Part 2B Data Interpretation

In eukaryotes, proteins are targeted for degradation by the proteasome by ubiquitination. Ubiquitin is a 76-amino acid protein and ubiquitination requires action of three enzymes (E1, E2, and E3) (reviewed in Hershko et al. 1998.). In contrast, most bacteria, including *E. coli*, do not have a proteasome. Prof. Cousteau’s group has found that *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and other *Mycobacteria* species do have a proteasome similar to that found in eukaryotes. Substrates for the mycobacterial proteasome have been identified. However, homologs of ubiquitin, E1, E2 and E3 are not found in the mycobacterial genomes, and it remains unknown how these proteins are targeted to the proteasome. The following experimental data was obtained by Prof. Cousteau’s group. Review this data and answer the following questions. Please be specific in your answers.

**Panel A.** Results from an *E. coli* two-hybrid system experiments performed using the method described in KARIMOVA et al. 1998. *E. coli* (*cyta*) was transformed with combinations of plasmids encoding either of the two domains of *Bordetella pertussis* Cya, T25 ("plasmid 1") or T18 ("plasmid 2"), fused to test proteins. Pup is a full-length (64 amino acids) protein identified from a *M. tuberculosis* genomic T25 library with T18C-Mpa as bait. The protein Mpa is a subunit of the mycobacterial...
proteasome. Pup is a 26–amino acid fragment of Pup identified also from a M. tuberculosis genomic T25 library with T18C-Mpa as bait. ("+"), growth on minimal lactose agar. All strains grew on minimal glucose agar (not shown).

Panel B. Results from affinity chromatograph-based analysis of protein-protein interactions. A lysate of E. coli expressing recombinant Mpa ("input") was incubated with purified His6-Pup, purified SigE-His6, or a lysate of E. coli with expression vector only ("empty") on nickel-nitritriacetic acid Ni-NTA agarose. After incubation, the Ni-NTA agarose was collected by centrifugation and the supernatant ("flow through") was saved. After washing the collected agarose with buffer with low imidazole concentration, proteins were eluted from the agarose with buffer containing high imidazole concentration ("elution"). Input, flow through, and elution fractions were separated by 15% SDS–polyacrylamide gel electrophoresis (PAGE) and visualized with Coomassie Brilliant Blue (CBB). The same samples were also analyzed by anti-Mpa immunoblot (IB). Note that His6-Pup migrated (faster than expected) as ~15 kD protein in the SDS-PAGE. This observation is irrelevant to this exam and should not be taken into account for data analysis.

Panel C. Results from a Mycobacterium smegmatis two-hybrid system. These experiments were conducted using the mycobacterial protein fragment complementation system described in Singh et al. 2006. M. smegmatis was transformed with combinations of plasmids encoding either of the two domains of murine dihydrofolate reductase (mDHFR), ("plasmid 1") or ("plasmid 2"), fused to Pup, FabD (a known protein substrate of the mycobacterial proteasome), GCN4 (a Saccharomyces cerevisiae leucine zipper domain), or no other protein. ("+"), growth on antibiotic trimethoprim (Trim). Pup had weak interactions with GCN4 (f, l). All strains grew on media lacking Trim (not shown).

Panel D. FLAG-tagged proteins were enriched by immunoprecipitation with anti-FLAG antibodies from equal amounts of lysates of M. smegmatis with plasmids expressing FLAG-FabD and either empty vector or His6-Pup. Untagged FabD was the negative control. Samples were separated under denaturing conditions by 12% SDS-PAGE, and analyzed by anti-FLAG or anti-His 5 immunoblotting. FLAG-FabD migrated at the predicted size (arrow, left); the ~45-kD anti-His 6–reactive protein (asterisk, right) is only seen in mycobacteria producing FLAG-FabD and His6-Pup.

Q1) (a) Briefly, describe the E. coli two-hybrid system used in panel A and (b) indicate what information can be obtained from the results shown in this panel.

