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The protein truncation caused by fusion of PEP-1 peptide and protective roles of transduced PEP-1-MsrA in skin cells

Tae-Hyung Lee[#], *Seung Hee Choi & Hwa-Young Kim*[*]
Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu 705-717, Korea

PEP-1 peptide has been used for transduction of native protein into mammalian cells. This work describes the findings that the fusion of PEP-1 to target proteins led to protein truncation likely in a non-protein-specific manner. Approximately 75% of PEP-1-MsrA fusion protein was truncated in the N-terminal region of MsrA between Lys-27 and Val-28 during expression in *Escherichia coli* and purification. This large protein truncation was also observed in another PEP-1 fused protein, PEP-1-MsrB2, in the N-terminal region of MsrB2. The full-length PEP-1-MsrA protein was rapidly transduced into keratinocyte cells within 15 min. The transduced PEP-1-MsrA was functionally active and could protect skin cells against oxidative stress- and ultraviolet radiation-induced cell death. Collectively, our data demonstrated the protective roles of MsrA in skin cells and, moreover, may raise a concern of protein truncation caused by fusion of PEP-1 about the general use of this peptide for protein transduction. [BMB reports 2011; 44(4): 256-261]

INTRODUCTION

PEP-1 is a peptide carrier for the delivery of biologically active proteins into mammalian cells (1). It is an amphipathic, 21-residue peptide (KETWWETWWTEWSQPKKKRKV) that consists of three domains: a hydrophobic Trp-rich motif (KETWW ETWWTEW), a spacer (SQP), and a hydrophilic Lys-rich motif (KKKRKV). This peptide carrier facilitates the rapid (<10 min) and highly efficient transduction of non-denatured (native) target proteins into cells, is very stable in physiological buffer, and is not cytotoxic (1). These attributes make PEP-1 peptide a potentially useful carrier for protein transduction, especially protein therapy. Direct fusion of PEP-1 peptide to target proteins has been successfully used for transduction of many native proteins into mammalian cells, including superoxide dismutase, catalase, and heat shock protein 27 (2-4). In the PEP-1 fusion proteins reported so far, PEP-1 peptide was fused to N-terminus of target proteins. The reported PEP-1 fusion proteins also contained a His-tag in the N-terminal region for purification.

Methionine sulfoxide reductases (Msrs) catalyze the reduction of methionine sulfoxide to methionine (5, 6). Cyclic methionine oxidation/reduction is thought to be one of important defense mechanisms against oxidative stress (7) and the antioxidant roles of Msrs have been detailed (8-10). Two distinct Msr families have evolved for the stereospecific reduction of methionine sulfoxide residues in proteins. MsrA reduces the *S*-form of methionine sulfoxide, whereas MsrB only reduces the *R*-epimer. Mammals contain a single MsrA and three MsrB proteins (MsrB1-B3) (11). Skin serves as the first line of defense in protecting from the deleterious effects of ultraviolet (UV) radiation and other environmental oxidants. The MsrA and MsrB proteins are expressed in epidermal cells including keratinocytes and melanocytes (12, 13), but the protective roles of these proteins have not been demonstrated in skin cells.

The present study describes the findings that large portions of PEP-1 fused Msr proteins were truncated at the N-terminal regions of Msrs during expression in *Escherichia coli* and purification. This protein truncation induced by PEP-1 fusion seemed to be not protein-specific. In addition, this study demonstrated the protective roles of MsrA in skin cells against oxidative stress and UV damage.

RESULTS

Expression and purification of PEP-1-MsrA and -MsrB2 fusion proteins: truncation of proteins by PEP-1 fusion

A PEP-1-MsrA fusion protein, which is N-terminal PEP-1 fused to MsrA with a C-terminal His-tag, was generated (Supplementary Fig. S1A), expressed in *E. coli* BL21(DE3) by IPTG induction, and purified using a Talon-metal affinity column. Samples obtained from each purification step were subjected to SDS-PAGE (Fig. 1A). Unexpectedly, two forms of PEP-1-MsrA were detected in the eluted fraction (lane 6). A 31 kDa protein band (arrow *a*) corresponded in size to the full-length form of PEP-1-MsrA fusion protein, compared with MsrA con-

