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Extragenic Suppression Analysis of TS Mutations Using Sec61p

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Abstract

During synthesis, secretory and membrane proteins are cotranslationally translocated into the lumen of the endoplasmic reticulum through an aqueous gated channel. Proper folding, degradation, and transport of many polypeptides depend on a diverse set of helper proteins termed chaperone. I hypothesize that Sec 61p is a membrane chaperone, which actively directs membrane protein folding.

1. Introduction

The folding of membrane and secretory proteins is a complex process that occurs in the Endoplasmic Reticulum and involves a number of discrete reactions facilitated by specific folding enzymes and a group of endoplasmic reticulum – specific molecular chaperones. Deviations in the folding and assembly of proteins are believed to be an important cause of disease. Molecular chaperones are present in virtually all types of cells and in most cellular compartments; they perform several biological functions. Some are thought to be involved in the transport of proteins into various cellular organelles. Given the complexity of the folding reactions and the need for conformity in the process, it is not surprising that folding flaws play a part in many diseases. All proteins start out on a ribosome as a linear sequence of amino acids. This sequence must fold during and after the synthesis so the protein can take up its native conformation. The folding pathway of a protein is extremely complicated and not all research has been completed on its process, but it is scientifically evident that the folding process is facilitated by the action of specialized proteins called chaperones.

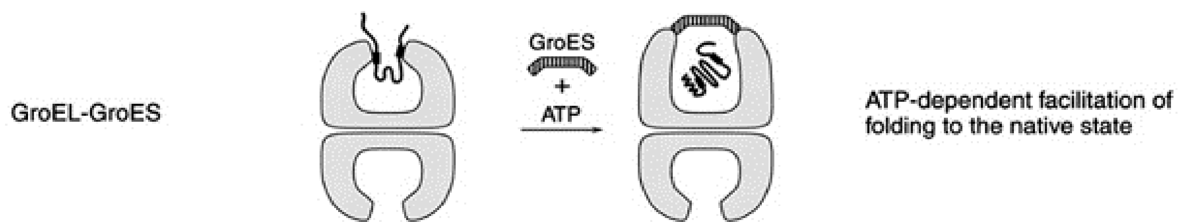
Signal Hypothesis (Blobel)

The signal hypothesis defines how numerous newly synthesized and distinct proteins are targeted to specific intracellular membranes either for translocation across them or for asymmetric integration.(1) A protein that is to be integrated into the membrane uses a signal sequence to open the channel. (protein conducting channels). Blobel hypothesized that organelle proteins contain signal sequences that direct

them to their proper destination. His research found that each kind of cell organelle has its own characteristic signal sequence, a cellular "zip code" that directs it to its proper address within the cell. Signal sequences are nothing more than certain amino acids which, when strung together, allow proteins to be specifically recognized by certain machinery in the cell. Two interacting proteins have stretches of amino acids that fit together like a lock and key.

Translocon Chaperonin Hypothesis (Smith)

Proper folding, degradation and transport of many polypeptides depend on a diverse set of helper proteins called chaperones. Chaperones fold a wide range of proteins. During this process, some proteins are folded correctly; others however, can be "misfolded" and be the cause of aging and disease. The correct folding of proteins is a critical step in determining their subsequent fate.(2) If not folded correctly the "misfold" may lead to serious problems in the accomplishments of its biological function. Particular chaperones are involved in the transport of proteins into various cellular organelles and some help cells survive stresses such as heat shock and glucose starvation. GroEL with its cochaperonin GroES capture, encapsulate and release their substrates in cycles driven by ATP binding and hydrolysis. (3)



GroEL/S acts as a general chaperone by providing substrates in a protective environment and allowing folding to proceed. GroEL/S is able to assist and in some cases substantially accelerate the folding of a number of substrates, suggesting a catalytic role for the chaperonin. (3) Cotranslational protein translocation across and integration into the membrane of the endoplasmic reticulum (ER) occur at sites termed translocons. Translocons are composed of several ER membrane proteins that associate to form an aqueous pore through which secretory proteins and lumenal domains of membrane proteins pass from the cytoplasm to the ER lumen. Intracellular transport refers to the movement of substances across membranes or organelles inside the cell. The major cellular routes of Intracellular transport are: A) The biosynthetic pathway, which is responsible for the transport of proteins from the ER to the extracellular space. This is formally known as "secretion". B) The endocytic pathway, which is responsible for the uptake of molecules from the extracellular milieu to be used in cellular metabolism. The focus of this research is the biosynthetic pathway. Most membrane proteins involved in secretion are synthesized in the ER whose luminal environment is especially suited to facilitate the proper folding of the synthesized protein and the initial steps of protein glycosylation. Sec61 is a translocon subunit that is responsible for both forward and retrograde transport of proteins across the ER membrane. (4) The Sec61 mediates cotranslational protein import into the ER in both yeast and mammalian cells.(4) Therefore we believe that the translocon is capable of directing TMP folding and correcting TMP misfolding. Research has shown that in most cases, misfolded proteins are eventually degraded. Studies of the degradation of misfolded secretory and membrane proteins have revealed that they can be extruded from the ER into the cytosol through the same

