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Evidence of post-translational modification of the tumor suppressor maspin under oxidative stress

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Abstract. Maspin, identified as a 42 kDa unique tumor suppressive serine protease inhibitor (serpin), has multifaceted biological functions by interacting with various target molecules under physiological and pathological conditions including oxidative stress. However, the type of post-translational modification that confers the specific binding affinity of maspin to target molecules, such as glutathione S-transferase (GST), has not been determined. The aim of this study, therefore, is to analyze the molecular heterogeneity of maspin and to identify its modifications in the normal mammary epithelial cell line, MCF-10A, which is known to express the maspin protein abundantly, using electrophoretic analysis. Conventional SDS-PAGE analysis of MCF-10A cell extracts showed that endogenous maspin is composed of both an intact form observed as a 42 kDa band and a smaller form observed as a 36 kDa band. Interestingly, a brief exposure of MCF-10A cells to 10 mM hydrogen peroxide (H_2O_2) led to the appearance of a novel endogenous maspin form, as demonstrated by non-denaturing PAGE and non-reducing SDS-PAGE. Two-dimensional sequential non-reducing/reducing SDS-PAGE supported that this novel form was generated by intramolecular disulfide-bonded linkage under oxidative stress, and this oxidized maspin form also existed under physiological conditions. Furthermore, a glutathione (GSH) bead pull-down assay revealed that the intramolecular disulfide-bonded maspin lost its binding activity to endogenous GST, indicating that intramolecular disulfide-bonded maspin might have some distinct properties under oxidative stress, although the precise biological significance of this modification remains elusive. In conclusion, we have shown that maspin undertakes different modifications under oxidative stress.

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Introduction

Maspin was formally identified as a 42 kDa unique tumor suppressive serine protease inhibitor (serpin) in normal breast epithelial cells (1). Many biochemical studies have demonstrated that maspin has multifaceted biological functions under physiological and pathological conditions including oxidative stress (2-4). In the last few years, approximately 20 molecules have been reported as maspin-interacting candidate molecules (5). For example, Yin *et al* (6) reported that maspin may regulate oxidative stress-induced reactive oxygen species (ROS) generation by interacting with endogenous glutathione S-transferase (GST). However, it remains unknown what regulates maspin's specific binding affinity to GST or to other target proteins *in vivo*.

Generally, protein modifications are known as one of the important regulatory factors for a protein's specific binding properties. Among the protein modifications, much attention has recently been paid to the significance of the cysteine-targeted oxidation in the regulation of protein function under conditions of oxidative stress (7,8). Since maspin is a single gene-derived 42 kDa non-inhibitory type of serpin with eight cysteine residues (9), it is postulated that post-translational modifications including cysteine-targeted oxidation may contribute to maspin's molecular mechanism of action.

Oxidative stress, a cellular condition during which ROS far exceed the antioxidant defenses (10), has been implicated in numerous pathophysiological conditions and in aging (11). ROS can cause specific protein modifications that lead to a change in the activity or function of the oxidized protein (12). Considering the recent report that maspin regulates the cell's response to oxidative stress by direct interaction with endogenous GST (6), it is possible that maspin is one of the crucial proteins which function specifically under oxidative stress-induced pathological conditions.

This evidence prompted us to analyze the post-translational molecular heterogeneity of maspin under oxidative stress. The aim of this study is to analyze the post-translational modifications of maspin and to investigate their effect on

