An Analysis of the Role of RegA in Pathogenesis and Ras Signaling

Abstract:

Dictyostelium discoideum is a excellent eukaryotic model organism for signal transduction research purposes. It is a free living amoeba, which under nutritional stress utilizes two complex signaling pathways for multicellular development. The primary signaling pathways mechanism that *Dictyostelium* utilizes are dependent on the secondary messenger's cAMP and cGMP for amplifying the signal and activating important downstream signaling proteins such as PKA. Phosphodiesterase's are one mechanism that *Dictyostelium* utilizes for the regulating cAMP and cGMP levels that drive signaling. *Dictyostelium* uses a total of 7 different cAMP and cGMP phosphodiesterase's for signaling. RegA is a phosphodiesterase that *Dictyostelium* utilizes for managing intracellular cAMP levels in the Erk2 signaling pathway. RegA plays a critical role in the Erk2 signaling pathway, however; the role of RegA in pathogenesis and Ras signaling is not currently well understood. Multiple sequence alignment was used to provide significant insight into sequence conservation of RegA and its role in pathogenesis. Sequence conservation was observed in several of RegA's phosphorylation sites in many different eukaryotic pathogens. This suggests that eukaryotic pathogens may utilize many of the same mechanisms for controlling intracellular cAMP levels. To evaluate the role of RegA in Ras signaling, a RegA- mutant cells were labeled with a GFP Ras binding domain (RBD) reporter gene, stimulated with folate, and observed to determine differences in the response to chemotaxic stimulation. We didn't observe any significant difference suggesting that RegA is not essential in Ras signaling.

Introduction:

Dictyostelium discoideum is both a single cell and multicellular organism primarily found in soil samples around the world. *Dictyostelium* is an amoeba widely used in scientific signal

transduction studies because of its ability for cell to cell communication. Communication between sovereign cells allows the organism to undergo development and move systematically as a single unit. It is a unique organism with the ability to form into an aggregate that develops into a fruiting body. *Dictyostelium* rarely forms into an aggregate when the environmental conditions are optimal for growth but, when conditions change and the nutrients are needed for survival become scarce or nonexistent, cells will aggregate together to form a multicellular organism that develops spores. These spores remain dormant until resources become plentiful once again. As an aggregate *Dictyostelium* uses signaling mechanisms to communicate between its cells allowing them to function together as a single unit, where each cell has a specific role in specific regions of the aggregate. These signaling mechanism are also found in many other eukaryotes. Many of the signaling proteins found in other eukaryotic organisms are also present in *Dictyostelium. Dictyostelium* is widely used as a model organism for studying cellular communication and signal transduction because of this molecular conservation.

Some genetic diseases are the result of defects in cellular signaling and signal transduction that affect cell function, growth, differentiation, or movement. In pathogenic diseases, many eukaryotic pathogens also have signal transduction pathways and these might function similar to those in *Dictyostelium*. This signaling might allow pathogens to communicate between their cells so they can reproduce and increase the effectiveness against their host and its immune responses; because of this similarity *Dictyostelium* is an excellent source for identifying how pathogens effect host cells/organisms, the signal pathways between them, and their ability to proliferate new cells. Pathogens must be able proliferate and form an effective infection while evading the host's immune system to be considered successful. Cellular signaling is one mechanism that a pathogen could utilize to achieve this. *Dictyostelium* is likely to share many of the same

signaling pathways with other eukaryotic pathogens. This conservation allows *Dictyostelium* discoideum to be a useful model for analyzing the similar communication strategies that many eukaryotic pathogens extensively use to infect their host. Analyzing some of these pathways in *Dictyostelium* and comparing them to those present in other pathogenic organisms could be useful in expanding our understanding of mechanisms these pathogens use to establish an effective infection.

