Characterization of β-heavy Spectrin Self-interaction

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Abstract

Spectrin is a protein which serves to form the cytoskeletal network in cells for structural support. The protein forms tetramers consisting of two α and β subunits. This study's objective is to investigate the possibility that β-heavy spectrin functions independently of α-spectrin to form higher order structures contrary to the classical tetramer-based structure found in erythrocytes. An *in vitro* western blot overlay method is utilized to observe a self-interaction of β-heavy spectrin. A self-interaction was observed, offering new insight into how spectrin may function in biological processes and generate networks.

Introduction

First discovered in 1968, spectrin was found to be a defining part of the membrane skeleton in red blood cells, where it maintains a structural role to form and shape erythrocytes (Marchesi and Steers, 1968). The structural role of spectrin was thought to be its only function, but further research has revealed that non-erythroid spectrin plays a role in many more biological processes. Some of these processes include cell polarity, the organization and formation of cell adhesion complexes, and protein trafficking (Zarnescu and Thomas, 1999; Xiuli An *et al*., 2008; De Matteis and Morrow, 2000).

In *Drosophila*, there are three isoforms of spectrin: α, β, and the larger $β_H$-spectrin. Vertebrates possess multiple spectrin isoforms in the forms of αI, αII, and βI through βV. Spectrin functions and forms networks through its several binding domains and interactions with other proteins. α-spectrins contain a Src homology 3 (SH3) domain as well as a C-terminal calmodulin related Ca^{2+} binding site. β-spectrins possess an N-terminal actin binding domain as well as ankyrin binding sites. In addition to the extended length on $β_H$-spectrin, these spectrins also differ from β in that they possess an SH3 domain and lack ankyrin binding sites (Bennet and Baines, 2001). These subunits organize into a cytoskeletal network by forming heterodimers in which α spectrin subunits connect to β spectrin in an antiparallel, side-to-side fashion (Fig. 1). These α/β heterodimers then connect to form tetramers in which the N-terminus of each α subunit connects to the C-terminus of each β subunit in the head-to-head interaction (Fig. 1). This tetramerization allows the formation of long spectrin protein chains, which make up the membrane skeleton network.
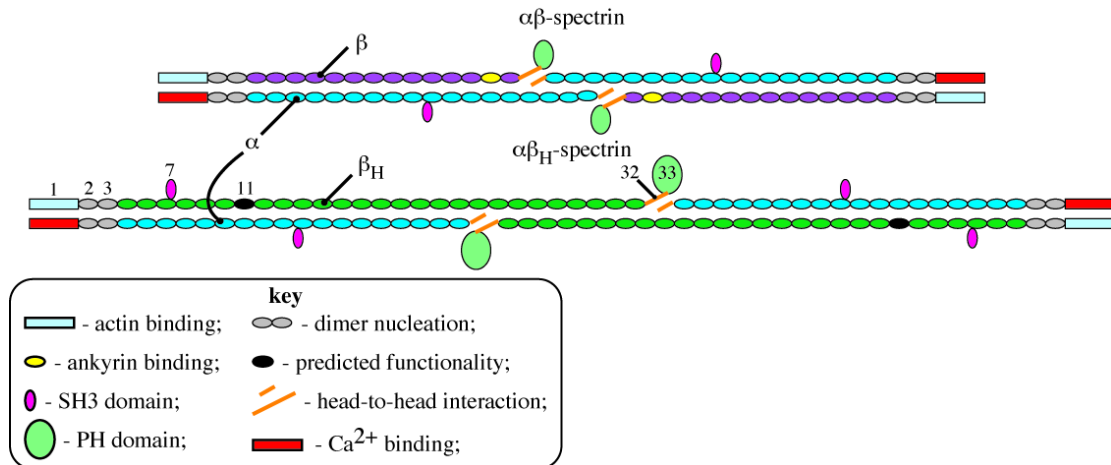
Figure 1. Spectrin Tetramerization and Binding Domains (Schematic provide by G. Thomas and used with permission)