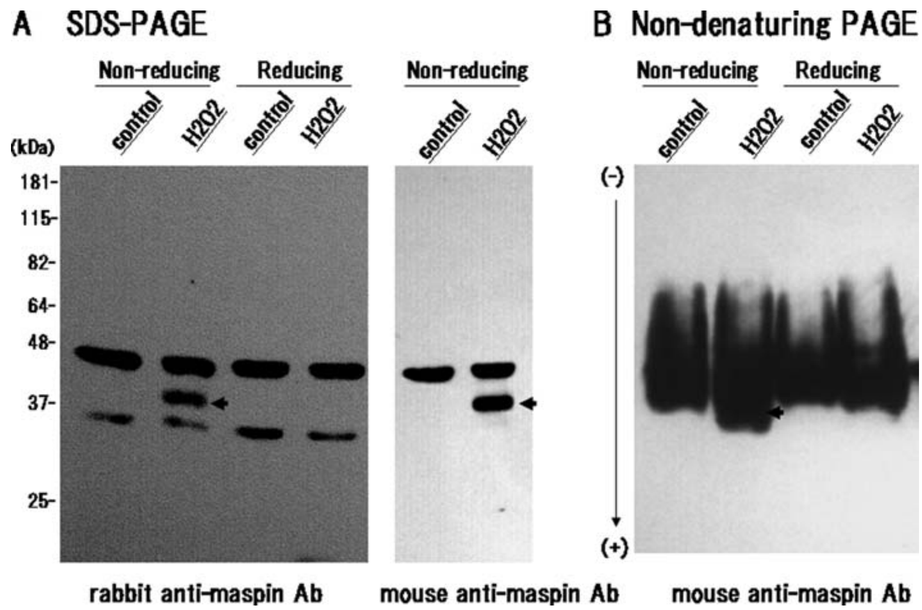

Figure 1. Effect of H_2O_2 treatment on the electrophoretic patterns of maspin. (A) SDS-PAGE analysis. The control and H_2O_2-treated MCF-10A cell extracts were subjected to SDS-PAGE under non-reducing and reducing conditions. After electrophoresis, the proteins were transferred to the PVDF membrane, and then detected by immunostaining using rabbit anti-maspin antibody or mouse anti-maspin antibody. (B) Non-denaturing PAGE analysis. Non-denaturing PAGE of H_2O_2-treated MCF-10A cell extract combined with mouse anti-maspin antibody showed a higher mobility band of maspin, as indicated by the arrow, compared to the intact form of maspin, which diminished under reducing conditions.

maspin's binding affinity in H_2O_2-treated MCF-10A human mammary cells, using various electrophoretic analyses. The present study shows for the first time that intramolecular disulfide-bonded maspin exsists in MCF-10A cells under oxidative stress. We also discuss the possible biological role of intramolecular disulfide-bonded maspin.

Materials and methods

Materials. The molecular mass marker proteins were obtained from Invitrogen. The Western blotting system was purchased from Pierce (Rockfold, IL, USA). The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody and swine anti-rabbit IgG were from Sigma (St. Louis, MO, USA). The polyvinylidene difluoride (PVDF) membranes were from Atto (Tokyo, Japan).

Cell culture. MCF-10A human mammary cells (CRL-10317; American Type Culture Collection) were cultivated as a monolayer in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) containing 5% donor horse serum, 20 μg/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 10 μg/ml insulin, 500 μg/ml hydrocortisone, 50 U/ml penicillin, and 50 μg/ml streptomycin in 95% air and 5% CO_2 at 37˚C, as previously described (13). The medium was changed every two days.

H_2O_2 treatment. Cells were treated with a 10 mM H_2O_2 insult for 5 min, washed three times with PBS, and then incubated in ice-cold PBS with 40 mM iodoacetamide (IA) for 5 min to prevent thiol-disulfide exchange and inhibit post-lysis oxidation of free cysteines. For preparation of the cell extracts for electrophoresis, cells were lysed with a modified RIPA buffer (50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM ß-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or with non-denaturing lysis buffer (20 mM Tris pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4, 2.5 mM pyrophosphate, 10 mM ß-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) for non-denaturing PAGE. The cell extracts were cleared by centrifugation and the protein concentration was determined by the Bradford method.

Non-denaturing PAGE. Non-denaturing PAGE was performed using 10% polyacrylamide gels without denaturants at a constant current of 10 mA/slab as previously described (14), with a modification. Namely, non-denaturing PAGE in this study utilized a Tris/glycine buffer system with the same pH value of 6.8, for stacking and separating gels, instead of different pH values.

Two-dimensional sequential non-reducing/reducing SDS-PAGE. To elucidate the changes in disulfide bond formation following ROS exposures, two-dimensional sequential non-reducing/reducing SDS-PAGE electrophoresis was carried out as previously described (7), by using slab-type polyacrylamide gels for SDS electrophoresis in the first dimension under non-reducing conditions, followed by slab-type polyacrylamide gels for SDS electrophoresis in the second dimension under reducing condition at a 10 mA/slab constant current.

Western blot analysis. After electrophoresis was completed, proteins were transferred to PVDF membranes with a semidry type blotting apparatus (Horizblot; Atto, Japan) using standard Tris-base (25 mM), glycine (195 mM), 20% (v/v) methanol

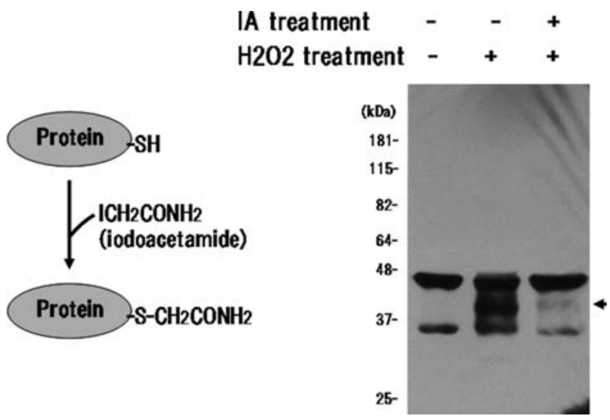

Figure 2. Effect of iodoacetamide (IA) on the appearance of a novel maspin form in H_2O_2-treated MCF-10A cells. Non-reducing SDS-PAGE analysis combined with rabbit anti-maspin antibody showed a novel maspin band between the intact and the 36 kDa processed isoforms of maspin in H_2O_2-treated MCF-10A cells. However, in IA-pretreated MCF-10A cells, H_2O_2 treatment did not yield this novel maspin form.

