5.08J Biological Chemistry II (Spring 2016)
Problem Set #7

1. Biocatalysis has emerged as a general approach to asymmetric synthesis in organic chemistry. Recently the unique protonation machinery of a squalene /hopene cyclase (SHC, Fig. 1) was evolved and engineered to function as a Bronsted acid catalyst in water. The success of the approach was highlighted by the stereospecific synthesis of a variety of cyclohexanoids (Fig. 5). One of the active areas of research today is to harness nature's catalytic machineries to generate unique catalysts.

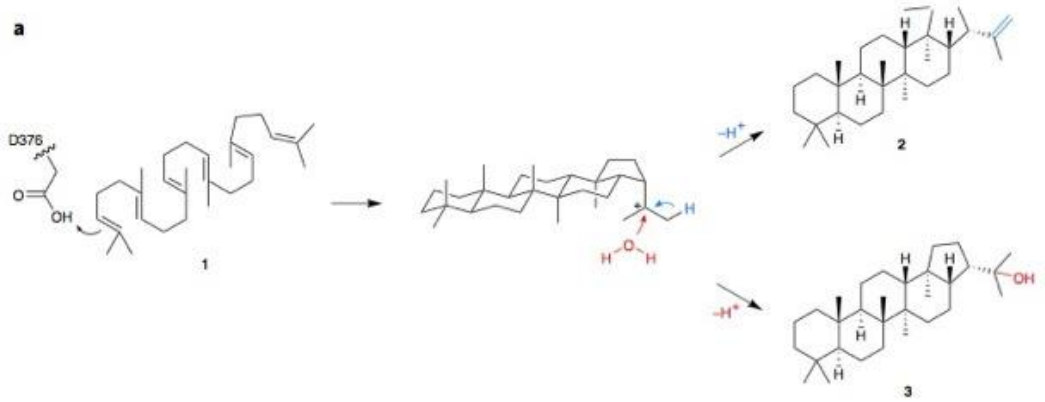
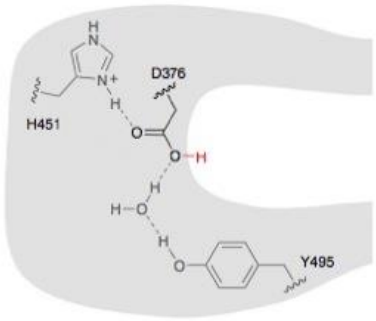


Fig 1 SHC catalyzes the polyene cyclization of squalene (1) to pentacyclic products hopene (2) and hopanol (3). [Recall from Lecture, the formation of lanosterol from the epoxide of squalene.]

The catalytic machinery of SHC is shown in Fig.2 where D376 plays a key role in the cyclization process. Shown in Fig. 3 is the active site of SHC colored in gray. The Bronsted acid D376 is shown in gray sticks (red oxygens).



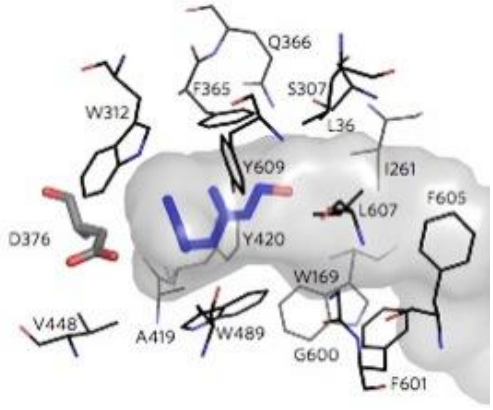


Fig 2. (left) Active site machinery of SHC and Bronsted acid D376
Fig 3. (right) The active site with its shape highlighted in gray. D376, the putative Bronsted acid, is shown in gray sticks with red oxygens and geraniol shown in blue sticks, is modeled into the active site.

A library of 34 single variants of SHC was generated by site-directed mutagenesis and screened using the monoterpenoid geraniol 4 or the (S)-6, 7-epoxygeraniol 7 revealing mutant proteins that had excellent catalytic activity and high stereoselectivity. In the case of the conversion of 4

to **6**, a single point mutation was sufficient to convert an almost inactive, wt-enzyme into a practical, useful catalyst.

