Interaction between Notch and its ligands Delta and Serrate

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Abstract

The purpose of this study is to investigate the interaction between Notch and its ligands Delta and Serrate. The ultimate goal of this research is to obtain structural and kinetic data for this interaction. This report will only cover the preliminary results and the procedures involved. Through the use of PCR and Gel Eletrophoresis Notch EGF segments #10-13 were amplified and confirmed. The PCR products were gel purified and inserted into a TA vector. These products were sent for sequencing. A cDNA from adult *Drosophila* was created. The EBD region of Delta was amplified and confirmed with a DNA gel. Delta was also purified and inserted into a vector. These products were sent for sequencing. The Notch EGF segments were successfully amplified and inserted into a TA vector. As for Delta, it was confirmed that our plasmid and construct were expressed. The results showed that Delta was part of the cDNA library created. With the current results the further progress will be made in which both Delta and Notch will be expressed in bacteria, purified and refolded properly.

Introduction

Notch is a single transmembrane protein first identified in *Drosophila* (Sakamoto 2005) with an extracellular and intracellular domain. *Drosophila* Notch contains 36 Epidermal Growth Factor like repeats (ELRs), with each ELR encompassing 36 amino acids. An ELR is characteristic of six conserved cysteines, which form 3 disulfide bonds. In *Drosophila*, ELRs 11 and 12 have been shown to be "necessary and sufficient" for the interaction between Notch and its ligands. To further support the fact that ELRs 11 and 12 are essential for ligand binding, *Drosophila* Notch was replaced with *Xenopus* Notch in which normal interaction between Delta/Serrate remained (Fleming 1998) Also, ELRs 11 and 12 specifically have been conserved throughout all vertebrates.

Cell communication controls cell growth, survival, fate and differentiation. Notch and its receptors play an important role in cell fate and differentiation throughout species ranging in complexity from Drosophila to Humans (Schweisguth 2004). Calcium binding appears to be crucial for cell differentiation with binding sequences found in both Notch and Delta. Upon the binding of Delta/Serrate to Notch immediate protolytic cleavage occurs. The intracellular portion translocates to the nucleus where it interacts with a CSL (CBF-1/Suppressor of Hairless/Lag-1) DNA binding protein. This ultimately results in transcriptional changes in the involved cells.

Glycosylation plays a key role in the interaction between Notch and its ligands Delta and Serrate. Notch is rare in the fact that it undergoes O-fucosylation. Notch is modified by O FucT which adds a fucose to a serine or threonine. This fucosylation site can be further modified by the action of the enzyme Fringe, which adds a GlyNac residue to the existing fucose. The O-fucosylation sites found within the ELR 12 is known to be essential for ligand binding. Previous

studies have shown that with the addition of the GlcNac Notch is more likely to bind to Delta rather than Serrate. When OFucT1 acts upon Notch it preferentially binds to Serrate over Delta. Both Delta and Serrate are modified by O-fucose also (Haltiwanger 2004).

Various diseases result from a disruption in Notch signaling. In humans a defect in Notch signaling is linked to the cause of CADASIL, T-cell leukemia, and other types of cancers. (Notch signaling in development and disease) CADASIL (cerebral autosomal dominant adult onset ateriopathy) is connected to a mutation in Notch3. This mutation is described as a loss or addition of cysteine residue in the EGF domain. This loss or addition of the cysteine residue disrupts the cell signaling due to the build up Notch on the cell surface mainly affecting vascular smooth muscle cells. Recent studies have also shown that discrepancies in Notch signaling lead to multiple sclerosis. In *Drosophila* one of the results of interrupted Notch signaling is the notched wing phenotype. Also in the case of *Drosophila* when Delta is overexpressed extensive wing structure is apparent.

