BIOL463 – Fall 2016

Elena Giuchici
Dec. 2, 2016

The Role of phosphate transport gene Pho88 on regulation of other PHO genes.

Background

Although the exact function of the protein product of pho88 in Saccharomyces cerevisiae is not known, research indicates that pho88 may have an effect on the positive and negative regulation of the phosphate uptake pathway in response to different concentrations of phosphate in the intracellular and extracellular environments. When the sequencing of Pho88 and other phosphate metabolism proteins was completed by Feldmann et al., Pho88 was found to be a 21kDa protein consisting of 188 amino acid residues and various hydrophobic and hydrophilic domains (1994). The structural prediction of Pho88 that was generated included 51.60% hydrophobic, 28.72% hydrophilic, and 19.68% neutral amino acids. Based on this information, researchers predicted that Pho88 is a membrane protein involved in phosphate transport (Cherry et al., 2012). Using techniques outlined by Eisenberg in 1984, researchers Yompakdee et al. generated and analyzed the hydrophobicity plots of PHO88 and PHO86. They found that the predicted hydrophobicity plot of PHO88 suggested that, like PHO86 it is also also likely a membrane bound protein. These researchers later confirmed that Pho88 encodes a membrane protein that has a functional location in the endoplasmic reticulum. It has been suggested that Pho88 plays a role in the regulation or maturation of secretory proteins acting in the phosphate transport pathway (Hurto et.al. 2007).

Regulatory activity on the phosphate uptake pathway is largely controlled by the protein product of Pho81. PHO81 is a primary receptor for detecting inorganic phosphate (Pi) signals from the environment and functions as an inhibitor under low phosphate conditions (Oshima 1997). It is both necessary and sufficient to inhibit activity of PHO80, PHO85, and other regulatory proteins in the phosphate uptake pathway (Mouillion et. al. 2006). PHO80 and PHO85 function as inhibitors of PHO4, a positive regulator of PHO5 and the rest of the PHO regulon. Through this pathway, expression of many phosphate transport genes including the Pho5 gene, which encodes the major repressible acid phosphatase (rAPase) in yeast, is stringently controlled by the level of inorganic phosphate (Pi) in the environment (Yompakdee et. al. 1996). The protein product of pho5 serves as an important phosphate scavenger under low phosphate conditions (Hurto et.al. 2007). The phosphate transport pathway in yeast is shown below.

