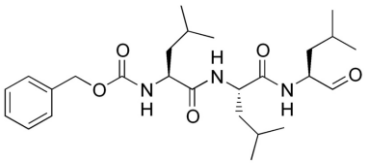
5.08J Biological Chemistry II (Spring 2016)
Problem Set 8 Chemistry 5.08

This PS is focused on HMGR regulation and revisits the PCSK-9 paper.

1. HMG-CoA reductase (HMGR) is a 97 kDa integral membrane glycoprotein localized to the ER. The N-terminal membrane domain has eight transmembrane helices and is sufficient to regulate the enzyme's stability. At the time of Experiment 1 described below, the signaling pathway or pathways for the degradation of HMGR in response to metabolic cues were not understood. Experiment 2 describes experiments to further address this issue that were carried out in 2014. Review the model for controlling HMGR activity presented in your class notes.

Experiment 1 was carried out to determine the fate of HMGR in the presence and absence of sterols and in the presence or absence of a proteasome inhibitor, MG-132. The results of these experiments are shown in Figures 1 and 2 and are described in the figure legends associated with each of the experiments.

Digression:



MG-132 is a cell permeable proteasome inhibitor with a K_d of 4 nM.

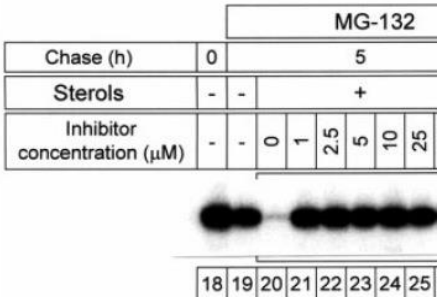


Figure 1: Effect of MG-132 proteasome inhibitor on the amounts of HMGR in a pulse-chase experiment. LP-90 cells were pulse-labeled for 30 min with 150 mCi of [^{35}S]-Met and either lysed immediately (lane 18) or chased for 5 h in the absence of sterols (lane 19) or in the presence of sterols (lanes 20-25) and the indicated concentration of the proteasome inhibitor. Cells were lysed in lysis buffer in the presence of the detergent deoxycholate and the lysates were centrifuged for 30 min at 16,000 x g to remove cell debris. HMGR was immunoprecipitated from the supernatant fraction with an antibody (Ab) targeting the HMGR membrane domain and analyzed by SDS PAGE and fluorography(Phosphorimager technology where the phosphor detects radioactivity, see recitation 2/3 notes).

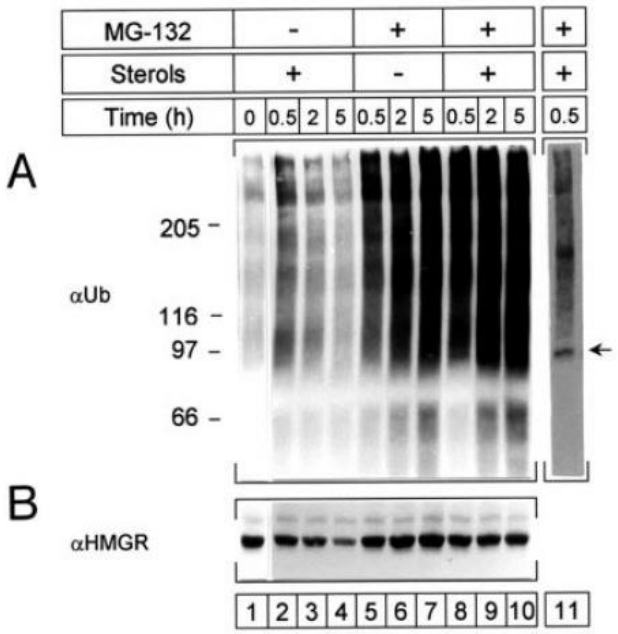
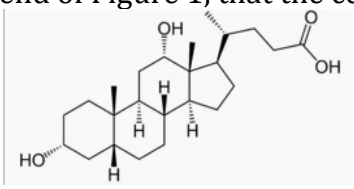


Figure 2: Effect of Sterols on modification of HMGR in chinese hamster ovary (CHO) cells. CHO cells were treated with sterols (25-hydroxycholesterol and cholesterol), MG-132, or both for the indicated time periods. The cells were lysed in lysis buffer, the lysate was centrifuged at 16000 x g to remove cell debris, and HMGR was immunoprecipitated from the supernatant with antibodies against the HMGR membrane domain. Immune complexes were resolved by SDS-PAGE (5 to 15% gradient gel) and blotted onto a nitrocellulose membrane. Ubiquitin conjugates were detected with anti-Ubiquitin monoclonal antibodies (αUb) from mice as the primary antibody and HRP-conjugated anti-mouse-IgG antibodies from goats as the secondary antibody and color developed using HRP. In lane 11 of part A and in all lanes of B, the membrane was stripped and reprobed with A9 monoclonal Ab to HMGR (arrow indicates HMGR band).

