Biochemical and Biophysical Research Communications 371 (2008) 22–27

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Suppression of protein L-isoaspartyl (D-aspartyl) methyltransferase results in hyperactivation of EGF-stimulated MEK-ERK signaling in cultured mammalian cells ☆

Sakurako Kosugi [a], Takemitsu Furuchi [a], Masumi Katane [a], Masae Sekine [a], Takuji Shirasawa [b,1], Hiroshi Homma [a,*]

[a] Laboratory of Biomolecular Science, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
[b] Research Team for Molecular Biomarkers, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

ARTICLE INFO

Article history:
Received 6 March 2008
Available online 31 March 2008

Keywords:
Protein L-isoaspartyl (D-aspartyl) methyltransferase
Isomerization
Racemization
Protein repair
Epidermal growth factor
MAPK cascade

ABSTRACT

L-Aspartyl (L-Asp) and L-asparaginyl residues in proteins isomerize or racemize to D,L-isoaspartyl (D,L-isoAsp) or D-aspartyl (D-Asp) residues during protein aging. These atypical aspartyl residues can interfere with the biological function of the protein and lead to cellular dysfunction. Protein L-isoaspartyl (D-aspartyl) methyltransferase (PIMT) is a repair enzyme that facilitates conversion of L-isoAsp and D-Asp to L-Asp. PIMT deficient mice exhibit accumulation of L-isoAsp in several tissues and die, on average, 12 days after birth from progressive epileptic seizures with grand mal and myoclonus features. However, little is known about the molecular mechanisms by which accumulation of the aberrant residues leads to cellular abnormalities. In this study, we established PIMT-knockdown cells using a short interfering RNA expression system and characterized the resultant molecular abnormalities in intracellular signaling pathways. PIMT-knockdown cells showed significant accumulation of proteins with isomerized residues, compared to control cells. In the PIMT-knockdown cells, Raf-1, MEK, and ERK, members of the MAPK cascade, were hyperphosphorylated after EGF stimulation compared to control cells. These results suggest that PIMT repair of abnormal proteins is necessary to maintain normal MAPK signaling.

L-Aspartyl residues (L-Asp) and L-asparaginyl residues in proteins racemize or isomerize to D-aspartyl and D,L-isoaspartyl residues (D,L-isoAsp) under physiological conditions during the process of protein aging [1–5]. These posttranslational modifications may result in a dysfunctional protein, since these modifications change the conformation of the protein backbone as well as the number of carbon atoms [6–8]. Protein L-isoaspartyl (D-aspartyl) methyltransferase (PIMT) facilitates the conversion of these isomerized aspartyl residues to L-Asp.

PIMT-knockout mice die at an average age of 12 days after birth from progressive epileptic seizures with grand mal and myoclonus features [9]. *Escherichia coli* cells in which the gene encoding PIMT is disrupted are sensitive to heat shock and oxidative stress [10]. In addition, overexpression of PIMT in *Drosophila melanogaster* increases the lifespan of the mutant flies [11]. These studies suggest that PIMT is able to repair abnormal proteins *in vivo* in which racemized or isomerized Asp residues accumulate during protein aging or under conditions of cell stress. However, little is known about the molecular mechanisms by which accumulation of the aberrant residues leads to pathological abnormalities such as neurogenerative disorders.

PIMT-knockout mice display abnormal arborization of the dendrites of pyramidal neurons and an increased brain size [9]. Furthermore, splenocytes and $CD4^+$ T cells from PIMT-knockout mice exhibit increased proliferation in response to mitogen and receptor-mediated stimulation, compared to wild-type mice [12]. These studies suggest that PIMT deficiency (and the resultant accumulation of deleterious proteins) increases cell prolifer-

Abbreviations: DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; isoAsp, isoaspartyl residues; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PIMT, protein L-isoaspartyl (D-aspartyl) methyltransferase; RKIP, Raf kinase inhibitory protein; rPIMT, recombinant PIMT; SAM, *S*-adenosyl-L-methionine; siRNA, short interfering RNA.

☆ Some of the results described in this communication were presented orally at the 127th Annual Meeting of Pharmaceutical Society of Japan held in March 28th, 2007 [29].

* Corresponding author. Fax: +81 3 5791 6381.
E-mail address: hommah@pharm.kitasato-u.ac.jp (H. Homma).
[1] Present address: Juntendo University School of Medicine, 3-3-10-201, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.03.109

ation by affecting components of signaling pathways. In this study, we first established several lines of PIMT-knockdown cells using a short interfering RNA (siRNA) expression system, and characterized the resultant molecular abnormalities in intracellular signaling pathways (in particular, the mitogen-activated protein kinase (MAPK) cascade) in response to epidermal growth factor (EGF) stimulation.