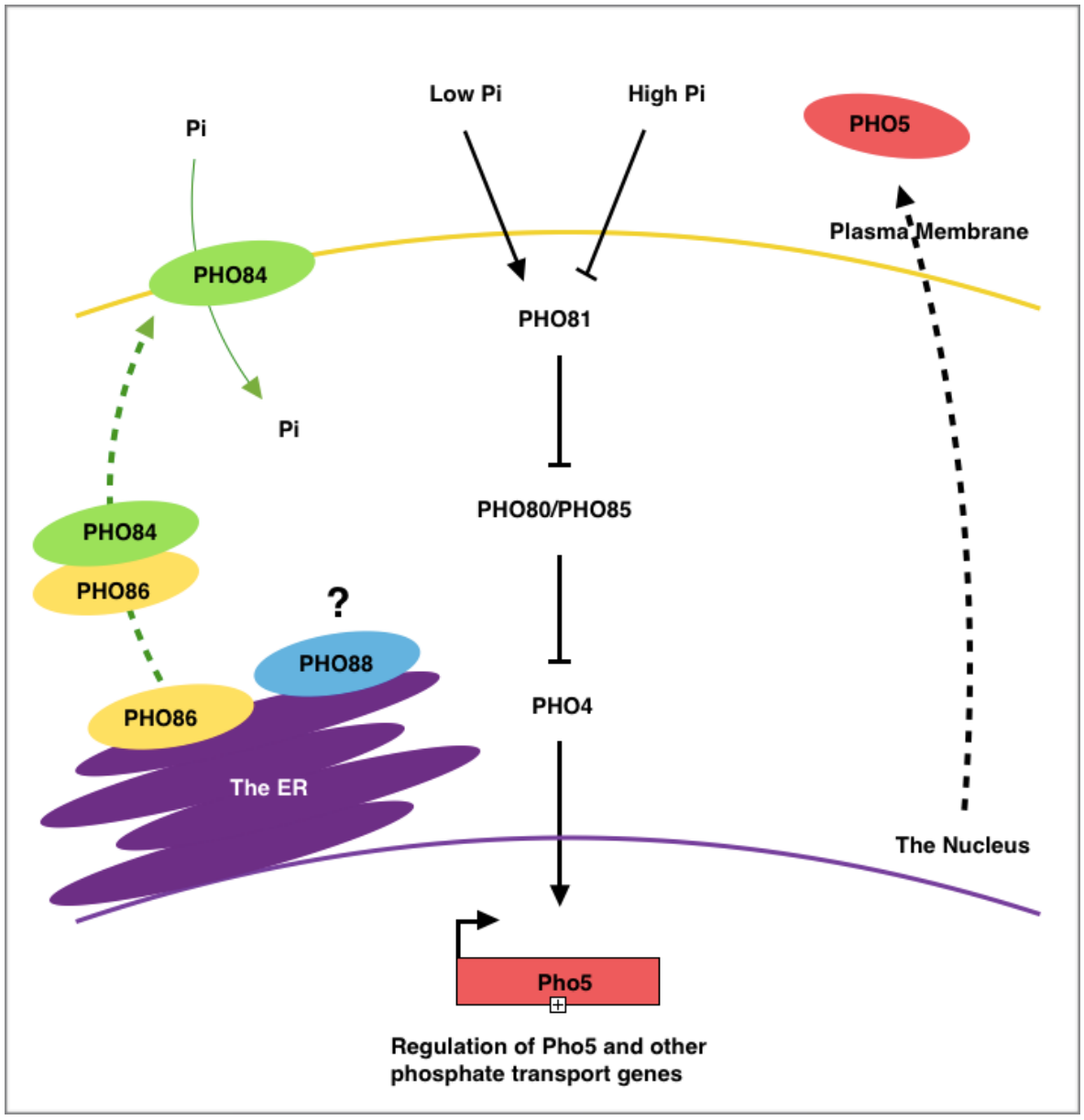

Fig. 1 Phosphate Transport Pathway in Yeast. Pho88 is localized at the endoplasmic reticulum along with PHO86. PHO84 is localized at the plasma membrane and serves to uptake inorganic phosphate from the extracellular environment. PHO5 scavenges for sources of phosphate in the environment and liberates inorganic phosphate molecules that can then be taken up by PHO84. Under low phosphate conditions, the PHO regulon is "turned on" (expressed), and under high phosphate conditions the PHO regulon is "turned off".

The gene Pho88 has shown great sequence similarity to Pho86 and mutants of each gene have also showed great phenotypic similarity, suggesting that these genes may have similar functions. One theory about the interactions between these two genes is that

Pho88 and Pho86 may function by binding to the phosphate transporter Pho84 to promote its maturation or trafficking to the plasma membrane. Research suggests that Pho86 functions as an active aid in transporting Pho84, while Pho88's role in this mechanism is unknown (Miller 2005). This theory would place Pho88 as an indirect regulator of phosphate transport genes since Pho84 is involved in transporting Pi into the cell. The Pi increase would then induce a change in transcriptional activity through the other known transcription factors downstream in the phosphate transport pathway.

In addition, when researchers studied the double knockout mutant of Pho86 and Pho88, they found that the mutant showed enhanced synthesis of the Pho5 rAPase under high-Pi conditions (Yompakdee et.al. 1996). This response is the opposite of what occurs in wild-type yeast cells, and is the key piece of literature that suggests Pho88 (and Pho86) may function to regulate other phosphate transport genes. This is the theory that I will be investigating through the various experiments in my final project.

Question

To what extent can the phosphate transport pathway gene, Pho88, regulate the expression of other PHO genes?

I ask this question specifically about Pho88, but my investigation innately contains questions regarding this protein's possible interactions with PHO86 and/or other components of the phosphate transport pathway.

