Biochemical and Biophysical Research Communications 434 (2013) 162–165

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Tespa1 protein is phosphorylated in response to store-operated calcium entry

Takahiro Fujimoto [a,b], Hiroshi Matsuzaki [a], Masatoshi Tanaka [c], Senji Shirasawa [a,b,*]

[a] Department of Cell Biology, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
[b] Central Research Institute for Advanced Molecular Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
[c] Department of Urology, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

ARTICLE INFO

Article history:
Received 25 February 2013
Available online 26 March 2013

Keywords:
Tespa1
Posttranslational modification
Phosphorylation
Store-operated calcium entry
KRAP
IP_3R

ABSTRACT

We previously reported that Tespa1 (thymocyte-expressed, positive selection-associated gene 1) protein expressed in lymphocytes physically interacts with IP_3R (Inositol 1,4,5-trisphosphate receptor), a Ca^{2+} channel protein spanning endoplasmic reticulum (ER) membrane. However, the biochemical characterization of Tespa1 protein remains unknown. In this study, we have found that Tespa1 protein was post-translationally modified upon intracellular Ca^{2+} increase in thymocytes. Through the analyses using various inhibitors, store-operated Ca^{2+} entry (SOCE) was found to be an essential factor for the Tespa1 protein modification induced by T cell receptor (TCR)-stimulation. Remarkably, the Ca^{2+}-dependent Tespa1 protein modification was restored by *in vitro* protein phosphatase treatment, indicating that this modification was due to phosphorylation. Moreover, we examined whether Ca^{2+}-dependent phosphorylation of Tespa1 protein would affect the physical association between Tespa1 and IP_3R proteins, revealing that physical association of these proteins is maintained regardless of the presence or absence of phosphorylation of Tespa1. In addition, KRAP protein which represents substantial amino acid sequence homology to Tespa1 was also posttranslationally phosphorylated by intracellular Ca^{2+} increase in HCT116 human colon cancer cells and HEK293 human embryonic kidney cells, suggesting that common signaling mechanism(s) may contribute to the molecular modification of Tespa1 and KRAP in different cellular processes. All these results suggested a novel molecular modification of Tespa1 and the existence of the regulatory pathway that SOCE affects the Tespa1–IP_3R molecular complex.

1. Introduction

Intracellular Ca^{2+} is a universal second messenger controlling numerous biological processes [1,2]. In T-lymphocytes, antigen binding to the T cell receptor (TCR) promotes the transient release of Ca^{2+} from endoplasmic reticulum (ER) stores through the activation of IP_3R (inositol 1,4,5-trisphosphate receptor), and this leads to prolonged Ca^{2+} influx across the plasma membrane, a process termed store-operated Ca^{2+} entry (SOCE) [3–8]. Sustained Ca^{2+} influx via SOCE results in the activation of Ca^{2+}-dependent enzymes and transcription factors.

Tespa1 (thymocyte-expressed, positive selection-associated gene 1) was firstly identified as a critical gene responsible for T cell development in the thymus [9]. We previously reported that Tespa1 protein possessed substantial amino acid sequence homology to KRAP and that this protein was highly expressed in T and B lymphocytes, and physically interacted with IP_3R [10]. IP_3R functions as the Ca^{2+} release channel on ER membranes [11–15] and IP_3R itself is regulated by many intracellular modulators including Ca^{2+}, phosphorylation, and associated proteins [16–20]. However, the biochemical characterization of Tespa1 protein remains unknown.

In this study, we identified a novel molecular modification of Tespa1, i.e. Tespa1 protein was phosphorylated in response to SOCE in primary T lymphocytes and an immortalized T lymphoblastic leukemia cell line, Jurkat cells. Moreover, phosphorylated Tespa1 protein was maintained in the molecular complex with IP_3R, thereby indicating the existence of the regulatory pathway that SOCE affects the Tespa1–IP_3R molecular complex which is a critical component of intracellular Ca^{2+} store.

2. Materials and methods

2.1. Animals

All animals used in this study were treated in accordance with the guidelines of Fukuoka University.

