Identifying the interaction between Calcineurin and Nuclear Factor of Activated T Cells (NFAT)

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Section 102, October 21, 2019

Abstract

The protein NFAT (nuclear factor of activated T cells) is a transcription factor that is crucial for developmental processes and T lymphocyte signaling (Luo et. al 1996, Wesselborg et. al. 1996). It has been observed that calcineurin plays a role in NFAT partitioning between the cytoplasm and nucleus. When calcineurin is activated, it acts as a phosphatase that promotes the translocation of NFAT into the nucleus (Crabtree and Schrieber 2009). The present study tries to uncover a specific sequence to which calcineurin binds and interacts with NFAT. This is performed via a binding assay using truncated NFAT sequences and visualized by immunoblotting. Results suggest that the binding region is localized in either the 118-228 amino acid region or 368-414 amino acid region.

Introduction

NFAT (nuclear factor of activated T cells) is a transcription factor that plays a critical role in cytokine gene transcription and other developmental processes (Luo et. al. 1996). NFAT is translocated into the nucleus with the aid of calcineurin, which is activated by an intracellular pathway that increases Ca^{2+} concentration. The Ca^{2+}-calcineurin-NFAT signaling transmits signals to the nucleus and is required for developmental events such as axon growth, cardiac morphogenesis, neural crest diversification, and more. NFAT signaling malfunction is associated with abnormalities such as Down's syndrome, diabetes, and cardiac hypertrophy (Crabtree and Schreiber 2009). In response to increasing Ca^{2+} concentration, calcineurin phosphatase is activated by calmodulin, which promotes NFAT translocation into the nucleus by removal of phosphate groups. This uncovers a nuclear localization sequence that allows NFAT to be recognized by nuclear pore complexes that mediate entry into the nucleus (Crabtree and Schreiber 2009, Park, Uesugi, and Verdine 2000). In the same process, calcineurin also blocks a nuclear export signal, which further promotes nuclear entry. Calcineurin is inhibited by immunosuppressive drugs such as cyclosporin A and FK506, which prevent dephosphorylation of NFAT and as a result its ability to translocate into the nucleus (Wesselborg et. al. 1996). Thus, the interaction between calcineurin and NFAT must be critical in T lymphocyte signaling, however a deeper understanding of NFAT-calcineurin binding is necessary. Previous work has shown that calcineurin interacts with the first 415 amino acids of NFAT and its dependence on calcium presence (Luo et. al. 1996). Further studies determined the presence of calcineurin subunits that interact with NFAT, denoted A and B. The present study attempts to determine the region within the NFAT regulatory domain that is required for interaction with calcineurin. This is done through a binding assay with truncated NFAT sequences.

Materials and Methods

To determine the binding domain of calcineurin on the NFAT protein, a binding assay was performed after cloning of the NFAT gene. To begin, PCR of different NFAT truncations (**Fig. 1**)