Questions:

1. You are given in the figure legend of Figure 1, that the cell lysis buffer contained deoxycholate. Its structure is: 

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Why was deoxycholate included in the lysis buffer?
2. Describe the conclusion(s) that can be drawn from the data in Figure 1.
3. In Figure 2 lanes 1-4 (no MG-132) provide an explanation for why there are so many high molecular weight species observed?
4. Describe conclusions that can be drawn from this experiment.
5. Describe how the αUb-HRP-anti-mouse antibody sandwich works to allow visualization of the proteins of interest, in this case ubiquitin.
6. Given what you have learned about the mechanism of the proteasome, propose a mechanism by which MG-132 inhibition might occur. What may be the issues with using an aldehyde as an inhibitor in general in humans and with MG-132 specifically?

7. Are the data presented in Figures 1 and 2 consistent with the model for HMGR regulation that you learned about in class and your reading?

2. From the data described above there are NO molecular details, that is information about the proteins E1, E2 and E3 that must be involved in this process. In fact, the model in part 7 should include gp78 and Ubc7 which are E3 and E2 proteins involved in ERAD (endoplasmic reticulum associated degradation).

Experiment 2: Studies in the last decade have shown that there are three E3 protein complexes involved in ERAD, one of which includes gp78. Song et al reported that gp78, which they showed was associated with Insig1, is responsible for degradation of HMGR in response to sterols. They also reported that another ERAD E3, TRC8, interacts with Insig1 and also plays a role in HMGR degradation.

Recently Tsai et al studied mouse embryonic fibroblasts (MEFs) from a liver-specific gp78 knockout mouse. Initially they carried out the experiment shown in Figure 3.

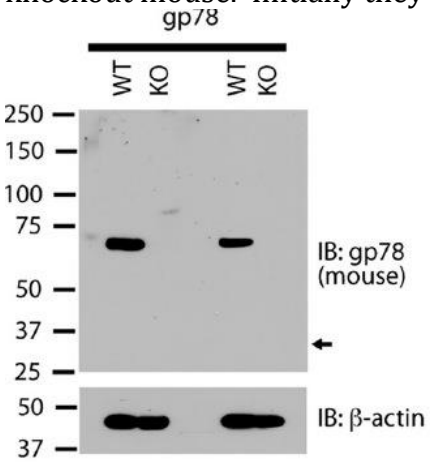

Figure 3: Levels and length of gp78 in two different sets of primary MEFs (from two separate pregnancies of mice) were examined because of the complexity of the targeting strategy for the knockout of gp78 in order to determine if the knockout experiment was successful. Ignore the black arrow in the Figure. The β-actin serves as a loading control (actin should be, and is, present at the same levels in WT and KO cells).

They then used ^{35}S pulse-chase metabolic labeling and immunoprecipitation to quantitatively assess the degradation of the endogenous HMGR in these MEFs. The cells were grown under complex conditions to maintain their viability (details have been omitted) and then pulse labeled for 30 min with [^{35}S]-Met and Cys. This labeling was followed by a chase in "cold" medium in the absence or presence of sterols. At indicated times (Figures 4 A and B), the endogenous HMGR was immunoprecipitated with antibodies to the transmembrane domain of HMGR (as described above).

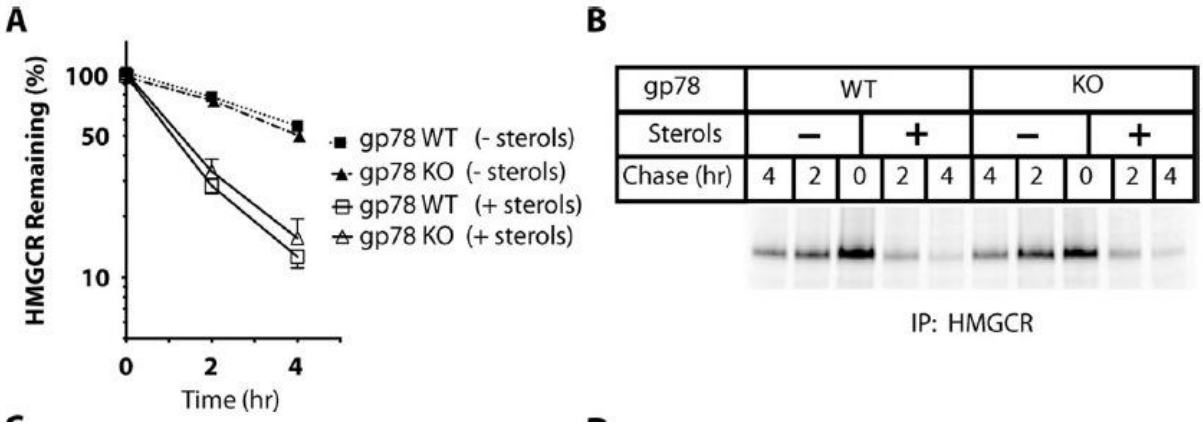

Figure 4 A and B. Results of pulse-chase experiments described above.