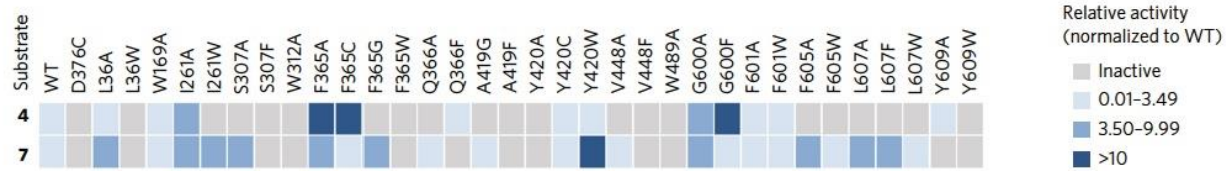


Fig 4 The generation and screening of a variety of point mutations of SHC for activity with either **4** or **7**.

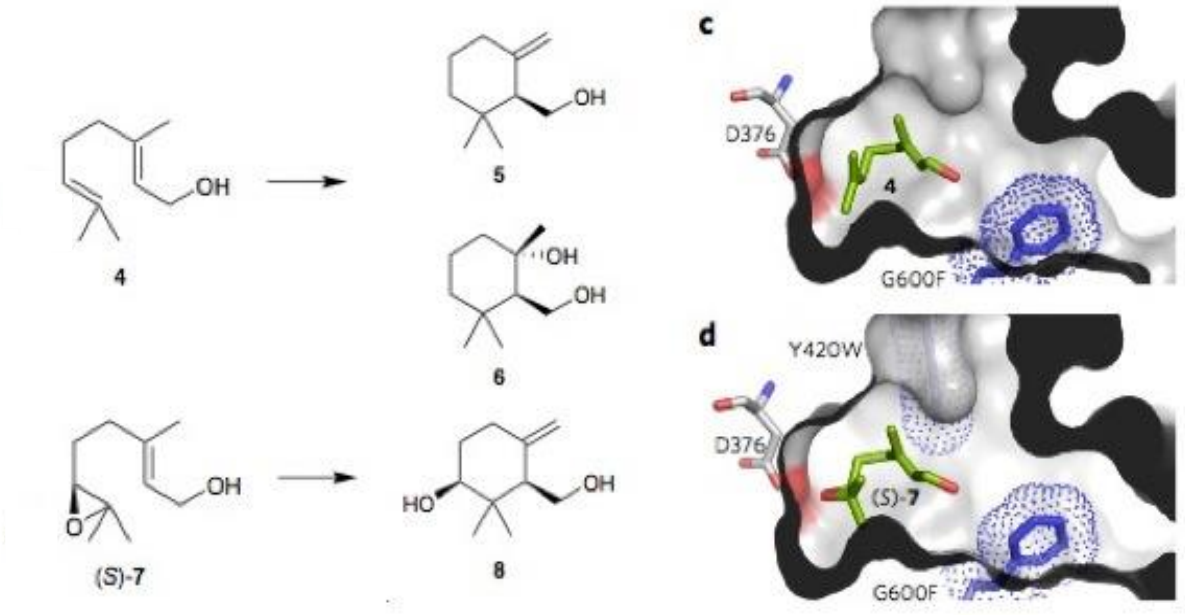


Fig 5. Cyclization of geraniol **4** and (S)-6, 7-epoxygeraniol **7**. The transformations of **4** to **5** and **6** and of **7** to **8** are shown above. The putative active conformation of 4 (c above) and 7 (d above) are modeled into the active site of SHC.

Questions:

1. Look at the active site machinery in Figs 2 and 3. Think about the basic rules for the chemistry of terpene reactions discussed in Lecture.
    a. What is unusual about D376 in Fig 2 and what might it tell you about its function?
    b. What is unusual about the active site cavity of the enzyme shown in Fig. 3 that will be important for catalysis and specificity? (Think about discussions in Lecture)
2. The reactions that the investigators were interested in catalyzing are shown in Fig 5. Propose a mechanism for the role of D376 in each of these reactions. Draw a mechanistic scheme (show the intermediates) for the conversion of **4** to **5** and **6** and for the conversion of **7** to **8**.

Summary: The distinct properties of enzymes involved in terpene biosynthesis offer many opportunities for their use as versatile catalysts taking advantage of our understanding of these types of reactions and our ability to make libraries of mutants that can be screened.

2. "The volumetric heating values of today's biofuels are too low to power energy-intensive aircraft, rockets and missiles." Recently dimers of the natural product pinene (Fig 6) has been shown to have a volumetric heating value similar to the tactical fuel JP-10. Several labs have thus focused their efforts on engineering *E. coli* to provide a sustainable source of pinene.