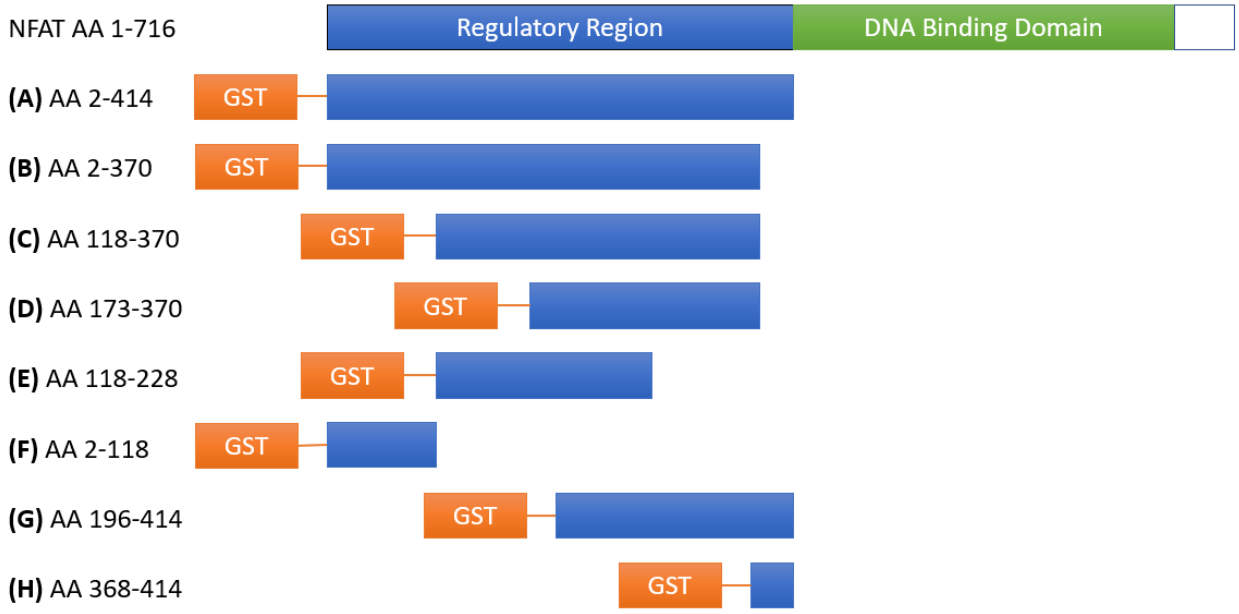

Figure 1: Schematic Diagram of the GST-NFAT fusion proteins cloned and expressed. GST-NFAT-A represented the entirety of the regulatory NFAT region. GST-NFAT-G was not directly tested.

was performed then separated via gel electrophoresis. Following separation and purification of the amplified DNA, the truncated sequences were inserted into pGEX plasmid vectors and transformed into competent *E. Coli* cells. The pGEX plasmid contains a GST sequence immediately prior to the NFAT truncated sequence in order to form a GST-NFAT fusion protein for purification purposes (**Fig. 2**). After cloning of the NFAT gene into *E. Coli*, the NFAT truncations were expressed via lac operon activation with IPTG.

Purification of NFAT-calcineurin complex

The *E. Coli* lysate was run through an affinity column with glutathione-linked agarose beads (100µL of lysate), and the flow through was collected. The column was washed four times with

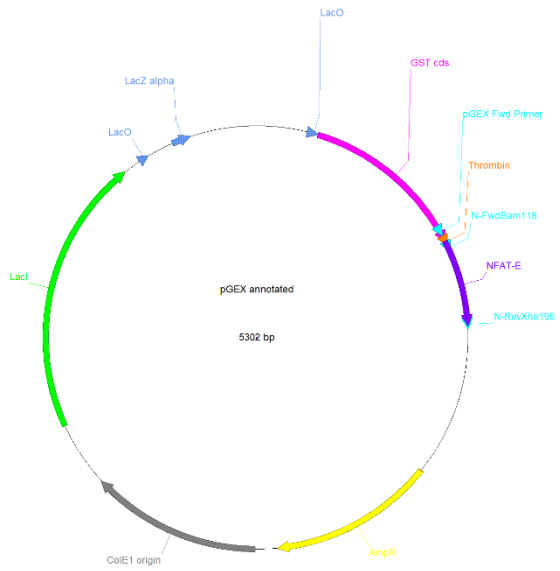

Figure 2: pGEX vector plasmid map. Key features include GST sequence preceding NFAT insert (this diagram illustrates NFAT-E). Thrombin as a GST-NFAT linker. Amp^r for positive selection screening, and Lac gene for expression promotion.

phosphate-buffered saline with 0.1% Triton X-100 (PBST). Protein concentration was assessed by adding 5µL of flow-through to 80µL of Bradford reagent and compared against a Bradford standard using known BSA concentrations. Columns were washed with 0.5mL Binding buffer (1.5mM Ca^{2+}) three times. The column was then incubated with 50µL of calcineurin in blocking solution (5% BSA, 1% Triton in Binding Buffer) at 4°C for 30 min, then washed four times with 0.5mL Binding Buffer. The proteins were then eluted using 50µL of 0.1M glutathione. Saved fractions of the lysate, flow-through, and peak elution (20µL each and 10µL 2X SDS sample buffer) were analyzed using SDS-PAGE through a 12% polyacrylamide running gel and 4% polyacrylamide stacking gel. The gels were run for 1.5 hours (120V until proteins reached stacking gel, then 200V through running gel) and incubated in Coomassie blue stain for 30 minutes.