The role of RegA and its homologs in eukaryotic pathogenesis:

Signal transduction pathways are composed of a series of signaling proteins which are organized into a cascade that transduces the signal. These pathways allow the organism to communicate between its cells and adapt to its changing surrounding. Specifically, *Dictyostelium* contains several phosphodiesterases that are vital for maintaining the signal transduction pathways. cAMP phosphodiesterases are enzymes that specifically degrade cAMP. Some of these proteins help to maintain intracellular cAMP levels which are necessary for driving the kinetics of signal transduction. cAMP is an important secondary messenger that can activate cAMP-dependent protein kinase (PKA) and possibly other signaling proteins. cAMP activation of PKA allows this protein kinase to phosphorylate other proteins that are important for metabolism, gene expression, and cell movement. Levels of cAMP are regulated through a variety of mechanisms. cAMP is produced by adenylyl cyclases and degraded by phosphodiesterases. cAMP phosphodiesterases like RegA are one such mechanisms *Dictyostelium* discoideum uses to maintain cAMP levels. There are a total of 7 phosphodiesterase proteins found in *Dictyostelium*. Each of these has catalytic domain similar to RegA but some are cGMP specific, cAMP specific or capable of hydrolyzing both cAMP and cGMP. Many of these phosphodiesterase homologs are also found and conserved in other

eukaryotic organisms and pathogens. Currently, the function and role of RegA and cellular communication and development is not completely understood, however, a genetic analysis and comparison of RegA and the 6 other homologs might provide insight into the role of cAMP phosphodiesterase in pathogenesis, cellular signaling, and development.

The role of RegA in chemotactic signaling pathway:

While *Dictyostelium* has many different signaling pathways, the two best studied pathways are the pathways stimulated by the chemoattractants folate and cAMP. These pathways begin with G protein-coupled receptors (GPCR) on the cell surface. The receptors activate heterotrimeric G proteins that in turn stimulate downstream signaling components. Some of the downstream components are MAPKs and monomeric G proteins such as Ras proteins and these are thought to function in parallel pathways. The MAPK Erk2 is a negative regulator of the phosphodiesterase RegA. A simplified overview of the general signaling cascade is shown in Figure 1 below with folate acting as the stimulus. Upon detection of the stimulus the G-couple protein receptor (GCPR) will activate other proteins that eventually leads to adenylyl cyclase activity and cAMP production. The activation of the GCR increases the intracellular cAMP levels which in turn activates Protein Kinase (PKA). PKA is responsible for activating the pathways necessary for a specific biological process. Figure 1 demonstrates how PKA can be

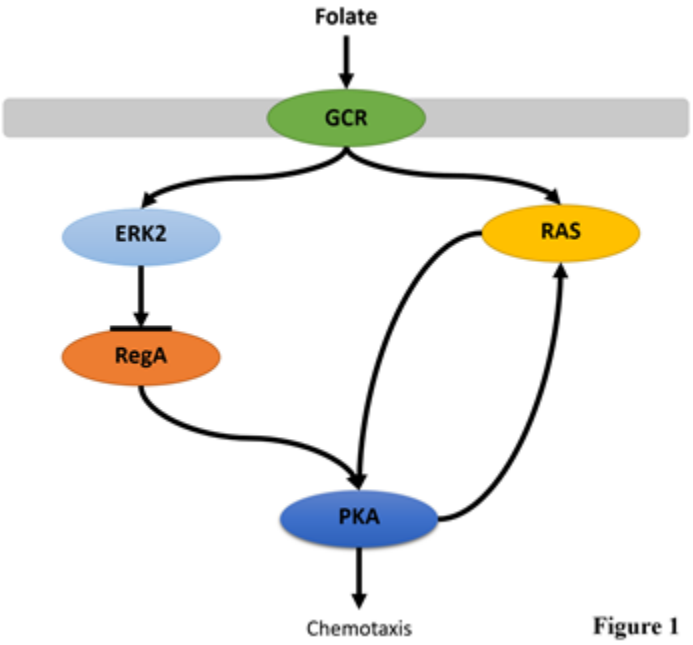


Figure 1

activated and result in a response regardless of which pathway is activated. A signal can be transduced down the cascade through either pathway, however, only the Erk2 pathway contains the cAMP phosphodiesterase, RegA. RegA is a critical protein for regulating intracellular cAMP levels. Previous research suggests that RegA is a necessary component of the Erk2 signaling pathway. (Hadwiger). Erk2 has the ability to bind to RegA and downregulate regulate RegA through phosphorylation (Hadwiger). RegA is critical portion of the Erk2 pathway, however, its absence may also have profound effects on other mechanisms including the Ras activity. A previous study has shown that the absence of PKA results in increased Ras activity in response to chemoattractant stimulation. Therefore, PKA is thought to regulate Ras activity through a negative feedback mechanism. The loss of RegA is expected to have the opposite effect because PKA activity should be high in this mutant.