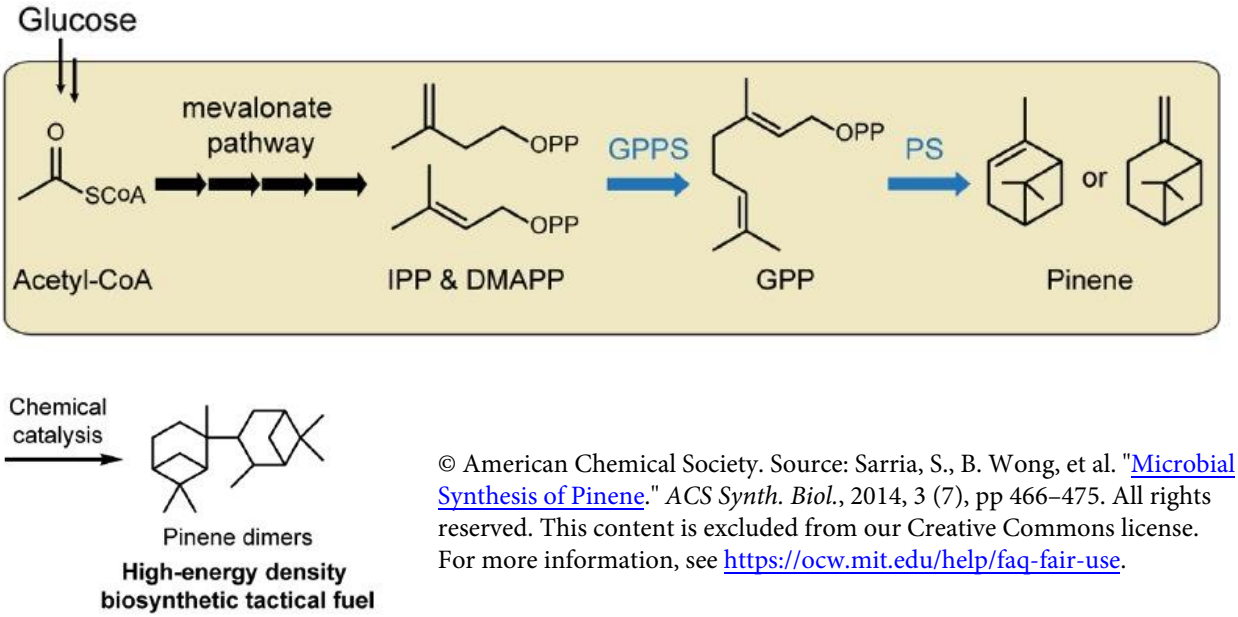


Fig. 6 Microbial synthesis of pinene using the mevalonate pathway to produce IPP and DMAPP and geranyl diphosphate synthase (GPPS) and pinene synthase (PS) to produce pinene.

Extensive experimentation was required to reduce toxicity associated with the high concentrations of GPP and the pinenes. GPPS and PS from many different organisms were examined and many problems associated with levels of protein expression were encountered. They found that the *A. grandis* PS resulted in the highest production of pinene, even though in *E. coli* it was one of the more poorly expressed PSs. Furthermore, the ratio of the two pinene isomers varied with GPPS in a way that is not understand. Work is progressing to try to optimize production of pinene and understand the basis for and fix the problems encountered.

Questions:

1. Propose a mechanism by which GPP might be converted to the two isomers of pinene. Show the intermediates that might be involved.
2. How might you design a rapid chemical quench experiment to detect your intermediates? Show design and the structures of the expected trapped species.

3. Brown, Goldstein and coworkers have studied the Scap protein in an effort to understand its function as a cholesterol sensor. The amino acid sequence and topology of the membrane domain of Scap is shown in Fig 7.

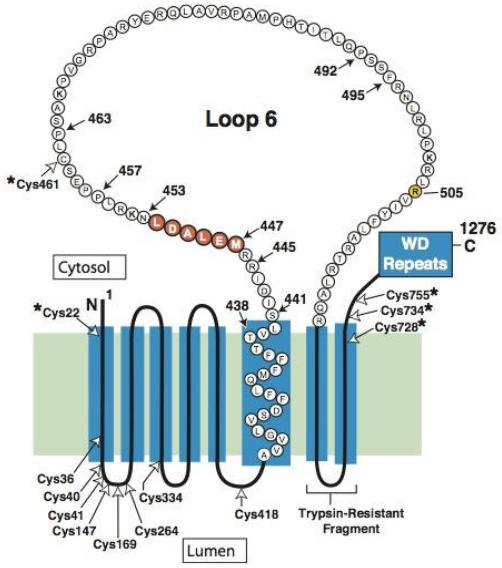


Fig 7 A cartoon representation of Scap. Loop 1 (between transmembrane helices 1 and 2 has many cysteines on the lumen side).

