FEBS Letters 587 (2013) 2952–2957

journal homepage: www.FEBSLetters.org

Cysteine mutations cause defective tyrosine phosphorylation in MEGF10 myopathy

Satomi Mitsuhashi [a,b], Hiroaki Mitsuhashi [c], Matthew S. Alexander [c], Hiroyuki Sugimoto [b], Peter B. Kang [a,*]

[a] Department of Neurology, Boston Children's Hospital and Harvard Medical School, Boston, MA 02115, USA
[b] Department of Biochemistry, Dokkyo Medical School, Tochigi 3210293, Japan
[c] Division of Genetics, Program in Genomics, Boston Children's Hospital and Harvard Medical School, Boston, MA 02115, USA

ARTICLE INFO

Article history:
Received 17 April 2013
Revised 27 June 2013
Accepted 1 August 2013
Available online 15 August 2013

Edited by Zhijie Chang

Keywords:
MEGF10
Myopathy
Tyrosine phosphorylation

ABSTRACT

Recessive mutations in *MEGF10* are known to cause a congenital myopathy in humans. Two mutations in the extracellular EGF-like domains of MEGF10, C326R and C774R, were associated with decreased tyrosine phosphorylation of MEGF10 in vitro. Y1030 was identified to be the major tyrosine phosphorylation site in MEGF10 and is phosphorylated at least in part by c-Src. Overexpression of wild-type MEGF10 enhanced C2C12 myoblast proliferation, while overexpression of Y1030F mutated MEGF10 did not. We conclude that MEGF10-mediated signaling via tyrosine phosphorylation helps to regulate myoblast proliferation. Defects in this signaling pathway may contribute to the disease mechanism of MEGF10 myopathy.

Structured summary of protein interactions:
c-Src physically interacts with **MEGF10** by anti tag coimmunoprecipitation (1, 2, 3, 4)

1. Introduction

Mutations in *MEGF10* were recently found to cause an autosomal recessive skeletal muscle disease [1–3]. MEGF10 myopathy patients experience progressive congenital muscle weakness and respiratory failure, without any signs of central nervous system dysfunction [2,3]. MEGF10 is expressed in satellite cells of skeletal muscle [4]. Satellite cells are muscle stem cells that play a key role in muscle growth and regeneration. Satellite cells normally remain quiescent, but activate upon muscle injury or exercise and undergo asymmetrical division, leading to self-renewal of the stem cell population and production of myogenic cells that differentiate into new muscle fibers. Satellite cells were found to be depleted in the skeletal muscle tissue of a MEGF10 myopathy patient [2]. Concordantly, it was reported that MEGF10 overexpression in C2C12 myoblasts enhances cell proliferation and that knockdown of MEGF10 in muscle fibers leads to a reduction of satellite cells due to premature differentiation [4]. MEGF10 is also highly expressed in the central nervous system [5]. In neuronal tissue, MEGF10 has been reported to play roles in neuronal cell engulfment [6,7], amyloid-β protein uptake [8] and retinal neuron spacing [9].

MEGF10 is a single transmembrane protein that has 17 EGF-like domains in the extracellular N-terminus and a C-terminal cytoplasmic domain with 13 tyrosine residues that may be involved in signal transduction [10]. MEGF10 has two mammalian homologues, MEGF11 and MEGF12 (also known as PEAR1 and Jedi-1), and one orthologue in *Drosophila melanogaster*, *draper*. Tyrosine phosphorylation of MEGF11 and MEGF12 has been reported to contribute to platelet activation [11,12] and glial phagocytosis of apoptotic neurons [6,7,13], and tyrosine phosphorylation signaling of draper is important for phagocytic activity [13,14]. Recently, it has also been reported that MEGF10 and MEGF12 are tyrosine phosphorylated and regulate phagocytosis of apoptotic neurons via the Src family kinase-Syk pathway [7]; similar phosphorylation and regulation patterns have been reported for draper [11,14]. We hypothesized that MEGF10 participates in a signaling pathway that is important for satellite cell function. However, there have been no studies of MEGF10 signaling in muscle and little is known about the mechanism of disease for MEGF10 myopathy.

To date, MEGF10 myopathy has been reported in 7 families from different populations (Fig 1A) [2,3,15]. First, five families were reported to possess various homozygous nonsense mutations or compound heterozygous nonsense and missense mutations, all showing severe phenotypes [2]. In one of these severely-affected families, a heterozygous C774R mutation was paired with a heterozygous P442HfsX9 nonsense mutation. Three of the patients died in infancy due to respiratory complications. Later, we reported a sixth family with a milder phenotype who harbored the compound heterozygous missense mutations C326R and C774R, located at the second cysteine of the 6th EGF-like domain and the 4th cysteine of

* Corresponding author. Address: Department of Neurology, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA, USA. Fax: +1 617 730 0366.
E-mail address: peter.kang@childrens.harvard.edu (P.B. Kang).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.08.002