Spectrin mutations cause improper erythrocyte network formation, and the compromised networks result in weak and fragile membranes. Humans with these mutations exhibit hereditary hemolytic anemias and spinocerebellar ataxia (Bennet and Healy, 2008). Spectrin is recognized as an essential non-erythroid protein. However, recent studies on the $\alpha spec^{R22S}$ mutation, in which the tetramerization of α and β subunits is disrupted, have revealed that the tetramerization of spectrin in non-erythrocyte cells is not crucial. In fact, *Drosophila* $\alpha spec^{R22S}$ mutants complete normal development with only minor abnormalities (Khanna *et al*., 2014). Some of these observed abnormalities included compromised fusome integrity during oogenisis, fewer individuated synaptic boutons in the nueromuscular junction, and irregular shaping and position of cuprophillic cells in the midgut. Despite these abnormalities, viable adult flies were produced. The evidence suggests that the tetramer network is not essential for the major function of spectrin. This would suggest that a new model needs to be developed for the function and structure of the spectrin membrane skeleton in non-erythroid cells and that the importance of tetramerization in erythrocytes is a special case. One possibility is that spectrins may form a network that differs from the erythrocyte network to function in non-erythroid cells.

A recent discovery related to spectrin binding was made by Samantha Papal *et al*., (2013) during an investigation of βV-spectrin, the β_H ortholog in humans. In this paper, *in vitro* and *in vivo* evidence demonstrated a homodimeric interaction between repeats 26-30 and 29-30 of βV (Papal *et al*., 2013). The proposed model arising from this observation is that βV has the ability to form homodimers through its C-terminal region. This model is supported by *in vitro* western blot evidence, which demonstrated a self-interaction between βV protein fragments. Homodimer formation is also supported by *in vivo* evidence of α independent function of βV along opsin trafficking routes in photoreceptor cells. The discovery of this interaction suggests that there are α independent functions for βV and that new molecular pathways could result in network formation in a system where βV spectrin is the key component. Since βV is the human ortholog for β_H, I set out to investigate whether this self-interaction is also present in the *Drosophila* protein using the *in vitro* method of a western blot overlay.

Methods

Polymerase chain reaction (PCR) and Cloning

PCR was performed using the primers 3'-AGCGGATCCTCTCACCAATTGCAGGAGT TCCTGCGCAGTCTCTA-5' (top primer) and 3'-ATGCGAATTCTTACTGGTGACGGAATA GCTGCTCAA-5' (bottom primer) to amplify the region S25-32 of β-heavy using the preexisting cDNA p3F1 as a template. The 2.47kb PCR product (Fig. 3) was then purified by running it on an agarose gel and the band was extracted using a Qiagen gel extraction kit (Germantown, Maryland). This was followed by digestion with the restriction enzymes BamHI and EcoRI and ligation into similarly digested pGEX-4T-1 plasmid to create a fused open reading frame for GST-S25-32. Next, this plasmid was transformed into *Escherichia coli* (*E. coli*) strain XL10. The transformed bacteria containing the positive clone were grown overnight to stationary phase for plasmid extraction using a Qiagen maxi prep kit. This DNA was sequence verified. Following verification, the plasmid was transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL (Agilent Technologies, Santa Clara, CA) for expression using standard methods. These *E.coli* were optimized for induction for the process of protein expression and purification.

Expression and Purification

6 X 600 mL cultures of BL21 cells containing the GST-S25-32 fusion were grown to log phase and induced for 5 hours at room temperature using 1mM IPTG. After centrifugation, the pellets were combined and re-suspended together in lysis buffer (50 mL, 50mM Tris-Cl, pH 8.0, 50mM NaCl, 1mM β-mercaptoethanol, 5mM EDTA, 150µM PMSF, 1µg/ml of leupeptin, 1 µg/ml of pepstatin, 1 µg/ml of diisopropyl fluorophosphate). The bacteria were then sonicated (6 times for two minutes each) on ice to extract the fusion protein. The sonicated sample was centrifuged and the supernatant was mixed with glutathione agarose beads and tumbled for 1 hour at $4^°$C. Next, the beads were packed into a column and washed with 1X PBS + EDTA/PMSF (PBEP, 10 mM NaPO4, 130 mM NaCl, pH 7.3, 1 mM β-mercaptoethanol, 5 mM EDTA, and 150 µM PMSF), followed by PBEP without PMSF. Finally, the fusion protein was eluted with G buffer (50 mL, 50 mM Tris-Cl, pH 8.0, 10 mM reduced glutathione, 1 mM β-mercaptoethanol, 5mM EDTA, 1µg/ml of pepstatin).