Summary of what you have learned in class and from required reading: Scap is as a cholesterol (Ch) sensor that regulates transport of SREBP from the ER membrane to the Golgi where it gets processed to become a transcription factor (TF). When Ch levels are low in the ER membrane, SREBP binds to the WD domain of Scap and Scap is in a conformation where the MELADL sequence in loop 6 (red, Fig 7) recruits proteins associated with COPII coated vesicle formation. This process facilitates budding of the ER membrane and both Scap and SREBP move to the Golgi. Once in the Golgi, the SREBP is processed by two proteases that generate the soluble transcription factor 75,000 Da from the full length SREBP which is 115,000 kDa. The TF then moves from the cytosol to the nucleus and activates genes involved in fatty acid, cholesterol, and triglyceride production.

When Ch levels are high on the other hand, Scap binds to an ER integral membrane protein Insig, the MELADL sequence becomes sequestered and Scap and SREBP remain in the ER membrane. **The experiments and data presented below were designed to understand the mechanism behind Scap sensing of Ch, more specifically, the role of Loop1 in this process**.

Experimental information is given in Figures 8 through 12. Loop 1 contains 245 amino acids (40-284) and faces the ER lumen. Several constructs of Loop 1 only have been designed and expressed. In addition, [^{3}H] Ch is available which can be analyzed by scintillation counting. In some cases the results with the different Loop 1 constructs are compared with the results from the full length (FL) Scap and FL-Scap-mutants. The experimental design is described in each of the Figure legends.

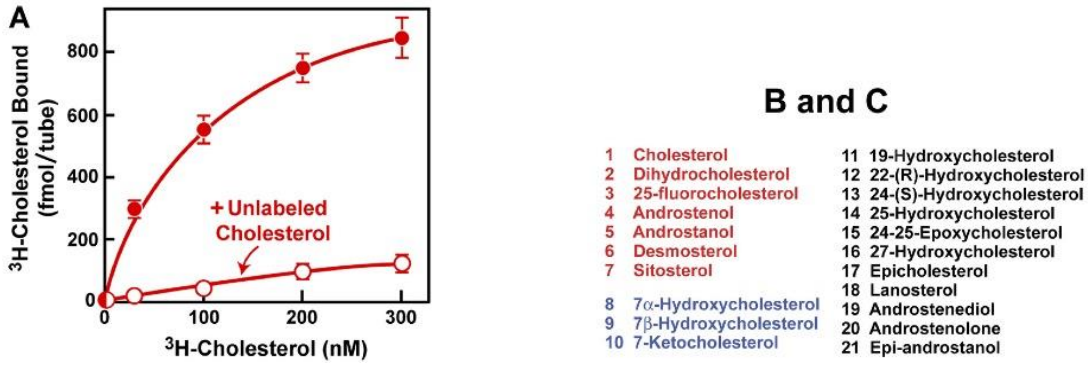


Fig 8 A. [^{3}H] Sterol binding to His$_6$-Scap (Loop1) where Loop 1 is residues 46-269. The control is identical to the experiment except that unlabeled cholesterol is added. **Fig 8B and C** are a list of the unlabeled sterols used in Figure **9B** and **9C** below (note there is no 9A). These sterols are used to displace [^{3}H]-cholesterol bound from the different Scap constructs.