2.2. Reagents

Anti-Tespa1 antibody was described before [10]. Mouse monoclonal anti-IP_3R3 antibody (610313) was from BD Transduction

* Corresponding author at: Department of Cell Biology, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Fax: +81 92 864 3865.
E-mail address: sshirasa@fukuoka-u.ac.jp (S. Shirasawa).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.02.128

Laboratories. Rabbit polyclonal anti-actin antibody (A2066) was from Sigma. Anti-human CD3 antibody (OKT3) was from BioLegend. Ionomycin, PMA (phorbol 12-Myristate 13-acetate), forskolin, cycloheximide, BAPTA-AM, and thapsigargin were from Sigma. BTP2 was from Calbiochem. Nifedipine was from R&D systems.

2.3. Cell culture and cell preparation

Jurkat cells were cultured at 37 °C with 5% CO_2 in the growth medium (RPMI 1640 containing 10% fetal calf serum and penicillin–streptomycin–glutamine). Jurkat cells were stimulated with 10 μg/ml of anti-CD3 antibody in the growth medium for 30 min at 37 °C. Thymocytes from the C57BL/6 mouse were depleted of erythrocytes by hypotonic lysis solution, and then stimulated in the growth medium supplemented with ionomycin (0.5 μg/ml), PMA (100 nM), forskolin (10 μM), or thapsigargin (5 μM) for 30 min at 37 °C. When cells were pretreated with inhibitor, cells were incubated in the growth medium containing cycloheximide (10 μg/ml), BAPTA-AM (5 μM), EGTA (5 mM), BTP2 (3 μM), $NiCl_2$ (100 μM), or nifedipine (1 μM) for 15 min prior to the stimulation. HCT116 cells and HEK293 cells were cultured as described before [21].

2.4. In vitro protein phosphatase assay

Cells were extracted in the buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40). After removing cell decries by centrifugation at 20,000g, the extracts were supplemented with NEBuffer Pack for Protein Metallophosphatases, $MnCl_2$, and λ protein phosphatase (NEW ENGLAND BioLabs) according to the manufacture's instruction, and then incubated for 30 min at 30 °C.

2.5. Western blotting and immunoprecipitations

Western blotting and immunoprecipitations were performed as described before [21,22].

3. Results and discussion

3.1. Ca^{2+}-dependent posttranslational modification of Tespa1 protein

To explore the possibility that Tespa1 protein is modified by a particular signaling pathway, we treated thymocytes with ionomycin (an ionophore which raises the intracellular Ca^{2+} level), PMA (phorbol 12-Myristate 13-acetate which activates protein kinase C), or forskolin (an activator of adenylate cyclase, the enzyme that converts adenosine triphosphate into cyclic adenosine monophosphate) *in vitro*, and examined Tespa1 protein by immunoblotting (Fig. 1A). In comparison with that treated with DMSO as a control, Tespa1 protein was appeared to be retarded toward a high molecular weight area on the immunoblot upon the treatment with ionomycin but not PMA or forskolin, suggesting that Tespa1 protein was modified by a signaling pathway raised by an intracellular Ca^{2+} increase. To check whether the Tespa1 protein modification upon ionomycin treatment is a posttranslational modification, we utilized a CHX (cycloheximide which is a protein synthesis inhibitor). Tespa1 protein modification was induced by the treatment of thymocytes with ionomycin even in the presence of CHX (Fig. 1B), showing that the Tespa1 protein modification did not require the *de novo* protein synthesis and should be posttlanslationally processed. Next, to examine whether the Tespa1 protein modification upon ionomycin treatment is mediated by an intracellular Ca^{2+} increase, thymocytes were pretreated with the cell-permeable Ca^{2+} chelator (BAPTA), revealing that the Tespa1 protein modification induced by ionomycin was totally quenched by BAPTA treatment