transfer buffer. The PVDF membranes were stained by an immunochemical technique consisting of the following procedures. After blocking the membrane with skimmed milk dissolved in Tris-buffer saline (TBS), pH 7.5, the membrane was incubated with two antibodies, specific for maspin, a rabbit anti-maspin antibody (13) and a mouse anti-maspin antibody (BD Pharmingen) at a 500-fold dilution. Then, the membrane was incubated with HRP-conjugated rabbit anti-mouse IgG or swine anti-rabbit IgG antibody. Finally, maspin was detected using the ECL Western blotting detection system (Pierce) according to the manufacturer's instruction.

Glutathione (GSH) bead pull-down assay. To detect maspin using interaction with endogenously expressed GST, a GSH affinity pull-down assay was carried out as previously reported (6). Briefly, cell extracts were prepared by lysing H_2O_2-treated cells in modified RIPA buffer, and incubated with 50 μl of GSH-Sepharose 4B beads (50% slurry) for 1 h at 4˚C. The beads were washed six times with PBS containing 0.5% Tween-20, and the bound proteins were eluted with reduced glutathione buffer, denatured in SDS sample buffer without reducing agents such as ß-mercaptoethanol or DTT, and subjected to non-reducing SDS-PAGE combined with Western blot analysis using a rabbit anti-maspin antibody.

Results

Electrophoretic identification of a novel form of maspin in H_2O_2-treated MCF-10A cells. As shown in Fig. 1A (left panel), conventional SDS-PAGE Western blot analysis identified two maspin bands (42 kDa and 36 kDa) in MCF-10A cells extract with the rabbit anti-maspin antibody. These bands correspond to the intact and smaller isoforms of endogenous maspin, respectively, as previously described (15). On the other hand, the mouse anti-maspin antibody recognized only the 42 kDa band of intact maspin. Under non-reducing condition, a novel form of maspin could be detected as a band which had migrated between the 42 kDa and 36 kDa bands of maspin in H_2O_2-treated MCF-10A cells, but not in control cells. This novel form was detected by Western blot analysis using the mouse anti-maspin antibody (Fig. 1A, right panel), indicating that this maspin form was derived from intact maspin, rather than the 36 kDa smaller isoform of maspin. Since we attempted to focus on the novel form of maspin generated under oxidative stress in this study, we also checked the possibility that the mouse anti-maspin antibody could detect the novel form of maspin under non-denaturing PAGE, in addition to non-reducing SDS-PAGE. As shown in Fig. 1B, this novel band was detected as a higher mobility band compared to the intact maspin. Then, to confirm the contribution of the cysteine-residue oxidization on the generation of the novel form of maspin, we pretreated cells with IA, which blocked the thiol (SH) residues (see Fig. 2, left panel), before cells were exposed to H_2O_2 exposure. As shown in Fig. 2 (right panel), the novel maspin form did not appear in the IA-pretreated MCF-10A cells before H_2O_2 exposure, suggesting that cysteine residue is essential for the novel oxidized form of maspin.

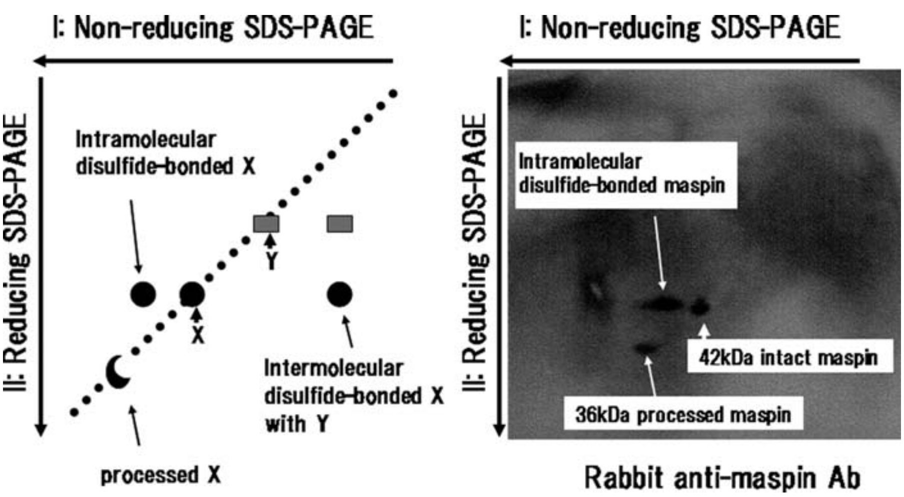

Figure 3. Identification of a novel maspin form in H_2O_2-treated MCF-10A cells on two-dimensional sequential non-reducing/reducing SDS-PAGE electrophoresis.

