Modeling and Experimental Characterization of IgE Receptor Signaling Year 2

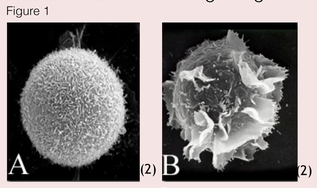
Introduction

More than 20% of all Americans have allergies. While many medications are helpful, they may have side effects and may not work for everybody. Our project focuses on the initiation of allergic reactions to further understand it with the hopes of potentially finding a more effective solution.

Mast Cell Undergoing Allergy Response

Mast cells (below) are a type of immune cell involved in the allergy signal cascade. When an allergen binds to a mast cell, the cell undergoes a series of internal signal cascades, resulting in the degranulation of the cell (B). The membrane of the mast cell breaks apart and releases signaling chemicals such as histamines. Most modern medications stop allergic reactions after the intracellular mast cell signaling.

Figure 1



(2) Healthy mast cell (2) Degranulated mast cell

Initiation of Mast Cell Signal Cascade

Allergens are recognized by IgE molecules – a mediatory molecule between the allergen and the mast cell surface receptor.

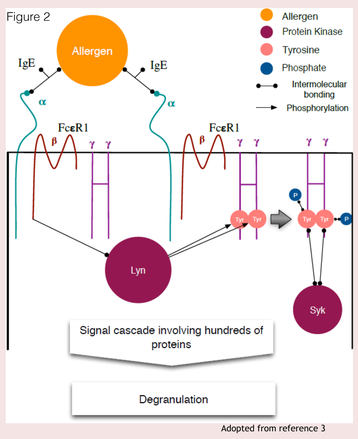
FcεRI receptor, a mast cell surface receptor, binds to allergen through IgE

Three subunits of FceR1

- α – binds to IgE
- β – localizes Lyn kinase
- γ – becomes phosphorylated (addition of a phosphate) by Lyn

Syk kinase binds to phosphorylated γ – initiation of signal cascade

Figure 2



Adopted from reference 3

Summary of Published Research

Amino acid sequence of γ-subunit

MIPAVILFLLLLVEEAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQV
RKADIASREKSDAVYTGLNTRNQETYETLKHEKPPQ

- Region important for signaling is highlighted in red (ITAM region) (5)
- Amino acid tyrosine (Y) which becomes phosphorylated is highlighted in purple

- Gamma phosphorylation needed for mast cell signal cascade initiation(5)
- Phosphorylation amount dependent upon concentration and duration of exposure to allergen(5)
- N-terminal tyrosine (P1) and C-terminal tyrosine (P2) phosphorylation timeline and amount not known
- Syk has two conserved SH2 domains, which means it requires two phosphorylated tyrosines in order to bind(26)
- Confirmation of computational models for this system has been limited due to lack of reagents for specific phosphorylation detection

Developing Antibodies to Detect Specific Gamma Phosphorylation

Aims for Year 2

- To further develop the N-terminal phospho-tyrosine specific antibody in order to more closely examine the phosphorylation kinetics of this tyrosine
- To obtain an antibody that can detect C-terminal tyrosine phosphorylation

Procedures

Antibodies are large Y-shaped proteins, which help to remove foreign substances, such as viruses and bacteria from our body. The antibody genes are naturally very diverse, and each individual one binds very specifically to its own target. The tips of the antibody molecule, known as the variable regions (vL-vH), provides this specificity. vL-vH regions can be linked together to create single chain fragment variable (ScFv), which can be used in antibody selections.

