Cellular Signalling 33 (2017) 30–40

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Enzymatic cleavage of myoferlin releases a dual C2-domain module linked to ERK signalling

Ann-Katrin Piper [a,b], Samuel E. Ross [a], Gregory M. Redpath [c], Frances A. Lemckert [a], Natalie Woolger [a,b], Adam Bournazos [a], Peter A. Greer [d], Roger B. Sutton [e,f], Sandra T. Cooper [a,b,*]

[a] Institute for Neuroscience and Muscle Research, Children's Hospital at Westmead, Sydney, NSW 2145, Australia
[b] Discipline of Child and Adolescent Health, Faculty of Medicine, University of Sydney, Sydney, Australia
[c] EMBL Australia Node in Single Molecule Science, School of Medical Science, University of New South Wales, Sydney, NSW, Australia
[d] Department of Pathology and Molecular Medicine, Queen's University, Division of Cancer Biology and Genetics, Queen's Cancer Research Institute, Kingston, ON K7L 3N6, Canada
[e] Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA
[f] Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

ARTICLE INFO

Article history:
Received 11 January 2017
Accepted 7 February 2017
Available online 10 February 2017

Keywords:
Myoferlin
Extracellular-signal-regulated kinase (ERK)
Breast cancer
Calpain
Proteolysis
Cell signalling

ABSTRACT

Myoferlin and dysferlin are closely related members of the ferlin family of Ca^{2+}-regulated vesicle fusion proteins. Dysferlin is proposed to play a role in Ca^{2+}-triggered vesicle fusion during membrane repair. Myoferlin regulates endocytosis, recycling of growth factor receptors and adhesion proteins, and is linked to the metastatic potential of cancer cells. Our previous studies establish that dysferlin is cleaved by calpains during membrane injury, with the cleavage motif encoded by alternately-spliced exon 40a. Herein we describe the cleavage of myoferlin, yielding a membrane-associated dual C2 domain *'mini-myoferlin'*. Myoferlin bears two enzymatic cleavage sites: a canonical cleavage site encoded by exon 38 within the $C2_{DE}$ domain; and a second cleavage site in the linker adjacent to $C2_{DE}$, encoded by alternately-spliced exon 38a, homologous to dysferlin exon 40a. Both myoferlin cleavage sites, when introduced into dysferlin, can functionally substitute for exon 40a to confer Ca^{2+}-triggered calpain cleavage in response to membrane injury. However, enzymatic cleavage of myoferlin is complex, showing both constitutive or Ca^{2+}-enhanced cleavage in different cell lines, that is not solely dependent on calpains-1 or -2. The functional impact of myoferlin cleavage was explored through signalling protein phospho-protein arrays revealing specific activation of ERK1/2 by ectopic expression of cleavable myoferlin, but not an uncleavable isoform. In summary, we molecularly define two enzymatic cleavage sites within myoferlin and demonstrate 'mini-myoferlin' can be detected in human breast cancer tumour samples and cell lines. These data further illustrate that enzymatic cleavage of ferlins is an evolutionarily preserved mechanism to release functionally specialized mini-modules.

1. Introduction

Myoferlin is a member of the ferlin family of Ca^{2+}-regulated vesicle fusion proteins [1]. In mammals, there are six ferlins: dysferlin (fer1L1), otoferlin (fer1L2), myoferlin (fer1L3), fer1L4, fer1L5 and fer1L6 [2]. Ferlin proteins have a distinctive structure, with a C-terminal transmembrane anchor and 5–7 tandem cytoplasmic C2 domains [2], which are Ca^{2+}-regulated lipid and protein binding domains [3]. Proteins that bear C2 domains have varied roles, often involving Ca^{2+}-triggered membrane binding events [4]. The ferlins, synaptotagmins, double C2-like-domain-containing proteins (DOCs), extended synaptotagmin-like proteins and synaptotagmin-like proteins (SLPs) are C2 domain-containing proteins that have been functionally associated with vesicle fusion [5]. Mutations in the dysferlin (*DYSF*) and otoferlin genes (*OTOF*) cause inherited disorders in humans linked to defective vesicle fusion. Mutations in the dysferlin gene cause an inherited late-onset form of muscular dystrophy [6,7], believed to be partially due to defective vesicle fusion required for muscle membrane repair [8]. Mutations in otoferlin cause congenital deafness [9,10], due to defective synaptic exocytosis [11] and vesicle replenishment [12] at the ribbon synapse of the inner ear.