Methodology:

The cAMP phosphodiesterase homologs were analyzed in various different eukaryotic pathogens and compared to those present in *Dictyostelium* discoideum. The gene names for the seven cAMP phosphodiesterase homologs was obtained through blank's previous study (Bader). The sequences of these genes were then obtained through Dictybase, a genome database for *Dictyostelium.* These sequences were then compared with homologs in other eukaryotic pathogens to identify sequence similarities and differences using the local sequence alignment search tool, BLAST. A list of eukaryotic organisms were selected because of their similarity to *Dictyostelium* and their pathogenic potential. The following eukaryotic pathogens were chosen based on this criteria: Acanthamoeba, Entamoeba, Giardia, Naegleria, Plasmodium, and Trypanasoma. A BLAST sequence search of these pathogens was performed for each cAMP phosphodiesterase homolog. The sequences of these organisms were compiled and

compared for each homolog using the multiple sequence alignment tool, Clustal Omega. These alignments were used for establishing a phylogenetic relationship. A phylogenetic tree for each homolog was created using Clustal Omega. Organisms were also highlighted in the phylogenetic tree based on whether they were free-living organisms like *Dictyostelium* or not. The multiple sequence alignments were also used to determine sequence conservation between the different organisms. The program Boxshade was used on the multiple sequence alignments to identify conservation in the sequences. The Boxshade data was analyzed to identify areas of conservation significance. The phylogenetic and sequence conservation data obtained was evaluated to determine the significance of RegA and other cAMP phosphodiesterase's in pathogenesis.

To investigate the role of RegA in chemotactic signaling pathway, cells that lack RegA can be labeled with a GFP-RBD reporter gene. The GFP-RBD reporter binds to activated Ras associated with the plasma membrane in chemoattractant stimulated cells. To express the reporter gene in the mutant cells the cultures were grown in shaking cultures of HL-5 media to optimize plasmid transformation through electroporation. When an acceptable quantity of the stock *Dictyostelium* cells has been grown then a new strain can be created through transformation of plasmid DNA into the cells; to increase the likeliness of the cells taking up the free DNA electroporation shall be used. The GFP-RBD plasmid (plasmid stock number 1187) was added to both RegA null and wild-type cells, KAX3, and then electroporated. The experimental and control group should both be electroporated at 1.3kv to create holes in the membrane of *Dictyostelium* cells so that free DNA has a greater chance of being taken up and incorporated by the cells. The plasmid 1187 expresses a reporter gene that specifically binds to the activated Ras protein. When exposed to blue light the reporter fluoresces green and this can

be detected using fluorescence microscopy. Besides the reporter gene the plasmid also contains a gene providing resistance to the drug G418 and this allows for the selection of transformants. Following several days after electroporation, G418 needs to be administered to both the control and experimental cultures. The addition of the antibiotic G418 kills off any of the remaining cells that failed to take up the free DNA. This should totally kill all the cells in the control groups and all but the newly mutated cells in the experimental group. The drug selection allows us to isolate fluorescent clones of regA- null and KAx3 cells expressing the GFP-RBD reporter. The control group is essential in determining the success of the transformation of the plasmid DNA. Once the transformed colonies have grown, clones were picked from the plates and examined for fluorescence intensity. The transformed cells should be grown in HL5 media and continued to be placed under drug selection.

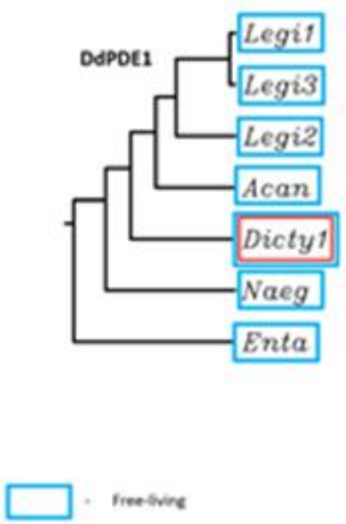
Results:

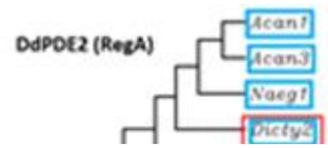
Phosphodiesterases are important signaling proteins that regulate cAMP and cGMP levels in cellular responses. RegA is one such signaling mechanism that *Dictyostelium* uses for regulating signal transduction. Signaling is a very critical mechanism in pathogenesis, therefore, it can be assumed that eukaryotic pathogens would also require molecular mechanisms similar to *Dictyostelium* for regulating cAMP and cGMP levels. *Dictyostelium* utilizes a total of 7 different phosphodiesterases, including the cAMP-specific phosphodiesterase RegA, in signaling (Bader et al.). The seven cAMP/cGMP phosphodiesterases are labeled DdPDE1- DdPDE7 with RegA being DdPDE2 (Bader et al.). A genetic sequence search revealed 4 of these phosphodiesterases were present in many different eukaryotic pathogens genome as well. Sequences from many different eukaryotic species were then compiled for these 4 phosphodiesterases and analyzed

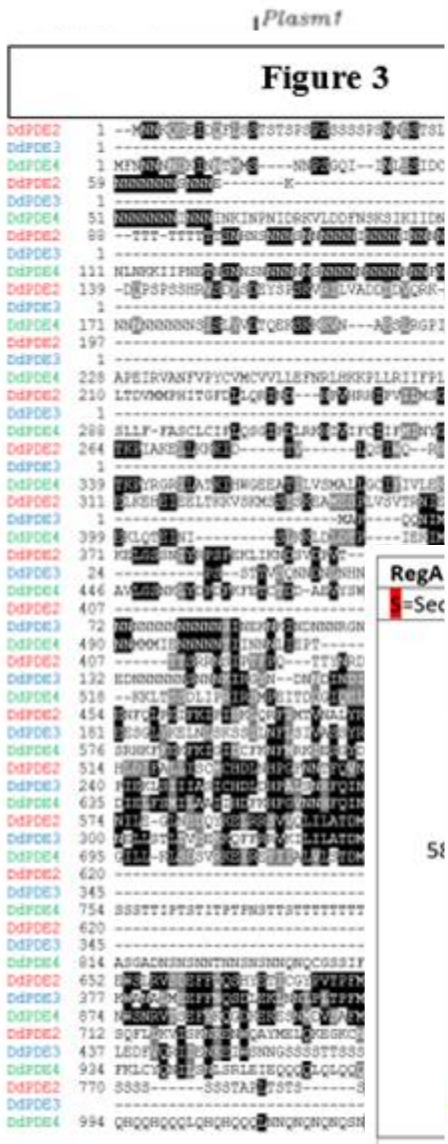
phylogenetically. A phylogenetic tree is shown above for each of these 4 phosphodiesterases (Figure 2). The phylogenetic trees can be used to infer phosphodiesterases phylogenetic relationships across different eukaryotic organisms. Genomic sequence analysis is another technique that can be used to identify the mechanisms that eukaryotic pathogens utilize for regulating signaling. A sequence analysis revealed that eukaryotic pathogens utilize many of the same mechanisms for degrading intracellular and extracellular cAMP and cGMP. All seven of the phosphodiesterases were located in other eukaryotic pathogens except DdPDE7. However, only four of the phosphodiesterases were highly conserved across many different eukaryotic species. RegA was the greatest conserved phosphodiesterase. It was was found in every eukaryotic pathogen tested, however, it should be noted that DdPDE1, DdPDE3, and DdPDE4 was also found in many of the same eukaryotic pathogens tested. The sequences for DdPDE2, DdPDE3, and sDdPD4 were analyzed using the multiple sequence alignment tool boxshade to identify areas of conservation. Sequence conservation was primarily located towards the carboxyl-terminal half in these phosphodiesterases. Conservation was primarily concentrated towards in the last 200 amino acids of the protein sequences for these 3 phosphodiesterases. The areas that contained sequence conservation are shaded in the figure shown to the left and these correspond to the catalytic domain (Figure 3). All three phosphodiesterase showed similar areas of conservation across different species. Several of RegA's different phosphorylation sites were also analyzed to determine if sequence conservation also existed in these locations . Current research suggests that several of these sites may play a significant role in regulation of cAMP and cGMP. Figure 4 shows the sequence analysis for these phosphorylation sites. The binding site for the MAPK Erk2 was another location that was analyzed for sequence conservation. Erk2 is an important regulatory mechanism for controling the activity of RegA. Erk2 has the ability to

