FEBS Letters 582 (2008) 2993–2997

The α-kinases TRPM6 and TRPM7, but not eEF-2 kinase, phosphorylate the assembly domain of myosin IIA, IIB and IIC

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Received 6 May 2008; revised 29 May 2008; accepted 1 July 2008

Available online 7 August 2008

Edited by Giulio Superti-Furga

Abstract TRPM6 and TRPM7 encode channel-kinases. While these channels share electrophysiological properties and cellular functions, TRPM6 and TRPM7 are non-redundant genes raising the possibility that the kinases have distinct substrates. Here, we demonstrate that TRPM6 and TRPM7 phosphorylate the assembly domain of myosin IIA, IIB and IIC on identical residues. Whereas phosphorylation of myosin IIA is restricted to the coiled-coil domain, TRPM6 and TRPM7 also phosphorylate the non-helical tails of myosin IIB and IIC. TRPM7 does not phosphorylate eukaryotic elongation factor-2 (eEF-2) and myosin II is a poor substrate for eEF-2 kinase. In conclusion, TRPM6 and TRPM7 share exogenous substrates among themselves but not with functionally distant α-kinases.

Structured summary:
MINT-6700314:
GNA1 (uniprotkb:Q96EK6) and *GNA1* (uniprotkb:Q96EK6) *bind* (MI:0407) by *X-ray crystallography* (MI:0114)

Keywords: α-Kinase; TRPM6; TRPM7; Myosin II; Phosphorylation

1. Introduction

TRPM6 and TRPM7 are bifunctional proteins encoding a TRP cation channel fused to an α-kinase and represent the only two proteins in the mammalian genome with this particular architecture. A critical issue in understanding the biological function of TRPM6 and TRPM7 is to identify the substrates for each kinase.

Within the mammalian α-kinase family, TRPM6 and TRPM7 are most closely related to each other. In addition to similarity in primary structure, TRPM6 and TRPM7 have comparable electrophysiological properties as well as cellular functions. Physiological Mg^{2+} and Mg^{2+}-nucleotide concentrations inhibit TRPM6 and TRPM7 channels [1,2] and these channels play a key role in Mg^{2+} homeostasis [3–5]. However, TRPM6 and TRPM7 also have unique roles in human biology. Overexpression of TRPM6 cannot rescue cell growth arrest due to ablation of TRPM7 [6] and the disease familial hypomagnesemia with secondary hypocalcemia, which is caused by mutations of TRPM6, progresses despite the expression of TRPM7 in both the intestine and kidney [3,4]. Thus, TRPM6 and TRPM7 have unique functions even though they share many structural and electrophysiological features. Are these functional differences related to a distinct set of substrates for each kinase?

In contrast to channel function, little is known about the substrates for TRPM6 and TRPM7 kinases. To date, no exogenous substrates have been identified for TRPM6, whereas TRPM7 phosphorylates annexin I and myosin IIA heavy chain [7–9]. In addition to myosin IIA, mammalian cells also express myosin IIB and IIC. These three non-muscle myosin II isoforms are well conserved throughout the entire protein and share certain cellular functions but there is increasing evidence for non-redundant roles for each isoform [10]. Whether TRPM7 phosphorylates the heavy chains of all three myosin II isoforms remains unknown. Therefore, we compared the substrate specificity of TRPM6, TRPM7 and a functionally distant α-kinase, eukaryotic elongation factor-2 (eEF-2) kinase, by assessing the phosphorylation of non-muscle myosin IIA, IIB and IIC.