Variants in the myoferlin gene (*MYOF*) have not been implicated in inherited disease, however, emerging evidence links the expression of

Abbreviations: DOCs, double C2-like-domain-containing proteins; SLPs, synaptotagmin-like proteins; ERK, extracellular-signal-regulated kinase; pERK, phosphorylated ERK; MAPK, mitogen-activated protein kinase; DYSF, dysferlin; MYOF, myoferlin; OTOF, otoferlin; CAPN1, calpain-1; CAPN2, calpain-2; CAPNS1, calpain small subunit-1; KD, knock-down; KO, knockout; LV, lenti virus.

* Corresponding author at: Institute for Neuroscience and Muscle Research, Children's Hospital at Westmead, Sydney, NSW 2145, Australia.
E-mail address: sandra.cooper@sydney.edu.au (S.T. Cooper).

http://dx.doi.org/10.1016/j.cellsig.2017.02.009
0898-6568/

myoferlin mRNA and protein to the metastatic potential of cancer cells. In breast cancer and renal cell cancer, myoferlin mRNA expression is upregulated in tumours compared to control tissues [13]. Myoferlin protein levels are upregulated in high-grade pancreatic adenocarcinoma compared to low-grade pancreatic adenocarcinoma or adjacent tissue [14], and myoferlin levels were also elevated in breast ductal adenocarcinoma and breast lobular carcinoma, compared to adjacent control breast tissue [15]. Targeted myoferlin knockdown using siRNA injected into murine tumours grown from Lewis lung cells engrafted into C57BL/6 mice reduced tumour size by almost 50% [16]. Furthermore, shRNA-mediated myoferlin knock down in MDA-MB-231 breast cancer cells was associated with reduced cell migration velocity, and increased cell-matrix and cell-cell interactions *in vitro*, and reduced proliferation and localized invasive behaviour in mouse xenografts [17,18].

Thus, emerging evidence implicates myoferlin in cancer progression and metastasis, with the underlying mechanism still to be elucidated. Several studies show myoferlin regulates growth factor receptor recycling and endocytosis [15,19–22]. Altered growth factor signalling is linked to the progression and pathogenesis of different carcinoma types [23,24], and tumours often secrete autocrine acting growth factors [25]. Given the established roles of ferlin proteins in dynamic membrane remodelling, including endocytosis and secretion, it is plausible that myoferlin regulates signalling events in cancer cells that contribute to proliferation and metastasis.

Our previous studies demonstrate that dysferlin is cleaved by calpains-1 and -2 in response to the Ca^{2+} influx caused by membrane injury. Calpain cleavage of dysferlin releases a C-terminal fragment of ~72 kDa, mini-dysferlin$_{C72}$, bearing two C2 domains anchored by the transmembrane domain, with broad structural homology to the synaptotagmin family of vesicle fusion proteins [26,27]. As antibodies directed against N-terminal epitopes are unable to detect dysferlin at injuries sites, data suggest the C-terminal mini-dysferlin$_{C72}$ is the species recruited to sites of membrane injury, where it may function as a specialized membrane repair module [26,27]. Our previous studies also demonstrated other ferlin paralogues could be cleaved by calpains *in vitro* [27].

Our interest in myoferlin was piqued by reports showing myoferlin as a doublet band in western blots when detected with N-terminal epitope specific antibodies [16,28–30]. This was reminiscent of the N-terminal calpain-cleavage fragment we reported for dysferlin [26,27]. Herein, we provide evidence that myoferlin is also proteolytically cleaved in breast cancer tumours and cell lines, releasing a C-terminal 'mini-myoferlin' fragment. We molecularly characterize two myoferlin cleavage sites in close proximity at the fifth C2 domain, C2$_{DE}$ (between C2$_D$ and C2$_E$, and not annotated by PFAM and/or SMART for all ferlin paralogues). Further we show that ectopic expression of the cleavable canonical myoferlin isoform, but not an uncleavable isoform, leads to increased ERK1/2 phosphorylation (extracellular signal–regulated kinase), providing a potential connection between myoferlin cleavage and the MAPK/ERK pathway linked to cancer cell progression.