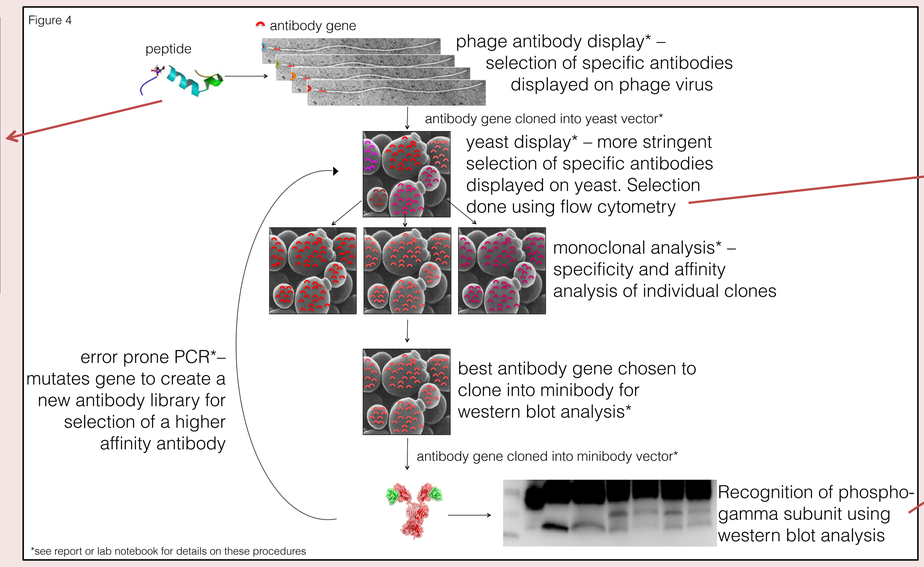
Amino acid sequence of the peptide ITAM region of the gamma subunit used as target for antibodies

Table 1

Fcγ-2 phosphorylation sites	
γ-P0	ASREKSDAVYTGLNTRNQETYETLKH E
γ-P1	ASREKSDAVYTGLNTRNQETYETLKH E
γ-P2	ASREKSDAVYTGLNTRNQETYETLKH E
γ-P12	ASREKSDAVYTGLNTRNQETYETLKH E

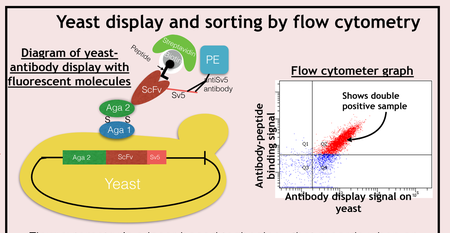
Peptides are a specific section of the gamma subunit. Phospho-tyrosines are shown in red. These peptides were used as antigens or targets for the antibody library.

Figure 4



*see report or lab notebook for details on these procedures

Yeast display and sorting by flow cytometry



Flow cytometry is a laser-based technology that records when an antibody is displayed on yeast (x-axis) and recognizing the peptide (y-axis). This allows sorting of yeast that contain specific antibodies.

Recognition of the phospho-gamma subunit

- Western blot analysis was used to assess recognition of the whole gamma subunit rather than the peptides
- Rat basophilic leukemia (RBL) cells are very similar to human mast cells. It can be stimulated using an allergen called dinitrophenyl (DNP-BSA)
- RBL cells are stimulated at different concentrations(14), then lysed, or exploded, at different times(14) after stimulation and antibody detects the phospho-gamma subunit within the cell lysate

Results

Last year

- Selected an antibody that detects phosphorylation of the N-terminal tyrosine (specific to the P1 peptide). Western blot analysis showed phosphorylation after one minute of allergen stimulation and dephosphorylation after four minutes.
- Antibody selection for a C-terminal phospho-tyrosine specific antibody (P2 specific) was ongoing

This year

N-terminal phospho-tyrosine antibody (P1 peptide specific antibody)

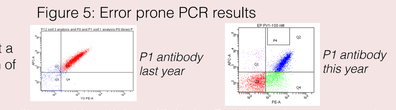
- Error prone PCR of the previous antibody resulted in antibody library with a higher affinity to the P1 peptide* (Figure 1)
- Yeast sorting and monoclonal analysis enabled selection of antibody with 50X better binding affinity than last year's antibody* (Figure 2)
- New antibody was used for western blot analysis to study the effect of concentration and time exposure of the allergen on phosphorylation (Figure 3A)
- Western shows constant phosphorylation of P1. Second band appears as a result of P2 phosphorylation. Both tyrosines are still phosphorylated after 10 minutes (Figure 3A)
- Concentration of stimulant seems to have more influence on gamma phosphorylation than time. At 2 minutes with 20nM allergen stimulation, a second band appears. However at 2nM and 200nM, the second band only appears at 5 minutes. This shows a bell curve effect of concentration on P2 phosphorylation. (Figure 3A, 3B)
- Other experiments verified that antibody is truly detecting the phosphorylated tyrosine, not the unphosphorylated gamma chain*