Visualization of NFAT-calcineurin binding

The elution fractions were analyzed separately using SDS-PAGE (for Western Blot) against a calcineurin control (10µL loaded of each sample). After electrophoresis of the gel, a Western sandwich was assembled, and proteins were transferred onto a nitrocellulose membrane at 50V for 1.5 hours. The nitrocellulose membranes were then stained using Ponceau S, then washed with 10mL of PBST. The membranes were then incubated in 5 mL of proprietary blocking solution

mixture for 5 minutes, followed by 1 hour in mouse anti-calcineurin antibody in 5% BSA in PBST. The membranes were then washed three times in 10mL PBST (5 min wash), then incubated for 30 min in 5mL of goat anti-mouse immunoglobin conjugated to horseradish peroxidase (HRP) diluted in 5% BSA in PBST. After washing, the membranes were then exposed to 5mL of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine to give blue chromogenic signal.

Results

Coomassie-stained polyacrylamide gels were illuminated with UV light and image-captured. The GST fractions (present on each gel) had a clear band at ~27 kDa corresponding to free GST. This is expected since GST fractions contained only GST and no NFAT protein. For GST-NFAT-X fractions, truncations that bound to calcineurin would contain multiple bands corresponding to NFAT-X, GST, and calcineurin. This was seen in fractions of NFAT-C, NFAT-D, and NFAT-F (**Fig. 3**). Depending on the size of the truncation, the NFAT band would materialize at different

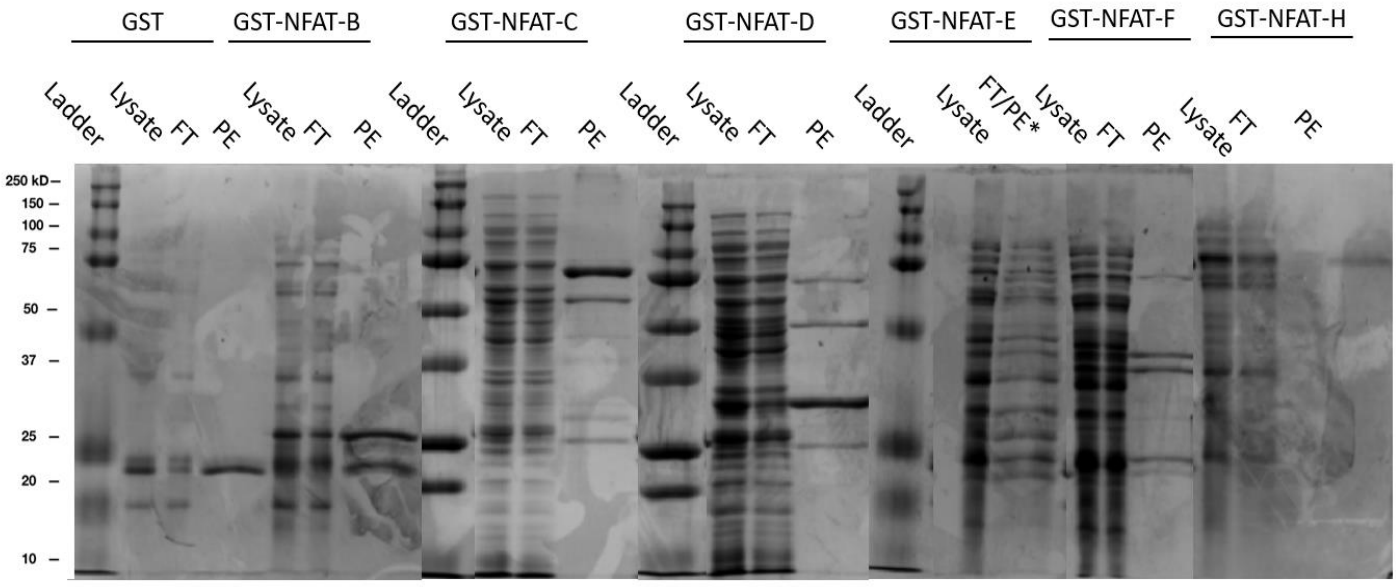

Figure 3: Coomassie-stained SDS-PAGE gels for GST-NFAT truncations. Lysate indicates unpurified *E. Coli* lysate with corresponding NFAT plasmid (2µL). FT indicates flow-through collected after running through column containing glutathione-linked agarose beads (2µL). PE indicates fraction collected after glutathione-elution that contained the most protein product as determined via Bradford assay (10µL). Note marked GST-NFAT-E lane indicating unintentional mixing of flow-through and peak elution.