2. Materials and methods

2.1. Constructs

Cloning of wild-type (WT) and kinase-dead HA-TRPM7-C and WT HA-TRPM6-C in pcDNA3, eEF-2 kinase in pGEX-2T and the TRPM7 kinase domain (TRPM7-cat) in pMAL-p2x were previously

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Abbreviations: eEF-2, eukaryotic elongation factor-2; LC-MS/MS, nanoliquid chromatography–tandem mass spectrometry; PTM, post-translational modification; WT, wild-type

0014-5793/$34.00
doi:10.1016/j.febslet.2008.07.043

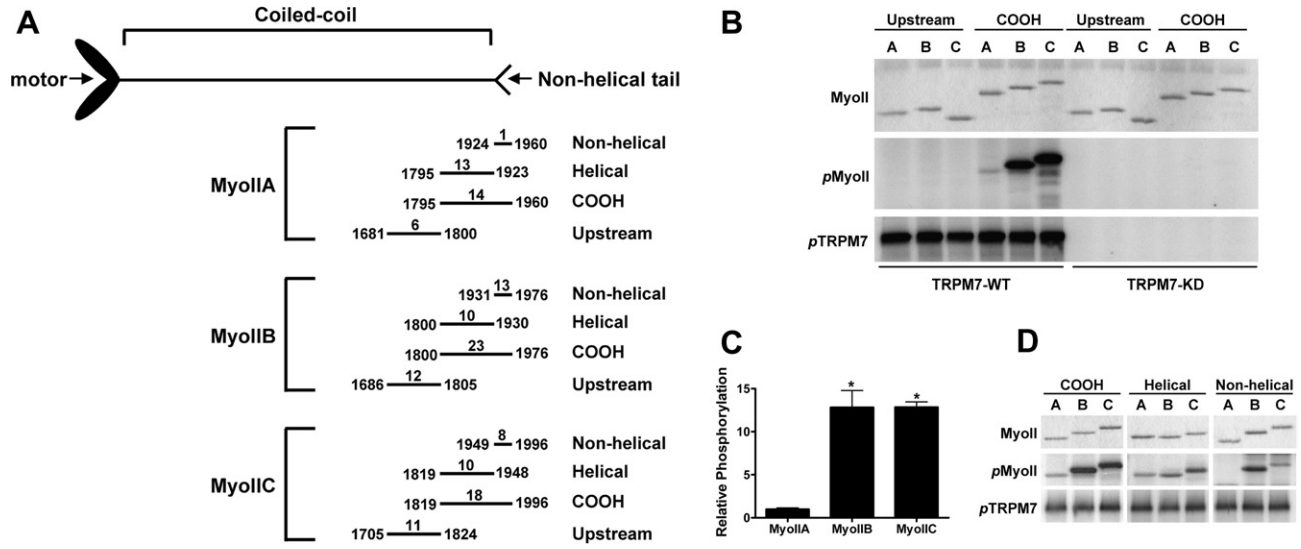


Fig. 1. Phosphorylation of myosin IIA, IIB and IIC heavy chains by TRPM7. (A) A schematic diagram of the regions of myosin IIA, IIB and IIC heavy chains expressed as GST-fusion proteins. The start and end amino acid is indicated on either side of the line. Above the line is the total number of threonine and serine residues within the fragment. (B) TRPM7 phosphorylates the COOH terminus of myosin IIA, IIB and IIC heavy chains. Top, coomassie blue stained gel; middle, autoradiogram of GST-myosin II; bottom, autoradiogram of autophosphorylated TRPM7. (C) Quantification of myosin II phosphorylation by TRPM7. The level of ^{32}P incorporation after a 30 min incubation with TRPM7 is reported relative to the degree of myosin IIA phosphorylation which was set to 1. (D) Deletion of the non-helical tail from the COOH terminus of myosin IIB and IIC heavy chains reduces their phosphorylation to levels observed in myosin IIA. Top, coomassie blue stained gel; middle, autoradiogram of GST-myosin II; bottom, autoradiogram of autophosphorylated TRPM7.

described [11–13]. GST-myosin IIA, IIB and IIC constructs (Fig. 1A) were generated by inserting PCR products into the *BamHI–Eco*RI sites in the pGEX-1N vector. All constructs were verified by DNA sequencing.

2.2. Cell culture

HEK293 cells were cultured in DMEM medium with 10% FCS. Cells were transfected using lipofectamine (Roche).

2.3. Purification of recombinant proteins

GST-myosin II proteins were expressed in *Escherichia coli* and purified by affinity chromatography on a glutathione-sepharose column using standard methods. HA-TRPM6-C and HA-TRPM7-C kinases were purified from mammalian cells by immunoprecipitation [11]. TRPM7-cat, eEF-2 kinase and eEF-2 were purified as previously described [12,13]. The identity and integrity of the recombinant proteins were verified by mass spectrometry.