Materials and methods

Materials. An anti-active MAPK (pTEpY) antibody was obtained from Promega (Madison, WI, USA). Anti-p44/p42 MAP kinase, anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2, and anti-phospho-c-Raf (Ser338) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti c-Raf was obtained from BD Biosciences (San Jose, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from American Research Products (Belmont, MA, USA). The anti-PIMT-I antibody was raised in rabbits immunized with a synthetic peptide corresponding to residues Trp^{222}-Lys^{226} of mouse PIMT-I, as described previously [13,14].

Cell culture. Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. The cells were passaged every 7 days.

Construction of siRNA expression plasmids. The H1 promoter was amplified by PCR using the following primers: H1pro-F, 5′-GGATCCGAGTGGTCTCATACA GAACTTATAAGATTCCCAAATCCAAAGACATTTCAC-3′; and H1pro-R, 5′-GTTTCTGT AAAGTGCAAATACCACTAAAGGGTCTTGTGTATCGCTGTACGTTTATAA-3′. These primers are complementary to each other at the underlined sequences and act as templates for one another. The H1 promoter was subcloned into pcDNA3.1(+) (Invitrogen, Carsbad, CA, USA) between EcoRI and BamHI and the CMV promoter was deleted from pcDNA3.1(+) by restriction enzyme digestion with NheI and BglII, followed by blunt ending with Klenow fragment and self-ligation. Hairpin-type siRNA-expression sequences were chemically synthesized and subcloned into H1pro/pcDNA3.1(+) between the BamHI and HindIII sites after annealing and phosphorylation with T4 kinase. Hairpin-type siRNA-expression sequences were as follows: hPIMT si-1 F, 5′-GATCCCATGGAATCATCAAGACAGATTCAAGAGATCTGTCTTGATGAT TCCATTTTTTTGGAAA-3′; hPIMT si-1 R, 3′-GGTACCTTAGTAGTTCTGTCTAAGTTCTC TAGACAGAACTACTAAGGTAAAAAAACCTTTTCGA-5′; hPIMT si-3 F, 5′-GATCCC GTGATGCTGGCTACAGACCTTCAAGAGAGGTCTGTAGCCAGCATCACTTTTTTGGAAA-3; hPIMT si-3 R, 3′-GGCACTACGACCGATGTCTGGAAGTTCTCTCCAGACATCGGTCGTAGT GAAAAAAACCTTTTCGA-5′; control-si F, 5′-GATCCCAATGACATAGACATGGCAATT CAAGAGATTGCCATGTCTATGTCATTTTTTTTGGAAA-3′; control-si R, 3′-GGTTACTG TATCTGTACCGTTAAGTTCTCTAACGGTACAGATACAGTAAAAAAACCTTTTCGA-5′. These sequences were designed to generate BamHI and HindIII cohesive ends at the 5′ and 3′ ends (underlined sites) when each F and R pair were annealed. The control siRNA is a randomized sequence.

Stable transfection of siRNA expression plasmids. Plasmid DNA was transfected into subconfluent HEK-293 cells using the *Trans*IT-293 transfection reagent (Mirus Bio Corp.) according to the manufacturer's instructions. Stable transfectants were selected by culturing in medium containing 1 mg/mL G418 (Promega) for 2–4 weeks.

Northern blotting. Total RNA (20 μg), extracted from HEK-293 cells using Isogen (Nippon Gene, Tokyo, Japan), was separated on 1% agarose gels containing 18% formaldehyde, blotted with 10× SSC onto a nylon membrane (Schleicher & Schuell), and hybridized to a digoxigenin (DIG)-labeled RNA probe recognizing PIMT. Hybridization was performed at 68 °C overnight with DIG Easy Hyb Granules (Roche Applied Science, Mannheim, Germany). Signals were detected using chemiluminescent detection with CDP-Star (Roche Applied Science).

Cloning, expression and purification of recombinant human PIMT. The human PIMT coding sequence was amplified from HEK-293 cDNA by RT-PCR and subcloned into pRSET-B (Invitrogen) between the BamHI and EcoRI sites. The primers used were: hPIMT–BamHI—F, 5′-TAAGGATCCGATGGCCTGGAAATCCGGC-3′; hPIMT–EcoRI—R, 3′-TGGTCCAGGTGGAAGTGAGAATTCGAA-5′. These sequences were designed to generate BamHI and EcoRI restriction sites at the 5′ and 3′ ends (underlined sites), respectively. *E. coli* BL21(DE3)pLysS transformed with the plasmid was cultured overnight with 0.01 mM isopropyl-β-D-thiogalactoside and solubilized using the Bug Buster Protein Extraction Reagent (Novagen, Madison, WI, USA). The His-tagged recombinant PIMT was purified by affinity chromatography using His GraviTrap (GE Healthcare, Buckinghamshire, UK). The protein fraction was eluted with buffer containing 200 mM imidazole and dialyzed at 4 °C for 6 h against 20 mM potassium phosphate, pH 7.5, 10% glycerol, and 5 mM 2-mercaptoethanol. The specific activity of the purified PIMT was 11 nmol/min/mg protein using the delta sleep-inducing peptide as the methyl acceptor.