positions based on the size of the specific truncation. During loading of the SDS-PAGE gel, GST-NFAT-E peak-elution fraction was loaded over GST-NFAT-E flow through. The resulting lane does not provide enough resolution to elucidate a clear result. After Coomassie staining, a Western blot was performed on the NFAT-X truncations against GST-NFAT-A control (which represents the length of the NFAT regulatory region). Under Ponceau staining (red stain), the proteins are clearly visualized and compared to the immunoblotted membrane. The Western blot detected the presence of calcineurin, with NFAT-A as the NFAT positive control, calcineurin (Cn) as an antibody positive control and GST as a negative control. The presence of bands in NFAT-A, NFAT-X, and Cn lanes indicate a possible binding region for calcineurin. This is seen in the lanes for NFAT-B, NFAT-C, NFAT-H (**Fig. 4a, b, f**).

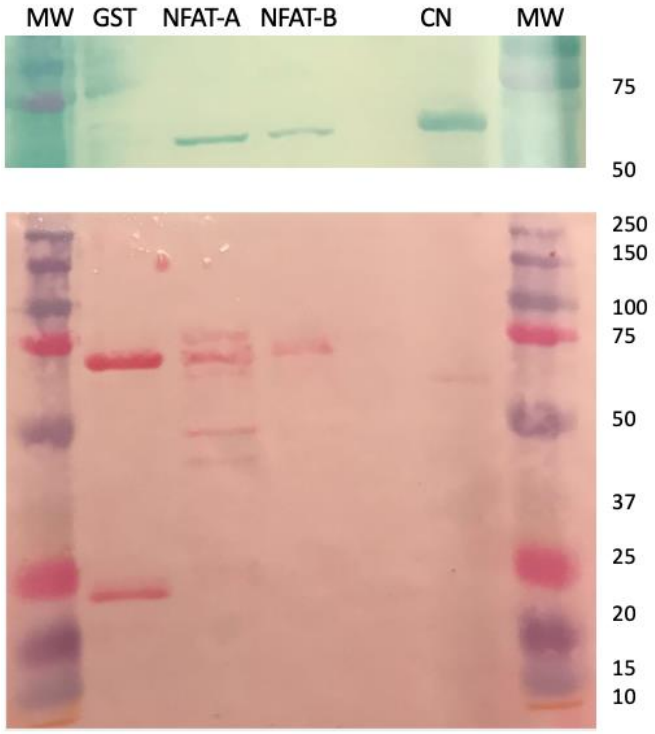

Figure 4a: GST-NFAT-B Note faint presence of Cn in Ponceau S stained membrane, but presence in immunoblotted membrane. Clear band in NFAT-B lane.

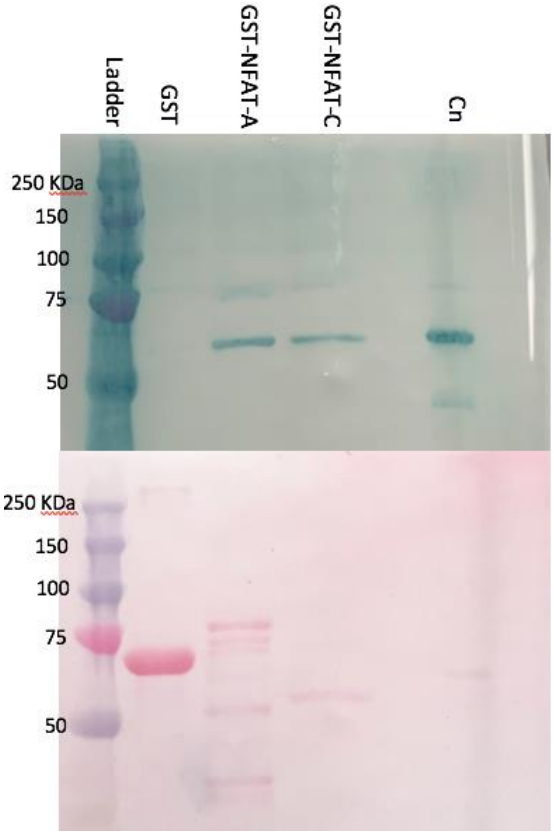

Figure 4b: GST-NFAT-C Note faint presence of Cn in Ponceau S stained membrane, but clear presence in immunoblotted membrane. Clear band in NFAT-C lane.