2.4. In vitro kinase assays

TRPM6 and TRPM7 kinases (20 ng) were dissolved in 50 μl IVK buffer (50 mM HEPES, pH 7.0, 4 mM $MnCl_2$, 2 mM DTT) with 2 μg of GST-fusion protein. Kinase reactions were initiated by adding 0.1 mM ATP in combination with 5 μCi of [γ-^{32}P]ATP and allowed to proceed for 30 min at 30 °C. Activity of eEF-2K was assayed as previously described [12]. The reactions were stopped by adding Laemmli buffer containing 40 mM EDTA and subjected to SDS–PAGE. Phosphorylated proteins were detected by autoradiography and quantified by phosphorimager analysis.

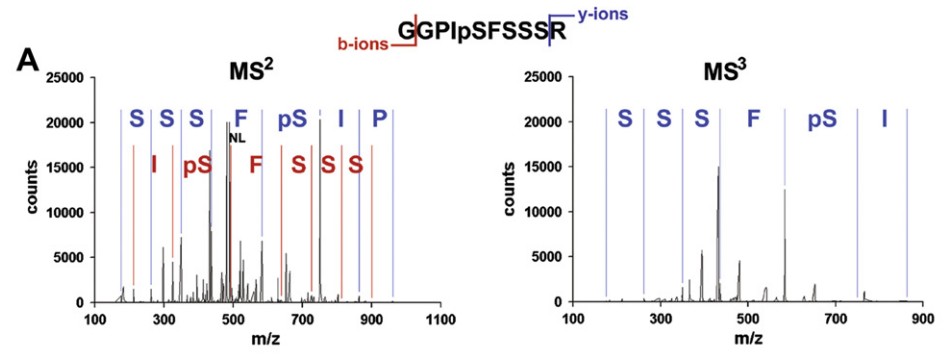
2.5. Mass spectrometry

Peptide identification experiments were performed using a nano-HPLC Agilent 1100 nanoflow system connected online to a 7-T linear ion trap Ion Cyclotron Resonance Fourier Transform mass spectrometer (Thermo Fisher, Bremen, Germany). Verification and site mapping of phosphorylated peptides was performed using the post-translational modification (PTM) algorithm implemented in MSQuant according to the procedure of Olsen et al. [14]. Phosphopeptides were identified with a 99%-significance threshold when the sum of the Mascot and PTM score was higher than 28 [14]. The delta PTM score, the difference between the highest and second highest PTM score, was set to be larger than 8.0 to exclusively report peptides with a mapped phosphorylation site. As a final verification step, peptides containing phosphorylation sites occurring only once or twice were verified by manual inspection of the MS^2 and MS^3 spectra.

3. Results

TRPM7 phosphorylates the COOH extremity of the myosin IIA heavy chain [9]. To examine whether TRPM7 also phosphorylates myosin IIB and IIC heavy chains, regions homologous to myosin IIA were purified as GST-fusion proteins (Fig. 1A). Incubation of these proteins with TRPM7 led to the phosphorylation of all three myosin II isoforms (Fig. 1B). Like myosin IIA, the phosphorylation sites in the myosin IIB and IIC heavy chains are located within the COOH terminus since upstream regions of the coiled-coil domain were not phosphorylated by TRPM7. However, after a 30 min reaction, ^{32}P incorporation by TRPM7 into myosin IIB and IIC was approximately 10-fold greater than for myosin IIA (Fig. 1C).

