the A-site (acceptor site); P is the P-site (peptidyl site) of ribosome; and the purple colored structure is ribosome.

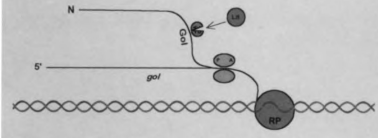
Model for the *cis*-inhibition of Translation by Lit Protease

No Lit Protease

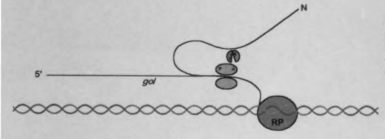


The Gol peptide binds to EF-Tu as it exits the ribosome

Plus Lit Protease
(e14 Lysogen)



Lit cleaves Gol peptide-bound EF-Tu



Cleaved EF-Tu with peptide attached enters A site of ribosome to which it is tethered, blocking translation in *cis*

Table

Table 1: The characteristics and references of bacterial strains and plasmid constructs used in this article.

STRAIN		
JM109DE3	JM109, *E.coli* K carrying λ DE3 with T7 RNA polymerase under LacUV5 promoter.	Promega, Inc.
DH5	supE44hsdR17recA1endA1gyrA96 thi-1relA1.	Low 1968; Meselson and Yuan 1968; Hanahan 1983
PLASMID		
pET30PZ1	*gol* coding sequence from the *Pst*I site in gene 23 to the *Hind*III site at the Δ1 deletion cloned between the *Pst*I and *Hind*III sites of pET30b.	This work (Snyder, L.)
pGEX2T-*tuf*A	*tuf*A gene cloned into pGEX2T under *tac* promoter	Parmeggiani laboratory.