Answer (a) The bacterial two-hybrid system allows for an in vivo screening and selection of functional interactions between two proteins. This genetic test is based on the reconstitution, in an Escherichia coli cya (adenylate cyclase deficient) strain, of a signal transduction pathway that takes advantage of the positive control exerted by cAMP. Two putative interacting proteins are genetically fused to two complementary fragments, T25 and T18, that constitute the catalytic domain of Bordetella pertussis adenylate cyclase. Association of the two-hybrid proteins results in functional complementation between T25 and T18 fragments and leads to cAMP synthesis. Cyclic AMP then triggers transcriptional activation of catabolic operons, such as lactose or maltose, that yield a characteristic phenotype (such as lactose fermenting, growth on lactose). In this genetic test, the involvement of a signaling cascade offers the unique property that association between the hybrid proteins can be spatially separated from the transcriptional activation readout.

Answer (b) A fusion protein that encoded the last 26 amino acids of Rv2111c (prokaryotic ubiquitin-like protein, here referred to as “Pup”) interacted with the Mpa (Mycobacterium proteasome ATPase) bait fusion. Full-length Pup also interacted with the Mpa. Mpa also interacts with itself.
Q2) (a) What information can be obtained from the results shown in panel B? (b) What might have been the purpose of including SigE-His6 (a His6-tagged *Salmonella typhimurium* protein) in the experiment shown in this panel and what information was obtained from inclusion of SigE-His6 in the experiment, which could not be derived from panel A?

**Answer (a)** Recombinant Mpa is able to interact in vitro with His6-Pup bound to nickel-nitrilotriacetic acid (Ni-NTA) agarose. The interaction is non-covalent in nature (no band for Pup-Mpa fusion is detected).

**Answer (b)** SigE-His6 is a *Salmonella typhimurium* protein that is similar in size and charge to Pup. This is a control protein used to investigate the degree of specificity of the interaction of Mpa and Pup. Mpa did not interact with SigE-His6, thus suggesting that the Mpa-Pup interaction is specific. The results shown in panel A do not permit one to assess the specificity of the interaction.

Q3) (a) Briefly, describe the mycobacterial protein fragment complementation system used in panel C and (b) indicate what information can be obtained from the results shown in this panel.

**Answer (a)** This two hybrid system, termed mycobacterial protein fragment complementation (M-PFC), permits to study protein–protein association in mycobacteria. The system is based upon the functional reconstitution of two small murine dihydrofolate reductase (mDHFR) domains independently fused to two interacting proteins. When two mycobacterial interacting proteins are independently fused with domains of mDHFR, functional reconstitution of the two mDHFR domains can occur in mycobacteria, thereby allowing for to selection of mycobacteria resistance against trimethoprim (TRIM). Despite the fact that both prokaryotic and eukaryotic DHFRs are targets of the antifolate drug TRIM, mammalian DHFR has a ~12,000-fold lower affinity for TRIM than does bacterial DHFR. The selective targeting of mycobacterial DHFR by TRIM is the basis of the *in vivo* assay and allows for the identification of recombinant mycobacteria expressing functional mDHFR by screening for growth in the presence of TRIM concentrations that inhibit endogenous the mycobacterial DHFR.

**Answer (b)** The results demonstrate a strong positive interaction between Pup and the proteasome substrate FabD (malonyl coenzyme A acyl carrier protein).

Q4) Data not shown herein have demonstrated that Pup can be found covalently attached to FadD under specific conditions. What specific data from the above experiments supports this fact and why?

**Answer.** The fact that His5-specific antibodies detected a purified ~45-kD species when FLAG-FabD and His6-Pup were coproduced in mycobacteria (Panel D). This ~45-kD species, probably representing a Pup-FabD complex, that is highly stable because it was maintained under reducing and denaturing conditions. The formation of a stable complex between the substrate FabD and Pup is reminiscent of the covalent attachment of ubiquitin to proteasome substrates in eukaryotes.