Sequence alignment of the myosin II isoforms revealed that the helical tail is highly conserved between all three isoforms but the non-helical tail differs significantly in length and sequence (see Fig. 2B). The non-helical tails of myosin IIB and IIC contain a short stretch of amino acids rich in serines and threonines that is absent in the myosin IIA isoform. We therefore generated GST-fusion proteins where the helical and non-helical tails were separated from one another. The myosin IIB and IIC non-helical tails were phosphorylated by TRPM7, whereas the myosin IIA non-helical tail was not phosphorylated (Fig. 1D). Moreover, removal of the non-helical tail from the COOH terminus of myosin IIB and IIC reduced the levels of phosphorylation to those observed in myosin IIA (Fig. 1D). Thus, differential phosphorylation of the non-helical tail by



B

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                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                      5          15         25         35         45         55
hMyoIIA 1795 QEMEGTVKSK YKASITALEA KIAQLEEQLD NETKERQAAC KQVRRTEKKL KDVLLQVDDE 1854
hMyoIIB 1802 QELEGAVKSK FKATISALEA KIGQLEEQLE QEAKERAAAN KLVRRTEKKL KEIFMQVEDE 1861
hMyoIIC 1819 GEEDAGARAR HKMTIAALES KLAQAEEQLE QETRERILSG KLVRRAEKRL KEVVLQVEEE 1878

                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                     65         75         85         95        105        115
hMyoIIA 1855 RRNAEQYKDQ ADKASTRLKQ LKRQLEEAEE EAQRANASRR KLQRELEDAT ETADAMNREV 1914
hMyoIIB 1862 RRHADQYKEQ MEKANARMKQ LKRQLEEAEE EATRANASRR KLQRELDDAT EANEGLSREV 1921
hMyoIIC 1879 RRVADQLRDQ LEKGNLRVKQ LKRQLEEAEE EASRAQAGRR RLQRELEDVT ESAESMNREV 1938

                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....
                    125        135        145        155        165        175
hMyoIIA 1915 SSLKNKLRRG D-LPFVVPRR MAR----KGA G-DGSDEEVD GK-ADGAEAK ---PAE--   1960
hMyoIIB 1922 STLKNRLRRG GPISFSSSRS GRRQLHLEGA SLELSDDDTE SKTSDVNETQ ---PPQSE   1976
hMyoIIC 1939 TTLRNRLRRG P-LTFTTRTV RQVFRLEEGV ASDEEAEEAQ PGSGPSPEPE GSPPAHPQ   1995
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Fig. 2. Mapping of TRPM7 phosphorylation sites in myosin IIA, IIB and IIC heavy chains by nanoLC-MS/MS. (A) A representative MS^2 and MS^3 spectra for a myosin IIB peptide phosphorylated on Ser-1935 (*m/z* observed of parent ion was 537.7342, mass accuracy 0.38 ppm, +2 charge state; NL indicates neutral loss of H_3PO_4 which triggers acquisition of MS^3 spectrum). The b^+-ion series is indicated in red, whereas the y^+-ion series is blue. (B) Alignment of the COOH termini of myosin IIA, IIB and IIC heavy chains. The helical tail is in normal font and the non-helical tail in italic. Residues phosphorylated by TRPM7 are in bold and underlined.

TRPM7 accounts for the differences in phosphorylation of the myosin II isoforms.

TRPM7 phosphorylates mainly serines in myosin IIA and IIB but threonines in myosin IIC (data not shown). We mapped the phosphorylation sites in all three non-muscle isoforms by mass spectrometry (Fig. 2 and Supplemental Data). TRPM7 phosphorylates Thr1800, Ser1803 and Ser1808 in the coiled-coil domain of myosin IIA. This part of the heavy chain is also phosphorylated in myosin IIB and IIC. In contrast to Ser1803, which is only conserved in myosin IIB (S1812), Ser1808 is conserved and phosphorylated in both myosin IIB (T1817) and IIC (T1833). In addition to the coiled-coil domain, TRPM7 phosphorylates numerous serines in the myosin IIB and threonines in the myosin IIC non-helical tails. Nanoliquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis determined with high probability that multiple residues in both myosin IIB and IIC are phosphorylated by TRPM7 in this region.