Detection of isoaspartate residues in HEK-293 cells by SDS–PAGE and [^{3}H]fluorography. HEK-293 cell lysates were prepared by incubating cell pellets on ice for 60 min in lysis buffer containing PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Roche Applied Science), followed by passage through a 20-gauge needle. Insoluble material and nuclei were removed by centrifugation at 10,000g and 4 °C for 10 min, and the lysates were stored at −80 °C until use. Protein concentration in the lysate was determined by the method of Bradford. Methylation of proteins in the HEK-293 cell lysate was carried out at 37 °C for 10 min, in a reaction mixture containing 100 mM sodium phosphate, pH 6.8, 1 mM EGTA, 0.004% sodium azide, 0.16% Triton X-100, 14 μM purified PIMT, and 1.8 μM [^{3}H]methyl-SAM (3.15 TBq/mmol, PerkinElmer). Reactions were terminated by addition of 3× SDS–PAGE sample buffer (pH 6.8). After heating at 50 °C for 5 min, samples were subjected to SDS–PAGE on Novex NuPAGE 4–12% gradient gels using MES running buffer (Invitrogen). Electrophoresis was carried out at 100 V at 4 °C to minimize methyl ester hydrolysis. Gels were stained with Coomassie Brilliant Blue, and subjected to fluorography using the Amplify Fluorographic Reagent (GE Healthcare). The intensity of each band was quantified using NIH Image software Ver.1.63.

EGF stimulation. HEK-293 cells were plated at 9×10^6 cells/well in six-well plates and grown in culture medium for 27 h at 37 °C. EGF (1 μg/ml, Sigma, St. Louis, MO, USA) was added to the medium and the cells were further incubated at 37 °C for the indicated times. Incubations were stopped by the addition of ice cold PBS.

Western blotting. Whole cell lysates were prepared by incubating cultured cells in 2% SDS at 100 °C for 10 min. Protein concentration in the lysates was determined by the Lowry method. The proteins in the cell lysates were then separated on polyacrylamide gels ranging from 8% to 12.5%, depending on the proteins to be analyzed. After electrophoresis, the proteins were electrotransferred to a PVDF membrane. Antibody–antigen complexes were detected by enhanced chemiluminescence (ECL, GE Healthcare) and visualized by exposure to X-ray film (Lumi-Film Chemiluminescent Detection Film, Roche). After chemilu-

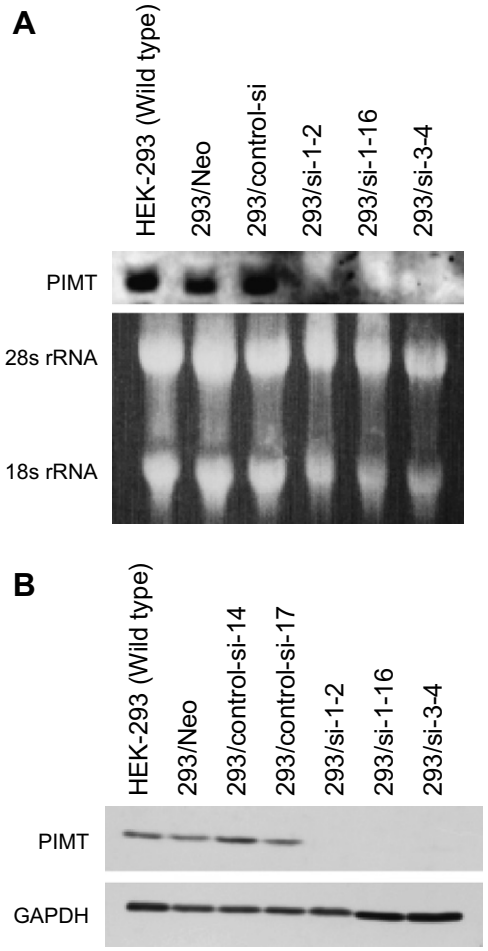

Fig. 1. Expression levels of PIMT in PIMT-knockdown HEK-293 cells. (A) Expression level of PIMT mRNA in each cell line was determined by Northern blotting with a DIG-labeled RNA probe for human PIMT (upper panel). The abundance of 28S and 18S rRNA is shown as a loading control (ethidium bromide staining, lower panel). Each lane contains 20 μg of total RNA. 293/si-1-2 and 293/si-1-16 are independently isolated clones. (B) Expression levels of PIMT protein in each cell line were analyzed by Western blotting with anti-PIMT-1. As a loading control, levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also determined. Each lane contains 30 μg of protein.

minescent detection, the membrane was stripped and reprobed with the indicated antibodies under the same conditions. The intensity of each band was quantified as described above.

