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Research paper

Characterization of isoform expression and subcellular distribution of MYPT1 in intestinal epithelial cells

Juan-Min Zha [a,b,1], Hua-Shan Li [b,1], Yi-Tang Wang [c], Qian Lin [b], Min Tao [a,*], Wei-Qi He [b,*]

[a] Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou 215006, China
[b] Cambridge-Suda (CAM-SU) Genome Resource Center, Soochow University, Suzhou 215123, China
[c] Department of Pathology, The University of Chicago, Chicago 60637, USA

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ABSTRACT

The regulation of intestinal epithelial permeability requires phosphorylation of myosin regulatory light chain (MLC). The phosphorylation status of MLC is regulated by myosin light chain phosphatase (MLCP) activities. The activity of the catalytic subunit of MLCP (PP1cδ) toward MLC depends on its regulatory subunit (MYPT1). In this study, we revealed the presence of two MYPT1 isoforms, full length and variant 2 in human intestinal (Caco-2) epithelial cells and isolated intestinal epithelial cells (IECs) from mice. In confluent Caco-2 cells, MYPT1 was distributed at cell–cell contacts and colocalized with F-actin. These results suggest that MYPT1 isoforms are expressed in intestinal epithelial cells and MYPT1 may be involved in the regulation of intestinal epithelial barrier function.

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1. Introduction

A single layer of epithelial cells lines the inner surface of the intestine. These cells form a barrier which supports nutrient, water transport, and prevents microbial contamination (Turner, 2009; Shen et al., 2011). Defects in epithelial barrier function are present in both Crohn's disease and ulcerative colitis and are thought to be central to development and progression of these diseases. It has shown that contraction of perijunctional actomyosin ring (PAMR), which lies within the cell immediately adjacent to the tight junctions, is a critical regulator of epithelial barrier dysfunction in inflammatory bowel disease (IBD) (Clayburgh et al., 2004; Su et al., 2009; Su et al., 2013). The contractile activity of actomyosin can be regulated by the reversible phosphorylation of the myosin regulatory light chain (MLC). The phosphorylation level of MLC is tightly regulated by the balanced activity between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Kamm and Stull, 2001; He et al., 2008; He et al., 2013).

Tumor necrosis factor-α (TNF-α) upregulates intestinal epithelial MLCK expression and, in turn, increases MLC phosphorylation and tight junction permeability (Clayburgh et al., 2005; Wang et al., 2005; Ye and Ma, 2008). Relevant observations show that (i) MLCK expression and MLC phosphorylation are increased in human IBD and correct with the degree of inflammatory activity in patients (Blair et al., 2006), (ii) intestinal epithelial expression of constitutively active MLCK (CA-MLCK) enhances experimental IBD progression (Su et al., 2009), and (iii) knockout of the epithelial long MLCK isoform attenuates experimental IBD (Su et al., 2013). Despite this potentially crucial role for myosin phosphatase, it has never been studied in IBD.

Myosin phosphatase was first identified as a phosphatase in purified preparations of smooth muscle myosin (Ito et al., 2004). It is composed of three subunits: PP1cδ, the catalytic component; MYPT1, the regulatory component; and a 20-kD protein, M20, of unknown function (Ito et al., 2004; Matsumura and Hartshorne, 2008; Grassie et al., 2011). PP1cδ activity is markedly reduced in the absence of MYPT1, which confers specificity by targeting PP1cδ to myosin. Myosin phosphatase plays a significant role in regulation of smooth muscle function (He et al., 2013).

Multiple isoforms of MYPT1 were reported from several species and various tissues (Dirksen et al., 2000; Xia et al., 2005; Kim et al., 2012). These MYPT1 variants are products of alternative splicing from a single gene (Matsumura and Hartshorne, 2008; Grassie et al., 2011). MYPT1 is expressed in smooth muscle and non-muscle cells, including Hela, HEK293, and endothelial cells (Hirano et al., 1999; Xia et al., 2005; Kim et al., 2012). However, it is unknown whether MYPT1 is expressed in intestinal epithelial cells. Remarkably, little is known regarding MYPT1 function in epithelia and in IBD. In this study, we demonstrated the expression of MYPT1 variants both in cultured human intestinal

Abbreviations: IECs, intestinal epithelial cells; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1; MLC, myosin regulatory light chain; IBD, inflammatory bowel disease; TNF-α, tumor necrosis factor-α.