Like TRPM7, TRPM6 phosphorylates the COOH terminus of the different myosin II isoforms (Fig. 3A) with a 10-fold greater incorporation of ^{32}P in myosin IIB and IIC. Again, this difference is due to the phosphorylation of the non-helical tails of myosin IIB and IIC by TRPM6 (data not shown). Our LC-MS/MS experiments indicated that TRPM6 phosphorylates the same sites as TRPM7 in the different myosin II isoforms (data not shown). Moreover, mutation of the phosphosites Thr1800, Ser1803 and Ser1808 to alanine reduced the phosphorylation of myosin IIA by TRPM6 and TRPM7 to background levels (Fig. 3B). Finally, we found that TRPM7 cannot phosphorylate eEF-2 and conversely, eEF-2 kinase poorly phosphorylates non-muscle myosin II isoforms, showing that these distantly related members of the α-kinase family each phosphorylate specific substrates in vitro (Fig. 4).

4. Discussion

An important conclusion from our investigation is that TRPM6 and TRPM7 share exogenous substrates with themselves but not with α-kinases having distantly related functions such as eEF-2 kinase. We demonstrated that TRPM6 and TRPM7 phosphorylate the different myosin II isoforms on identical residues. Moreover, TRPM7 failed to phosphorylate eEF-2 and eEF-2 kinase only weakly phosphorylated myosin II. While the differences between TRPM7 and eEF-2 kinase are consistent with a recent study on peptide substrate specificity of α-kinases [15], our results contrast with another study by Schmitz et al. [6] who concluded that TRPM6 and TRPM7 have different substrate specificities since TRPM6 cross-phosphorylates TRPM7 but not vice versa. Our studies differ in two important ways: (1) we examined the phosphorylation of unrelated exogenous substrates, whereas Schmitz et al. [6] investigated the level of cross-phosphorylation by both kinases and (2) we measured the incorporation of ^{32}P into our substrates while Schmitz et al. [6] used a pan phospho-threonine antibody to detect phosphorylation. TRPM6 and TRPM7 undergo massive autophosphorylation of the Ser/Thr-rich domain predominantly on serine residues [6,11,13]. Therefore,

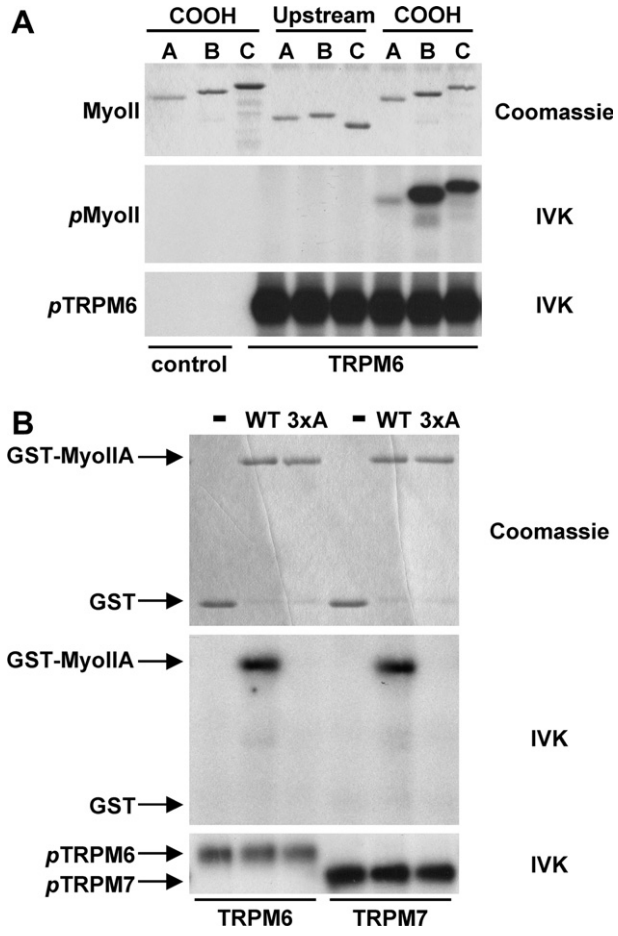

Fig. 3. Myosin II phosphorylation by TRPM6. (A) TRPM6 phosphorylates the COOH terminus of myosin IIA, IIB and IIC. (B) TRPM6 phosphorylates the same residues in myosin IIA as TRPM7. Thr1800, Ser1803 and Ser1808 were mutated to alanine to generate GST-myosin IIA COOH 3xA (aa 1795–1960).