Results

Isolation of PIMT-knockdown cells

To investigate the physiological role of PIMT, we generated cell lines with reduced levels of endogenous PIMT using siRNA. We selected two sites in the hPIMT gene as targeting sequences for construction of PIMT-short-hairpin RNA plasmids: +130 to +151 (si-1) and +162 to +183 (si-3). As a negative control, we designed a scrambled sequence (control-si), which consisted of the same nucleotides as si-1, but in randomized order. Hairpin-type siRNA expression plasmids were constructed using the mammalian expression vector pcDNA3.1(+) as described in Materials and methods. These siRNA expression plasmids (si-1 and si-3) were transfected into HEK-293 cells, and stably transfected cells were isolated by G418 selection. To confirm the effects of the siRNA expression plasmids on the PIMT expression levels in these isolated cells, we performed Northern blot analysis using a DIG-labeled probe against human PIMT mRNA. As shown in Fig. 1A, three clones, 293/si-1-2, 293/si-1-16, and 293/si-3-4 showed significant decreases in PIMT mRNA levels compared to wild-type HEK-293 cells. We also isolated control cell lines which were stably transfected with a CMV promoter-eliminated pcDNA3.1(+) empty vector (293/Neo) or a sequence-randomized siRNA expression plasmid (293/control-si). These control cells expressed levels of PIMT mRNA comparable to wild-type cells (Fig. 1A). In Fig. 1B, protein levels of PIMT were examined by Western blot analysis using an anti-PIMT-1 antibody. In agreement with the Northern blot analysis, the PIMT protein levels of the transfectants 293/si-1-2, 293/si-1-16, and 293/si-3-4 were markedly reduced compared to wild-type and control HEK-293 cells.