Method and Materials

Plasmids containing full length *Drosophila* Notch clone and Delta clone were received from Dr. Wu-Min Deng (Department of Biology, Florida State University). Notch EGF segments #10 through 13 were spliced out. The following represents the forward primer for ELR #10: 5' `TGCCACCTGG ACGATGCCTG TACCTCG 3'.` The reverse primer at the end of EGF #13 was 5' `ATTGATTTGA CATCGTGCGC CCGTAAAGC 3'.` The PCR reaction for Notch was carried out for 35 cycles with a denature of 94 degrees Celsius for 1 min, annealing of 62 degrees for 45 seconds, and an elongation of 72 degrees for 2 min. Magnesium screening was utilized. Next, a 2% agarose DNA gel was run with the PCR products to verify that the desired product

was present. The products were gel purified using the QIAquick Gel Extraction Kit Protocol found on pg. 23 of the QIAquick Spin Handbook (07/2002). Ligation was conducted in which 4uL of the PCR product, 1uL of the salt solution, and 1uL of the topo vector was combined in a tube. This mixture incubated at room temperature for 5 minutes. Next, 2uL of the ligation reaction was placed in a tube containing competent cells. This mixture was kept on ice for 5 minutes and then heat shocked at 42 degrees Celsius for 30 seconds. The tube containing the mixture was immediately returned to ice and 250uL of room temperature SOC medium was added. These tubes were then placed in the shaker at 200 rpm for 1 hour at 37 degrees Celsius.

LB media was made according to Invitrogen Topo TA Cloning Version R (8 April 2004) Instruction manual pg. 23. Ampicillin was the antibiotic used with a final concentration of 50ug/mL. The LB plates were made according to the same protocol used for the LB media as stated above. For each of the six PCR products 2 plates were spread with two different volumes: 25uL, 50uL. The plates were then placed in an incubator at 37 degrees Celsius overnight. From each plate 2 colonies were chosen and each colony places in a separate tube containing 3mL of LB media and 1.5uL of amp. These tubes were placed in an incubator/shaker for 24 hours at 37 degrees Celsius. The solutions were then centrifuged at 4000g for 15 minutes at 4 degrees C. The supernatant was then discarded. A QIA mini prep procedure was then followed according to the QIAprep Miniprep Handbook (03/2003), QIAprep Spin Miniprep Kit Protocol pg.22. These products were then sent for sequencing (DNA Sequencing Facility at Florida State University).

A cDNA library was created in which total RNA was extracted. *Drosophila* were received from Dr.Wu-Min Deng (Biology Department at Florida State University). The *Drosophila* were frozen at -20 degrees Celsius for 5 minutes and then immersed in liquid N_2. The *Drosophila* were then transferred to a homogenizer and 1 mL of TRIzol reagent (Invitrogen)

was added. The *Drosophila* were then crushed. The resulting solution was centrifuged for 10 minutes at 10000 G at 4 degrees Celsius. The supernatant was removed and transferred to a new tube to which 200uL of chloroform was added. The mixture was shaken by hand for 15 seconds. The aqueous layer was removed and placed in a new tube. To the tube 1mL of isopropanol was added and then allowed to sit at room temperature for 10 minutes. This tube was then centrifuged at 10,000 G for 10 minutes. The pellet was washed with 75% EtOH and place in the refrigerator overnight. The solution was then centrifuged at 7,500 G for 5 minutes. The solution was then placed in the speedvac to dry. Finally, the RNA was dissolved in RNase free water and incubated at 55 degree Celsius for 10 minutes until the RNA went into solution. Superscript II RT (Invitrogen SuperScript II protocol published in 2003, reviewed 11 Nov 2003) reaction was completed.

Primers were also designed for the EBD region of Delta. The forward and reverse primers represented as the following in respective order: 5'CACAGTTCCG GCAGCTTTGA GTTG 3', 5'ACAGTAATCG CCCTGCCATC CG 3'. The PCR conditions were as follows: denature at 94° C for 1 minute, 59° C for 45 seconds, and 72° C for 2 minutes. These steps were carried out for 35 cycles. A 2% agarose DNA gel was run in verify that the product was present along with the cDNA created. The products were then purified using the QIAquick PCR Purification kit protocol on pg. 18 of the QIAquick Spin Handbook (07/2002). A QIA mini prep procedure was then followed according to the QIAprep Miniprep Handbook (03/2003), QIAprep Spin Miniprep Kit Protocol pg.22. These products were sent for sequencing (DNA Sequencing Facility at Florida State University).