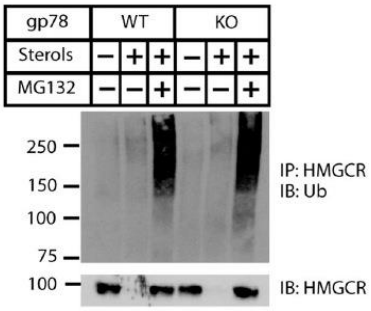

Figure 4C. (IP = Immunoprecipitation; IB = Immunoblot/Western blot)

In the experiment whose results are shown in Figure 4C, the cells were allowed to accumulate HMGR by media manipulation, followed by addition of sterols (where indicated) in the presence or absence of the proteasome inhibitor MG-132. After 60 min, the cells were lysed and HMGR was immunoprecipitated with HMGR antibodies raised in rabbit. The immunoprecipitates were sequentially immunoblotted with mouse monoclonal antibodies to ubiquitin (top part) and HMGR (bottom part).

Questions:

1. Describe what the data in Figure 3 tell you about the gp78 knockout mouse.
2. Describe what the results in Figure 4 A-C tell you about the studies with embryonic fibroblasts relative to the model for HMGR regulation.
3. The results given above are different from the earlier reported studies of Song et al. How might the differences between this study and the earlier study be resolved? I am not asking for details, but for how you would go about determining the basis for the differences between the two studies, which would in turn, provide the impetus for future experiments.

3. In recitation 8, you read the paper in which Crispr-cas9 was used to remove the PCSK-9 protein from 3T3L1 cells and from mouse liver cells. This question is focused on the experimental details described in that paper. The gene for *pcsk-9* is shown in Figure 5.

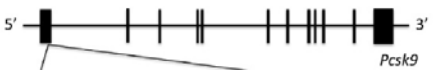

Figure 5. *pcsk-9* gene where the black rectangles are the targeted exons within this gene: exon one and exon two are at the far left and far right of the gene.

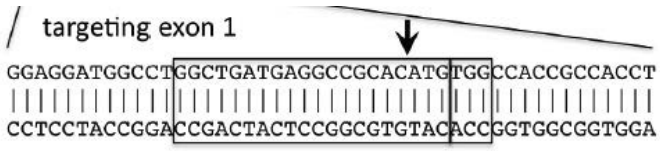

Figure 6. The target for the gRNA from exon 1

Both exon 1 and exon 2 of *pcks-9* (Figure 5) were targeted for guide RNA design with the choice for exon 1 targeting shown in Figure 6. Shown in Figure 7 are the results of the experiment to determine which exon (one vs two) would be best target.

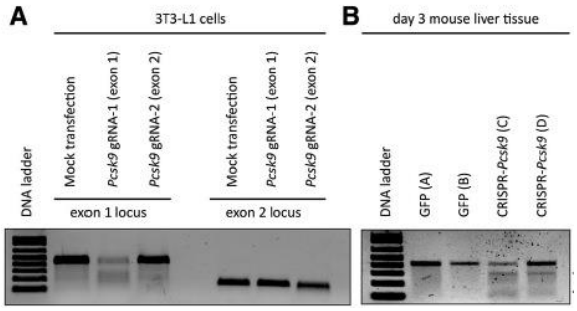

Figure 7. Target effects in mouse cells and livers subsequent to CRISPR-cas9 expression. **A.** Surveyor assays performed with genomic DNA from 3T3-L1 cells transfected with Cas9 and guide RNA targeting either exon 1 (gRNA-1) or exon 2 (gRNA-2). **B.** Surveyor assays performed with genomic DNA from liver samples taken from mice from mice 3 days after receiving a control adenovirus expressing GFP or adenovirus expressing Cas9 and gRNA-1. In panel B, there are two GFP controls from two different mice (A and B) and experiments with tissue from two different mice (C and D).

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Finally, PCR amplification and Sanger sequencing of the target site of many samples of mouse liver genomic DNA subsequent to the CRISPR-cas9 studies gave results, some of which are shown in Figure 8.

Figure 8. Sequencing of liver genomic DNA based on the target from the gRNA sequence.

Questions:

1. Why were the exons and not the introns targeted by Ding et al ?
2. The targeted region of exon 1 is shown in Figure 6. There are three features of this target DNA that are highlighted: the large rectangular box, the small box and the arrow. Given your understanding the Crispr-cas 9 technology describe why these three features are highlighted and why they are essential for the successful targeting *pcks-9*.
3. The first experiments described in the paper determined whether exon 1 or exon 2 would be the target of choice. An agarose gel is shown in Figure 7 describing the results. Describe what you observe in Figure 7 A and B and the how these observations led to the next sets of experiments.
4. On target and off-target DS DNA cleavage is a major concern with the Crispr-cas 9 technology. What experiments using methods similar to those described in Figure 7 were carried out to determine off-target cleavage?
5. The Crispr cas9 technology leads to ds DNA cleavage that can be repaired in a number of ways. What are the two general mechanisms of repair? Explain how the data shown in Figure 8 was obtained and what it tells you about which mechanism of repair predominates in this particular set of experiments based on the data shown.

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