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channel involved in importing the proteins into the ER during synthesis.(6) Before the TCH (translocon chaperone hypothesis) it was evident that after the reverse translation back to the cytoplasm, misfolded proteins become attached to ubiquitin molecules and are subsequently degraded by the proteasome. Our studies on yeast and TS/CS mutants could reveal another "end" for the misfolded protein. Recent data has indicated that the translocon is capable of inverting and secreting a single pass membrane protein. (4) Using Sec61p, it is thought that this translocon subunit participates in the entrance of proteins into the ER and in their removal. (4)

Fusion of the Signal Hypothesis with the Chaperone Hypothesis

The translocon is active in recognizing and orienting transmembrane helices. (4) Proteins to be transported within cells contain structural information that guides them to their destined destination. The main secretion pathway uses a heterotrimeric protein complex or translocase which becomes a conduit that newly created proteins can pass through and then continue folding. It is obvious that the structure of a protein and its ability to carry out its correct function are very tightly linked, such that small structural defects can produce a number of diseases. These include genetic diseases such as cystic fibrosis and sickle cell anemia, which are caused by single residue deletion and mutation respectively, rendering the protein incapable of its normal function. If the membrane chaperonin has a part in correct folding, and in misfolding then it seems obvious that the translocon can have actual interaction with each protein it translocates, and correct the misfold. If, in the signal hypothesis, each protein has a particular destination within proper folding, then it can be hypothesized that an incorrect signal can be corrected within the membrane chaperone and cause the protein to reach its correct "zip code". Crucial to the test of this hypothesis is an empirical demonstration that Sec61p directs the folding of TMS proteins or TM protein classes. So far Thomas & Simon (4) have only shown permiscuous activity of the translocon to a single artificial substrate, while phenotypic assays they have completed suggest the possibility that the mutant alleles of Sec61 differentially effect folding. They have not identified targets of the mutant alleles. It is the aim of this research to provide a mechanistic basis for the translocon chaperone hypothesis. Despite considerable progress in understanding the mechanisms of protein folding a new question has arisen as a result of this "fused hypothesis" research. If the Sec61p is a membrane chaperone that can actively direct membrane folding, then is it not feasible that the TMP can correct misfolding? This is the ultimate aim of the Translocon Chaperone Hypothesis.

2. Materials and Methods

Explanatory

Our research intends to create a library of TS mutants for use in suppressor genetic screens. This library will be a resource for researchers elsewhere for proteomic studies of protein confirmation.
TS mutants are typically generated by random mutagenesis, followed by detailed screening procedures. Research has shown that many TS mutations identify sites in the polypeptide chain that are critical for the

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folding or maturation of the protein. (7) The desired initial result of this research is to view a TS frequency of 2-5%.

Strains

BY4741 a derivation of the Hansen telemorph
Mata, his3, leo2, met15, ura3

Growth Conditions

YPD was used to maintain and propagate yeast in Petri dishes. YPD is made of :

2% Agar
5g Yeast Extract
10g Bacto Peptone
10g Dextrose

Yeasts grow well on a minimal medium containing only glucose and certain salts. Adding protein and yeast extract cause accelerated growth. (cells divide every 90 minutes)

Mutagenesis

UV mutagenesis was initially performed according to the Corbett lab protocol. (5) Two attempts were made using the strict protocol. No results were produced with the initial conditions. The following modifications were made:

1. Distance was adjusted from 4 cm high to 10 cm high.

2. Time sets were adjusted from 0, 1 minute, 2 minutes and 3 minutes to 0, 15 sec., 30 sec., 45 sec., and 60 sec.

3. UV wavelength was adjusted to its lowest setting at 252 nm.

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4. Initially the lids of the dishes were left off, in the final set the lids were left on to give fluence.

Conditions for testing the TS

1. The first plate grown serves as master plate.
2. Two replica plates are made by replica plating.
3. One plate is placed at 37 degrees for 2-3 days.
4. Another plate is placed at 30 degrees for 2-3 days.
5. The plates are then compared (counted) for differential colony growth.

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3. Results and Discussion

The first mutagenesis attempt was done with the UV source extremely close to the yeast. This resulted in a complete "burning" of the sample and no results could be retrieved. The second attempt at mutagenesis was done at a distance of 4 cm. Some colonies survived but the majority were "burned". The third endeavor to create TS mutants was irradiated at a distance of 10 cm, with a shorter time of irradiation. This resulted in successful colony cultivation. The first successful mutant results are shown in ***Appendix A***.

