Lecture 34
5/10/04

Review (not required) on ubiquitination in Biochem J (2004) 379, 513-25

Final will be 9-12 am on Mon May 17th

Proteosome

ClpXClpP
See lecture 33 for more experiment details
Titan-ssrA -> global and local stability of protein- is this related to ATP consumption in unfolding and translocation to the proteosome?

See model on p. 14 of handout 4a
Also see location of titan mutations in structure on handout 4c

Unfolding is related to stability of protein- it takes more ATP to unfold a more stable protein- Many ATP's are needed

"The whole is greater than the sum of the parts"
Both ClpX and ClpP are needed for efficient unfolding and translocation

Use soluble, denatured titan-ssrA to look at ATP required for just translocation (already unfolded)

All experiments looking at the kinetics of ATP hydrolysis and the total amounts of ATP consumed per molecule of titan degraded were done in the steady-state. To get information on whether energy from ATP binding or hydrolysis is used- to effect denaturation or unfolding and translocation would need pre-steady state analysis and a method to assay for partial unfolding or partial translocation.

Conclusion from the titan experiments:
1.The rate determining step is either unfolding or translocation- depends on the stability of the protein
2.Proteolysis and product release (product is 6-10 amino acids peptides) are not rate-limiting- they are fast
3. wild type titan with ssrA tag, ClpXClpP uses 640 ATPs to unfold 1 protein

Rate constants	Unfolding	Translocation
Wt titan-ssrA	0.29 min^{-1}	4.3 min^{-1}
Mutant (near c-term, Disrupts local stability)	11 min^{-1}	4.3 min^{-1}

Remember that the rate constant for proteolytic product formation is 4.3 min^{-1}

The number of ATPs tells you about the use of mechanical force in the problem of unfolding and translocation. The need for so many cycles of ATP hydrolysis to denature a single titan-ssrA suggests strongly that unfolding is a stochastic process that requires a repeated or interative application of force.

IV. **How do you target proteins for degradation**??
We will look at two systems

1. procaryotes- ssrA tag- tmRNA
2. eucaryotes – post-translational modification by ubiquitin and the N-end rule (polyubiquitination)

SsrA-
The tmRNA (also sometimes called ssrA)- directs modification of proteins where biosynthesis of the polypeptide is stalled on the ribosome. This modification, which adds 11 amino acids targets the truncated polypeptide for degradation by ClpXClpP.
Examples:

1. Quality control for mRNA that has been hit with a nuclease, has no stop codon and the mRNA just ends. How do you unclog the ribosome?
2. Stalling at rare codons (or anything that obstructs translation such as damage to the base or an abasic site where the base is cleaved from the sugar)

Need an adaptor protein-> SmpB that interacts with the ssrA tagged peptide and targets it to the AAA+ ClpX to the proteosome

TmRNA-> adds ssrA polypeptide tag to the stalled protein giving an ssra tagged peptide that interacts with SmpB adaptor protein and targets it to proteosome for degradation

See handout 4c for structure of tmRNA (transfer/messenger RNA)
The top of the tmRNA looks strikingly like the $tRNA^{ala}$ (with no anticodon)
In fact, the ala tRNA synthatase (RS), if given Ala and ATP, can charge tmRNA with alanine

The alanine charged tmRNA goes into the "A" site on the ribosome, adds Ala to end of stalled peptide chain. There is no codon/anticodon interaction and therefore binding to the A site is proposed to be based on shape selection of the tmRNA (molecular mimicry, remember structures of EF-G, EF-Tu•tRNA and RF1 and 2---all bind to the A site) allows binding to the "A" site. The delivery of alanine charged tmRNA uses EF-Tu!
Handout 4c shows the interaction of tmRNA (called ssrA) with the stalled ribosome

The mRNA part of the tmRNA codes for the rest of the ssrA tag (10 additional amino acids followed by a stop codon and these are added on after the alanine
Protein-ala-10-more-aminoacids = protein-ssrA

How all this occurs and the conformational changes required are still an active area of research