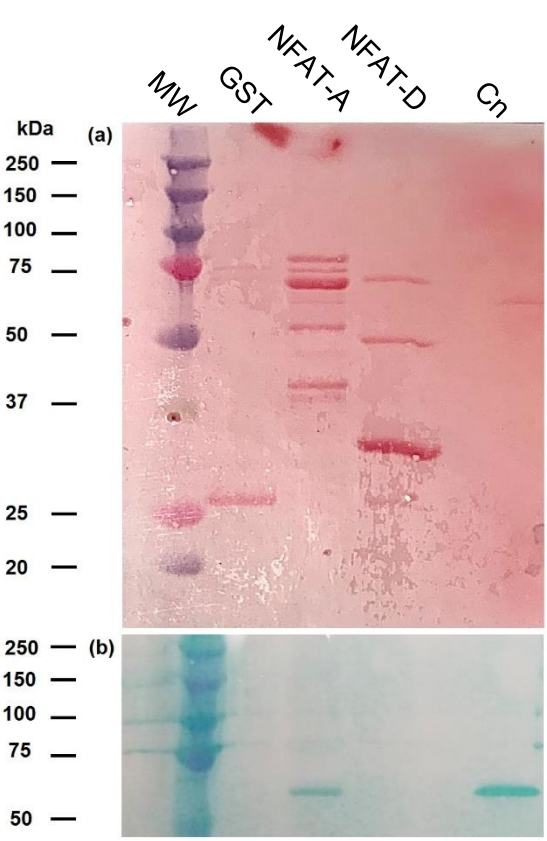

Figure 4c: GST-NFAT-D No calcineurin detected in immunoblot (bottom)

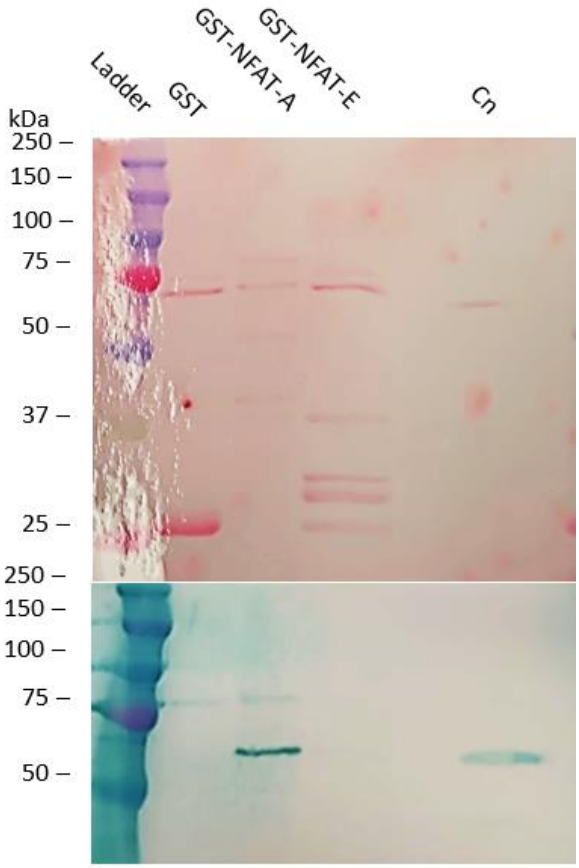

Figure 4d: GST-NFAT-E No calcineurin detected in immunoblot (bottom)

Calcineurin positive control was faint under Ponceau stain for most membranes but visible at ~60-65kDa. The membrane for NFAT-C Ponceau stain had an extremely faint calcineurin positive control but is visible after immunoblotting. In addition, the membrane for NFAT-H had multiple extraneous bands that were visible in all lanes. This could possibly be due to too much primary antibody being added or insufficient washing.