* Corresponding authors.
E-mail addresses: mtao@medmail.com.cn (M. Tao), whe@suda.edu.cn (W.-Q. He).

[1] These authors contributed equally to this work.

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epithelial monolayers and in mouse intestinal epithelia. The subcellular localization of MYPT1 was examined in cultured confluent epithelial cells.

2. Materials and Methods

2.1. Cell culture

Caco-2, Caco-2BBe, HT-29, HCT116, A549, Hela, HepG2, HEK293T, U2OS, L929, and RAW264.7 were maintained in Modified Eagle's Medium (DMEM) (Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA). The medium was replaced every 2–3 days.

2.2. Animals

C57BL/6 mice were maintained in mouse facility of Soochow University. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Soochow University. The isolation of intestinal epithelia was performed as described previously (Su et al., 2013). Briefly, 5–10 cm of small intestine was dissected, opened, and flushed with calcium/magnesium-free Hank's buffered saline solution (CMF-HBSS, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 141 mM NaCl, 5.6 mM D-glucose, pH 7.4). The intestine was cut to small pieces and incubated for 30 min with CMF-HBSS containing 10 mM DTT at 4 °C. The small pieces were transferred to CMF-HBSS containing 1 mM EDTA and incubated for 1 h at 4 °C. Epithelial cells were separated by vigorous shaking.

2.3. RNA isolation and reverse transcription

Cell pallets or tissues from C57BL/6 mice were homogenized in Trizol (Invitrogen). Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, USA). Reverse transcriptase PCR was performed with the iScriptTM cDNA Synthesis Kit (Bio-Rad, USA). The primers used for characterizing MYPT1 and its isoforms were designated as human variant 2 (P1, forward): 5′-AGC TCA GTT AAT GAA GGA TCA ACG-3′; human variant 2 (P2, reverse): 5′-GAT CAG TTA ATG CTC CCT GTG-3′; mouse variant 2 (mouse P1, forward): 5′-AGT TCG ATC AAT GAA GGA TCC ACT-3′; mouse variant 2 (mouse P2, reverse): 5′-GAT CAG TCA GTG TTA CCC CCT GTG-3′; human/mouse variant 1 (P3, forward): 5′-GGT CGC TCT GGA TCA TAC AG-3′; human/mouse variant 1 (P4, reverse): 5′-GAG CTC TTC TTC CAT TTC AG-3′; mouse exon 24 splicing isoform (P5, forward): 5′-ATT CCT TGC TGG GTC GCT CTG C-3′; mouse exon 24 splicing isoform (P6, reverse): 5′-ATC AAG GCT CCA TTT TCA TCC-3′.

2.4. Immunoblotting

Caco-2 monolayers were lysed in SDS–extraction buffer (50 mM Tris, pH 8.8, 2% SDS, 5 mM EDTA) and then reduced in Laemmli sample buffer. SDS–PAGE and transfer to polyvinylidene difluoride (PVDF) membrane were performed as described previously (Buschmann et al., 2013). Targets of interest were probed with primary antibodies against MYPT1 (Millipore, USA) and β-actin (Sigma-Aldrich, USA), followed by horseradish peroxidase–conjugated secondary antibodies, as described (Zha et al., 2011; He et al., 2008, 2011, 2013). Protein was detected by chemiluminescence.

2.5. Immunofluorescence staining and microscopy

Caco-2 monolayers were maintained and plated on coverslips as described previously (Buschmann et al., 2013). To differentiate the cells, the media were changed every 2–3 days for two weeks. Caco-2 cells were then fixed with 1% paraformaldehyde. Cells were washed with PBS and permeabilized with 0.1% Trion X-100 in PBS. After another around of PBS washes, cells were stained with anti-MYPT1 antibody (Millipore), followed by species-specific secondary antisera conjugated to Alexa Fluor 488 (Invitrogen), Phalloidin conjugated to Alexa Fluor 594 (Invitrogen), and Hoechst 33,342 (Life Technologies, USA). Images were collected using an Olympus FV1000 confocal microscope (Olympus, Japan). Line-Scan analysis was performed by ImageJ software (National Institutes of Health, USA).