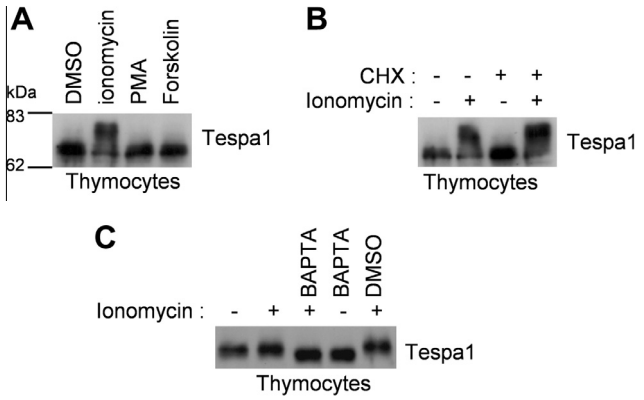

Fig. 1. Ca^{2+}-dependent posttranslational modification of Tespa1 protein. (A) Immunoblot of Tespa1 protein from thymocytes treated with DMSO, ionomycin, PMA, or forskolin for 30 min. (B) Immunoblot of Tespa1 protein from thymocytes treated with or without ionomycin for 30 min after the pretreatment with or without CHX. (C) Immunoblot of Tespa1 protein from thymocytes treated with or without ionomycin for 30 min after the pretreatment with or without BAPTA. DMSO was used as a control reagent. Pretreatment was performed 15 min prior to each stimulation. Data are representative of three independent experiments.

(Fig. 1C). Taken together, Tespa1 protein was posttranslationally modified upon intracellular Ca^{2+} increase in thymocytes.

3.2. Posttranslational modification of Tespa1 protein is mediated by store-operated Ca^{2+} entry

To address the mechanism of the Ca^{2+}-dependent posttranslational modification of Tespa1, we utilized an acute T lymphoblastic leukemia cell line, Jurkat cells, in which TCR-stimulation triggers IP_3R-mediated Ca^{2+} efflux from intracellular Ca^{2+} stores followed by SOCE from extracellular environment. In Jurkat cells, TCR-stimulation by using an anti-CD3 antibody induced Tespa1 protein modification in the presence or absence of CHX (Fig. 2A), indicating that TCR-stimulation triggers the posttranslational modification of Tespa1 protein in Jurkat cells. Through the applications of various inhibitors such as BTP2 [a cell-permeable analog of 3,5-bis(trifluoromethyl) pyrazole which specifically inhibits store-operated Ca^{2+} influx], $NiCl_2$ (T-type Ca^{2+} channel blocker), nifedipine (L-type Ca^{2+} channel blocker), or EGTA (ethylene glycol tetraacetic acid which is a cell-impermeable Ca^{2+} chelator), the Tespa1 protein modification upon TCR-stimulation was inhibited by BTP2 and EGTA but not $NiCl_2$ or nifedipine (Fig. 2B), suggesting that SOCE is the critical mediator for the Tespa1 protein modification. Additionally, as EGTA inhibited the Tespa1 protein modification induced by TCR-stimulation, it was found that Ca^{2+} efflux from intracellular stores was not able to modify Tespa1 protein without extracellular Ca^{2+} influx. To further address the relevance of SOCE to the Tespa1 protein modification, Jurkat cells were treated with thapsigargin [sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor] which induces passive Ca^{2+} leak from intracellular store and the subsequent SOCE from extracellular environment, revealing that Tespa1 protein modification was induced by the thapsigargin treatment (Fig. 2C, top). The Tespa1 protein modification induced by thapsigargin was inhibited by pretreatment with BTP2 (Fig. 2C, top), indicating that SOCE itself ,but not Ca^{2+} leak from intracellular store, was required for the Tespa1 protein modification. Furthermore, we treated thymocytes with thapsigargin in the presence or absence of BTP2, revealing that Tespa1 protein modification was induced by thapsigargin and the modification was inhibited by pretreatment with BTP2 (Fig. 2C, bottom). Taken together, posttranslational modification of Tespa1 protein induced by TCR-stimulation was mediated by SOCE and this mechanism of