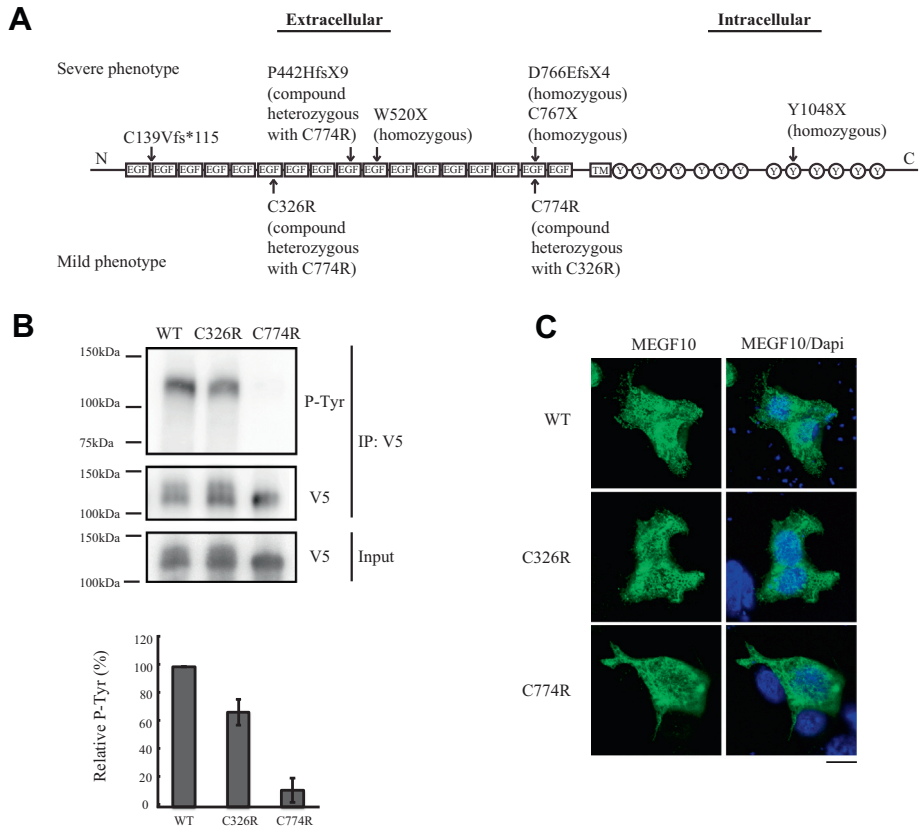

Fig. 1. Tyrosine phosphorylation in C326R and C774R mutant MEGF10. (A) Diagram of the MEGF10 protein domains with arrows indicating previously reported human patient's mutations [2,3,15]. Upper mutations are from individuals with severe disease phenotypes. Lower mutations are from three patients in one family with a mild phenotype. Note that the C774R mutation has been associated with both severe and mild phenotypes. EGF: EGF-like domain, TM: transmembrane domain, Y: tyrosine residue. (B) HEK293T cells were transfected with wild type, C326R mutant, and C774R mutant MEGF10 tagged with V5. Cell lysates were immunoprecipitated with anti-V5 antibody, then subjected to immunoblotting with anti-phosphotyrosine (P-Tyr) antibody or anti-V5 antibody. The C774R mutant shows a greater defect in tyrosine phosphorylation than the C326R mutant. IP V5: V5 tagged immunoprecipitated lysates. Western blot densitometry of the bands shows decreased tyrosine phosphorylation in C326R and C774R. Quantitative analysis was normalized against intensities of immunoprecipitated V5 bands (data are relative to wild type MEGF10, $n = 5$). (C) Wild type, C326R mutant, and C774R mutant MEGF10 constructs were transfected into 293T cells. One day after transfection, cells were fixed and stained with anti-V5 antibody (green). Representative cells are shown. The left column shows MEGF10 staining with anti-V5 antibody. The right column shows merged images of DAPI-labeled nuclei (blue) with the images on the left. Mutant proteins show the same subcellular localization pattern as the wild type proteins. Scale bar: 10 μm.

the 16th EGF-like domain, respectively [3]. The three affected individuals in this family are now in their third decade, and one is still ambulatory. They have milder respiratory complications than the initial cohort of 5 families, only requiring non-invasive nocturnal ventilatory support. These observations suggest that the C774R mutation is as deleterious as a nonsense mutation, while the C326R mutation may be less consequential. Recently, a seventh family with a homozygous frameshift deletion of exon 7 in *MEGF10* was reported to display a severe phenotype [15].

The current study investigates the tyrosine phosphorylation signaling of MEGF10, using constructs representing the C326R and C774R mutations and tyrosine phosphorylation deficient mutations. MEGF10 tyrosine phosphorylation contributes to MEGF10-induced muscle cell proliferation, potentially explaining the loss-of-function mechanism of this disease.

2. Material and methods

2.1. Construction of expression vectors for MEGF10 and mutants

The V5-tag sequence was inserted into *XhoI*- and *XbaI*-sites in pCS2(+). The human MEGF10 cDNA was cloned by PCR using the TOPO TA Cloning Kit (Life Technologies Corporation) then subcloned into pCS2(+)-V5. Human c-Src cDNA was generously provided by Professor Shoichi Ishiura (The University of Tokyo, Tokyo, Japan). The Myc-tagged c-Src was generated by subcloning c-Src into pcDNA3.1-Myc vector (Life Technologies Corporation). Various MEGF10 mutants, including C326R, C774R, Y1030F and Y1030D, deletion mutants, and the kinase-inactive dominant negative variant of c-Src (K298R) [16] were generated by site-directed mutagenesis as previously described [17]. Primers used in this study are listed in Supplementary Table 1.

2.2. Cell culture

Human embryonic kidney (HEK) 293T cells (GenHunter Corporation) and mouse myoblast C2C12 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corporation) supplemented with 10% and 20% fetal bovine serum (FBS, Atlanta Biologicals) respectively, penicillin (100 units/ml, Sigma), and streptomycin (100 μg/ml, Sigma). All cells were maintained in a 5% CO_2 incubator at 37 °C.

2.3. Immunoprecipitation

The constructs were transfected into HEK293T cells or C2C12 cells using Lipofectamine 2000 reagent (Life Technologies Corporation) and Fugene HD (Promega), respectively, according to the manufacturer's protocols. Cells were collected 24 h after transfection and lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na_3VO_4) containing a