*Corresponding author. Tel: 82-53-620-4347; Fax: 82-53-654-6651; E-mail: hykim@ynu.ac.kr
[#]Present address: Department of Biopharmaceuticals and Herbal Medicine Evaluation, Korea Food & Drug Administration, Cheongwon, Chungbuk 363-951, Korea
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trol protein (lane 7, arrow *b*). Another 25 kDa protein band (arrow *c*) may correspond to a truncated form of PEP-1-MsrA. Indeed, the full-length and truncated forms were verified by Western blot analysis using anti-MsrA antibody (Fig. 1B). The ratio of truncated PEP-1-MsrA to full-length form in the purified sample was calculated to be approximately 3:1 with a densitometric analysis. The enzyme activity of total PEP-1-MsrA fusion protein was similar to that of MsrA control protein. The truncated form of PEP-1-MsrA was also observed in the cell crude extracts (lane 1-3), suggesting that cleavage of PEP-1-MsrA already occurred during expression in *E. coli* cells. The PEP-1-MsrA fusion protein contains a C-terminal His-tag. As the truncated form was also able to bind to the His-tag affinity column, its cleavage site was suggested in the N-terminal region of PEP-1-MsrA. Indeed, the truncated PEP-1-MsrA could be detected in Western blot by using anti-His antibody (Fig. 1C). To predict the cleaved site of PEP-1-MsrA protein, SDS-PAGE was performed to compare the size of the truncated form with that of a short form of MsrA that lacked 1-46 residues (Fig. 1D). The truncated form of PEP-1-MsrA migrated in between the full-length and the short forms of MsrA, suggesting that the cleaved site is within the first 46 residues of MsrA protein.

To test whether truncation of protein by PEP-1 peptide fusion was specific for MsrA protein, PEP-1 peptide was N-terminally fused to MsrB2, another Msr protein, resulting in a PEP-1-MsrB2 fusion protein with a C-terminal His-tag (Supplementary Fig. S1B). The PEP-1-MsrB2 fusion protein was expressed in *E. coli* and purified using the Talon-metal affinity column, and SDS-PAGE analysis was performed (Fig. 2A).

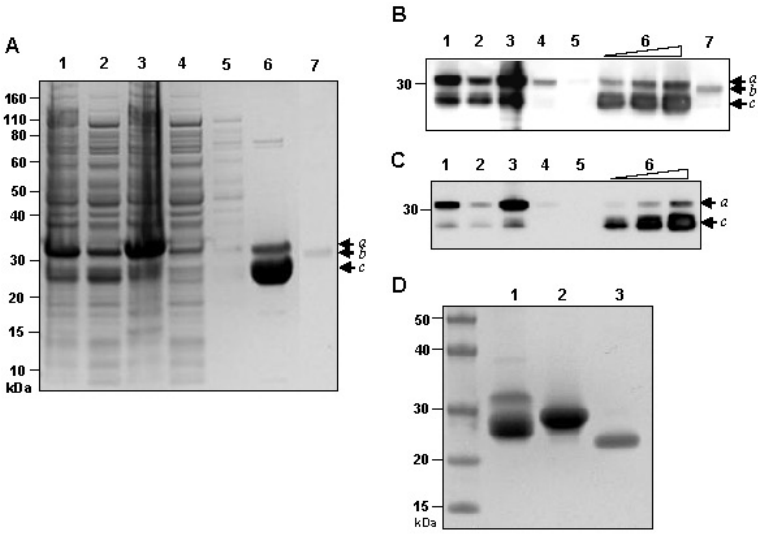

Fig. 1. Expression and purification of PEP-1-MsrA fusion protein. PEP-1-MsrA fusion protein was expressed in *E. coli* and purified using a Talon-metal affinity column. Crude extracts and purified samples were analyzed by SDS-PAGE (A) and were also subjected to Western blot analysis with anti-MsrA (B) and anti-His antibodies (C). Full-length PEP-1-MsrA is indicated by arrow *a*, MsrA control by arrow *b*, and truncated PEP-1-MsrA form by arrow *c*. Lane 1, total crude extract; lane 2, supernatant; lane 3, pellet; lane 4, flow-through fraction; lane 5, washed fraction; lane 6, eluted protein fraction; lane 7, purified MsrA control protein. (D) Size comparison of PEP-1-MsrA with full-length and short forms of MsrA. Lane 1, purified PEP-1-MsrA; lane 2, full-length MsrA; lane 3, short form of MsrA (deletion of 1-6 residues).