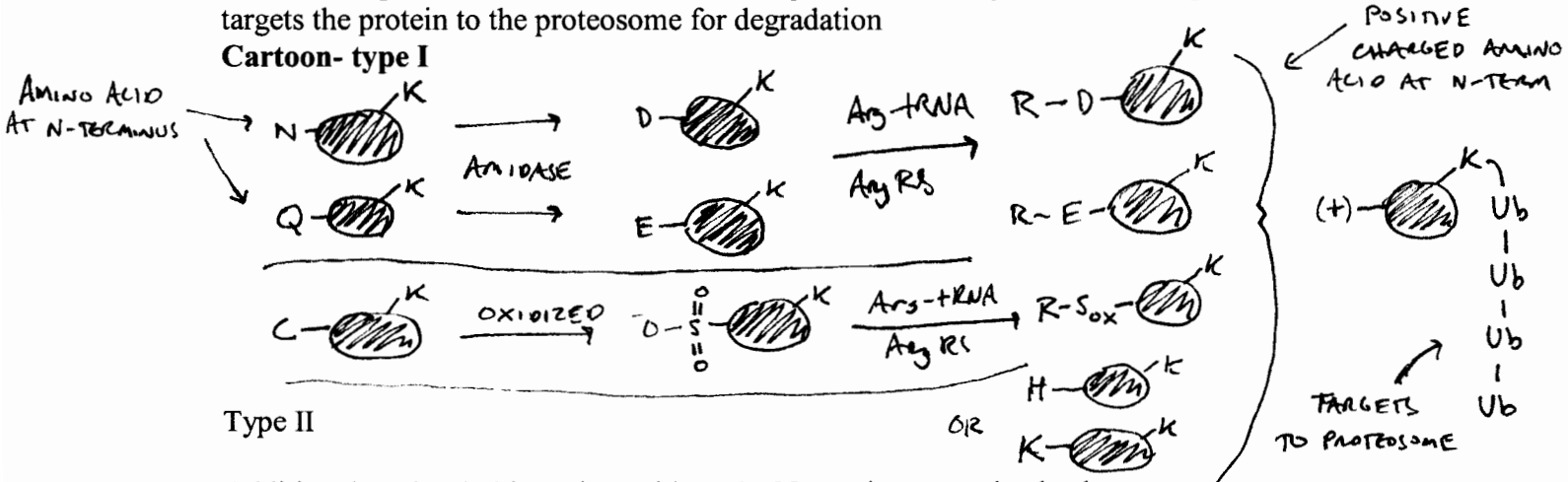
Targeting in Eucaryotes
Half life of proteins in cells varies from seconds to days.
Varshavsky- studied what determines protein lifetime and came up with the "N-end rule"
N-end rule= the N-terminal amino acid determines the lifetime of the protein-
In yeast, a destabilizing residue at the N-terminus and a lysine that will become poly-ubiquitinated determines the rate of degradation
Unstable N-terminal amino acid leads to poly-ubiquitination which then leads to rapid degradation by the proteosome. The mechanism will be discussed subsequently

Every organism has a different N-end rule- different destabilizing amino acids, different degradation systems. We are only discussing the yeast system.

Type I

Q, or N at N-terminus may be converted to D or E by an amidase
C at N-term may be oxidized
Then an R may be added to the N-terminus of D,E, or oxidized C by the Arg-tRNA + Arg-RS. All of these proteins now have an R at their N-terminii.
These modified proteins and R, H, or K, (positively charged amino acids) naturally occurring at the N-terminus are rapidly degraded by the ubiquitin-dependent pathway.
About 4 ubiquitins are added onto a K in the protein to be degraded, this ubiquitin chain targets the protein to the proteosome for degradation

Cartoon- type I



Type II

Additional Hydrophobic amino acids at the N-terminus can also lead to polyubiquitination and protein degradation. These proteins are probably recognized by a different ubiquitin ligase E3. As we will see, yeast has >100 E3s.

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Ubiquitin (Ub) is a 76 amino acid protein.
The C-terminus of Ub has two glycines (crucial for chemistry that will be discussed subsequently)
Only when multiple ubiquitins are attached is a protein targeted for degradation
Ubiquitin is as prevalent a posttranslational modification, and it may even be as common as post-translation modification by phosphorylation. In many cases only a single Ub or a

Ub like protein is attached. The function of attaching a single Ub will be highly varied but you can imagine it could modulate activity, effect protein-protein interactions etc. For us we are only concerned with polyubiquitination and protein degradation.

MODEL

Ubiquitin ligase (E3) determines substrate specificity-> interacts with protein with destabilizing N-term amino acid. As noted above there are >100 Ub ligases.
E1 + Ub + ATP-> E1-Ub + AMP; E1-Ub ubiquitinates E2; E2-Ub ubiquitinates the protein with the unstable N-terminus interacting with an E3; Several ubiquitins (four) are added. This polyUb targets the protein for degradation by the proteosome.
In yeast, there is 1 E1, 13 E2, and over 100 E3 proteins. E1 has a >>>> turnover number than E2 or E3 and that is why only one is needed.

Drawing of model