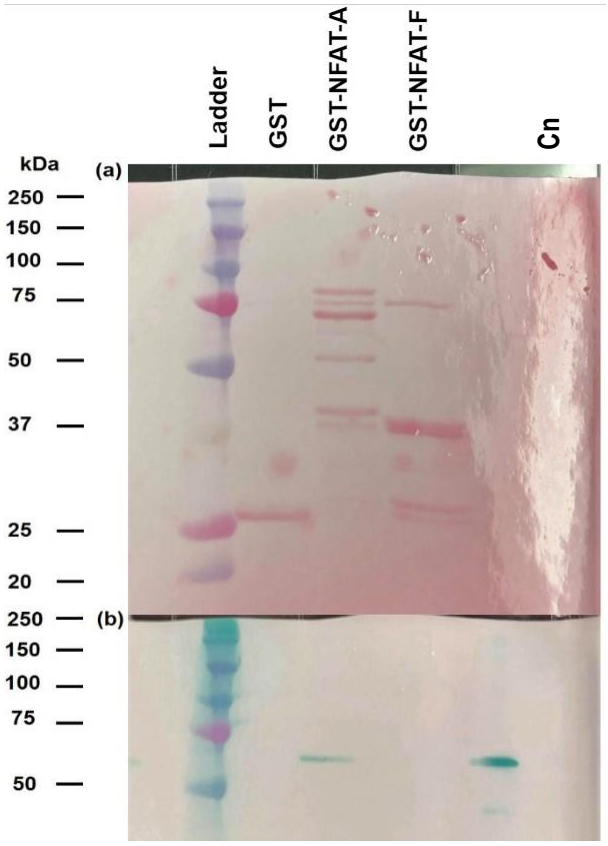

Figure 4e: GST-NFAT-F No calcineurin detected in immunoblot (bottom)

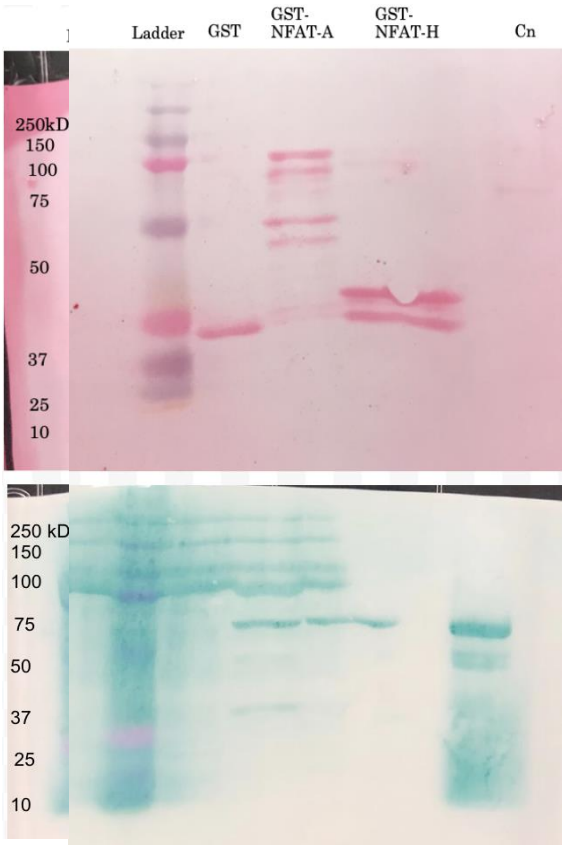

Figure 4f: GST-NFAT-H Note presence of multiple bands in immunoblot (bottom) not visible in Ponceau S stain (top)

Discussion

The intracellular division of NFAT between the cytoplasm and nucleus is controlled by phosphorylation of the regulatory domain on the N-terminal of NFAT. It is known that the phosphatase calcineurin promotes the translocation of cytoplasmic NFAT into the nucleus by exposing nuclear localization signals and masking nuclear export signals on the NFAT protein. Medical motivation for understanding the binding of calcineurin and NFAT stems from the importance of NFAT signaling in T lymphocytes. This analysis presents evidence for the specific region within the NFAT regulatory domain that calcineurin binds. This was accomplished by expressing truncated NFAT proteins and performing a binding assay with calcineurin. NFAT truncations were expressed via transformation of *E. Coli* with a pGEX plasmid vector containing

GST-thrombin-NFAT sequence. The GST-NFAT fusion was purified via affinity chromatography to glutathione-linked agarose beads. Before eluting the protein product, soluble calcineurin was added to the column. NFAT truncations that contained the necessary calcineurin-binding sequence would bind the calcineurin and elute as a GST-NFAT-calcineurin complex.

Coomassie Assay

The eluted fractions were first analyzed via Coomassie staining. A GST-NFAT-calcineurin complex would appear as multiple bands on an SDS-PAGE gel, indicating a possible calcineurin binding. Specifically, a clear band in ~60kDa region would indicate the presence of calcineurin. Though GST-NFAT-C, D, and F have multiple bands that could correspond to calcineurin, NFAT, and GST respectively, these results are contradictory due to the lack of overlap between NFAT-D and NFAT-F regions. GST-NFAT-C has a clear band in the 60kDa region that likely corresponds to calcineurin, especially since NFAT-C is approximately the same size as GST (~27kDa). GST-NFAT-D has a faint band at ~60kDa, however more peculiarly is the presence of a strong band in the ~30kDa region which is larger than the size of the NFAT-D truncation (~21kDa). A possibility is a mobile shift as a result of GST failing to be cleaved and separated from the NFAT protein during digestion. Though faint, based on Coomassie alone GST-NFAT-H could have a possible calcineurin interaction as indicated by a faint band in the ~60kDa region. During the assay of GST-NFAT-E, results were unclear due to the accidental combination of flow-through and peak elution into the same lane. As a result, the SDS-PAGE was uninterpretable due to the large presence of unknown and nonspecific proteins.