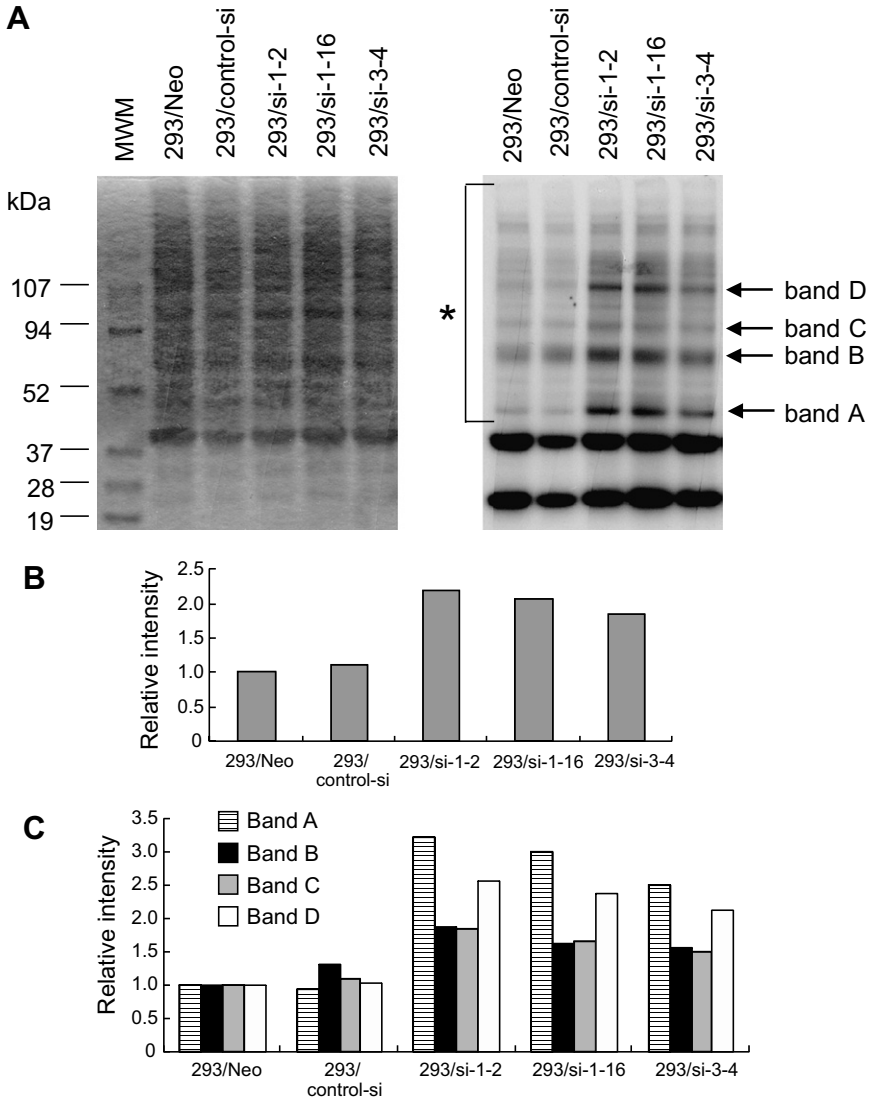

Fig. 2. Accumulation of isomerized or racemized proteins in PIMT-knockdown HEK-293 cells. Total cell lysates (40 μg protein) from each line of HEK-293 cells were incubated with recombinant human PIMT and [^{3}H]methyl-SAM to methylate isomerized or racemized aspartyl residues, as described in Materials and methods. Subsequently, methylated proteins were subjected to SDS–PAGE and fluorography. (A) Coomassie brilliant blue stained gel. No differences in protein composition between cell lines were apparent, and similar amounts of protein were present in each lane. (B) Proteins methylated by PIMT were visualized by fluorography. (C) Band intensities in the upper area of the fluorogram (B: ∗) were measured in each lane, using NIH Image, and normalized by the intensity of the band in the Coomassie brilliant blue-stained gel (A). The band intensity of each cell line is presented relative to the 293/Neo intensities. (D) The intensities of the protein bands A–D in the fluorogram (B) were measured in each lane using NIH Image, and normalized to the intensity of the corresponding band in the Coomassie brilliant blue-stained gel (A).

Accumulation of racemized or isomerized proteins in PIMT-knockdown cells

We next investigated whether racemized or isomerized proteins accumulated in PIMT-knockdown cells (Fig. 2A–D). Cell lysates prepared from PIMT-knockdown cells or control cells were incubated with recombinant PIMT (rPIMT) in the presence of [^{3}H]methyl-SAM. Subsequently, the proteins were separated by SDS–PAGE to detect [^{3}H]methyl groups incorporated into the proteins by the rPIMT-catalyzed reaction using fluorography (Fig. 2). Densitometric analysis showed that the incorporation of [^{3}H]methyl groups into total protein was approximately twofold higher in the PIMT-knockdown cells, 293/si-1-2, 293/si-1-16, and 293/si-3-4, than in the control cells, 293/Neo and 293/control-si (Fig. 2C). Further quantitation of several major bands demonstrated that the intensities of bands A, B, C, and D in these PIMT-knockdown cells displayed approximately 3.0-, 1.7-, 1.7-, and 2.4-fold increases, respectively, compared to those of control cells (293/Neo) (Fig. 2D). These results indicate that silencing of PIMT expression results in the accumulation of proteins with racemized or isomerized residues in HEK-293 cells

Hyperactivation of EGF-induced signaling in PIMT-knockdown cells

We subsequently investigated the effect of PIMT suppression on the MAP kinase cascade, which is involved in regulation of cell growth and differentiation. PIMT-knockdown cells and control cells were stimulated with 1 μg/mL EGF for 0, 5, 10, or 30 min at 37 °C and the amount of activated ERK1/2 was then detected by Western blot analysis using an anti-pTEpY motif antibody, followed by quantitation of band intensities. Fig. 3A and B show that EGF induced the transient phosphorylation of ERK1/2 with peak phosphorylation at 5 to 10 min after stimulation in both control (293/Neo and 293/control-si) and PIMT-knockdown (293/si-1-16) cells. In the PIMT-knockdown cells, however, the ERK1/2 phosphorylation was markedly enhanced compared to control cells. Quantitation of total ERK levels relative to those of GAPDH demonstrated that the total ERK levels did not change during the course of the experiment (data not shown).

ERKs are activated through phosphorylation by the upstream kinase MEK. To determine whether hyperphosphorylation of ERKs in the PIMT-knockdown cells was caused by increased activation of MEK, we examined the activated levels of MEK after EGF stimulation. In both control and PIMT-knockdown cells, transient phos-