Thrombin Titration, Cleavage, and GST Subtraction

Thrombin titration was performed on 50 µL aliquots of GST fusion protein that were digested with 50 µL of decreasing concentrations of thrombin (Sigma) mixed with PBEP with no PMSF. The thrombin ratios ranged from 1:1 to 1:10,000 and the titration was repeated in conditions of 0.15 M NaCl (Fig. 2, A and B). By running the titration samples on a gel, it was observed that the thrombin ratio of 1:10 in 0.15 M NaCl yielded maximum cleavage with little fragmentation of protein. Thrombin (400 µL) was added to a 20 mL sample of protein at a ratio of 1:10 with a 0.15 M NaCl concentration and incubated for 3 hours at $37^°$C to cleave off the GST and eventually isolate the S25-32 $β_H$ fragment (Fig. 2C). The digest was stopped by the addition of PMSF (80 µL) and chilled until dialysis with PBEP. Once dialyzed, glutathione agarose beads were added to the sample and tumbled for 1 hour at $4^°$C. The sample was then packed into the column and the isolated S25-32 flow-through was collected. The GST was eluted

out of the column with G buffer and the column was washed with PBEP. This process was repeated to maximize the removal of GST from the isolated S25-32 protein sample.
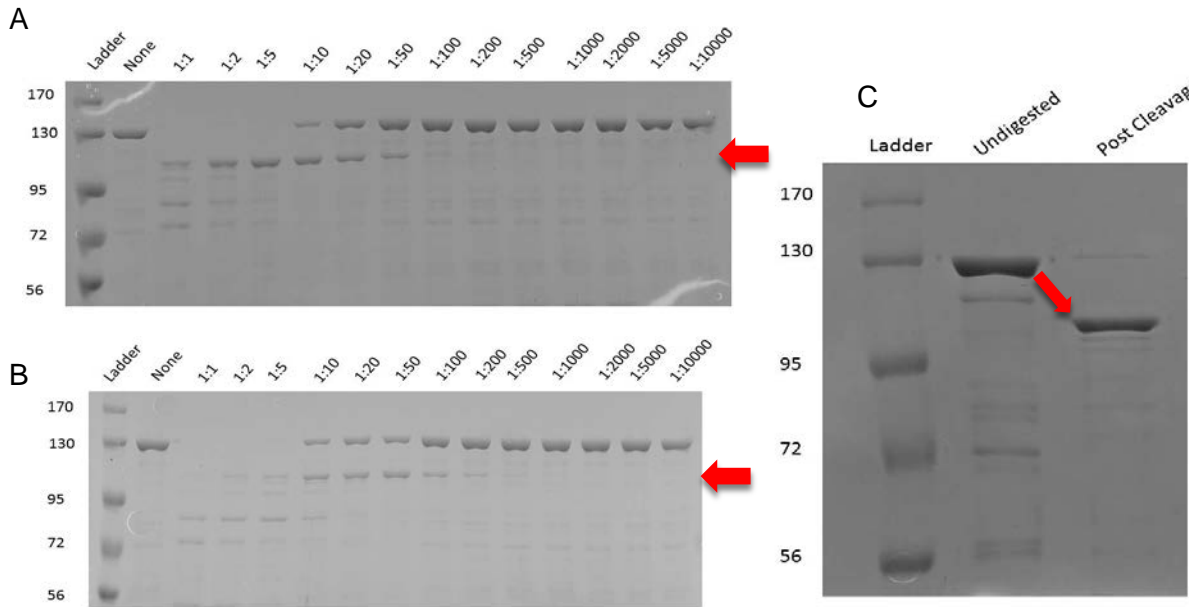

Figure 2. Thrombin Titration and Cleavage Results
(A) Thrombin titration performed with 0.15 M NaCl (B) Thrombin titration performed in absence of NaCl (C) Large-scale thrombin cleavage of fusion protein divulges successful cleavage with expected band at ~95kDa.

