

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.

Expression and purification of CFM2 and Filamin A repeat 10 domain

A thesis presented in partial fulfilment of the requirements for the degree of Master of
Science in Biochemistry at Massey University, Manawatu, New Zealand.

Ben Waite

2012

Abstract

Filamins are a group of proteins that interact with over 60 other proteins. Mutations to the Filamin A gene results in a spectrum of disorders including Otoplatodigital spectrum disorder type 1, Otoplatodigital spectrum disorder type 2, Frontometaphyseal dysplasia, Melnick-Needles syndrome and Periventricular Nodular Heterotopia. All cases of Melnick-Needles syndrome can be accounted for by mutations in repeat 10. Using a yeast-2 hybrid assay Professor S.P Robertson identified the protein FAM101A (the protein is alternatively named CFM2) that associated with Filamin A repeat 10. CFM2 was found to interact with itself in a yeast-2-hybrid screen, suggesting homo-dimerisation properties in addition to Filamin A repeats 10 and 21 binding affinity. If CFM2 dimerises and binds to repeat 10 and 21 it is possible that Filamin A's function will alter, thus altering the properties of the cytoskeleton. To investigate the interaction between Filamin A repeat 10 and CFM2, each was subcloned into an *E.coli* plasmid vector fused to a purification tag. Purification of CFM2 failed due to misfolding, this upholds later work that claims CFM2 cannot fold correctly without the presence of vertebrate Filamin. Filamin A repeat 10 purification went well but the fusion was unable to be concentrated without precipitating out of solution. Also the GST purification tag could not be cleaved without secondary cleavage products forming. Pull-down of C2C12 mouse fibroblast cell lysate using the GST-Filamin A repeat 10 fusion as the probe did not identify any other proteins that bind Filamin A repeat 10.

Acknowledgements

I would like to thank my supervisor Dr Andrew Sutherland-Smith for his support and guidance throughout my research.

A big thank-you to the post-graduates of the Institute of Molecular Biosciences, with special thanks to the post-graduates of Dr Andrew Sutherland-Smith and Dr Gill Norris. Being able to get help and share the success and failures of our respective projects makes the failures more bearable and the successes sweeter.

Thanks to my family. Your love and support has been a great motivator and enabler for me.

Abbreviations

AMP	ampicillin
LB	Luria broth
<i>E.coli</i>	<i>Escherichia coli</i>
MQ	milli-q
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
APS	ammonium persulfate
SDS	sodium dodecyl sulfate
DTT	dithiothreitol
DNase	deoxyribonuclease
cDNA	complementary deoxyribonucleic acid
TEMED	N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
BME	2-Mercaptoethanol
PPU	Precision plus protein unstained (Bio-rad)
FLNAR10	Filamin A repeat 10
IPTG	isopropyl β -D-1-thiogalactopyranoside
dNTP	deoxyribonucleotide triphosphate
MSC	mesenchymal stem cell
ABD	actin-binding domain
OPD1	Otoplatodigital spectrum disorder type 1
OPD2	Otoplatodigital spectrum disorder type 2
FMD	Frontometaphyseal dysplasia
MNS	Melnick-Needles syndrome
F-actin	filamentous actin
G-actin	globular actin
PVNH	Periventricular Nodular Heterotopia
EtBr	ethidium bromide
Ig	Immunoglobulin

Table of Contents

Abstract	i
Acknowledgements.....	ii
Abbreviations	iii
Table of Contents	iv
List of Tables.....	vii
List of Figures	ix
1 Introduction	1
1.1 Cytoskeleton.....	1
1.2 Intramembranous Ossification.....	2
1.3 Otoplatodigital syndrome spectrum disorders.....	2
1.4 Filamins.....	3
1.5 CFM2.....	7
1.6 Filamin A repeat 10 - CFM2 interaction hypothesis.....	9
2 Materials and Methods.....	11
2.1 Materials.....	11
2.1.1 DNA manipulation.....	11
2.1.2 Cell culturing	11
2.1.3 Protein manipulation	11
2.1.4 General chemicals and equipment	12
2.2 Methods	16
2.2.1 Competent cells	16
2.2.1.3 Competent Cell Transformation	16
2.2.2 Polymerase Chain Reaction	16
2.2.3 DNA analysis using ethidium bromide staining of agarose gel.....	17
2.2.4 DNA Sub-cloning	18