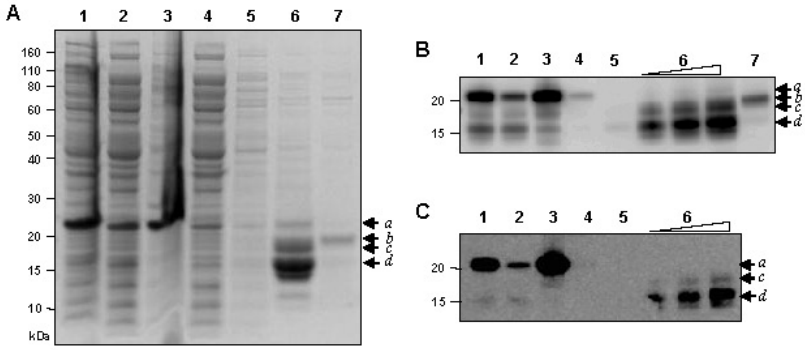

Fig. 2. Expression and purification of PEP-1-MsrB2 fusion protein. PEP-1- MsrB2 fusion protein was expressed in *E. coli* and purified using a Talon-metal affinity column. Crude extracts and purified samples were analyzed by SDS-PAGE (A) and also subjected to Western blot analysis with anti-MsrB2 (B) and anti-His antibodies (C). Full-length PEP-1-MsrB2 is indicated by arrow *a*, MsrB2 control by arrow *b*, and two truncated PEP-1-MsrB2 forms by arrow *c* and *d*. Lane 1, total crude extract; lane 2, supernatant; lane 3, pellet; lane 4, flow-through fraction; lane 5, washed fraction; lane 6, eluted protein fraction; lane 7, purified control MsrB2 protein.

Together with Western blot analyses using anti-MsrB2 and anti-His antibodies (Figs. 2B and 2C), at least two truncated forms of PEP-1-MsrB2 were observed in the purified sample (lane 6, arrows *c* and *d*) as well as the full-length fusion form (arrow *a*). The Western blot analyses also indicated that the truncation sites of PEP-1-MsrB2 were in the N-terminal regions of MsrB2 protein when compared with MsrB2 control (arrow *b*). The ratio of truncated PEP-1-MsrB2 form to full-length form was much higher than that of PEP-1-MsrA. The truncated forms of PEP-1-MsrB2 were also observed in the crude extracts (lane 1-3). Taken together, the protein truncation induced by PEP-1 fusion was suggested to be not protein-specific.

Determination of cleavage sites of PEP-1-MsrA and -MsrB2 fusion proteins

To determine the truncation sites of PEP-1-Msr fusion proteins, N-terminal amino acid sequencing was performed. The truncated PEP-1-MsrA protein was resolved by SDS-PAGE and subjected to N-terminal amino acid sequence analysis. The identified sequence was VISAE in MsrA protein, indicating that the cleavage site was between Lys-27 and Val-28 in the N-terminal region of MsrA. The two truncated PEP-1-MsrB2 proteins (arrows *c* and *d* in Fig. 2A) were also subjected to N-terminal amino acid sequence analysis. Of these two, the upper truncated protein (corresponding to the arrow *c* protein) was successfully N-terminally sequenced, while the lower truncated protein (the arrow *d* protein) was not. The identified sequence from the upper truncated protein was LRGLP. Thus, one of the at least two cleavage sites in PEP-1-MsrB2 was determined to be between Ala-7 and Leu-8 in the N-terminal region of MsrB2.