Pho88 is gene related to the phosphate transport pathway in yeast, but as no research was done specifically on this gene, and therefore it is definitely poorly understood. Specifically, I have found that the pho88 mutant has many phenotypic similarities to pho86, another phosphate transport gene. Both of these proteins have proven to localize in the ER membrane of yeast, suggesting possible interaction or interaction in the same pathway. More importantly however, an up regulation in certain phosphate transport genes was seen in the double mutant of pho88 and pho86 under high phosphate conditions. This affect on gene regulation is the opposite of what occurs in wild-type yeast, and therefore it suggests that pho88 and/or pho86 could influence gene regulation. However, researchers have not tested pho88 alone to see if it can affect gene regulation.

Experimental Approach

The goal of the experiments in this project is to investigate the role pho88 in positive and negative regulation of the PHO regulon under various phosphate conditions. To do this, I will comparing the Pho88 mutant with the Pho86 mutant, and the Pho86/88 double mutant under low, normal, and high phosphate conditions. By performing this experiment, I will hopefully be able to distinguish Pho88's role in gene regulation from that of Pho86. By comparing both single mutants to the double mutant, I can determine

whether or not Pho88 has a functionally redundant role to Pho86, or if it has an entirely novel function.

The phosphate uptake pathway shows that strong regulation of phosphate uptake is dictated by Pi concentrations in the environment. I will be testing the three mutants, and a wild-type control under low, normal, and high phosphate conditions and comparing the cellular response in phosphate gene regulation. To measure the effect of each gene on the PHO regulon, I will be detecting the activity of the Pho5, a repressible acid phosphatase that is secreted from the cell. By choosing a secreted enzyme, I will not need to break the cell membrane in order to detect levels of the protein. Instead, I can simply run an enzyme activity assay using the supernatant of pelleted cell culture.

In parallel with this enzyme activity assay to compare PHO gene regulation between mutants, I will perform a co-immunoprecipitation experiment. Co-immunoprecipitation is a technique performed to identify protein-protein interactions. By using target protein-specific antibodies, it will allow me to capture other proteins that are bound to the target protein. I will perform a co-immunoprecipitation on Pho88 to collect any protein complexes it may form. These protein complexes can then be analyzed to identify binding partners, and perhaps some aspects of the function of the target protein.

For the scope of this final project, it is unlikely that I will be able to determine the detailed mechanism of how Pho88 acts on the phosphate uptake pathway and the other genes involved. I should, however, be able to discover new information on Pho88's regulatory role and its interactions with other proteins.

Significance

Because this project may give greater insight into the phosphate transport pathway in yeast, it could have major effects on drug development and amplification in yeast. Recently, a knew field has opened up in drug development where genetically modified yeast are used to produce pharmaceuticals and precursors to certain drugs. The yeast themselves are used for the development and amplification of the drug and molecules. Just recently, in 2015, researchers in the Smoke lab at Stanford have made an incredible accomplishment in synthetic biology. The team created a strain of genetically engineered yeast using plant, bacterial, and rodent genes that could convert sugar into thebaine, the key opiate precursor to morphine and other strong narcotic painkillers. Smolke's team says that by tweaking the yeast pathways, medicinal chemists may be able to produce more effective, less addictive versions of opiate painkillers (Galanie *et al*., 2015). Inorganic phosphate is a very key molecule in enzymatic reactions and intracellular metabolic pathways. Therefore, regulating the balance of inorganic phosphate in yeast can be critical when developing methods to synthesize drugs using yeast metabolic pathways.

Hypothesis

From the little information that is known about Pho88, I hypothesize that it does have a role in regulating the PHO regulon and all of the phosphate transport genes that it contains. I suspect that Pho88 and Pho86 play redundant, but additive roles in the phosphate transport pathway. In response to the phosphate conditions inside and outside the cell, Pho88 and Pho86 must interact with some molecule such as a transcription factor that can directly affect PHO gene transcription. One possible interactor of Pho88 is Pho4, a sequence specific DNA-binding RNA polymerase II transcription factor that activates expression of genes involved in the response to phosphate starvation such as PHO5, which relocates to the nucleus during phosphate starvation. In this sense, Pho88 could indirectly regulate the expression of the Pho5 gene and other PHO genes by relaying information between the Pi receptors or detectors at the cell membrane and a transcription factor such as Pho4 in the nucleus, to establish a complete signal cascade.