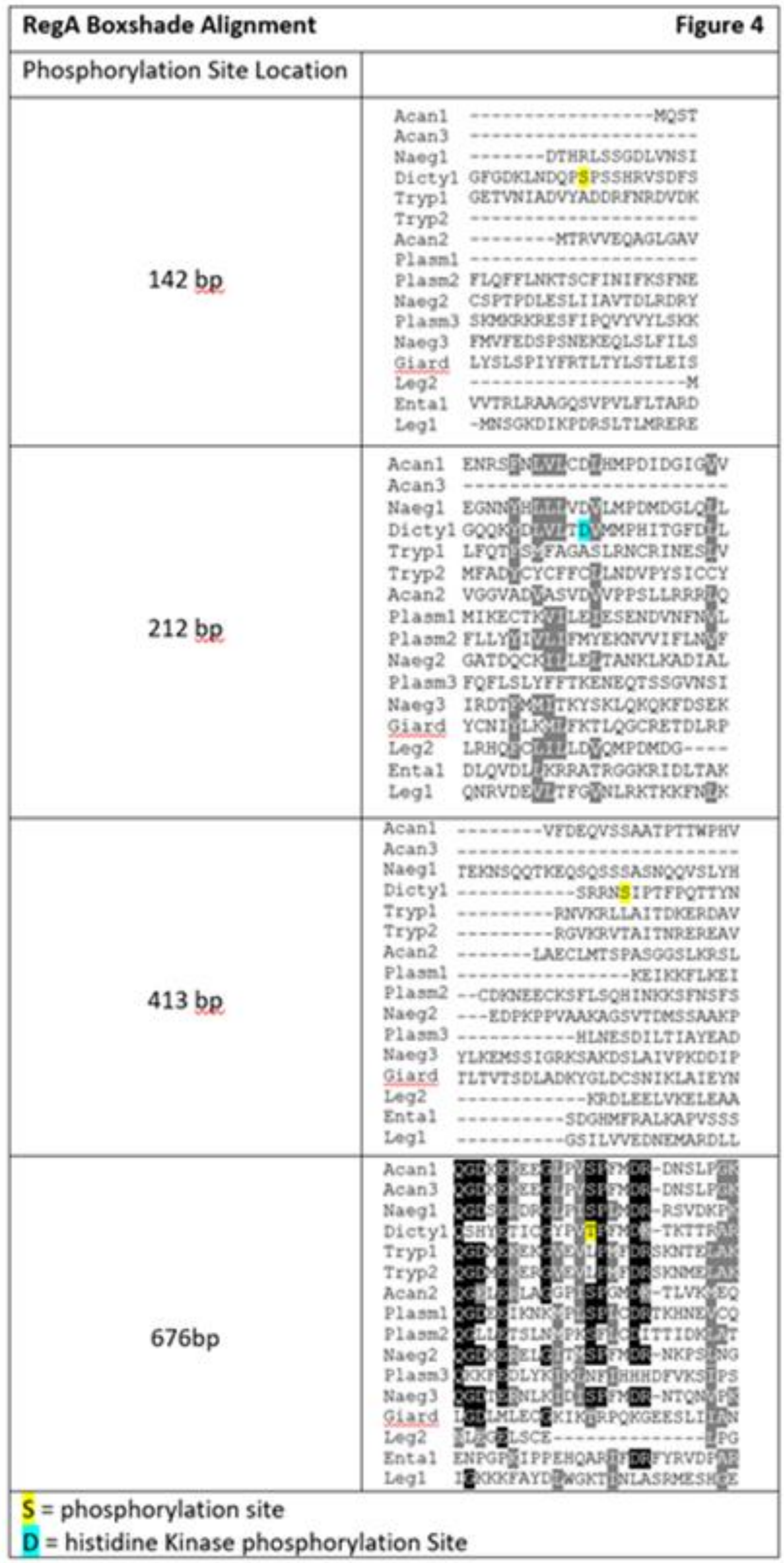
bind to RegA and regulate its activity. Figure 5 shows conservation of the sequence at this

location. An analysis of these unique locations may provide additional insight into the role of phosphodiesterases in pathogenesis.