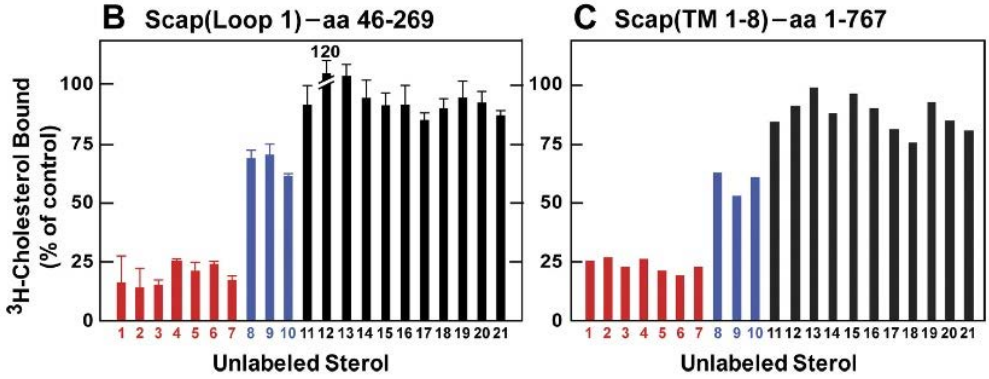


Fig 9. Comparison of the sterol specificity of [^{3}H]cholesterol binding to Scap(Loop 1, **B**) and Scap (TM1-8, where TM1-8 indicates full length Scap with 8 transmembrane helices, **C**). The assay uses scintillation counting to determine the amount of [^{3}H]cholesterol that remains bound to the small domain (**left panel, Scap(Loop 1)-aa 46-269**) of Scap and **right panel**, **full length** Scap (**Scap(TM 1-8), aa 1-767**).

Alanine scanning site-directed mutagenesis, SDM, is a method used to replace the natural amino acid with Ala at various positions within the protein of interest, in this case Scap Loop 1 (aa 47-248) and to examine the consequences of this substitution on the processing of SREBP into the TF. These Ala scanning mutants within the Loop 1 (47-248) region of Scap were each transfected into Chinese hamster ovary cells (CHO) cells that lack Scap. The cells contained a plasmid (pSRE-firefly luciferase) with a steroid responsive element (SRE) placed in front of the firefly luciferase gene. When the gene is transcribed and translated into luciferase, the luciferase activity can be easily measured even at very low amounts of protein by light emission in the presence of ATP. Thus, the researchers could assay in a sensitive fashion, the transcription factor activity of SREBP. The data presented in Fig 10 are a subset of mutants examined for luciferase activity in the presence and absence of cholesterol.

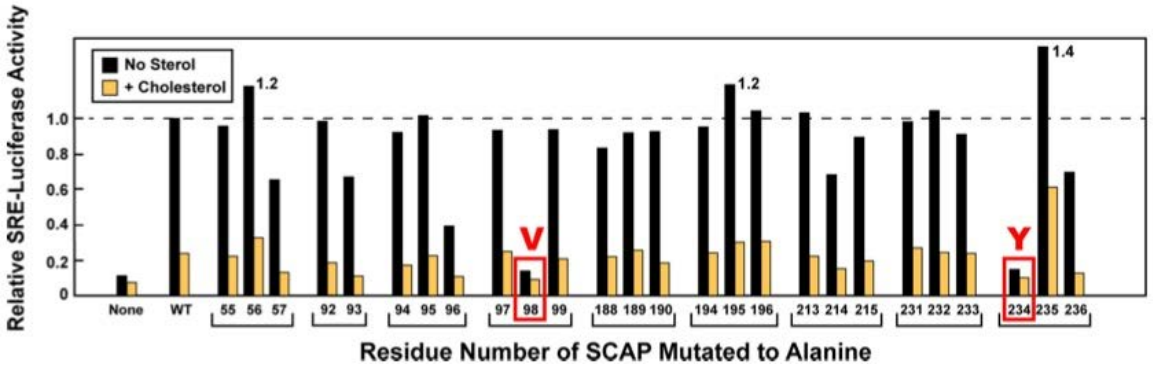


Fig 10. Alanine scanning in which site-directed mutagenesis, SDM, was used to replace the natural amino acid with Ala at various positions within Scap loop 1 (aa 47-248) and to examine the consequences of this substitution on the processing of SREBP into the TF. The SRE has been engineered in front of the gene that codes for luciferase. Thus, liberation of the TF activates the transcription of the luciferase gene and ultimately the production of protein that generates light that can be detected in a continuous assay in the presence of ATP.

To determine if the results observed with the Scap Loop1 construct are similar to the full length wt-Scap, the full length Y234A mutant was made and studied as described in Figure legend 11.