In Vitro Western Blot Protein Interaction Assay

100 µL of GST subtracted β_H S25-32 was run on a wide-lane 7% SDS page gel, transferred onto nitrocellulose paper, and sliced into strips. Individual strips were blocked (20 mM Tris pH 7.5, 150 mM NaCl, 5% nonfat milk), and rocked overnight at 4°C in various dilutions of uncleaved GST-S25-32 fusion protein. Following the overlay, the membrane was washed in incubation solution (4 x 5 minutes, 20 mM Tris pH 7.5, 150 mM NaCl, 5% nonfat milk, 0.1% Tween) and incubated in anti-GST primary antibody (GE Health Care) at a 1:500 dilution for 1 hour at room temperature. Following primary antibody incubation, the blot was washed in incubation solution (4 x 5 minutes) and incubated in secondary antibody (HRP donkey anti-goat) for 1 hour at room temperature. The blot was then washed in Tris Buffered Saline with Tween (4X 5 minutes, 20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween) and an ECL chemiluminescence system was used for detection.

Results

Through an *in vitro* western blot overlay method, Papal *et al.*, (2013) was able to identify self-interaction between repeats 26-30 of βV. I used PCR to amplify segments of the β_H sequence which are equivalent to this region. Amplification, using the primers described in the methods section and plasmid p3F1 as a template, resulted in the expected band of 2.467 kb (Fig. 3A). The primers used to amplify the fragment also added the restriction sites for BamHI and EcoRI for the purpose of cutting with these enzymes and cloning into pGEX-4TI, which was similarly digested (3B). After I performed the digest and ligated together the vector and PCR fragment, the plasmid was transformed into XL10 *E.coli*. The resulting colonies were then tested for successful plasmid intake. Analysis of several clones revealed that the insert was successfully transformed into colony #161 of the XL10 cell line, as indicated by the 2.4 kb band (Fig. 3C).
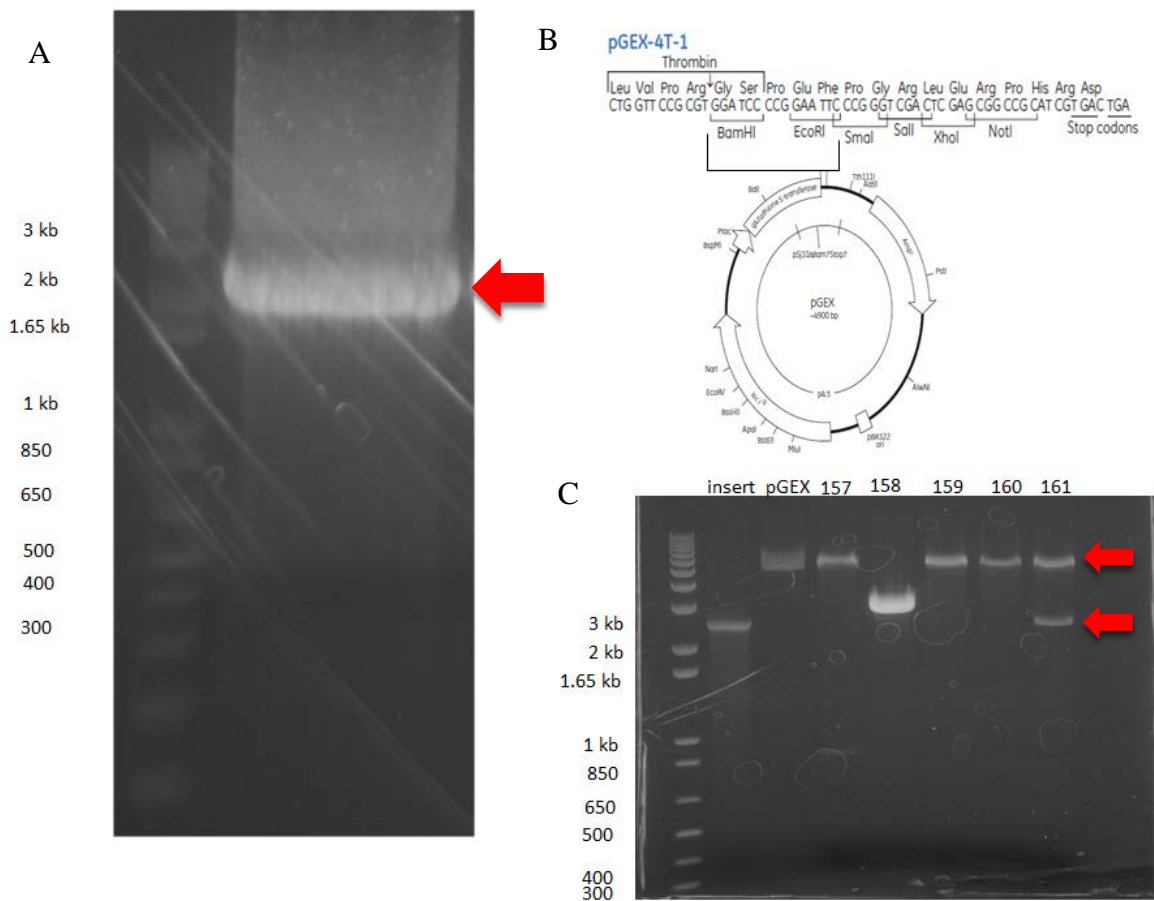