Evidence in Support of Hypothesis

In the absence of Pho88, cells were only able to acquire very low levels of Pi that were much lower than normal, but were sufficient enough for growth. This evidence suggests that pho88 plays a role in regulating either the expression, or membrane trafficking of the phosphate transport genes related to Pi uptake. One example of these is the protein product of pho5, a repressible acid phosphatase (rAPase) which serves as an important phosphate scavenger under low phosphate conditions (Hurto et.al. 2007). The double knockout of Pho86 and Pho88 showed an enhanced synthesis of the pho5 rAPase under the high-Pi condition (Yompakdee et.al. 1996). However, these researchers only tested the double mutant of pho88 and pho86, and not either protein independently.

Predictions or Possible Results and Discussion

If my hypothesis is correct, a mutation in Pho88 alone should be insufficient to cause any change in expression of phosphate transport genes (change being a deviation from the expression patterns observed in wild-type yeast). However, when both Pho88 and Pho86 are mutated or knocked out, the expression of Pho genes should be different or opposite to that of wild-type yeast. Recall that this is what was discovered in the double knockout of Pho86 and Pho88, where an enhanced synthesis of the pho5 rAPase occurred under the high-Pi condition (Yompakdee et.al. 1996). I would expect to see this increase in expression of Pho5 in the double mutant under high phosphate conditions only, in the enzyme activity assay experiment. Single mutants should show the same low levels of Pho5, as will be seen in the wild-type. I also hypothesize that a co-immunoprecipitation experiment would reveal interactions between Pho88 and a known transcription factor of the PHO regulon, such as Pho4.

If my hypothesis is incorrect, Pho88, alone may be sufficient to cause a change in expression of other phosphate transport genes. Pho88 may serve a completely different functional role than that of Pho86. In the enzyme activity assay, Pho88 single mutants could show an increase in Pho5 expression under high phosphate conditions. In addition, a co-immunoprecipitation experiment would reveal no interaction between Pho88 and any transcription factor capable of regulating the PHO regulon. These results would show that PHO88 alone is sufficient to trigger a change in gene expression of the PHO regulon, and does not require binding or interaction between any other genes.

Methods

Detection and determination of the repressible acid phosphatase activity:

I will measure the expression of the PHO genes indirectly by measuring the activity of Pho5, a secreted enzyme of the PHO regulon. In a reaction tube, 1.5mL of acetate buffer (pH = 4), 0.5 mL of 0.4M 4-nitrophenyl phosphate (substrate of Pho5) solution and 0.5 mL of supernatant (containing Pho5) will be added. The tube will be placed in a 37 °C waterbath for 10 minutes to allow Pho5 to function. Immediately after the incubation period, the reaction will be stopped by adding 0.5 mL 1M Tris Buffer (pH = 8.8) with 0.4M Sodium Phosphate. The high pH of the Tris buffer stops the reaction by inhibiting activity of Pho5. The high levels of inorganic phosphate in the Tris buffer inhibit any alkaline phosphatase activity from occurring once the pH is raised. Lastly, the alkaline environment established by the Tris Buffer induces a color change in the nitrophenol molecule that is liberated by Pho5 during the reaction period. These levels of liberated 4-Nitrophenol Phosphate will then be determined spectrophotometrically at a wavelength of 420 nm.

The amount of 4-nitrophenol liberated will act as a measure of phosphatase activity, and therefore Pho5 levels. This measure will be taken at low, normal, and high phosphate conditions for each of the single mutants, as well as the Pho88 double mutant, and a wild-type yeast control.

Co-immunoprecipitation:

Protocol taken from: Golemis E. (2002) Protein-protein interactions : A molecular cloning manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. ix, 682.