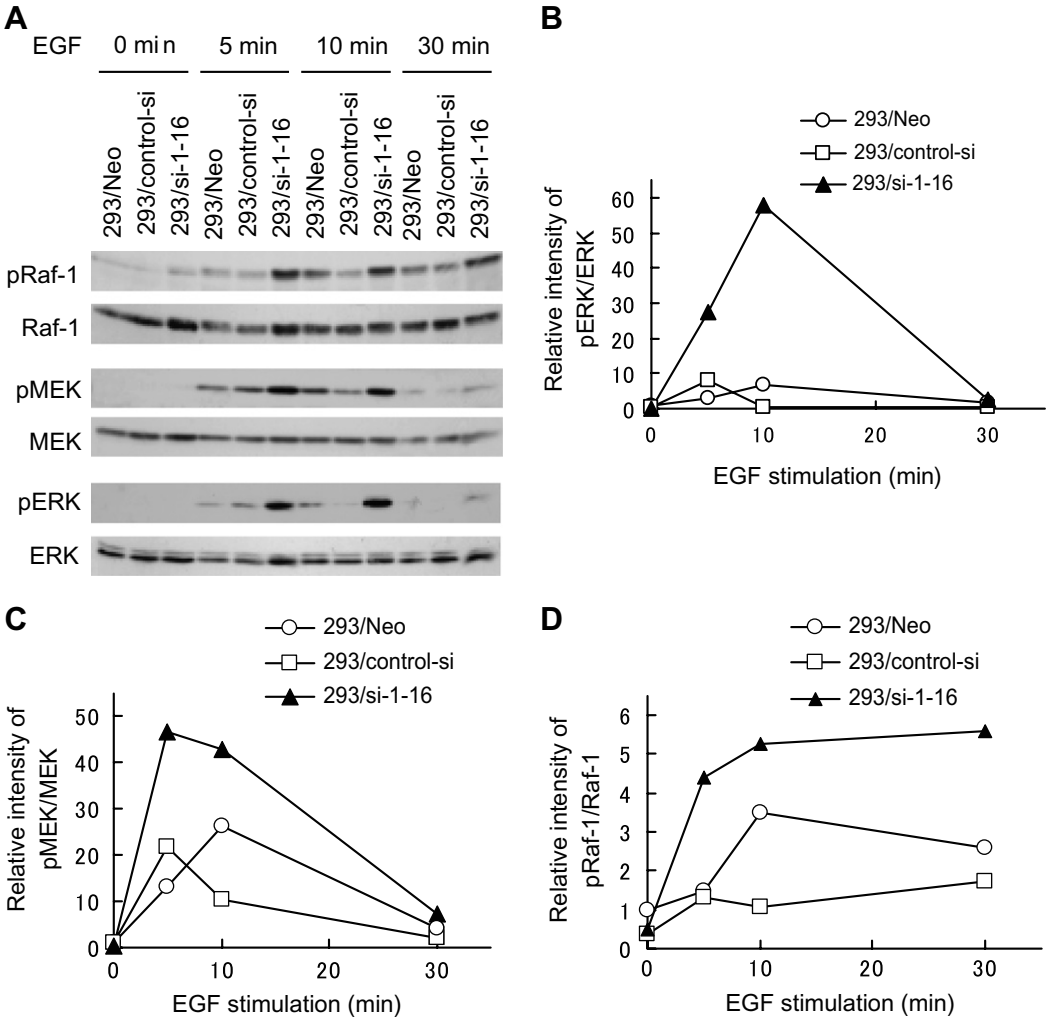

Fig. 3. EGF-stimulated Raf-1, MEK-1/2, and ERK-1/2 activation is increased in PIMT-knockdown cells. (A) HEK-293 cells (293/Neo, 293/control-si, and 293/si-1-16) were stimulated with 1 μg/mL EGF for 0, 5, 10, or 30 min, and the cell lysates were immunoblotted with the indicated antibodies. Each lane contains 15 μg of total cell lysate. Similar results were obtained in three separate experiments. (B–D) Phosphorylation levels of ERK-1/2 (B), MEK-1/2 (C) and Raf-1 (D). The intensities of phosphorylated ERK-1/2 (pERK), MEK-1/2 (pMEK) and Raf-1 (pRaf-1) were quantified using NIH Image. The total (phosphorylated and unphosphorylated) level of each protein was also measured, and a ratio (phosphorylated level/total level) was calculated.

phorylation of MEK was observed with the highest levels at 5–10 min after stimulation, and hyperphosphorylation of MEK was demonstrated in the PIMT-knockdown cells (Fig. 3A and C). Since MEK is activated by Raf-1, we next examined Raf-1 activation after EGF stimulation. Since phosphorylation of Raf-1 at Ser338 is essential for its activation by Ras as well as its recruitment to the plasma membrane [15], we used an antibody against Ser338 phosphorylated Raf-1 to detect activated Raf-1. As shown in Fig. 3A and D, Raf-1 was quickly phosphorylated, within 5–10 min, and remained phosphorylated up to 30 min after EGF stimulation both in control and PIMT-knockdown cells. The phosphorylation levels of Raf-1 in the PIMT-knockdown cells were consistently higher for up to 30 min after EGF stimulation, compared with levels in control cells. Similar results were obtained with the other PIMT-knockdown cells, 293/si-1-2 and 293/si-3-4 (data not shown). These lines of evidence suggest that repair of abnormal protein(s) by PIMT is necessary to maintain normal MAPK signaling.

Discussion

Immunoblot analysis in this study revealed that upon EGF stimulation, Raf-1, MEK-1/2 and ERK-1/2, components of the MAPK cascade, were phosphorylated to significantly higher levels in PIMT-knockdown cells than in control cells (Fig. 3). The MAPK cascade has been reported to play important roles in the regulation of dendritic arborization of neurons in the brain [16]. Thus, abnormal dendritic arborization in pyramidal neurons, a major phenotype of the PIMT-knockout mice [9], may result from hyperactivation of the MAPK cascade in the knockout mice.