Figure 3. PCR and Cloning Results. (A) PCR product ran on 1% gel with expected band size of 2.46 kb. (B) Model of pGEX-4TI cloning vector with restriction enzyme sites (C) Insert with expected size of 2.46 kb was revealed by diagnostic digest to have successfully transformed into colony 161 when compared with S25-32 and pGEX-4TI positive loading control.

I retransformed the plasmid containing β_H S25-32 into the BL21 cell line for the purpose of expression. β_H GST::S25-32 is expected to have a band weight of 120 kDa. Test inductions with the BL21 cell line confirm this band weight and show that strong induction takes place

within a relatively short time (Fig. 4A). Induction time was shown to have little impact on the yield of fusion protein after 5 hours as the band intensities at five and ten hours are similar (Fig. 4A). I then conducted solubility tests to optimize the solubility of the protein in lysis buffer. Tests demonstrated that induction at 37°C results in an insoluble protein which remains in the pellet (Fig. 4B). Since flies live at ambient temperature, I decided to test the induction at room temperature which might improve the proper folding and conformation. Solubility tests at room temperature supported this prediction and yielded a highly soluble protein (Fig. 4C and D). From this result, it can be inferred that proper folding conformation of the GST::S25-32 fusion protein requires an optimal temperature range based on the environment in which the protein would normally function. Additionally, induction time was shown to have little effect on the solubility and yield of the protein (Fig. 4C and D).
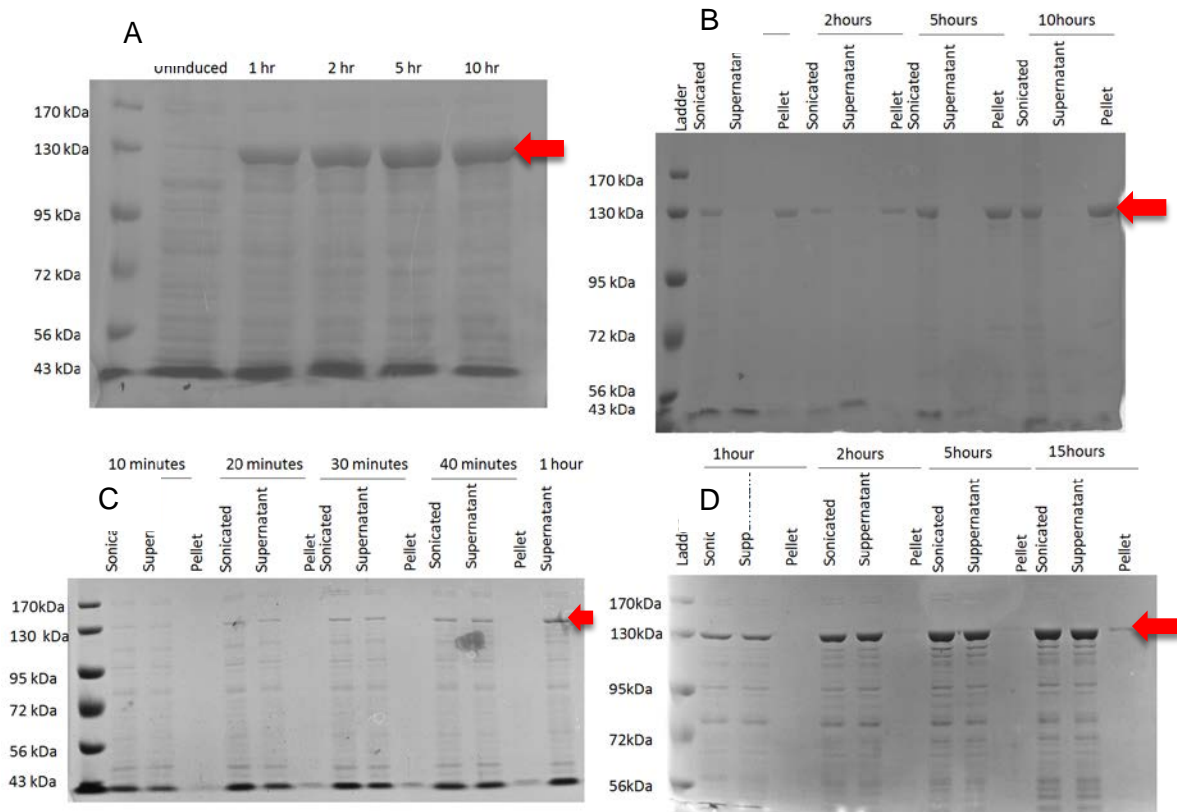