Transduction of PEP-1-MsrA fusion protein into keratinocyte cells

Next, the transduction ability of PEP-1-MsrA fusion protein was evaluated, because 25% (significant fraction) of purified protein sample contained full-length PEP-1-MsrA form. The purified PEP-1-MsrA fusion protein was added to HaCaT human keratinocyte cells at 4 μM for various times (15-75 min) and then the intracellular levels of transduced proteins were analyzed by Western blotting. MsrA protein was also used for control. The transduced PEP-1-MsrA was detected within 15 min and its levels were gradually increased up to 60 min (Supplementary Fig. S2), while the control MsrA protein was not transducible as previously described (14). To evaluate whether the transduced PEP-1-MsrA can be enzymatically active, MsrA activity was measured using 60 min-treated cell extracts; 80% increase in the activity was evident by the protein transduction (Supplementary Fig. S2). Together, these results indicated that PEP-1-MsrA fusion protein could be rapidly transduced into HaCaT keratinocyte cells (<15 min) as a competent form.

The dose dependency of the transduction of PEP-1-MsrA was then analyzed. Keratinocytes were treated with various concentrations (0-8 μM) of the fusion protein for 1 h, and the levels of the transduced proteins were assayed by Western blotting. The amounts of transduced PEP-1-MsrA proteins detected were elevated as the protein concentrations increased (Supplementary Fig. S3A). Also, MsrA activities were increased in a concentration-dependent manner, showing a twofold increase at 8 μM treatment (Supplementary Fig. S3A). Notably, the truncated form of PEP-1-MsrA fusion protein was also detected in transduced cells, suggesting that protein cleavage of PEP-1-MsrA could occur during protein transduction in keratinocyte cells.

The protein stability of PEP-1-MsrA transduced into keratinocyte cells was determined. The PEP-1-MsrA fusion protein was added to the culture medium of HaCaT cells at a concentration of 8 μM for 1 h. The transduced cells were then washed with PBS buffer and further incubated for various periods (1-24 h). Western blot analysis showed that the levels of intracellular transduced PEP-1-MsrA were reduced to 15% at 1 h post-transduction and further declined over the period of observation (Supplementary Fig. S3B). At 12 h post-transduction of the protein, the level of transduced PEP-1-MsrA was almost non-detectable.

Protective effects of transduced PEP-1-MsrA on the viability of keratinocytes under oxidative stress and UV irradiation

To determine whether transduced PEP-1-MsrA proteins can function in keratinocyte cells, cell viability under oxidative stress was ascertained. HaCaT cells were pretreated with 8 μM PEP-1-MsrA for 1 h, washed, and further incubated in the presence of 400-1,000 μM H_2O_2 for 2 h. Pre-treatment of MsrA protein was used as a control. As shown in Fig. 3A, when the cells pre-treated with the control MsrA protein were exposed to in excess of 600 μM H_2O_2, cell viability was significantly reduced. In contrast, when pretreated with PEP-1-MsrA, significant increases (40-70%) in cell viability were observed at 600-1,000 μM H_2O_2. These results demonstrated that protein transduction of PEP-1-MsrA could protect keratinocytes against oxidative stress-induced cell death. The tumor suppressor p53 induces cell death through transcription-dependent and -independent mechanisms (15). Acetylation of p53 enhances its transcriptional activity and stability. To further understand the protective role of PEP-1-MsrA at the molecular level, the levels of p53 and acetylated-p53 were assayed (Fig. 3B). While the expression levels of p53 remained unchanged, the acetylated-p53 level was reduced by PEP-1-MsrA transduction under oxidative stress. This result suggested that transduced PEP-1-MsrA protects cells against oxidative stress by inhibiting the proapoptotic function of p53.

The next experiment tested whether protein transduction of PEP-1-MsrA can protect cells against UV-irradiated damage. Keratinocyte cells were pretreated with PEP-1-MsrA for 1 h and irradiated with UVB. MsrA protein was also used for control in these experiments. As shown in Fig. 4A, in the pre-treated cells with PEP-1-MsrA, transduction of PEP-1-MsrA significantly elevated the viability of UVB-damaged cells irradiated at 20 and

30 mJ/cm^2. Also, when cells were first irradiated with UVB and even post-treated with PEP-1-MsrA for 1 h, the PEP-1-MsrA transduction protected cells from UVB-damaged cell death induced by 20 mJ/cm^2 irradiation (Fig. 4B).