3. Results

3.1. Two isoforms of MYPT1 exist in Caco-2 cells

MYPT1 isoforms are expressed in many cell types other than smooth muscle cells. Previous studies reported that two isoforms of human MYPT1 exist in Hela, HEK293, and human endothelial cells (Hirano et al., 1999; Xia et al., 2005; Kim et al., 2012). To examine the expression of myosin phosphatase target subunit 1 (MYPT1) in intestinal epithelial cells, extracts were subjected to immunoblot analyses using the anti-MYPT1 antibody. Two isoforms were detected in both human intestinal (Caco-2) epithelial monolayers and isolated intestinal epithelial cells (IECs) from mice, which is consistent with other reported human cells (Fig. 1A).

To further identify these two isoforms in epithelial cells, we designed two sets of primers to detect the existence of previously reported human MYPT1 variant 1 (GenBank accession number AF458589) or variant 2 (GenBank accession number AY380574). As shown in Fig. 1B, primer 1 (P1) and primer 2 (P2) were designed to verify the presence of MYPT1 variant 2. Primer 3 (P3) and primer 4 (P4) were designed to verify the presence of MYPT1 variant 1. P1 and P2 generated two bands corresponding to the MYPT1 full length (490 bp) and variant 2 (322 bp) (Fig. 1C). However, in the PCR reaction with the set of P3 and P4, the longer band (288 bp) corresponding to MYPT1 full length was detected, but the smaller band (183 bp) corresponding to MYPT1 variant 1 was not detected in Caco-2 cells (Fig. 1C). These data demonstrated that full length and variant 2 of MYPT1 were expressed in Caco-2 cells while variant 1 of MYPT1 was absent. Sequence results confirmed that full length and variant 2 of MYPT1 expressed in Caco-2 cells were identical to previously reported human full-length MYPT1 (GenBank accession number D87930) and variant 2 (GenBank accession number AY380574).

The expression of MYPT1 isoforms was also investigated in other human epithelial and non-epithelial cell lines. Intestinal epithelial cell lines, Caco-2 BBe, HT-29, and HCT116 express both full length and variant 2 of MYPT1 (Fig. 2A). These two isoforms were also detected in other non-epithelial cell lines including A549, Hela, HepG2, HEK293T, and U2OS (Fig. 2A). MYPT1 variant 1 was not expressed in none of these cell lines (Fig. 2B).

3.2. Two isoforms of MYPT1 exist in isolated mouse epithelial cells

Although two isoforms of MYPT1 were detected in isolated mouse and rat smooth muscles (Zhang and Fisher, 2007; Chen et al., 2015), the expression of MYPT1 isoforms was not carefully examined in mouse tissues. Mouse primer 1 (mouse P1) and primer 2 (mouse P2) were designed to verify the existence of full length and variant 2 of MYPT1. Both isoforms of MYPT1 were detected in mouse fibroblasts L929 and mouse monocyte/macrophages RAW 264.7 (Fig. 3A). Consistently, small intestine and colon tissues containing smooth muscle and laminae propria expressed both isoforms (Fig. 3B). To verify the expression of MYPT1 isoforms in pure intestinal epithelial cells (IECs), RT-PCR was performed in IECs isolated from mouse small intestine. As shown in Fig. 3B, IECs from small intestine also expressed both isoforms.

The expression of MYPT1 variants was also investigated in various mouse tissues, including heart, skeletal muscle, spleen, mesenteric lymph node, testis, cerebrum, cerebellum, lung, kidney, and bladder. Interestingly, variant 2 but not full-length MYPT1 was detected in spleen, mesenteric lymph node, and testis (Fig. 3B).

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