Figure 4. Transformed BL21 cells are shown to induce successfully and GST::S25-32 fusion protein is soluble when induced at room temperature. (A) Induction is shown to be successful with a band weight of 120 kDa and high concentrations of protein after only 1 hour. (B) Induction at 37°C reveals that the fusion protein is insoluble and remained in the pellet. (C) Induction at short time and room temperature reveals that the protein is soluble and remained in the supernatant. (D) Induction at room temperature for long periods of time reveals that the protein is nearly completely soluble and will remain in the supernatant as long as induction takes place at room temperature.

After a large-scale induction was performed, I extracted the fusion protein from the induced bacteria sample and isolated it using glutathione agarose beads, as described in the methods section. Some loss of protein was shown in the pellet and flow-through samples, but

despite these losses a significant amount of purified protein was eluted from the column (Fig. 5A). Following the successful purification, I went on to the cleavage and subtraction of GST from the S25-32. The subtraction was shown to be effective as the gel exposes no GST band at 25 kDa in the second flow-through sample.
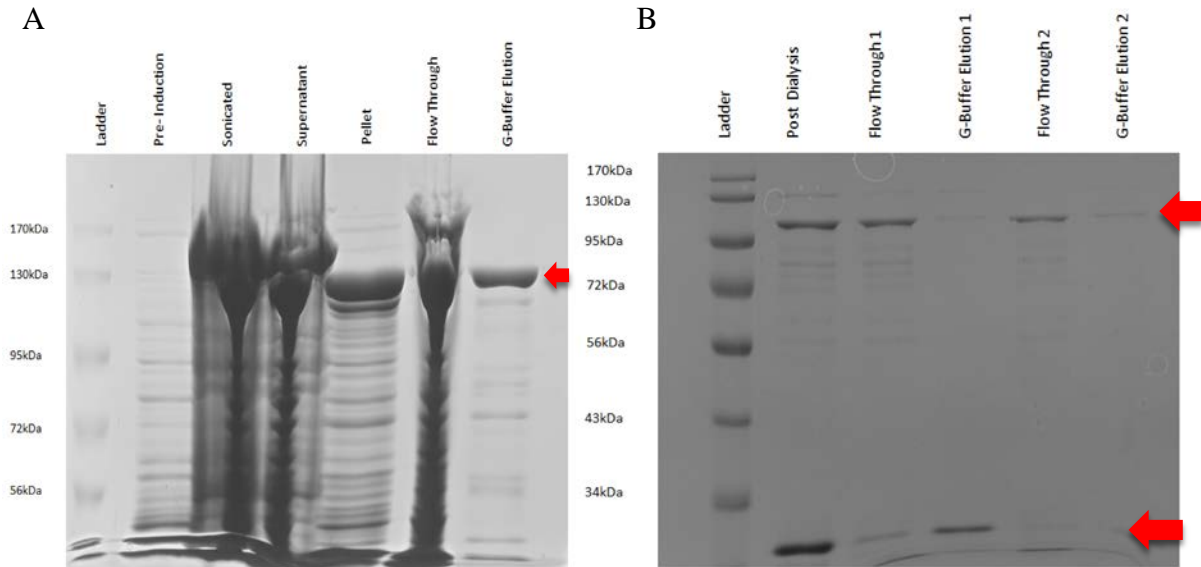

Figure 5. Protein purification and thrombin cleavage was shown to be successful. (A) Purification of GST S25-32 fusion protein was shown to be successful with some loss of protein in the flow-through and pellet. (B) Gel reports that thrombin cleavage and isolation of S25-32 from GST was successful after two flow-through collections.