DISCUSSION

PEP-1 is a competent delivery peptide for native protein transduction (1). In this study, a PEP-1-MsrA fusion construct with a C-terminal His-tag was designed and expressed in *E. coli*. Unexpectedly, a significant amount of PEP-1-MsrA protein (75% of purified sample) was processed between Lys-27 and Val-28 in the N-terminal region of MsrA. The truncations of protein by PEP-1 fusion were also observed in PEP-1-MsrB2 fusion protein, where one of the processed sites was between Ala-7 and Leu-8 in the N-terminal region of MsrB2. The truncated PEP-1-MsrA fusion protein was even observed in transduced keratinocyte cells. In contrast, protein cleavage of native MsrA and MsrB2 proteins was not detected when purified from *E. coli* (Figs. 1D and 2A). Together, the findings demonstrate that fusion of PEP-1 peptide causes, in a non-sequence-specific manner, protein truncation of MsrA and MsrB2 in their N-terminal regions.

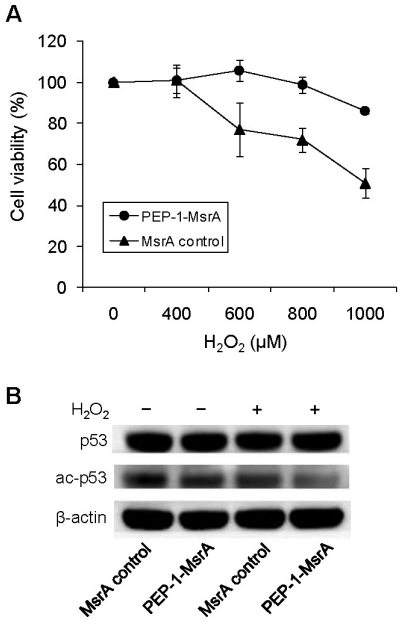

Fig. 3. Effects of transduced PEP-1-MsrA on cell viability and p53 levels under oxidative stress. (A) Cell viability. Hydrogen peroxide with the indicated concentrations was added to HaCaT cells for 2 h with 1 h-pretreatment of 8 μM PEP-1-MsrA or control MsrA. Cell viability was estimated with a colorimetric MTT assay. (B) p53 levels. Cells pretreated with 8 μM PEP-1-MsrA or control MsrA for 1 h were exposed to 800 μM H_2O_2 for 2 h. The levels of p53 and acetylated-p53 at K373/K382 were analyzed by Western blotting.

It is unknown how the PEP-fusion induces the protein truncation. Also, it is not clear whether protein truncation by PEP-1 fusion is specific for MsrA and MsrB2 proteins, or whether it generally occurs in other proteins. Many PEP-1 fused proteins, including superoxide dismutase, catalase, heat shock protein, pyridoxal-5'-phosphate phosphatase, and AMPK, have previously been developed and successfully used for protein transduction experiments (2-4, 16, 17). However, there have been no reports of the occurrence of protein truncation caused by PEP-1 peptide fusion. All previously reported PEP-1 fusion proteins contained an N-terminal His-tag. Thus, if protein truncation of the previous PEP-1 fusion proteins would occur, their truncated forms could not be isolated during purification procedures with His-tag affinity. Finally, if truncation by PEP-1 fusion really exists in other proteins, our results raise a potentially important concern about the general use of PEP-1 fused protein for transduction of protein.