The activated Ras reporter, GFP-RBD, was electroporated into wild-type and regA- cells. Clones of each strain were isolated and then examined using confocal microscopy. The level of fluorescence varied from cell to cell in both strains and this variation is likely due to the variability of plasmid copy number. The most intense fluorescences was observed at the membrane in some cells that appeared to be creating phagocytic cups. This fluorescent localization is consistent with the activation of Ras at these sites as previous described in other studies. However, we did not see a rapid translocation of cytoplasmic reporter to the membrane upon chemoattractant (folate) stimulation as previously reported by others.

Discussion:

Cell signaling is an important mechanism for pathogenesis. Eukaryotic pathogens require signal transduction pathways for interacting and responding to their environment. Many of the signaling components that are present in *Dictyostelium* are also found in other eukaryotic pathogens. RegA and other phosphodiesterases are one signaling molecule that is essential to *Dictyostelium* for regulating intracellular and extracellular cAMP and cGMP levels in signaling. RegA and several of the other phosphodiesterases are also found in many other eukaryotic pathogens. Currently, it is not completely understood how phosphodiesterases, like RegA, are important in pathogenesis; however, it can be assumed that they are an essential mechanism for eukaryotic pathogens in regulating cAMP and cGMP levels that are required for driving cAMP and cGMP dependent signal transduction. Phylogenetic relationships can be used make inferences on the importance of phosphodiesterases in pathogenesis. Phylogenetics is a technique that allows you to study the evolutionary relationship of two different organisms. This process can also be used to determine the evolutionary relationship of proteins and other

biomolecules. Two proteins that are closely related evolutionarily will most likely serve a similar function. Phosphodiesterases are enzymes that are essential in *Dictyostelium* for regulating signaling. The phylogenetic data in figure 2 demonstrates that phosphodiesterases are fairly similar in other eukaryotic pathogens to those seen in *Dictyostelium*. It can be inferred from this similarity that phosphodiesterases must also serve a similar function in other eukaryotic pathogens. A closer phylogenetic evaluation of figure 2 suggests that there is a difference between the phosphodiesterases of free-living and parasitic organisms. Free-living organisms are organisms that are not dependent on another organism for survival. *Dictyostelium* is one example of a free-living organism as it can live independently. *Plasmodium* is an example of an organism that is an of not free-living or parasitic organism because it requires a parasitic relationship with its host for survival. The phylogenetic evaluation of these sequences suggests that in free-living pathogens differ significantly from their non free-living counterparts. This difference suggests that phosphodiesterases found in parasitic organisms may have evolved differently than those in other free-living organisms. This difference may also be explained by the relative differences in the requirements for signaling between free-living and parasitic organisms. Free-living organisms require constant signaling to adapt and respond to their constantly changing environment. Host organisms provide parasitic organisms with a relatively stable environment that doesn't change as often. The difference in the environments of parasitic and free-living pathogens may explain the phylogenetic difference seen in these phosphodiesterases. This difference suggests that free-living and parasitic eukaryotic pathogens may utilize these mechanisms for regulating cAMP and cGMP levels differently.

Phylogenetics can also provide insight into the role of RegA orthologs in pathogenesis. A phylogenetic evaluation of RegA revealed that these organisms had the least phylogenetic

relationship to *Dictyostelium* and the other eukaryotic pathogens. This suggests that RegA may have been adapted and evolved differently evolutionarily in these organisms. The presence of conservation in a specific region of the sequences of the various phosphodiesterases suggests this region has an important catalytic or regulatory function. A closer evaluation through multiple sequence alignments of these phosphodiesterases revealed that sequence conservation was fairly similar between each eukaryotic organism. The similar conservation in the sequences of RegA, DdPDE3, and DdPDE4 can be seen in figure 3. The concentration of conservation towards the end of the protein sequence represents the catalytic. Even though these phosphodiesterases have different targets they still perform a similar function of degrading the phosphodiester bonds in cAMP and cGMP. The similarity in the conservation of the catalytic region of these phosphodiesterases most likely exists for this reason. The RegA sequences of *Entamoeba*, however, differed significantly from sequences found in the eukaryotic organisms. A phylogenetic evaluation of RegA revealed that these organisms had the least phylogenetic relationship to *Dictyostelium* and the other eukaryotic pathogens.