In order to look for self-interaction, I applied the western blot overlay technique (Papal *et al*., 2013) in which GST subtracted S25-32 was embedded in a nitrocellulose membrane and overlayed with GST::S25-32. The membranes were then incubated with primary and secondary antibodies to target the overlayed GST protein followed by imaging of the blot using HRP chemiluminescent detection of secondary antibody. Final imaging of a spaghetti western revealed that interaction did occur as the appearance of bands indicates that GST::S25-32 fusion protein bound to the GST subtracted S25-32 on the blot (Fig. 6A). It should be noted that the top band which appears in the spaghetti western is the result of GST::S25-32 contaminant in the GST subtracted sample of S25-32. The control strip with 0 fusion protein overlay reveals antibody targeting despite the expected absence of GST. The upper band also appears at the expected 120 kDa weight of GST::S25-32.
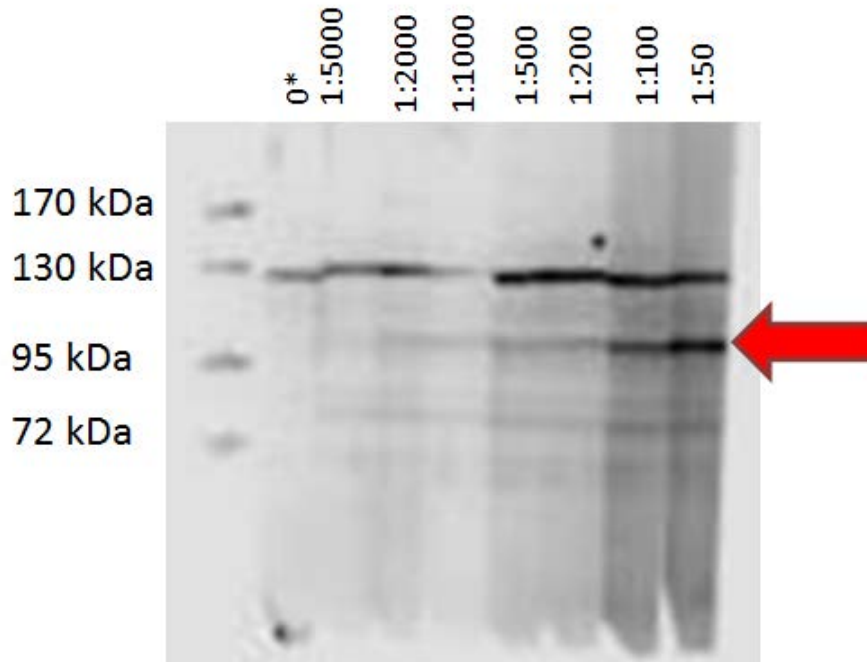
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Figure 6. Spaghetti Western Overlay of GST Subtracted β$_H$ S25-32 with β$_H$ GST::S25-32. (A) Blot reveals that GST::S25-32 fusion protein binds with GST subtracted S25-32 near predicted band weight of 95 kDa. Band intensity is shown to increase with increasing concentration of overlay.

Discussion

The *in vitro* western results indicate that self-interaction does occur within the region of terminal repeats of *Drosophila* β$_H$ spectrin (Fig. 6A). The appearance of a band in the blot indicates that the antibodies had targeted GST::S25-32 which was localized at 95 kDa due to a specific binding interaction with GST subtracted S25-32. This is in line with the results shown by Samantha Papal *et al*. (2013) in human βV. This finding could give a possible explanation as to how spectrin may have been able to form a network in R22S mutants and led to normal developmental pathways (Khanna *et al*., 2014). It is plausible that if network formation is still occurring in the absence of α/β dimerization, spectrin could function normally and still be able to cross link F-actin since the F-actin binding domain is located on the β subunits (Brenner and Korn, 1979; Karinch *et al*., 1990). If further supported, this new finding would significantly emphasize the importance of the β subunit for proper functionality of spectrin. The characteristic β$_H$ and βV independent network formation would support findings in vertebrates in which β-spectrin is reported to function in neurons and epithelial cells independent of α-spectrin (Papal *et al*., 2013; Dubreuill *et al*., 2000). If β$_H$ self-interaction is found to be conserved in both vertebrates and invertebrate species, further studies should attempt to identify examples of β$_H$ independent function in *Drosophila*.