2. Material and methods

2.1. Cell culture

HEK293 and MO3.13 cells were cultured in DMEM (Life Technologies) containing 10% FBS (Life Technologies). COS-7 and MEF cells were cultured in 1:1 DMEM:F12 (Life Technologies) containing 10% FBS. EVSA-T cells were cultured in MEM + 1xNEAA (non-essential amino acids) containing 10% FBS, MCF-7, BT-474 and MDA-MB-231 cells were cultured in RPMI 1640 (Life Technologies) containing 10% FBS. All media contained 50 μg/mL gentamicin (Life Technologies). Cells were harvested by scrape injury as described in Lek et al., 2013 [26].

2.2. SDS-PAGE and western blotting

Procedures were performed as described in [27].

2.3. Transfection

HEK293 cells were transfected using PEI (polyethylenimine Max, Polysciences) and COS-7 with Lipofectamine® LTX (LifeTechnologies) as described in [27].

2.4. Immunoprecipitation

Epitope-tagged ferlin constructs were immunoprecipitated from transfected HEK293 cells using anti-Myc antibody following the protocol described in [27].

2.5. In vitro calpain cleavage

Purified calpain-1 (porcine) and -2 (human) were purchased from Millipore. *In vitro* cleavage of the ferlin proteins was performed using a modified protocol from Mandic et al. [31]. Protein G–Sepharose–bound ferlin proteins were washed three times in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich; pH 7.5), 50 mM NaCl, and 1 mM $MgCl_2$ containing 2 mM $CaCl_2$. Diluted recombinant calpain was added directly to the protein G–Sepharose–bound ferlin proteins and incubated at 30 °C for 10s. and 2 min. as indicated. Digestion was quenched by reconstitution into 2× SDS loading buffer (4% SDS, 20% glycerol, 125 mM Tris, pH 7.4, and 10 mM DTT (Sigma-Aldrich)), and samples were heated to 94 °C for 3 min.

2.6. In vitro cathepsin L cleavage

Protein bound sepharose beads were transferred into trisacetate cleavage buffer (50 μL for each experimental condition) and split evenly into one tube for each condition used. 10 ng/μL purified recombinant human cathepsin L (952-CY, R&D systems) diluted in trisacetate buffer on ice was added to ferlin-bound Protein-G beads and incubated for the indicated time at 30 °C. Method adopted from Goulet et al., 2004 [32]. Afterwards samples were handled like described under *in vitro* calpain cleavage.

2.7. Proteome profiler

We have used the Proteome Profiler Human Phospho-MAPK (ARY002B) and the Mouse-RTK Array Kit (ARY014) from R&D systems and followed the company's instructions.

2.8. Antibodies

Antibodies used in western blotting included N-terminal anti-myoferlin (7D2, α-rabbit, 1:2000 abcam, ab76746, lot# GR209175-4), C-terminal anti-myoferlin (K16, α-goat, 1:200; Santa cruz, sc-51,367, lot# A6714), C-terminal anti-dysferlin (Hamlet-1, α-mouse, 1:5000; Leica Microsystems, Wetzlar, Germany, NCL-Hamlet, lot# 6045527), N-terminal anti-dysferlin (Romeo, α-rabbit, 1:1000; abcam, ab124684), mid region anti-dysferlin, (Fer-A, α-rabbit, 1:1000; Sigma-Aldrich, HPA021945, lot# R10883), anti-Myc (α-rabbit, 1:5000; abcam, ab9106, lot# GR130480-24), anti-β-tubulin (1:1000; Developmental Studies Hybridoma Bank, E7-c 8 M4, lot# 4/16/15), anti-GAPDH (α-mouse, 1:10,000; Merck, Millipore, MAB374, lot# 2742734), anti-FLAG M2 (α-mouse, 1:5000, Sigma Aldrich, F3165, lot# SLBH1191V), anti-phosphoERK1/2 (α-rabbit, 1:1000, P-p44/42 (T202/Y204) MAPK, Cell signaling, 4370P, lot#17), anti-ERK (α-rabbit, 1:1000, p44/42 MAPK ERK1/2, Cell Signalling, 4695P, lot#14), anti-phosphoAKT (α-rabbit, 1:1000, Cell signaling, (Ser473), 9271S, lot# 9), anti-CAPNS1 (Calpain reg (P1), α-mouse, 1:500, Santa cruz, sc-