Results

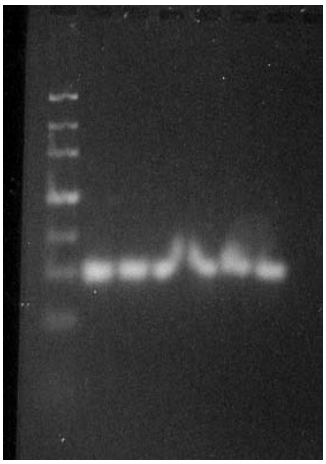

Figure 1. The above represents a 2% agarose gel for Notch EGF like repeats #10-13. The sixth visible band of the PCR DNA ladder 1 corresponds to 500 base pairs which most closely aligns with the products found in the adjacent lanes. Note that products in lanes 4, 5, and 6 are irregular in shape due to improper loading of the sample into the wells.

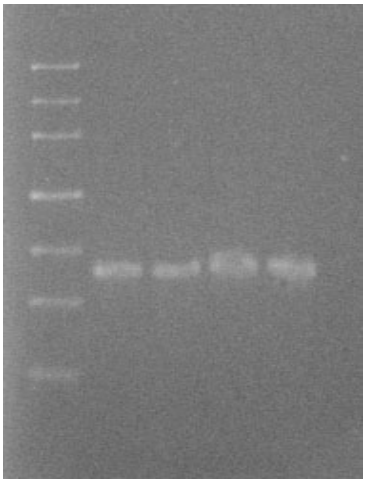

Figure 2. The 2% agarose gel represented above was processed for the EBD region of Drosophila Delta. The fifth and sixth bands of the PCR DNA ladder 1 correspond to 700 and 500 base pairs respectively, with the products in the following lanes lying between these bands. Lanes 2, 3 and 4 shown are products in the pUAST vector with the fifth lane being the cDNA.

Discussion

For Notch EGF segments #10-13 were successfully amplified as shown in Figure1. The expected length of our product was 463 base pairs. The products appeared in the correct position relative to the PCR DNA ladder with the closest band at 500 base pairs. Therefore, with this confirmation the Notch constructs were inserted into a TA vector and then sent for sequencing. Not all of the Notch products came back with the correct sequence but one of the 12 created did. The next step in this research will be to add the necessary restriction enzyme sites insert into a TA vector to cut with restriction enzymes. It will eventually be inserted into a pET vector and transformed. In summary the continuing work will include expression in bacteria, purification of the protein, and proper refolding will follow.

As Figure 2 shows that our construct and plasmid are present. The DNA gel confirms with the position of the products in relation to the PCR DNA ladder that it is the correct number of base pairs. Also, Figure 2 shows that the cDNA library that was created was a success because Delta within the cDNA library showed up at the same position. In the future we hope to follow the same procedure with Delta as with Notch in that it will be expressed in bacteria, the protein will be purified and refolded.

SPR (Surface Plasmon Resonance) and NMR (Nuclear Magnetic Resonance) will be used as tools to gather information concerning the structure and kinetics.

References

Fleming, Robert. 1998. “Structural conservation of Notch receptors and ligands” *Cell and Developmental Biology*. v. 9, 599-607.

Haltiwanger, R. and J. Lowe. 2004. “Role of Glycosylation in Development” *Annu.Rev. Biochem.* v. 73, 491-537.

Hambleton, S., N. Valeyev, A. Muranyi, V. Knott, J. Werner, A. McMichael, P. Handford, A. Downing. 2004. “Structural and Functional Properties of the Human Notch-1 Ligand Binding Region” *Structure* v. 12, 2173- 2183

Harper, JA., J. Yuan, JB. Tan. I. Visan, CJ. Guidos. 2003. “Notch signaling in development of disease” *ClinGenet* v. 64, 461-472

Joutel, A., F. Andreux, S. Gaulis, V. Domenga, M. Cecillon, N. Battail, N. Piga, F. Chapon, C. Godfrain, and E. Tournier-Lasserve. 2000. “The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients” *Journal of Clinical Investigation* v. 105(5), 597-605

Lai, Eric C. 2004 “Notch signaling: control of cell communication and cell fate” *Development.* v. 131, 965-973

Sakamoto K., S.C. Wang, K. Katsube, A. Yamaguchi. 2005. “Distinct roles of EGF repeats for the Notch signaling system” *Cell Research*. v. 302, 281-291.

Schweisguth, Francois. 2004. “Regulation of Notch Signaling Activity” *Current Biology.* v. 14, 129-138.