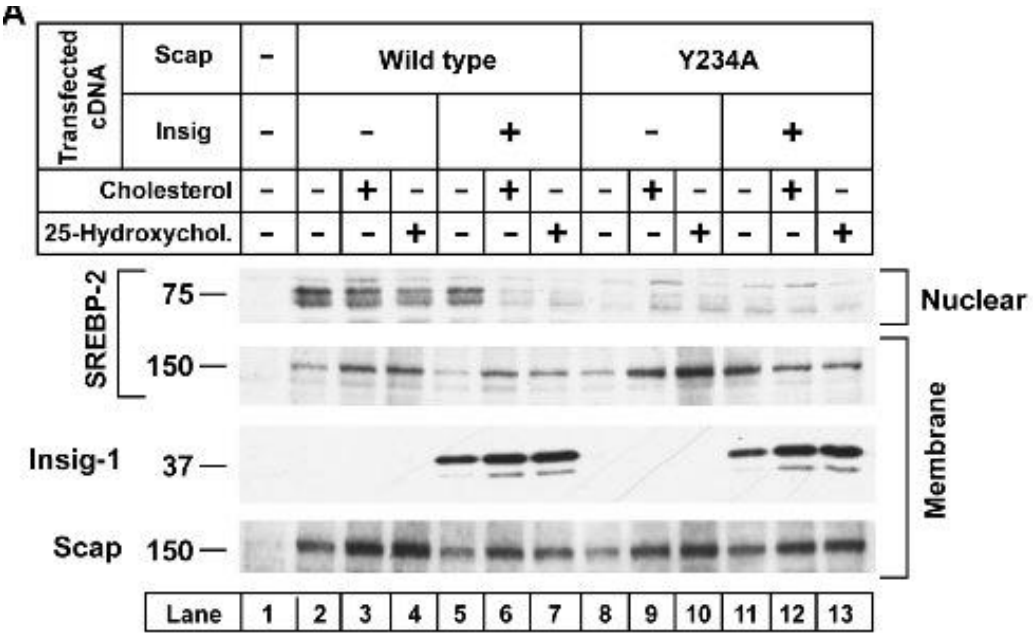


Fig 11: Biochemical characterization of the full length wt vs full length Y234A Scap (see Fig 10). Immunoblot of SREBP-2 in Scap-deficient cells transfected with WT or Y234A Scap (**full length versions**) in the absence or presence of transfected Insig-1 and in the absence or presence of cholesterol or 25-hydroxycholesterol. Nuclear or membrane indicate that separation of these cellular components has been achieved by centrifugation and then analyzed by SDS PAGE and western blotting. The TF part of SREBP moves to the nucleus and the rest remains in the ER membrane.

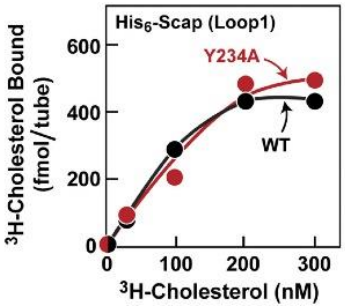


Fig 12. (His)$_6$-WT Scap (loop 1) and Y234A (His)$_6$-Scap (loop 1) were expressed and purified and cholesterol binding studied with the results shown.

Questions: **The experiments were designed to identify the mechanism of Scap sensing of cholesterol.**

1. A Loop 1 construct of Scap was prepared (46-269 residues) and contained a signal recognition sequence to target it to the ER and a (His)$_6$-tag. Isolation of this construct required detergent. The experiments in Fig 8 and 9 were carried out to compare its properties with full length (TM1-8) Scap. Describe what the results in these figures tell you.

2. The requirement for detergent solubilization potentially raises concerns. What would these concerns be? How might one further characterize this new construct?

3. The results of an alanine scanning experiment are shown in Fig 10 and 11. This method was developed by the Wells group in an effort to define potential sites critical for ligand binding and function. It involves making many many mutants where each residue or a cluster of residues is replaced with Ala. The assay developed involves taking advantage of the transcription factor (TF) properties of SREBP and its binding to SRE motifs. Given the model described in lecture for regulation of cholesterol levels, explain the observed results with the WT Scap in the presence and absence of cholesterol (Fig 10). The two aberrant mutants are boxed in red (Fig 10). Provide an explanation given these results. Provide an explanation for why Brown and Goldstein decided to study the Y234A-Scap in detail.

4. To test their model, the experiments described in Fig 11 were carried out. To think about this data look at the model for regulation of TF release and the role of cholesterol and then compare the results with the Y234A-Scap FL(full length) mutant. You are looking at western blots of different players under different conditions. Explain the data in each of the lanes of Fig 11 and how they relate to the WT model and the proposed role for Loop 1 from all of the data.

5. You are given that excellent antibodies (Ab) are available to all the players in cholesterol homeostasis. Given the experiments above and using an Ab to Insig-1 for immunoprecipitation (IP) and SDS-PAGE/western analysis of the IP, what proteins would you expect to see in the presence and absence of cholesterol? Explain each prediction.

5.08J Biological Chemistry II

Spring 2016