The mechanism by which hyperactivation of the MAPK cascade occurs in PIMT-knockdown cells upon EGF stimulation remains to be clarified. One possibility is that the function of the Raf kinase inhibitory protein (RKIP), also known as phosphatidylethanolamine binding protein, is disrupted by racemization or isomerization. Recently, RKIP was identified as one protein in the tissues of PIMT-knockout mice, in which accumulation of isomerized aspartyl residue(s) was detected by a proteomic analysis [17]. RKIP regulates EGF-stimulated Raf-1 activation by binding to the Raf-1 kinase domain and inhibiting Raf-1 phosphorylation [18,19]. Binding of RKIP to Raf-1 may decrease as racemized or isomerized residues accumulate, leading to EGF-stimulated hyperphosphorylation of Raf-1 in the PIMT-knockdown cells.

Alternatively, protein phosphatase activity may be reduced by racemization or isomerization. Increased phosphorylation of various signaling proteins has been observed in PIMT-knockout mice compared to wild-type mice [12,20]. Most protein phosphatases have broad substrate specificity [21], and one phosphatase can usually dephosphorylate a number of different substrates. Therefore, a reasonable hypothesis is that a protein phosphatase dysfunction may result in the hyperphosphorylation of many signaling proteins that are components of different signaling cascades. Clinical manifestations of the PIMT-knockout mice are similar to those of patients with Lafora's disease, which is caused by dysfunction of laforin, a dual-specificity phosphatase. [9]. Thus, the PIMT deficiency may also disrupt the function of a phosphatase such as laforin and cause progressive myoclonus epilepsy of the Lafora type.

Protein racemization or isomerization may also facilitate protein aggregation: isomerization of the β-amyloid peptide enhances its aggregation [22,23], and tau protein is a major target for deamidation/isomerization *in vivo* [24,25]. In the human eye, lenses containing cataracts show higher levels of αA-crystalline racemization and isomerization than lenses from young subjects [26–28]. Therefore, further clarification of the molecular mechanism by which PIMT deficiency results in cellular malfunctions would certainly contribute to the understanding not only of epileptic seizures, but also age-related disorders such as the Alzheimer's disease and cataract formation.

Acknowledgments

This research was supported in part by a Kitasato University Research Grant for Young Researchers and a Grant-in-Aid for Scientific Research (18590070) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] M.V. Paranandi, A.W. Guzzetta, W.S. Hancock, D.W. Aswad, Deamidation and isoaspartate formation during in vitro aging of recombinant tissue plasminogen activator, J. Biol. Chem. 269 (1994) 243–253.
[2] J. Najbauer, J. Orpiszewski, D.W. Aswad, Molecular aging of tubulin: accumulation of isoaspartyl sites in vitro and in vivo, Biochemistry 35 (1996) 5183–5190.
[3] N. Fujii, K. Harada, Y. Momose, N. Ishii, M. Akaboshi, D-Amino acid formation induced by a chiral field within a human lens protein during aging, Biochem. Biophys. Res. Commun. 263 (1999) 322–326.
[4] N. Fujii, L.J. Takemoto, Y. Momose, S. Matsumoto, K. Hiroki, M. Akaboshi, Formation of four isomers at the asp-151 residue of aged human alphaA-crystallin by natural aging, Biochem. Biophys. Res. Commun. 265 (1999) 746–751.
[5] D.W. Aswad, M.V. Paranandi, B.T. Schurter, Isoaspartate in peptides and proteins: formation, significance, and analysis, J. Pharm. Biomed. Anal. 21 (2000) 1129–1136.
[6] B.A. Johnson, E.D. Murray Jr., S. Clarke, D.B. Glass, D.W. Aswad, Protein carboxyl methyltransferase facilitates conversion of atypical L-isoaspartyl peptides to normal L-aspartyl peptides, J. Biol. Chem. 262 (1987) 5622–5629.
[7] T.V. Brennan, J.W. Anderson, Z. Jia, E.B. Waygood, S. Clarke, Repair of spontaneously deamidated HPr phosphocarrier protein catalyzed by the L-isoaspartate-(D-aspartate) *O*-methyltransferase, J. Biol. Chem. 269 (1994) 24586–24595.
[8] J. Lanthier, R.R. Desrosiers, Protein L-isoaspartyl methyltransferase repairs abnormal aspartyl residues accumulated in vivo in type-I collagen and restores cell migration, Exp. Cell Res. 293 (2004) 96–105.
[9] A. Yamamoto, H. Takagi, D. Kitamura, H. Tatsuoka, H. Nakano, H. Kawano, H. Kuroyanagi, Y. Yahagi, S. Kobayashi, K. Koizumi, T. Sakai, K. Saito, T. Chiba, K. Kawamura, K. Suzuki, T. Watanabe, H. Mori, T. Shirasawa, Deficiency in protein L-isoaspartyl methyltransferase results in a fatal progressive epilepsy, J. Neurosci. 18 (1998) 2063–2074.
[10] C. Li, S. Clarke, A protein methyltransferase specific for altered aspartyl residues is important in *Escherichia coli* stationary-phase survival and heat-shock resistance, Proc. Natl. Acad. Sci. USA 89 (1992) 9885–9889.
[11] D.A. Chavous, F.R. Jackson, C.M. O'connor, Extension of the *Drosophila* lifespan by overexpression of a protein repair methyltransferase, Proc. Natl. Acad. Sci. USA 98 (2001) 14814–14818.
[12] H.A. Doyle, R.J. Gee, M.J. Mamula, A failure to repair self-proteins leads to T cell hyperproliferation and autoantibody production, J. Immunol. 171 (2003) 2840–2847.
[13] T. Shimizu, T. Ikegami, M. Ogawara, Y. Suzuki, M. Takahashi, H. Morio, T. Shirasawa, Transgenic expression of the protein-L-isoaspartyl methyltransferase (PIMT) gene in the brain rescues mice from the fatal epilepsy of PIMT deficiency, J. Neurosci. Res. 69 (2002) 341–352.
[14] M. Ogawara, M. Takahashi, T. Shimizu, M. Nakajima, Y. Setoguchi, T. Shirasawa, Adenoviral expression of protein-L-isoaspartyl methyltransferase (PIMT) partially attenuates the biochemical changes in PIMT-deficient mice, J. Neurosci. Res. 69 (2002) 353–361.
[15] B. Diaz, D. Barnard, A. Filson, S. Macdonald, A. King, M. Marshall, Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling, Mol. Cell. Biol. 17 (1997) 4509–4516.
[16] V. Kumar, M.X. Zhang, M.W. Swank, J. Kunz, G.Y. Wu, Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways, J. Neurosci. 25 (2005) 11288–11299.
[17] V. Vigneswara, J.D. Lowenson, C.D. Powell, M. Thakur, K. Bailey, S. Clarke, D.E. Ray, W.G. Carter, Proteomic identification of novel substrates of a protein isoaspartyl methyltransferase repair enzyme, J. Biol. Chem. 281 (2006) 32619–32629.
[18] K. Yeung, T. Seitz, S. Li, P. Janosch, B. Mcferran, C. Kaiser, F. Fee, K.D. Katsanakis, D.W. Rose, H. Mischak, J.M. Sedivy, W. Kolch, Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP, Nature 401 (1999) 173–177.
[19] K. Yeung, P. Janosch, B. Mcferran, D.W. Rose, H. Mischak, J.M. Sedivy, W. Kolch, Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein, Mol. Cell. Biol. 20 (2000) 3079–3085.
[20] C. Farrar, C.R. Houser, S. Clarke, Activation of the PI3K/Akt signal transduction pathway and increased levels of insulin receptor in protein repair-deficient mice, Aging Cell 4 (2005) 1–12.