2.2.5 Ligation	19
2.2.6 Plasmid Isolation	19
2.2.7 DNA sequencing	19
2.2.8 Protein Expression	20
2.2.9 Cell Lysis	20
2.2.10 Protein size and mass analysis using SDS-PAGE	20
2.2.11 GST-fusion protein pull-down	21
2.2.12 Histidine-tag protein pull-down	22
2.2.13 GST fusion protein probe pull-down	23
3.1 Results and Discussion	24
3.1.1 Sub-cloning CFM2	24
3.1.1.1 Sub-cloning CFM2 PCR product into pProEX HTb	33
3.1.2 Expression of CFM2	48
3.1.3 Purification of CFM2	54
3.1.4 Sub-cloning FLNAR10	65
3.1.5 Expression of FLNAR10	80
3.1.6 Purification of FLNAR10	85
3.1.6.1 Preparation of Lysate	85
3.1.6.2 GST trap purification of FLNAR10	85
3.1.6.3 Thrombin digest of GST-FLNAR10	87
3.1.6.3 AKTA purification of GST-FLNAR10 thrombin digest products	93
3.1.7 Pull-down experiments with FLNAR10	98
4.1 Summary	103
4.1.1 CFM2	103
4.1.2 FLNAR10	103

4.1.3 FLNAR10 pull-down	104
4.1.4 Future work	105
5 References.....	106
6 Appendix	109

List of Tables

Table 1: Alignment of CFM2 isoforms.....	8
Table 2: Primers used in this project.....	13
Table 3: Plasmids used in this project.....	14
Table 4: <i>E.coli</i> strains used in this project.....	15
Table 5: PCR run cycle*	17
Table 6: Double digest of pProEX HTb and PCR product	33
Table 7: Ligation setup; pProEX HTb and HCFM2-non-truncated SF1.....	36
Table 8: Ligation setup; pProEX HTb and HCFM2-SF1-non-truncated with ratios	37
Table 9: Double digest mix for pProEX HTb+CFM2 plasmids #1 and #2.....	38
Table 10: Confirmation PCR of insert in plasmid mini prep #1 and #2.....	40
Table 11: Predicted digest size of pProEX HTb using NcoI and XhoI	47
Table 12: His-Trap Column loading washing and elution protocol.....	55
Table 13: His-Trap loading extended washing and elution setup.....	60
Table 14: Alignment of FLNAR10fragment with FLNAR10F3 and FLNAR10B2	72
Table 15: Double digest of pGEX 4T3 and PCR product FLNAR10	73
Table 16: Ligation of Double digested pGEX 4T3 vector and FLNAR10 insert.....	73
Table 17: Alignment of pGEX 4T3:FLNAR10 (Sequence 1) with pGEX 5` and pGEX 3` primers (Sequence 2)	77
Table 18: Alignment of pGEX 4T3:FLNAR10 (Sequence 1) with pGEX 5` and FLNAR10B2 primers (Sequence 2)	78
Table 19: GST-FLNAR10 predicted fusion protein sequence	82
Table 20: FASTA protein sequences of CFM2 isoforms	109
Table 21: FAM101A sequence in pGEMT vector	109

Table 22: Chromatogram of pProEX+CFM2 sequencing using HTRVS and m13REV primers	110
Table 23: Alignment of (Sequence 1) FAM101A mRNA with (Sequence 2) pProEX+CFM2 sequenced consensus	113
Table 24: Chromatogram of pGEX 4T3-FLNAR10 #5 and #6 plasmid sequencing using pGEX 5` primer	115
Table 25: Alignment of (Sequence 1) Sequencing of Colony #5 with (Sequence 2) pGEX-4T3:FLNAR10	118
Table 26: Alignment of (Sequence 1) Sequencing of Colony #6 with (Sequence 2) pGEX-4T3:FLNAR10	119