Being the outermost tissue of our body, skin is exposed to high amounts of UV radiation and other environmental oxidants. MsrA and MsrB proteins are present in skin cells including keratinocytes and melanocytes (12, 13), but their functional roles in these cells are unclear. In this work, PEP-1-MsrA fusion protein could be rapidly delivered (<15 min) into keratinocyte cells. The transduced PEP-1-MsrA protein was enzymatically active but not relatively stable (85% protein was de-

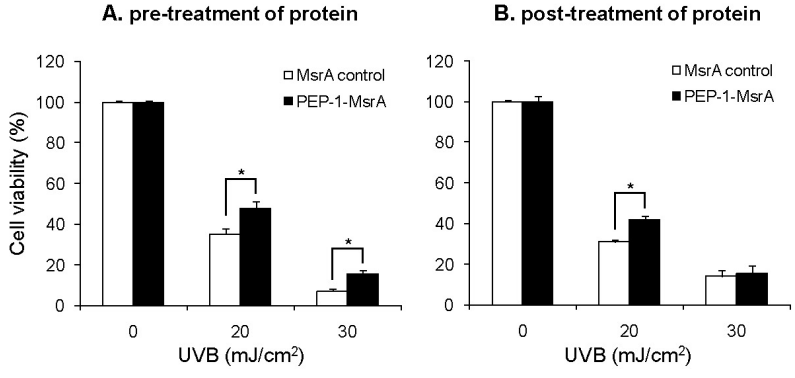

Fig. 4. Protective effect of PEP-1-MsrA transduction in keratinocyte cells under UV radiation. Cells were pretreated with PEP-1-MsrA or control MsrA proteins (8 μM) before UVB treatment with 20 or 30 mJ/cm^2 (A) or were post-treated with the proteins after UVB irradiation (B). Cell viability was analyzed with a colorimetric MTT assay. Statistical analysis was performed using a Student's *t*-test. P values of <0.05 were considered significant and indicated by asterisks.

graded within 1 h). Nevertheless, the transduced PEP-1-MsrA could increase the viability of keratinocytes under oxidative stress and, furthermore, could protect cells from UV-induced damage. To our knowledge, the present study first demonstrated the protective roles of MsrA in skin cells.

MATERIALS AND METHODS

Construction, expression, and purification of PEP-1-MsrA and -MsrB2 fusion proteins in *E. coli*

Sense and antisense oligonucleotides coding for PEP-1 peptide (5'-TATGAAAGAAACCTGGTGGGAAACCTGGTGGACCGAA TGGTCTCAGCCGAAAAAAAAACGTAAAGTGG-3' and 5'-AA TTCCACTTTACGTTTTTTTTTCGGCTGAGACCATTCGGTCCA CCAGGTTTCCCACCAGGTTTCTTTCA-3') were synthesized, annealed, and cloned into the *Nde*I/*Eco*RI sites of pET21b (Novagen). The resulting construct was named pPEP- 21b. To generate a PEP-1-MsrA fusion construct, a coding region of mouse MsrA was PCR-amplified using a pET-MsrA-His construct (18) as template and cloned into the *Eco*RI/*Xho*I sites of pPEP-21b. The resulting plasmid, designated pPEP-MsrA, encoded the PEP-1-MsrA fusion protein with a C-terminal His-tag (LEHHHHHH). A coding region of mouse MsrB2 was also PCR-amplified using a pET28 based construct (11) as template and cloned into the *Eco*RI/*Xho*I sites of pPEP-21b. The resulting construct, named pPEP-MsrB2, coded for the PEP-1-MsrB2 fusion protein with a C-terminal His-tag (LEHHHHHH).

The constructs pPEP-MsrA and pPEP-MsrB2 were separately transformed into *E. coli* BL21(DE3) cells. The transformed cells were grown in LB medium containing 100 μg/ml ampicillin at 37°C. When optical density at 600 nm reached 0.6-0.8, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. The cells were cultured for another 5 h at 30°C, harvested, and stored at −20°C until use. The cell pellets were resuspended in an extraction buffer [50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and lysed by sonication. The supernatant protein samples were separated by centrifugation, loaded on a Talon-metal affinity column (Clontech), washed with the extraction buffer, and eluted with a buffer comprised of 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, and 150 mM imidazole. The eluted proteins were dialyzed against 50 mM sodium phosphate (pH 7.5) and 50 mM NaCl. Concentrations of the purified proteins were determined by the Bradford method using a Bio-Rad protein assay reagent and bovine serum albumin as a standard. Full-length and short forms (deletion of 1-46 amino acids) of mouse MsrA and full-length mouse MsrB2 were also prepared as described previously (11, 18). These proteins contained a C-terminal His-tag (LEHHHHHH).