Western Blot

To further confirm the calcineurin-NFAT binding, a Western blot was performed using an anti-calcineurin primary antibody. In this assay, the presence of a band would indirectly confirm the

presence of calcineurin. This band was observed in GST-NFAT-B, C, and H. With GST-NFAT-C being a candidate in both assays, it is likely that the calcineurin binding region exists between amino acids 118-370. However, the evidence also suggests that GST-NFAT-H contains a binding region, which represents the last 46 amino acids of the domain, amino acids 368-414. It is possible that methodological errors could have produced a false positive, in which case the binding region is isolated within the GST-NFAT-C region. Given that the calcineurin band in the Coomassie stain was extremely light, it is possible that this could have been residual calcineurin leftover from previous washes. In addition, the Ponceau S stain of the nitrocellulose membrane didn't have a clear band in the calcineurin region that could predict the presence during immunoblotting. Lastly, the visible extraneous bands in the immunoblotted membrane suggests either too much antibody added or insufficient washing of the membrane. These could both make it likely to reveal a false positive. However, besides methodological error the NFAT-H region could contain a region that is *sufficient* for calcineurin binding. This would indicate that the full calcineurin binding region possibly exists between amino acids 118 and 414. Although the Coomassie stain did not provide much results, the Western blot for GST-NFAT-E showed clearly a lack of calcineurin. This is helpful since the entire NFAT-E region overlaps with the N-terminal region of NFAT-C. This suggests that the calcineurin-binding region in NFAT is localized between amino acids 228 and 414.

Analysis

Although this is a reasonable conclusion, a few contradictions arise in the results. If the calcineurin-binding region of NFAT is contained between amino acids 228-414, then it is expected that GST-NFAT-D also show evidence of calcineurin presence. However, this is not the case as demonstrated by the Western blot in **Figure 4c**. Coupled with the lack of calcineurin binding in

NFAT-E, it shouldn't be possible for NFAT-C to give a positive calcineurin-binding result. A possible explanation could be methodological error that led to false negatives for either NFAT-E or NFAT-D. If NFAT-E was a false negative due to methodological error then it's reasonable to conclude that the calcineurin binding region is between amino acids 118-228, and would align with the rest of the data assuming NFAT-H is a false positive. Alternatively, if NFAT-D were a false negative due to methodological error, then the calcineurin binding region is likely localized between amino acids 228-414. Given that methodological error occurred with GST-NFAT-E Coomassie assay, it is not unlikely that a methodological error occurred during Western blotting.

Interaction between Calcineurin and NFAT

With the data as is, it is possible to speculate that there are two binding regions for calcineurin, and that NFAT-C contains both or part of both. If calcineurin had the ability to bind the amino acid region from 368-414 sufficiently, but also bind a region from 118-173, it would be possible to obtain the data as given. This would mean that NFAT-H could bind since it's region is sufficient, but NFAT-E could possibly need the binding of the more upstream region in order to bind. NFAT-C would be able to bind both, as well as NFAT-B and NFAT-A. Given the current literature and studies on calcineurin and it's interaction with NFAT, it is unlikely that this is the case. Another possible explanation for the current data could be the result of prokaryotic expression of a eukaryotic gene, making it likely that post-transcriptional modifications of the NFAT sequence could yield a different protein sequence then the one obtained in this experiement. It could be possible that the *in vivo* NFAT calcineurin binding site might be located at the junction of two exons. As a result, this experiment would be limited since calcineurin might have bound to a non-specific binding region with similar binding motif. It is also possible that alternatively spliced NFAT proteins have varying calcineurin binding regions. Future work could attempt to separate

the binding assay from the purification, and expose calcineurin to NFAT only after GST-NFAT has been purified. Furthermore, expressing more truncations of NFAT could help narrow down the specific binding regions.

References

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