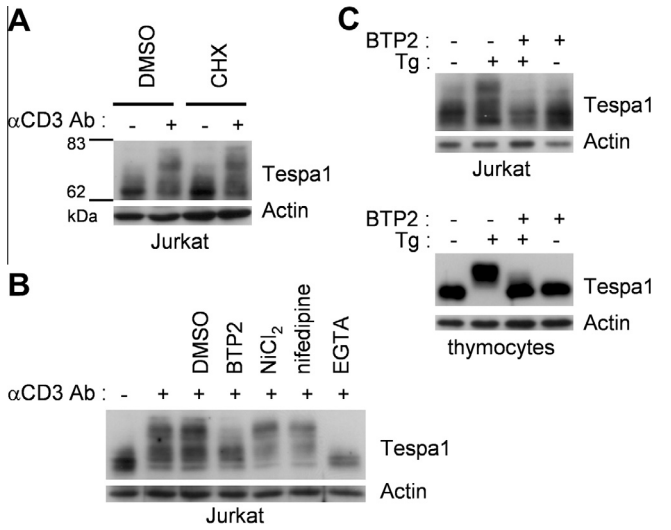

Fig. 2. Posttranslational modification of Tespa1 protein is mediated by store-operated Ca^{2+} entry. (A) Immunoblot of Tespa1 protein from Jurkat cells treated with or without anti-CD3 antibody (αCD3 Ab) for 30 min after the pretreatment with CHX or DMSO. (B) Immunoblot of Tespa1 protein from Jurkat cells treated with or without αCD3 Ab for 30 min after the pretreatment with DMSO, BTP2, $NiCl_2$, nifedipine, or EGTA. (C) Immunoblot of Tespa1 protein from Jurkat cells treated with or without thapsigargin (Tg) for 30 min after the pretreatment with or without BTP2 (top). Immunoblot of Tespa1 protein from thymocytes treated with or without thapsigargin (Tg) for 30 min after the pretreatment with or without BTP2 (bottom). Actin was detected as a loading control. Data are representative of three independent experiments.

the Ca^{2+}-dependent posttranslational modification of Tespa1 was well-conserved in the immortalized T lymphoblastic leukemia cell line, Jurkat, and primary T lymphocytes.

3.3. Ca^{2+}-dependent posttranslational modification of Tespa1 protein is due to phosphorylation

To analyze what kind of molecular modification of Tespa1 protein was induced upon intracellular Ca^{2+} increase, we performed *in vitro* phosphatase assay in which protein extracts from thymocytes stimulated by ionomycin were treated *in vitro* with λ protein phosphatase. Posttranslational modification of Tespa1 protein induced by ionomycin treatment was totally restored by *in vitro* phosphatase treatment (Fig. 3A), indicating that Ca^{2+}-dependent posttranslational modification of Tespa1 protein was due to phosphorylation.

As Tespa1 was previously reported to possess substantial amino acid sequence homology to KRAP [10], we next examined whether Ca^{2+}-dependent phosphorylation is a conserved molecular modification between Tespa1 and KRAP proteins. When we stimulated HCT116 human colon cancer cells or HEK293 human embryonic kidney cells with ionomycin, KRAP protein was appeared to be slightly retarded toward a high molecular weight area on the immunoblots compared with the control without the stimulation (Fig. 3B), suggesting that KRAP protein was modified upon the ionomycin treatment. The KRAP protein modification was quenched by pretreatment with BAPTA (Fig. 3B), showing that intracellular Ca^{2+} increase was responsible for the modification. Moreover, we performed *in vitro* phosphatase assay in which protein extracts from HCT116 cells or HEK293 cells stimulated by ionomycin were treated *in vitro* with λ protein phosphatase, revealing that modification of KRAP protein induced by ionomycin treatment was totally restored by *in vitro* phosphatase treatment (Fig. 3C). These results indicated that Ca^{2+}-dependent phosphorylation is a conserved molecular modification between Tespa1 and KRAP proteins, and may suggest that serine, threonine, and/or tyrosine residues conserved between these proteins are responsible for the phosphorylation.

3.4. Physical association of Tespa1 with IP_3R is maintained regardless of the presence or absence of phosphorylation of Tespa1

Because Tespa1 was previously reported to be an associated protein of IP_3R [10], we herein examined whether Ca^{2+}-dependent phosphorylation of Tespa1 protein would affect the physical association between Tespa1 and IP_3R proteins. Protein extracts from thymocytes cultured in distinct conditions, i.e. SOCE was induced or not, were subjected to co-immunoprecipitation assay, revealing that co-immunoprecipitation of Tespa1 and IP_3R3 proteins was detected regardless of the presence or absence of phosphorylation of Tespa1 (Fig. 4). This result indicated that the physical association of Tespa1 with IP_3R occurred in both conditions, non-stimulated and stimulated, and that the Ca^{2+}-dependent phosphorylation of Tespa1 could be a molecular tag reflecting the signaling status whether SOCE influences the Tespa1–IP_3R complex.