List of Figures

Figure 1: Structure of Filamin A repeat 10.....	4
Figure 2: Diagram of Filamin A dimer	6
Figure 3: Chou & Fasman algorithm prediction of Secondary structure of CFM2 isoforms.....	8
Figure 4: Diagram of proposed action of CFM2 on Filamin A.....	10
Figure 5: pGEM T Vector Map[34]	25
Figure 6: pProEX HTB Vector Map[35].....	26
Figure 7: Amplification products of pGEM-T: FAM101A-truncated and pGEM-T: FAM101A with HCFM2LF2 and HCFM2SF1 forward primers	28
Figure 8: Touchdown PCR amplification products of pGEM-T: FAM101A and pGEM-T: FAM101A-truncated with HCFM2LF2 and HCFM2SF1 forward primers	30
Figure 9: Touchdown PCR amplification products of pGEM-T: FAM101A and pGEM-T: FAM101A-truncated with HCFM2LF2, HCFM2LF3 and HCFM2SF1 forward primers.....	32
Figure 10: Double digest of pProEX HTb vector and PCR product (HCFM2-non-truncated SF1).....	34
Figure 11: Double digest of pProEX HTb+CFM2 plasmid mini preps.....	39
Figure 12: PCR amplification products of pProEX HTb+CFM2 #1 and #2 plasmids using HCFM2SF1 and HCFM2B1 primers.....	41
Figure 13: Colony PCR of pProEX HTb+CFM2 ligation product transformants using vector primers	43
Figure 14: Colony PCR of pProEX HTb+CFM2 ligation product transformants using insert primers	44
Figure 15: Colony PCR of pProEX HTb+CFM2 ligation product transformants using vector primers	46
Figure 16: Time expression trial of CFM2 short isoform non-truncated	49

Figure 17: Lysis buffer trial using overnight expression of CFM2 short isoform non-truncated.....	51
Figure 18: Lysis buffer trial 2 using overnight expression of CFM2 short isoform non-truncated.....	52
Figure 19: His-Trap purification of BL21 <i>E.coli</i> expressing CFM2 short isoform non-truncated.....	56
Figure 20: His Bead 1.5 mL Tube purification of BL21 <i>E.coli</i> expressing CFM2 short isoform non-truncated.....	58
Figure 21: His-Trap purification of BL21 <i>E.coli</i> expressing CFM2 short isoform non-truncated.....	61
Figure 22: Concentration and purification of Fraction 68 using 10 kDa cut-off concentrator	63
Figure 23: pREP4 Vector Map[36].....	65
Figure 24: pGEX 4T3 Vector Map[37]	66
Figure 25: Restriction map of FLNAR10F3 (5` to 3`) Showing restriction enzymes cutting maximum 1 time provided by Serial Cloner 1.3-11	68
Figure 26: Restriction map of FLNAR10F2 (5` to 3`) Showing restriction enzymes cutting maximum 1 time provided by Serial Cloner 1.3-11	68
Figure 27: Restriction map of FLNAR10B2 (5` to 3`) Showing restriction enzymes cutting maximum 1 time provided by Serial Cloner 1.3-11	68
Figure 28: Amplification products of pREP4: FLNA using repeat 10 domain primers....	69
Figure 29: Gradient PCR of pREP4: FLNA plasmid clones	71
Figure 30: Colony PCR of pGEX 4T3 + FLNAR10 ligate transformants using vector primer	75
Figure 31: Colony PCR of pGEX 4T3 + FLNAR10 ligate transformants using Vector and Insert primers.....	76
Figure 32: Time expression trial of GST-FLNAR10 fusion protein.....	81

Figure 33: Temperature expression trial of GST-FLNAR10 fusion protein	83
Figure 34: GST column purification of GST-FLNAR10 fusion protein from BL21 <i>E.coli</i> lysate	86
Figure 35: Thrombin digest of GST-FLNAR10 fusion protein from BL21 <i>E.coli</i> lysate	88
Figure 36: Thrombin digest of GST-FLNAR10 fusion protein on GST column.....	90
Figure 37: Thrombin digest products of GST-FLNAR10 fusion protein on GST column .	92
Figure 38: Purification of thrombin digest products using FPLC	94
Figure 39: Purification of thrombin digest products using FPLC	96
Figure 40: GST affinity purification of GST-FLNAR10 and GST.....	99
Figure 41: Fusion protein probe pull-down of C2C12 mouse myoblast cells	100
Figure 42: Fusion protein probe Pull-down of <i>E.coli</i> expressing CFM2 - cell lysate	101