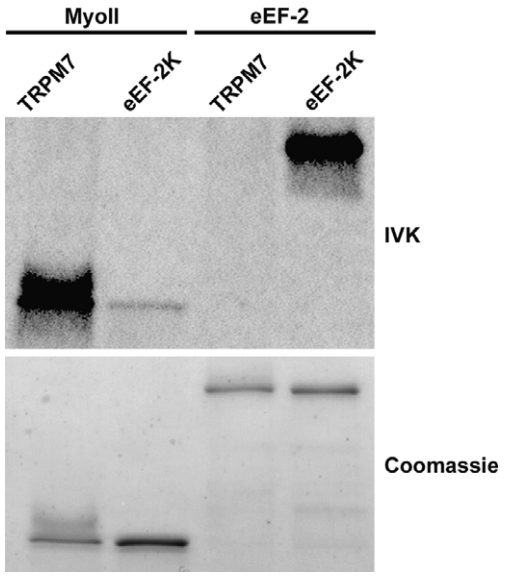

Fig. 4. Preferential phosphorylation of myosin II by TRPM7 and eEF-2 by eEF-2 kinase. Kinase reactions consisted of TRPM7-cat and eEF-2 kinase with either GST-myosin IIB COOH or eEF-2.

the use of the pan-phosphothreonine antibody may not provide an accurate account of the extent of cross-phosphorylation by the kinases. Our data clearly demonstrate that TRPM6 and TRPM7 share substrate specificity when it comes to phosphorylating exogenous substrates.

Although TRPM6 and TRPM7 phosphorylate all three non-muscle myosin II isoforms, we observed important differences between substrates. Most notably, both TRPM6 and TRPM7 phosphorylate the non-helical tail of myosin IIB and IIC but not that of myosin IIA. The presence of additional phosphoacceptor sites in this region of myosin IIB and IIC led to a 10-fold increase in ^{32}P incorporation in comparison to myosin IIA under the kinase reaction conditions used in this investigation (single time-point and substrate concentration). We would like to emphasize that these results do not necessarily mean that myosin IIB and IIC are better substrates for TRPM6 and TRPM7 than myosin IIA, as the phosphorylation occurs on different residues and important regulatory components present in mammalian cells may be absent from the in vitro kinase reactions. Additional experiments will be required to establish the circumstances under which TRPM6 and TRPM7 phosphorylate the different myosin II isoforms in mammalian cells.

The phosphorylation of the non-helical tails of myosin IIB and IIC by TRPM6 and TRPM7 was unexpected since α-kinases are thought to preferentially phosphorylate amino acids in the context of an α-helix, a distinguishing feature of this atypical protein kinase family [16]. However, characterization of a peptide library demonstrated that TRPM6 and TRPM7 preferentially phosphorylate peptides with a consensus sequence that adopts an α-helix structure under specific solvent conditions. Taking this information into consideration, an algorithm was designed to predict TRPM6 and TRPM7 phosphorylation sites in its substrates (Ryazanov et al., manuscript in preparation). This algorithm accurately predicted the phosphorylation of the myosin IIB and IIC non-helical tails. Currently, we are investigating the possibility that the TRPM6 and TRPM7 kinase domains assist in the shaping of these peptides into α-helices for subsequent phosphorylation.

Elucidation of the pathways that control myosin II function is critical to our understanding of the pathogenesis of human diseases involving defects in mechanobiology [10]. Recently, we demonstrated that TRPM7 promotes actomyosin relaxation in mammalian cells by phosphorylating myosin IIA [8,9]. Here, we further extend on these findings by showing that TRPM6 and TRPM7 phosphorylate the assembly domain of all three non-muscle myosin II isoforms. Both channel-kinases phosphorylate the same stretch of amino acids in the helical domain of all three myosin II isoforms. We recently demonstrated that this region regulates the assembly of myosin IIA filaments [9]. In addition to the helical domain, TRPM6 and TRPM7 phosphorylate a cluster of serines and threonines in the myosin IIB and IIC non-helical tails, respectively. This stretch of serine residues in myosin IIB is phosphorylated in vivo by PKCζ in response to EGF stimulation and is a critical determinant in regulating filament assembly and protein localization to the cortical cytoskeleton [17]. This raises the intriguing possibility that TRPM6 and TRPM7 may synergize with PKCζ to regulate myosin IIB filament stability.

