

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**A STRUCTURAL AND FUNCTIONAL INVESTIGATION INTO THE
FILAMIN A G2593E MUTATION: IMPLICATIONS FOR
NEUROLOGICAL DISEASE**

Submitted in partial fulfillment of a Master of Philosophy (Science) (Biochemistry) at the Institute of
Molecular BioSciences, Massey University, Pamerston North

MANICKAVASAGAR ANANDASAYANAN

2012

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Andrew Sutherland-Smith at the Center for Structural Biology, Institute of Molecular BioSciences, Massey University for his advice and encouragement throughout the course of this study and in the preparation of the thesis.

I thank Professor Stephen Robertson (University of Otago) for the kind gift of *filamin A* cDNA clone.

I acknowledge the generosity of my employers HortResearch Ltd, New Zealand for providing the necessary funds to conduct my research.

ABSTRACT

The dimeric F-actin cross-linking protein human filamin a (hsFLNa) is an actin binding protein, which modulates the properties of the actin cytoskeleton and plays a major role in maintaining the integrity of plasma membrane associated actin, the viscoelastic properties of the cytoplasm, endocytosis, cytoplasmic streaming, cell division and cell motility [McGough *et al*, 1998; Small *et al*, 2002; Popowicz *et al*, 2006]. Dimerization is crucial for actin cross-linking functions of filamins [Davies *et al*, 1980] and the most C-terminal repeat of hsFLNa (hsFLNa24) is sufficient for hsFLNa dimerization [Himmel *et al*, 2003].

Several mutations in *hsflnA* are associated with pathologies such as periventricular nodular heterotopia (PVNH) [Robertson, 2005]. In this study we examined the possible cause and effect of a G2593E mutation in hsFLNa24 in a male patient diagnosed with PVNH on protein functionality. This was done by comparing relevant biochemical properties of wildtype and mutant hsFLNa24 proteins. For this purpose, recombinant proteins were expressed from cloned *hsflnA24* coding sequence.

Full length wildtype *hsflnA24* (wt *hsflnA24*) was amplified from a cDNA library prepared from the PVNH patient. wt *hsflnA24* was used as the template to generate mutant *hsflnA24* (mt *hsflnA24*) by site directed mutagenesis. The amplified wildtype and mutant sequences were cloned and over-expressed in an *Eschericia coli* system. hsFLNa24 proteins were isolated from crude protein extracts by immobilized metal affinity chromatography, purified by gel filtration and concentrated.

Several features of the mutant protein indicate that it has a high-entropy, disordered structure. Thermal stability of the two proteins determined by melt curve analyses showed that mt hsFLNa24 is less stable than wt hsFLNa24, their respective melting temperatures being < 303 K and 317 K, respectively. The mutant protein also tended to aggregate during concentration and was prone to precipitation during low speed centrifugation.

The two proteins displayed different elution volumes in size exclusion chromatography; wt hsFLNa24 eluted at a volume characteristic of a dimer indicating that in its native

form wt hsFLNa24 exists as a dimer, while the mutant's elution volume suggests that in most probabilities it exists as a monomer with a slightly larger molecular size. The oligomerisation status of the proteins in solution was further confirmed in crosslinking assays using the chemical crosslinker ethylene glycol succinimido succinate ester (EGS). In assays where the proteins at a final concentration of 4 μ M were reacted with 1.3 mM EGS, the major form of wt FLNa24 was shown to be a high molecular weight dimeric species, while dimerisation was inhibited in mt hsFLNa24 and the protein exists predominantly in a monomeric state.

Both proteins were subject to various crystallizing conditions. Only wt hsFLNa24 produced diffracting crystals which have immunoglobulin (Ig)-like folds of the E-set superfamily [Murzin *et al*, 1995] with a predominantly β -sheet structure, where seven β -strands organized as two anti-parallel β -sheets of four (ABED) and three (CFG) strands, respectively, are arranged as a β -sandwich. The asymmetric unit consists of a dimer.

The dimer interface is formed by β -strands C and D of the monomers and the G2593 residue occurs in β -strand-C; it resides within the hydrophobic core of the dimer interface, where it is involved in a putative hydrophobic stacking. This glycine residue is highly conserved in most vertebrate filamins. Ablation of the G residue in conjunction with substitution by a polar residue is predicted to play a major role in disrupting the hydrophobic nature of the interface thereby inhibiting dimerisation. The inhibition effect arose probably more through unfavourable entropy change induced by substitution of the native G by an E residue than a reorganization of the dimer interface.

The actin cross-linking ability of filamins is ascribed to its dimerization mediated by the C-terminal repeats and the loss of the latter function may have serious implications to the patient harbouring the G2593E mutation. The relatively mild symptoms exhibited by the patient leads one to believe that there may be compensatory mechanisms in operation or that the basic premise that the most C-terminal repeat is important for filamin dimerisation is questionable.