Further studies are required in order to properly confirm and characterize the interaction occurring between β$_H$ subunits. If the interaction involves specific binding domains, an additional spaghetti western protocol should use higher concentration overlays of fusion protein in order to identify a point of saturation in which band intensity plateaus. Showing a point of saturation will

reveal the specificity of the self-interaction and whether it occurs through binding domains. In addition to *in vitro* western blotting evidence, additional protocols could also confirm self-interaction. One protocol which could potentially identify interaction is the use of chemical cross linking in which specific amino acid side chains, in close proximity of one another due to protein interaction, are covalently linked together (Life Technologies). Immunoprecipitation is another potential method in which anti-GST antibodies would be used to precipitate GST::S25-32 and anything binding it out of solution. In order to propose a specific model of where and how the binding occurs between two β_H chains, additional variants of β_H fusion proteins will need to be cloned, such as GST::S28-32 or GST::S30-32. In understanding the occurrence of self-interaction, the scientific community can develop better models as to how spectrin may form networks as well as explanations for observed α independent functions of β-spectrin.

References

1. An, X., Gauthier, E., Zhang, X., Guo, X., Anstee, D. J., Mohandas, N., and Chasis, J. A. (2008) Adhesive activity of Lu glycoproteins is regulated by interaction with spectrin. *Blood*. **112**, 5212–5218

2. Bennett, V., and Baines, A. J. (2001) Spectrin and Ankyrin-Based Pathways: Metazoan Inventions for Integrating Cells Into Tissues. *Physiological Reviews*. **81**, 1353–1392

3. Bennett, V., and Healy, J. (2008) Organizing the fluid membrane bilayer: diseases linked to spectrin and ankyrin. *Trends in Molecular Medicine*. **14**, 28–36

4. Brenner, S., and Korn, E. (1978) Spectrin-Actin Interaction. *Journal of Biological Chemistry*. **254**, 8620–8627

5. Crosslinking Protein Interaction Analysis *life technologies*. [online] https://www.lifetechnologies.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/crosslinking-protein-interaction-analysis.html (Accessed 30 July 2015)

6. De Matteis, M. A., and Morrow, J. S. (2000) Spectrin tethers and mesh in the biosynthetic pathway. *Journal of Cell Science*. **113**, 2331–2343

7. Dubreuil, R. R. (2000) Drosophila beta Spectrin Functions Independently of alpha Spectrin to Polarize the Na,K ATPase in Epithelial Cells. *The Journal of Cell Biology*. **149**, 647–656

8. Karinch, A., Zimmer, W., and Goodman, S. (1990) The Identification and Sequence of the Actin-binding Domain of Human Red Blood Cell β-Spectrin. *Journal of Biological Chemistry*. **265**, 11833–11840

9. Khanna, M. R., Mattie, F. J., Browder, K. C., Radyk, M. D., Crilly, S. E., Bakerink, K. J., Harper, S. L., Speicher, D. W., and Thomas, G. H. (2014) Spectrin Tetramer Formation Is Not Required for Viable Development in Drosophila. *Journal of Biological Chemistry*. **290**, 706–715

10. Marchesi, V. T., and Steers, E. (1968) Selective Solubilization of a Protein Component of the Red Cell Membrane. *Science*. **159**, 203–204

11. Papal, S., Cortese, M., Legendre, K., Sorusch, N., Dragavon, J., Sahly, I., Shorte, S., Wolfrum, U., Petit, C., and El-Amraoui, A. (2013) The giant spectrin V couples the molecular motors to phototransduction and Usher syndrome type I proteins along their trafficking route. *Human Molecular Genetics*. **22**, 3773–3788

12. Zarnescu, D. C., and Thomas, G. H. (1999) Apical Spectrin Is Essential for Epithelial Morphogenesis but Not Apicobasal Polarity in Drosophila. *The Journal of Cell Biology*. **146**, 1075–1086