In conclusion, our results are consistent with a role for TRPM6 and TRPM7 in regulating actomyosin contractility by phosphorylating myosin IIA, IIB and IIC. Establishing

the cellular context in which TRPM6 and TRPM7 control the activity of these different myosin II isoforms and its link to magnesium homeostasis remain challenges for future research.

Acknowledgements: We thank Niels Peterse for technical assistance. This work was supported by Grants from the Dutch Cancer Society to FNvL.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.043.

References

[1] Nadler, M.J. et al. (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. Nature 411, 590–595.
[2] Voets, T., Nilius, B., Hoefs, S., van der Kemp, A.W., Droogmans, G., Bindels, R.J. and Hoenderop, J.G. (2004) TRPM6 forms the Mg2+ influx channel involved in intestinal and renal Mg2+ absorption. J. Biol. Chem. 279, 19–25.
[3] Schlingmann, K.P. et al. (2002) Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. Nat. Genet. 31, 166–170.
[4] Walder, R.Y. et al. (2002) Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. Nat. Genet. 31, 171–174.
[5] Schmitz, C. et al. (2003) Regulation of vertebrate cellular Mg2+ homeostasis by TRPM7. Cell 114, 191–200.
[6] Schmitz, C., Dorovkov, M.V., Zhao, X., Davenport, B.J., Ryazanov, A.G. and Perraud, A.L. (2005) The channel kinases TRPM6 and TRPM7 are functionally nonredundant. J. Biol. Chem. 280, 37763–37771.
[7] Dorovkov, M.V. and Ryazanov, A.G. (2004) Phosphorylation of annexin I by TRPM7 channel-kinase. J. Biol. Chem. 279, 50643–50646.
[8] Clark, K. et al. (2006) TRPM7, a novel regulator of actomyosin contractility and cell adhesion. EMBO J. 25, 290–301.
[9] Clark, K. et al. (2008) TRPM7 regulates myosin IIA filament stability and protein localization by heavy chain phosphorylation. J. Mol. Biol. 378, 788–801.
[10] Clark, K., Langeslag, M., Figdor, C.G. and van Leeuwen, F.N. (2007) Myosin II and mechanotransduction: a balancing act. Trend Cell Biol. 17, 178–186.
[11] Clark, K., Middelbeek, J., Morrice, N.A., Figdor, C.G., Lasonder, E. and van Leeuwen, F.N. (2008) Massive autophosphorylation of the Ser/Thr-rich domain controls protein kinase activity of TRPM6 and TRPM7. PLoS ONE 3, e1876.
[12] Dorovkov, M.V., Pavur, K.S., Petrov, A.N. and Ryazanov, A.G. (2002) Regulation of elongation factor-2 kinase by pH. Biochemistry 41, 13444–13450.
[13] Ryazanova, L.V., Dorovkov, M.V., Ansari, A. and Ryazanov, A.G. (2004) Characterization of the protein kinase activity of TRPM7/ChaK1, a protein kinase fused to TRP ion channel. J. Biol. Chem. 279, 3708–3716.
[14] Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P. and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648.
[15] Crawley, S.W. and Cote, G.P. (2008) Determinants for substrate phosphorylation by Dictyostelium myosin II heavy chain kinases A and B and eukaryotic elongation factor-2 kinase. Biochim. Biophys. Acta.
[16] Ryazanov, A.G., Pavur, K.S. and Dorovkov, M.V. (1999) Alpha-kinases: a new class of protein kinases with a novel catalytic domain. Curr. Biol. 9, R43–R45.
[17] Rosenberg, M. and Ravid, S. (2006) Protein kinase Cgamma regulates myosin IIB phosphorylation, cellular localization, and filament assembly. Mol. Biol. Cell 17, 1364–1374.