CONTENTS

Acknowledgements	i
Abstract	ii
Abbreviations	iv
Contents	vi
List of figures	ix
<u>Chapter I - Introduction</u>	
1.1. Actin cross-linking proteins	1
1.2. Filamin structure	2
1.3. Filamin genes	5
1.4. Filamin proteins	7
1.4.1. Human filamin isoforms	7
1.4.2. Filamin orthologs	8
1.5. Human <i>fln</i> expression	11
1.6. Physiological functions of human filamins	13
1.6.1. Ligand binding	14
1.6.2. Receptor binding	15
1.6.3. Nuclear transport and transcription regulation	17
1.7. Mutations and pathological conditions	17
1.8. Dimerization	19
1.8.1. Structural basis of ddfln dimerization	19
1.8.2. Structural basis of hsfln dimerization	21
1.9. Aim of study	23
<u>Chapter II – Materials and methods</u>	
2.1. Primers for hsFLNa repeat domain 24 amplification	24

2.2. Amplification of wt hsFLNa24 insert	24
2.3. Cloning vector preparation	25
2.4. Cloning of wt hsFLNa24 fragments	25
2.5. Transformation	25
2.6. Site directed mutagenesis and production of mutant mt FLNa24 clones	26
2.7. hsFLNa protein expression	26
2.8. Protein purification and immobilized metal ion affinity chromatography	26
2.9. wt- and mt-hsFLNa protein isolation	27
2.10. Ethylene glycol bis(succinimido succinate) crosslinking assay	29
2.11. Western blotting	29
2.12. Protein melting temperature T_m	30
2.13. Protein crystallization and data collection	30
2.14. Protein modelling	31
<u>Chapter III – Results</u>	
3.1. Vector construction	34
3.1.1. pWt-flna24 vector preparation	34
3.1.2. pWt-flna24 vector validation	35
3.1.3. pMt-flna24 vector preparation	36
3.2. Conditions for scaled-up protein expression	37
3.3. Affinity chromatography	38
3.4. Redesigned vectors	40
3.5. hsflna protein isolation	40
3.5.1. Twin bands	41
3.6. Size exclusion chromatography (sec)	43
3.7. Crosslinking	45
3.8. Thermodynamic stability	46

3.9. X-ray crystallography	47
3.9.1. Crystallization	47
3.9.2. Protein modelling and crystal structure	48
 <u>Chapter IV – Discussion and conclusion</u>	
4.1. hsflna molecular architecture in the dimerization region	57
4.2. Determinants of vertebrate filamin repeat domain interaction	58
4.3. The g2593e missense mutation	59
4.4. Role of G2593E mutation in PVNH	61
4.5. Conclusions	62
<u>BIBLIOGRAPHY</u>	64

List of figures

Figure 1: Schematic of the human filamin A (hsFLNa) dimer	2
Figure 2: The immunoglobulin fold	4
Figure 3: Phylogenetic tree of CH domains	8
Figure 4 Alignment of hsFLNa24 and FR sequences of 10 species	9
Figure 5. The multifunctional nature of filamins	13
Figure 6: Filamin A binding partners and their location	16
Figure 7: Schematic of filamin A scaffolding	16
Figure 8: <i>hsflnA</i> mutations identified in various human genetic disorders	18
Figure 9: Topology of ddFLN rod domain 6	20
Figure 10: Dimerization of ddFLN rod domain 6	20
Figure 11: Crystallographic Dimer of Filamin C	22
Figure 11: MCS of pProEX HTb	24
FIGURE 12: Agarose gel image of PCR	34
Figure 13: Agarose gel image of plasmid digests	35
Figure 14: SDS-PAGE gel image of cell extracts	36
Figure 15: SDS-PAGE gel image of cell extracts at different temperatures	37
Figure 16: SDS-PAGE gel image of IMAC eluate fractions	39
Figure 17: A 12% SDS-PAGE gel of cell lysate and IMAC eluates	40
Figure 18: SDS-PAGE of fractions eluted at 500 mM imidazole	41
Figure 19: (A) Western blot (B) Duplicate SDS-PAGE of gel	42
Figure 20: Elution profiles of proteins after SEC	44
Figure 21. Crosslinking of hsFLNa24 proteins by EGS	45
Figure 22: Sequence alignment of FLN protein sequences	49
Figure 23: Ramachandran plot of hsFLNa24 residues	49
Figure 24: Topology diagram of hsFLNa24 monomer	50
Table 1: Elements of hsFLNa24 secondary structure	51
Figure 25A: Hydropathy plot of FLNa24	52
Figure 25B: Cartoon of FLNa24 superimposed on FLNc	52
Figure 26: Cartoon of hsFLNa24 superimposed on ddFLN6	53
Figure 27: Crystallographic dimer of hsFLNa24	54
Figure 28: The C strands of dimer interface - stick-and-ball model	55
Figure 29: Multiple sequence alignment of hsFLN sequences	55

Figure 30: Antiparallel C (left) and D (right) strands of the dimer interface 56

Figure 31 Model of the orientation of filamin chains in relation to FR24 57