In this study, we found that Tespa1 protein was postranslationally modified by phosphorylation in response to TCR stimulation-induced intracellular Ca^{2+} increase. SOCE was responsible for triggering the phosphorylation of Tespa1, whereas Ca^{2+} efflux from intracellular stores was not enough to cause the modification.

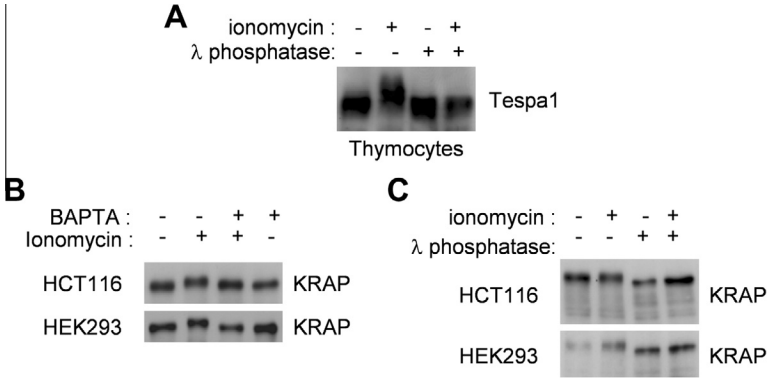

Fig. 3. Ca^{2+}-dependent posttranslational modification of Tespa1 protein is due to phosphorylation. (A) Immunoblot of Tespa1 protein after *in vitro* λ protein phosphatase (λ phosphatase) treatment of extracts from thymocytes treated with or without ionomycin. As the control experiment, extracts were incubated without λ protein phosphatase. (B) Immunoblot of KRAP protein from HCT116 cells or HEK293 cells treated with or without ionomycin for 30 min after the pretreatment with or without BAPTA. (C) Immunoblot of KRAP protein after *in vitro* λ protein phosphatase treatment of extracts from HCT116 cells or HEK293 cells treated with or without ionomycin. Data are representative of three independent experiments.

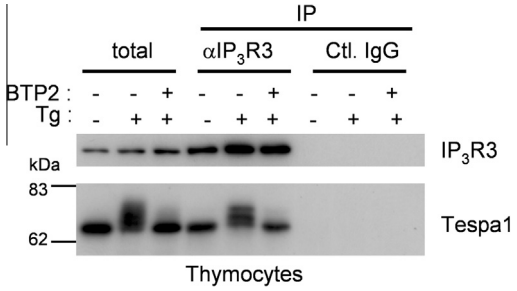

Fig. 4. Physical association of Tespa1 with IP_3R is maintained regardless of the presence or absence of phosphorylation of Tespa1. Anti-IP_3R3 (αIP_3R3) immunoprecipitations were performed using mouse thymocytes treated for 30 min with or without Tg in the presence or absence of BTP2 as indicated, followed by western blotting with anti-IP_3R3 or anti-Tespa1 antibodies. Total, total lysate; IP, immunoprecipitation; Ctl., control.

Many studies have shown that TCR-stimulation induces the transient release of Ca^{2+} from ER store through the activation of IP_3R, and this leads to prolonged Ca^{2+} influx across the plasma membrane, a process termed SOCE, which results in the activation of Ca^{2+}-dependent enzymes and transcription factors [3–8]. However, it remains unknown whether SOCE affects molecular components which are involved in the regulation of Ca^{2+} efflux from intracellular Ca^{2+} store. Here, our finding provided a novel molecular modification of Tespa1 and suggested the existence of the regulatory pathway that SOCE influences Tespa1–IP_3R molecular complex, a critical regulator of intracellular Ca^{2+} store. If SOCE positively or negatively regulates IP_3R functions, the regulatory pathway would become therapeutic targets for various diseases. Although exact elucidation of mechanisms underlying the SOCE-dependent phosphorylation of Tespa1 should await future studies, it is not likely that Ca^{2+}/calmodulin-dependent kinases (CAMKs) is responsible for the Tespa1 modification because of the absence of the inhibitory effect of CAMKs inhibitor against the Tespa1 modification (data not shown). Considering our finding that KRAP protein in epithelial cells is also phosphorylated in a similar manner to Tespa1, there might be conserved mechanisms phosphorylating KRAP and Tespa1 in epithelial cells and lymphocytes, respectively. Further investigations concerning the effects of these Tespa1 and KRAP protein modifications on IP_3R functions will provide better understanding of numerous biological processes.