The optimal range of success was considered to be between 30 seconds and 60 seconds. These particular time point plates were replica plated. Of replica 1 a 60 second and a 30 second were placed in 30 degree temperature for 2-3 days. A 60 second and a 30 second plate of replica 2 were placed at 37 degrees for 2-3 days. 37 degrees has been shown to be the optimal temperature to grow yeast cells. The 30 degrees set was used to grow TS mutants. The results after 2-3 days of temperature exposure are shown in ***Appendix B.*** The colonies that were at 37 degrees had a higher number of colonies than those that grew at 30 degrees. After scanning the plates and observing obvious TS and CS mutants the percentage achieved was as follows:

30 sec. Replica 1 30 degrees 7 TS mutants on plate of 144 colonies 5 %

60 sec.	Replica 1	30 degrees	8 TS mutants on plate of 214 colonies	4 %
60 sec.	Replica 2	37 degrees	3 CS mutants on plate of 243	1 %
30 sec.	Replica 2	37 degrees	2 CS mutants on plate of 187	1 %

The scanned results of this initial scaled down research is shown in ***Appendix C***. The results, though small were highly favorable. The percentage of TS mutants achieved on a small scale is a positive result.

4. Future Work

Gunter Blobel, the man behind the signal sequence hypothesis, once said that "to do science, one must never lose the hope of a child". It is with this type of hope that this research has been founded. Our future research consists of :

1. scaled up mutagenesis.
2. Confirm and authenticate TS and CS from the primary genetic screen.
3. Make frozen stocks of TS mutants.
4. Create mutagenized sec61 library
5. When 100 mutants are obtained the goal is to transform that mutant pool or mutant subpool of 10 each with a library of 40,000 mutant Sec61 sequences to achieve adequate representation of all mutant sequences in the Sec61 library , in all of the strains from the TS strain collection.

We observed positive results on a small scale. It is evident that a similar result could be achieved on the larger scale but with more results. The aim of the research was achieved. We developed a set of conditions for scaled up mutagenesis. We succeeded in obtaining a TS frequency of 2-5 %. We now need to make a library of mutant sec61 sequences to begin our suppression screen.

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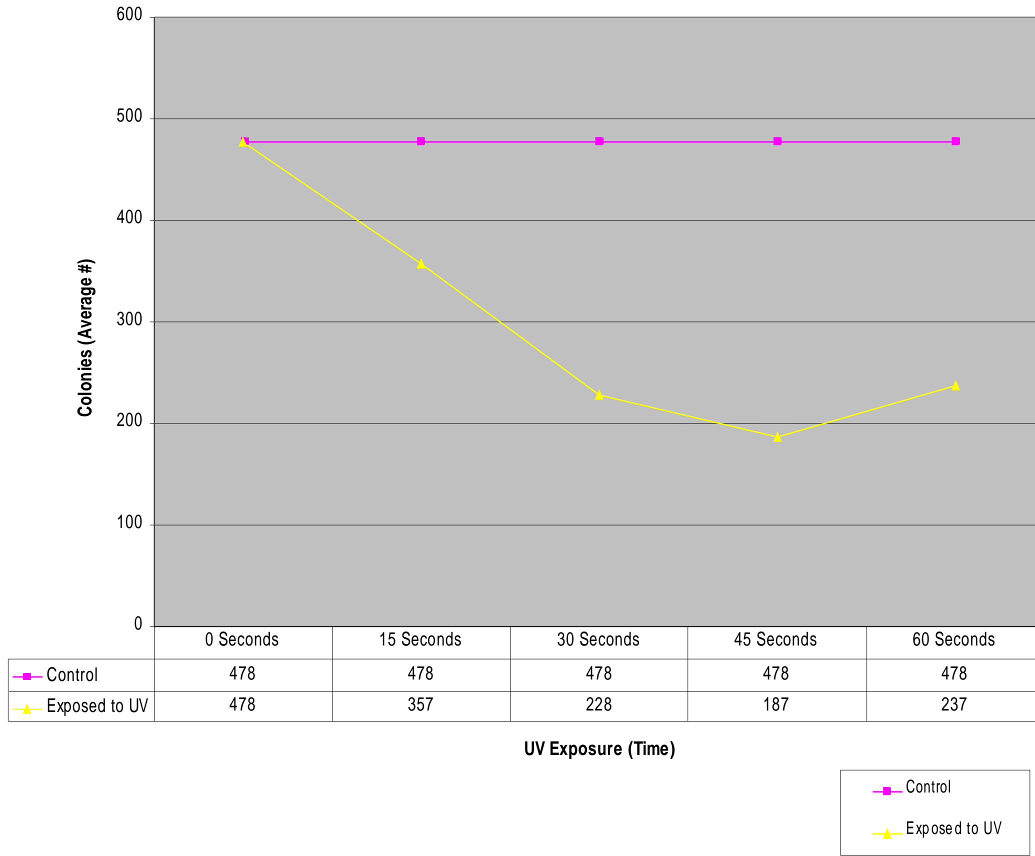
Appendix A



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Appendix B