[21] A. Ostman, F.D. Bohmer, Regulation of receptor tyrosine kinase signaling by protein tyrosine phosphatases, Trends Cell Biol. 11 (2001) 258–266.
[22] T. Tomiyama, S. Asano, Y. Furiya, T. Shirasawa, N. Endo, H. Mori, Racemization of Asp23 residue affects the aggregation properties of Alzheimer amyloid beta protein analogues, J. Biol. Chem. 269 (1994) 10205–10208.
[23] T. Shimizu, A. Watanabe, M. Ogawara, H. Mori, T. Shirasawa, Isoaspartate formation and neurodegeneration in Alzheimer's disease, Arch. Biochem. Biophys. 381 (2000) 225–234.
[24] A. Watanabe, K. Takio, Y. Ihara, Deamidation and isoaspartate formation in smeared tau in paired helical filaments. Unusual properties of the microtubule-binding domain of tau, J. Biol. Chem. 274 (1999) 7368–7378.
[25] A. Watanabe, W.K. Hong, N. Dohmae, K. Takio, M. Morishima-Kawashima, Y. Ihara, Molecular aging of tau: disulfide-independent aggregation and non-enzymatic degradation in vitro and in vivo, J. Neurochem. 90 (2004) 1302–1311.
[26] P.M. Masters, J.L. Bada, J.S. Zigler Jr., Aspartic acid racemisation in the human lens during ageing and in cataract formation, Nature 268 (1977) 71–73.
[27] N. Fujii, Y. Ishibashi, K. Satoh, M. Fujino, K. Harada, Simultaneous racemization and isomerization at specific aspartic acid residues in alpha B-crystallin from the aged human lens, Biochim. Biophys. Acta 1204 (1994) 157–163.
[28] N. Fujii, K. Satoh, K. Harada, Y. Ishibashi, Simultaneous stereoinversion and isomerization at specific aspartic acid residues in alpha A-crystallin from human lens, J. Biochem. (Tokyo) 116 (1994) 663–669.
[29] T. Furuchi, H. Homma, Role of isomerized protein repair enzyme, PIMT, in cellular functions, Yakugaku Zasshi 127 (2007) 1927–1936 (in Japanese).