A closer analysis of RegA reveals that there is also conservation in many of its phosphorylation sites. Phosphorylation is one mechanisms which organisms can use to control protein function and regulate biological processes. These phosphorylation sites are one mechanism *Dictyostelium* utilizes to regulate RegA activity. Phosphorylation sites for RegA were analyzed across multiple eukaryotic species to determine if conservation also existed in these regions. The presence of conservation in these phosphorylation sites would suggest that eukaryotic pathogens utilize the same mechanisms for regulating RegA as *Dictyostelium.* A closer evaluation of the sequences around these phosphorylation site is shown in figure 4. Significant sequence conservation was seen in sequences for the phosphorylation sites located at 212 bp. and 676 bp. This suggests that

many eukaryotic pathogens also use these same phosphorylation sites for regulating RegA activity. While sequence conservation was not seen in the phosphorylation sites at 142 bp. and 412 bp., amino acid conservation was seen at these phosphorylation locations for several of the eukaryotic pathogens. This suggests that eukaryotic pathogens may not utilize these phosphorylation sites for regulating RegA activity. The conservation of RegA phosphorylation sites in eukaryotic pathogens suggests these sites may also be important for pathogenesis. The controlling the activity of RegA is necessary for regulating the levels of intracellular cAMP necessary for signaling. Signaling is an important part of pathogenesis, therefore it can be inferred that any mechanisms that are important for regulating signaling may also be important for pathogenesis. Phosphorylation is one mechanism *Dictyostelium* uses to control RegA activity. The presence of conservation in the sequences for phosphorylation shows that many eukaryotic pathogens utilize similar phosphorylation sites for regulating RegA. This suggests that these phosphorylation sites may also be crucial in pathogenesis.

Phylogenetic and genomic evaluations of RegA and the other phosphodiesterases revealed that many of the same mechanisms found in *Dictyostelium* for managing cAMP and cGMP levels are also found in other eukaryotic pathogens. Phosphodiesterases like RegA are an important piece of signal transduction that allow *Dictyostelium* to interact and respond to stimulus and to its environment. A successful pathogen must also be able to interact and respond to stimulus and their environment. Signaling is an important mechanism in pathogenesis. Mechanisms such as RegA and other phosphodiesterases help an organism regulate signaling that are most likely also important for pathogenesis in other eukaryotic organisms.

Works Cited

Bader, S, et al. “Seven Dictyostelium Discoideum Phosphodiesterases Degrade Three Pools of CAMP and CGMP.” *Advances in Pediatrics.,* U.S. National Library of Medicine, 15 Feb. 2007, www.ncbi.nlm.nih.gov/pubmed/17040207.

Du, Qingyou, et al. “The Cyclic AMP Phosphodiesterase RegA Critically Regulates Encystation in Social and Pathogenic Amoebas.” *Advances in Pediatrics.,* U.S. National Library of Medicine, Feb. 2014, www.ncbi.nlm.nih.gov/pmc/articles/PMC3906536/.

Loomis, W F. “Cell Signaling during Development of Dictyostelium.” *Advances in Pediatrics.,* U.S. National Library of Medicine, 1 July 2014, www.ncbi.nlm.nih.gov/pubmed/24726820.

Petlick, Alexandra R., et al. “Protein Kinase A Regulates the Ras, Rap1, and TORC2 Pathways in Response to the Chemoattractant CAMP in Dictyostelium.” *Journal of Cell Science,* The Company of Biologists Ltd, 1 Jan. 2017, jcs.biologists.org/content/early/2017/03/15/jcs.177170.

Schwebs, D J, et al. “Loss of CAMP-Specific Phosphodiesterase Rescues Spore Development in G Protein Mutant in Dictyostelium.” *Advances in Pediatrics.*, U.S. National Library of Medicine, Feb. 2014, www.ncbi.nlm.nih.gov/pubmed/24511612.