C-terminal phospho-tyrosine antibody (P2 peptide specific antibody)

- An extra round of phage selection for the P2 antibody was done, then three rounds of yeast sorting were done*
- P2 selections did not yield a specific antibody.* No P2 antibody in original antibody library

*see report for details on these results

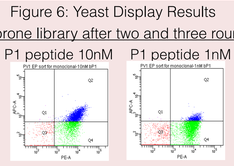
Figure 5: Error prone PCR results



EP PCR resulted in a library with a higher affinity for P1 (shown by a steeper slope)

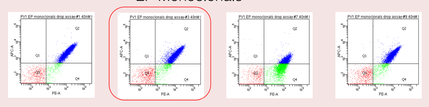
Figure 6: Yeast Display Results

Results for P1 error prone library after two and three rounds of yeast sorting



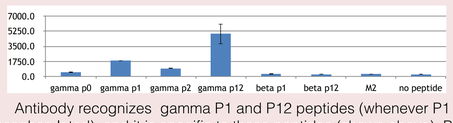
The populations above were sorted to use in monoclonal analysis.

EP Monoclonals



Four best clones from monoclonal analysis binding to the P1 peptide at 40nM. Circled antibody was chosen for detection of the whole gamma subunit using western blot analysis.

Specificity assay of circled antibody above

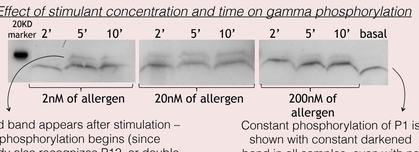


Antibody recognizes gamma P1 and P12 peptides (whenever P1 is phosphorylated), and it is specific to those peptides (shown above). Binding affinity of this new antibody is 50 times stronger than the antibody from last year.

Figure 7: Western Blot Analysis

- RBL cells were stimulated with 2nM, 20nM, and 200nM of allergen.
- Cells were lysed at 2 minutes, 5 minutes, and 10 minutes

Effect of stimulant concentration and time on gamma phosphorylation

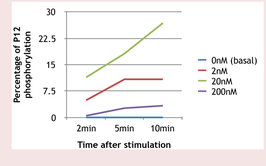


Second band appears after stimulation – P2 phosphorylation begins (since antibody also recognizes P12, or double phosphorylation)

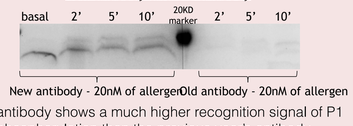
Constant phosphorylation of P1 is shown with constant darkened band in all samples, even with no stimulation (basal)

In 20nM sample, P2 phosphorylation begins at 2 minutes. In 2nM and 200nM samples, it begins at 5 minutes. This is the result of a bell curve effect of concentration on gamma phosphorylation.

Percentage of doubly phosphorylated gamma in cell lysate samples



Comparison of gamma recognition signal between new antibody and old antibody



New antibody shows a much higher recognition signal of P1 phosphorylation than the previous year's antibody

Research Goal

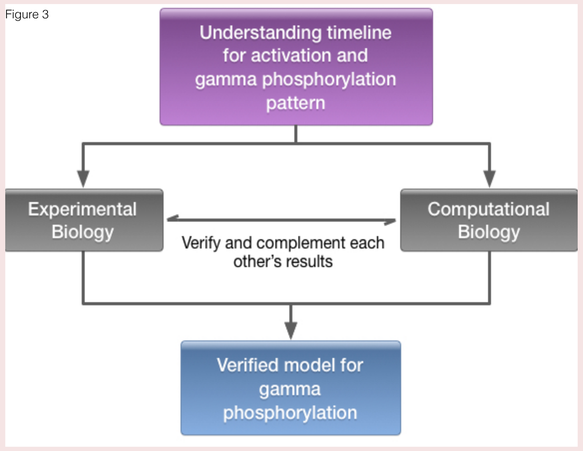
- To further understand gamma phosphorylation kinetics
- To develop a methodology for studying phosphorylation based signaling