SDS-PAGE and Western blot analyses

The purity of PEP-1-MsrA and -MsrB2 proteins was analyzed by SDS-PAGE using a NuPAGE 4-12% Bis-Tris gel (Invitrogen). Western blot analysis was performed using anti-MsrA, anti-MsrB2, or anti-His antibodies. The full-length and truncated PEP-1-MsrA or PEP-1-MsrB2 proteins were analyzed by SDS-PAGE and Western blot.

MsrA enzyme assay

The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 200 μM dabsylated methionine-*S*-sulfoxide, and purified or crude samples. The reaction was carried out at 37°C for 30 min and the reaction product, dabsyl-Met, was analyzed by an established HPLC procedure (11).

N-terminal amino acid sequencing

To determine the cleavage sites of truncated PEP-1-MsrA or PEP-1-MsrB2, N-terminal amino acid sequencing was performed. The purified PEP-1-MsrA or PEP-1-MsrB2 proteins were subjected to SDS-PAGE and the resolved proteins were transferred onto a polyvinylidene difluoride membrane. The transferred proteins were stained with a solution (0.1% Coomassie Blue, 40% methanol, and 1% acetic acid) and destained with 50% methanol. The truncated PEP-1-MsrA or PEP-1-MsrB2 bands were excised and their N-terminal amino acid residues were determined using the Procise 491 protein sequencer (Applied Biosystems) at the Korea Basic Science Institute.

Transduction of PEP-1-MsrA into keratinocyte cells

Human keratinocyte HaCaT cells (kindly provided by Dr. Tae-Yoon Kim, Catholic University, Korea) were seeded in wells of 6-well plates at a density of 1×10^6 cells/well and cultured for 22 h in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. The culture medium was replaced with 1 ml of fresh medium. Two hours later, the cells were treated with various concentrations of PEP-1-MsrA for 15-75 min, washed with phosphate-buffered saline (PBS), and harvested in PBS containing 1 mM PMSF. The harvested cells were lysed by sonication and the supernatant samples were subjected to enzyme assays and Western blot analysis.

Analysis of protein stability of transduced PEP-1-MsrA

HaCaT cells (2×10^6) seeded in 60 mm-diameter plates were cultured for 22 h and the culture medium was replaced with fresh medium. After 2 h, cells were treated with 8 μM PEP-1-MsrA for 1 h, washed three times with PBS to remove untransduced proteins, and transferred to fresh medium. The cells were further incubated and harvested in PBS containing 1 mM PMSF at 0, 1, 3, 6, 12, and 24 h culture. The harvested cells were lysed by sonication and the supernatant samples were subjected to Western blot analysis.

Cell viability assays under oxidative stress and UV irradiation

For determination of cell viability under oxidative stress,

HaCaT cells seeded in wells of 24-well plates at a density of 5×10^5 cells/well were cultured for 22 h and the culture medium was replaced with fresh medium. After 2 h, cells were pretreated with 8 μM PEP-1-MsrA for 1 h and washed three times with PBS prior to the addition of 400-1,000 μM hydrogen peroxide to the culture medium for 2 h. Cell viability was analyzed using an established colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. MsrA-treated cells were used for control. For the determination of cell viability under UV irradiation, HaCaT cells seeded in 6-well plates at a density of 1×10^6 cells/well were cultured for 22 h and the culture medium was replaced with fresh medium. Cells were pretreated with 8 μM PEP-1-MsrA for 1 h prior to irradiation with a UVB dose of 0-30 mJ/cm^2 (pre-treatment of PEP-1-MsrA). Also, cells were pretreated with UVB irradiation and 8 μM PEP-1-MsrA was transduced for 1 h (post-treatment of PEP-1-MsrA). After 24 h treatment, cell viability was estimated with the colorimetric MTT-based assay. Cells treated with MsrA were used for control.

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