Acknowledgments

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) – supported Program for the Strategic Research Foundation at Private Universities, a Grant-in-Aid for Young Scientists (B) Grant Number 24710265. This work was supported in part by funds (No.: 112503) from the Central Research Institute of Fukuoka University. We thank Takami Danno for technical assistance.

References

[1] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signaling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21.
[2] M.J. Berridge, Inositol trisphosphate and calcium signalling mechanisms, Biochim. Biophys. Acta 1793 (2009) 933–940.
[3] J.W. Putney, Capacitative calcium entry: from concept to molecules, Immunol. Rev. 231 (2009) 10–22.
[4] Y. Baba, T. Kurosaki, Physiological function and molecular basis of STIM1-mediated calcium entry in immune cells, Immunol. Rev. 231 (2009) 174–188.
[5] S. Feske, ORAI1 and STIM1 deficiency in human and mice. Roles of store-operated Ca^{2+} entry in the immune system and beyond, Immunol. Rev. 231 (2009) 189–209.
[6] V. Robert, E. Triffaux, M. Savigna, L. Pelletier, Calcium signalling in T-lymphocytes, Biochimie 93 (2011) 2087–2094.
[7] Y. Baba, T. Kurosaki, Impact of Ca^{2+} signaling on B cell function, Trends Immunol. 32 (2011) 589–594.
[8] S. Feske, E.Y. Skolnik, M. Prakriya, Ion channels and transporters in lymphocyte function and immunity, Nat. Rev. Immunol. 12 (2012) 532–547.
[9] D. Wang, M. Zheng, L. Lei, et al., Tespa1 is involved in late thymocyte development through the regulation of TCR-mediated signaling, Nat. Immunol. 13 (2012) 560–568.
[10] H. Matsuzaki, T. Fujimoto, T. Ota, et al., Tespa1 is a novel IP_3R-binding protein in T- and B-lymphocytes, FEBS Open Bio 2 (2012) 255–259.
[11] T. Jayaraman, E. Ondriasová, K. Ondrias, et al., The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling, Proc. Natl. Acad. Sci. USA 92 (1995) 6007–6011.
[12] A.A. Khan, M.J. Soloski, A.H. Sharp, et al., Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor, Science 273 (1996) 503–507.
[13] H. Sugawara, M. Kurosaki, M. Takata, et al., Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor, EMBO J. 16 (1997) 3078–3088.
[14] A.M. Scharenberg, L.A. Humphries, D.J. Rawlings, Calcium signalling and cell-fate choice in B cells, Nat. Rev. Immunol. 7 (2007) 778–789.
[15] N. de Souza, J. Cui, M. Dura, et al., A function for tyrosine phosphorylation of type 1 inositol 1,4,5-trisphosphate receptor in lymphocyte activation, J. Cell Biol. 179 (2007) 923–934.
[16] R.L. Patterson, D. Boehning, S.H. Snyder, Inositol 1,4,5-trisphosphate receptors as signal integrators, Annu. Rev. Biochem. 73 (2004) 437–465.
[17] I. Bezprozvanny, The inositol 1,4,5-trisphosphate receptors, Cell Calcium 38 (2005) 261–272.
[18] J.K. Foskett, C. White, K.H. Cheung, et al., Inositol trisphosphate receptor Ca^{2+} release channels, Physiol. Rev. 87 (2007) 593–658.
[19] K. Mikoshiba, IP_3 receptor/Ca^{2+} channel: from discovery to new signaling concepts, J. Neurochem. 102 (2007) 1426–1446.
[20] J.B. Parys, H. De Smedt, Inositol 1,4,5-trisphosphate and its receptors, Adv. Exp. Med. Biol. 740 (2012) 255–279.
[21] T. Fujimoto, M. Koyanagi, I. Baba, et al., Analysis of KRAP expression and localization, and genes regulated by KRAP in a human colon cancer cell line, J. Hum. Genet. 52 (2007) 978–984.
[22] T. Fujimoto, T. Machida, Y. Tanaka, et al., KRAS-induced actin-interacting protein is required for the proper localization of inositol 1,4,5-trisphosphate receptor in the epithelial cells, Biochem. Biophys. Res. Commun. 407 (2011) 438–443.