1. Hsp 70 chaperones assist a large variety of protein folding processes in the cell including folding of newly synthesized proteins in the cytosol, translocation of proteins into organelles such as the mitochondria and assembly and disassembly of protein complexes. The substrate specificity of Hsps is in part dictated by the co-chaperones, the Hsp40s. DnaK and DnaJ are examples of the Hsp70 and 40 respectively, discussed in class. In class we also briefly discussed the substrate specificity of DnaK, although we did not discuss the experimental methods that elucidated this specificity. This problem addresses the methods developed to address the specificity of DnaJ and its similarities to the specificity determined for DnaK. The methods are generally applicable to a variety of systems that interact with contiguous amino acids in peptides including the proteosome.

Fourteen proteins have been identified to interact with the DnaKJ system in vivo. The sequences of these proteins are known. A method was developed to screen 13-mer peptides bound to cellulose solid support. The peptides were synthesized and represent the complete sequences of the 14 proteins. The peptides synthesized overlap with adjacent peptides by 10 residues. Previous studies on both DnaJ and DnaK have demonstrated that these proteins can bind tightly to the appropriate peptides and that the binding is not affected by attachment to cellulose.

Several experiments have been carried out to address the question of substrate specificity of DnaJ and its similarities and differences with DnaK. You are given the following data. Peptides covering contiguous sequence space for four proteins (DnaA, λP, p53 and luciferase) have been generated and attached to cellulose by a (β-Ala)$_2$ spacer. DnaJ (50 nM) is allowed to bind and reach equilibrium with gentle shaking in Tris-HCl pH 7.6, 100 mM NaCl, 6.4 mM KCl, 0.05% Tween for 30 min at 25°C. The excess DnaJ is removed by washing. The peptide-bound DnaJ is electro-transferred on to a polyvinylidene difluoride (PVDF) membrane using a semi-dry blotter apparatus. The transferred DnaJ is detected using Dna J-specific polyclonal antibodies and an alkaline phosphatase-conjugated secondary antibody. The alkaline phosphatase catalyzes hydrolysis of a chemiluminescent substrate that can be measured using a fluoroimaging system. The relative intensities are normalized to the signal of a reference peptide (AKTLILSHLRFVV) that is set at 100.

The results of the blotting on peptides associated with the four proteins are shown in Figure 1. The screened peptides were ordered according to their affinity for DnaJ as determined by quantification of the fluoroimager signals. The affinities varied all over the place with high affinity defined as those > 40 and these peptides were further investigated. The composition of the tight binding peptides have been normalized relative to the entire library (black bars) and compared with a similar experiment carried out with DnaK (white bars). The results are shown in Figure 2 A, B.

Finally, a search was made for a consensus motif of the 62, DnaJ-tight-binding peptides. Hydrophobic cores were anchored with a large hydrophobic or aromatic residue at position 10 by shifting the sequences by up to two residues. The frequency of the acidic (white bars), large hydrophobic and aromatic (black bars) and basic residues (grey bars) at each position is given as a percentage. The results are shown in Figure 2C.

Finally a comparison of DnaJ and DnaK binding to peptides in luciferase is shown in Figure 3A. The Luciferase-derived peptides that were screened for DnaJ binding are represented as bars. The length of the bars corresponds to the affinity of the peptide for DnaJ. Black bars represent peptides that were previously classified as Dna K binders.

Figure 1   Figure 3

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Questions:
1. Describe the method for detection of bound DnaJ.
2. What does the data in Figure 1 tell you about the binding domain of DnaJ for peptides?
3. What does Figure 2B tell you about the specificity of DnaJ for specific amino acids? Does this observation make sense with the putative function for DnaJ in vivo? Why? How does the specificity of DnaJ for peptides compare with that of DnaK in this analysis?
4. What does the data in Figure 2C further tell you about the binding specificity of DnaJ?
5. What does the data with peptides generated from luciferase tell you about the specificity of DnaJ and DnaK?
6. Draw a model for the substrate binding site of DnaJ.

2. To probe metabolic pathways both genetics (gene knock outs) and chemical genetics (specific inhibitors of an enzyme catalyzed step in a pathway) can provide important information about the function of a specific pathway. The natural product, lactacystin isolated from *Streptomyces*, has been useful in thinking about the function of the proteosome. Lactocystin and its metabolites are shown in Figure 4.

One pathway that has been probed using lactocystin is the mechanism by which the body mounts its immune response to virus invasion. Specifically the viral proteins can be degraded and presented on the surface of T cells to trigger off an immune response. Major histocompatability complex (MHC I) class I molecules typically bind 8 to 9 residue peptides generated from viral proteins. Most of these peptides are generated by protein degradation in the cytosol. The peptides are transported in a specific fashion into the endoplasmic reticulum where the peptide, an MHC class I protein and β_2-microglobulin associate and the complex is transported through the Golgi apparatus to the plasma membrane where the peptide is exposed on the surface of the cell in complex with an MHC I protein. This process (antigen presentation) allows T lymphocytes to screen for cells that are synthesizing foreign and abnormal proteins. The mechanism required for generation of the class I-presented antigens has been recently studied with the results described below.

Lactocystin or a metabolite has been proposed to specifically inactivate the proteosome. To test this model [^{3}H]-lactocystin has been examined with crude cell extracts, proteosme fractions, and with purified 20S and 26S proteosome. After incubation, SDS-PAGE and fluorography revealed the results in Figure 5A. To analyze the results in more detail, the 20S proteosome was incubated with radiolabeled lactocystin and analyzed by two dimensional PAGE and identified by Western blotting with specific antibodies to the α or β subunits (Figure 5B). Note that the β subunits are: LMP2, LMP7, Y, X, Z, MECL-1 etc. In addition, the 20S and 26S proteosome has been studied using specific fluorgenic substrates for its muliple activities (Table 1).
Questions:
7. What does this data suggest about the target of lactocystin? Explain why the data in Figures 4 and 5 lead you to this conclusion.
8. Propose a mechanism for lactocystin given what we have discussed in class about the mechanism of proteases.

9. What does the data in Table 1 tell you about the lactocystin and its metabolite, β–lactone?

Given what you have learned about lactacystin, we are now ready to address the mechanism of peptide presentation on the surface of T cells. To examine this question the protein ovalalbumin was chosen as the precursor for peptide presentation. Antigen-presenting cells were first reacted with or without lactacystin and then infected with a recombinant vaccinia virus that expresses high levels of T7 RNA polymerase, and subsequently transfected with a plasmid containing ovalbumin cDNA under the control of a T7 promoter. After allowing time for antigen presentation, cells were fixed and tested for the presence of surface K_bMHC class I molecules containing the ovalbumin-derived peptide SIINFEKL, using an antigen specific monoclonal antibody. The results of these experiments using two different cell lines (E36.12.4 and DAP34.8) are shown in Figure 6. [Note LLnL is a peptide with an aldehyde at its C-terminus, a man-made protease inhibitor.] In Figure 6C, a cDNA containing a minigene to generate SIINFEKL was expressed in place of the gene for ovalbumin.

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Questions:
10. What do the data in Figure 6 suggest about the role of the proteosome in antigen presentation?
11. Suggest an additional controls might you think about, to further support your conclusion

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