Implications

- An explanation for how double phosphorylation occurs, and therefore how Syk binding and subsequent signal cascade is regulated
- There are many diseases that are associated with altered phosphorylation states or mutated kinases, such as cancers/tumors, inflammatory diseases, and diabetes(25)
- The methodology we used in this project is applicable for studying the phosphorylation mechanics of these diseases.
- With a better understanding of these signal cascades, we could potentially develop drugs to prevent them

Experimental Design

Figure 3



By using experimental and computational biology together, we can verify and complement each other's results, and use those results to obtain a final verified model of gamma phosphorylation. Additionally, each side of this project can aid the other in gaining results at a faster pace.

Continuation

Last year, the experimental side of the project had selected an antibody that could recognize when the N-terminal tyrosine was phosphorylated. Results indicated that the N-terminal tyrosine is phosphorylated after one minute of allergen stimulation, and dephosphorylation starts after four minutes of stimulation. A working model had been developed, however it was not fit nor detailed enough.

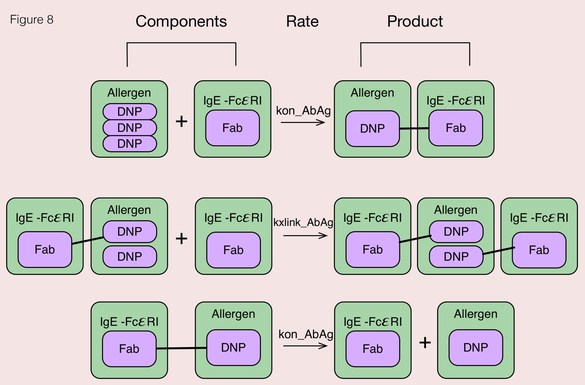
This year, we planned to select a C-terminal phospho-tyrosine specific antibody, as well as further develop the N-terminal phospho-tyrosine antibody from last year. This would allow the antibody to detect phosphorylation more accurately and at a lower concentration.
We will also develop the model further so that it is more detailed and it will fit to the experimental data more accurately.

Modeling of Gamma Phosphorylation

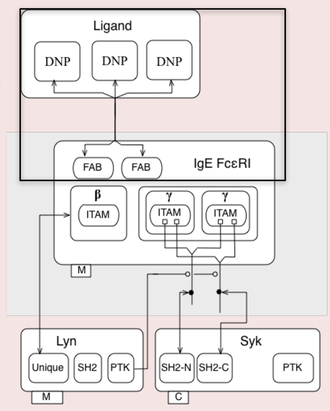
Method

A programming language called BioNetGen Language (BNGL) was used. BNGL is specifically designed for development of chemical kinetic models of cell signaling systems. BNGL is a rule-based programming language which entails specification of necessary and sufficient conditions for a reaction to occur, as well as parameters governing the rates of the reactions (10)

Figure 8



Pictured above are a few of the rules for ligand interactions graphically represented. The rule structure is very similar to a chemical equation. The diagram to the right is a full rule map of the rules in our program (13).



This diagram shows all the rules in the model that deal with the reactions in the introduction. We input the molecules of interest, rules for interactions, and parameters governing the rules in order to receive specified outputs, e.g.P1 phosphorylation.