DAY 1

Carefully wash cultured cells with pre-chilled PBS for 2 times. Add in cold RIPA lysis buffer. Scrape cells off into clean 1.5ml eppendorf tubes with a clean, cold scraper. Put them on a low-speed rotating shaker for 15 min at 4°C. Centrifuge at 14,000 g 4°C for 15min, transfer the supernatant to new tubes immediately. Wash protein A/G-agarose beads 2 times with PBS and make a 50% protein A/G agarose working solution (in PBS)

Add in 50% protein A/G agarose with ratio of 100µl for a 1ml sample solution. Shake on horizontal shaker for 10min, 4°C (This step aims to eliminate non-specific binding proteins). Centrifuge 14,000g at 4°C for 15min, transfer the supernatant to new tubes and discard protein A/G-agraose beads. Quantify total protein with BCA assay or other methods.

Dilute the total protein to 1µg/µl with PBS to decline the concentrations of detergents. If you feel the concentration of your target protein is low, you can dilute the total protein to 10µg/µl. (if it's high enough) Add in appropriate amount of primary antibody to approximately 500µl total volume. Slowly shake antigen-antibody complex on rotating shaker at 4°C for overnight.

DAY 2

Centrifuge 14,000g for 5s, keep the pellet and wash with pre-chilled washing buffer (or cold PBS) for 3 times. (800µl each). Collect the supernatant to proceed to SDS-PAGE, western-blot, or mass spectra analysis.

References

Cherry JM, Hong EL, Amundsen C, Balakrishnan R, Binkley G, Chan ET, Christie KR, Costanzo MC, Dwight SS, Engel SR, Fisk DG, Hirschman JE, Hitz BC, Karra K, Krieger CJ, Miyasato SR, Nash RS, Park J, Skrzypek MS, Simison M, Weng S, Wong ED (2012) Saccharomyces Genome Database: the genomics resource of budding yeast. Nucleic Acids Res. Jan;40(Database issue):D700-5. [PMID:22110037]

Eisenberg D. (1984) Three-dimensional structure of membrane and surface proteins. Annu Rev Biochem 53 : 595-623

Feldmann, H., Aigle, M., Aljinovic, G., André, B., Baclet, M. C., Barthe, C., … Boles, E. (1994). Complete DNA sequence of yeast chromosome II. The EMBO Journal, 13(24), 5795–5809.

Galanie, S., Thodey, K., Trenchard, I. J., Interrante, M. F., & Smolke, C. D. (2015). Complete biosynthesis of opioids in yeast. Science, 349(6252), 1095-1100. doi: 10.1126/science.aac9373

Golemis E. (2002) Protein-protein interactions : A molecular cloning manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. ix, 682.

Hurto, R. L., Tong, A. H. Y., Boone, C., & Hopper, A. K. "Inorganic Phosphate Deprivation Causes tRNA Nuclear Accumulation Via Retrograde Transport in Saccharomyces Cerevisiae." Genetics 176.2, 2007 pp. 841-852.

Miller, J. P., Lo, R. S., Ben-Hur, A., Desmarais, C., Stagljar, I., Noble, W. S., & Fields, S. "Large-scale identification of yeast integral membrane protein interactions." Proceedings of the National Academy of Sciences of the United States of America 102.34, 2005. 12123-12128.

Oshima, Y. "The Phosphatase System in Saccharomyces Cerevisiae." Genes & Genetic Systems, 72.6, 1997. pp. 323-334.

Mouillon, J., Persson, B. L. "New Aspects on Phosphate Sensing and Signalling in Saccharomyces Cerevisiae." FEMS Yeast Research, 6.2, 2006. pp. 171-176.

Yompakdee, C., Ogawa, N., Harashima, S., & Oshima, Y. “Molecular & General Genetics: A Putative Membrane Protein, Pho88p, Involved in Inorganic Phosphate Transport in Saccharomyces Cerevisiae." Springer, 251.7, 1996.

Toh-E, A., & Oshima, Y. (1974). Characterization of a dominant, constitutive mutation, PHOO, for the repressible acid phosphatase synthesis in Saccharomyces cerevisiae. Journal of bacteriology, 120(2), 608-617.