Advantages of modeling

- Each component can be manipulated and its effect on the system can be studied
- Most effective interference point for inhibitory drug molecule can be investigated
- Simulations performed without expensive reagents
- Provide guidance and information to experimental biology
- Receives parameters from experimental biology

BioNetFit

- BioNetFit is a fitting application made to automate the process of fitting a model to experimental data.
- BioNetFit offers many algorithms for fitting: genetic algorithm, differential evolution, and particle swarm optimization

Table 2

Algorithms		
Genetic Algorithm	Particle Swarm	Differential Evolution
define population		
in each iter.: generation	a particle is a parameter	each parameter is an agent
after a number of generations, best fit is decided	particles move towards a "best fit"	agents move, if the new position is "better" it is kept

- depending on algorithm, user chooses amount of generations and runs
- BNF then parses the model file
- it looks for "free" variables with the definition of variablename__FREE__
- Next, the algorithm that was chosen runs for the amount of generations and runs
- it picks the best fit parameters and provides you with the model file with the parameters

Results

- The model has the basal n terminal tyrosine phosphorylation in agreement with the experimental data
- The fits that are graphed were run for 1000 generations of 100 permutations
- The simulation also mutates the original values of the free variables in order to find better fits
- We also see an drop in phosphorylated P12 while a the slope of the P1 graph is always positive
- The simulation is well fit to the experimental P1 data and we have predictive P2 data
- We have also predicted data for a higher concentration of allergen (20nM rather than 2nM)

Figure 9

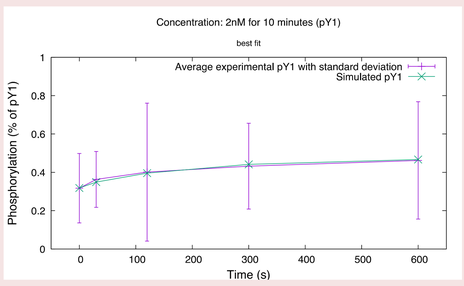


Figure 11

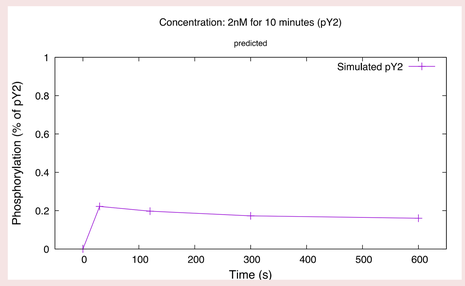


Figure 10

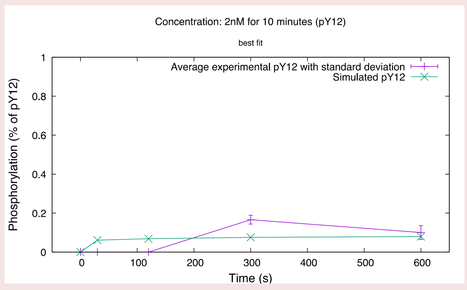
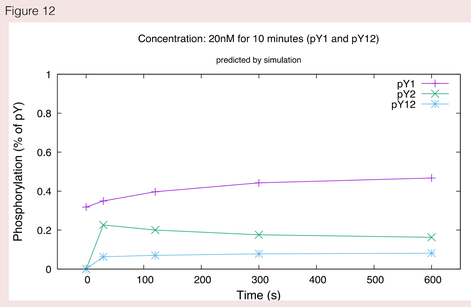


Figure 12



Conclusion

- Much closer to understanding the phosphorylation patterns of the FcεR1 gamma chain and the mechanisms by which Syk binds to gamma
- Developed a brand new antibody that is sequenced and phosphorylation specific to the gamma chain which has shown that the N-terminal tyrosine is constantly phosphorylated, and the C-terminal tyrosine becomes phosphorylated a few minutes after allergen stimulation
- Built a model that reflects the experimental data above and, once verified, could extrapolate phosphorylation patterns at other allergen concentrations to study the effects of that

With this project, we have developed a brand new methodology to study cascades based on post-translational modifications like phosphorylation. Using experimental and computational biology together allows each independent field to have separate results that can verify each other, and they can combine forces to find solutions faster.

Next Steps

- Obtain a crystal structure of the antibody-P1/P12 peptide complex to study interacting amino acids which may aid in designing a P2 specific antibody or a potential pharmaceutical molecule
- We would like to use the model to predict more data about this system including P2 and the effect other molecules may have on this system

References and acknowledgements in report