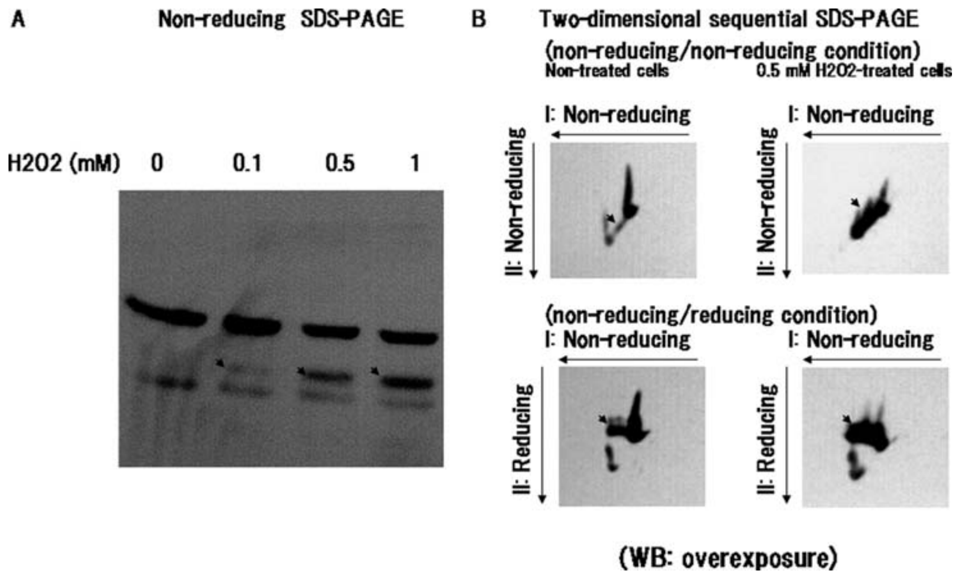

Figure 4. Dose-dependent expression pattern of oxidized maspin in various concentrations of H_2O_2-treated MCF-10A cells. (A) Non-reducing SDS-PAGE pattern of maspin isoforms in MCF-10A cells after treatment with various doses of H_2O_2. (B) Two-dimensional sequential non-reducing/non-reducing SDS-PAGE electrophoretic pattern of maspin in control or 0.5 mM H_2O_2-treated MCF-10A cells (upper panel). Two-dimensional sequential non-reducing/reducing SDS-PAGE electrophoretic pattern of maspin in control or 0.5 mM H_2O_2-treated MCF-10A cells (lower panel). The oxidized maspin is indicated by the arrow.

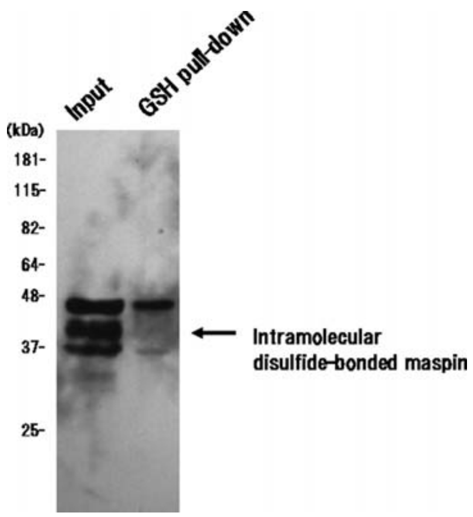

Figure 5. GSH bead pull-down assay. In the GSH bead pull-down assay, the band of the intramolecular disulfide-bonded maspin (arrow) was not detected in the products interacted with endogenous GST, while the native maspin isoforms, 42 kDa and 36 kDa bands, were clearly detected on non-reducing SDS-PAGE combined with Western blot analysis using the rabbit anti-maspin antibody.

Two-dimensional sequential non-reducing/reducing SDS-PAGE pattern of maspin in H_2O_2-treated MCF-10A cells. To estimate whether intramolecular or intermolecular disulfide-bonded linkage was formed during generation of novel form of maspin as indicated in Fig. 3 (left panel) according to the previous method (7), two-dimensional sequential non-reducing/reducing two-dimensional SDS-PAGE analysis was carried out. As shown in Fig. 3 this novel form of maspin was generated by intramolecular disulfide-bonded linkage under oxidative stress. On the other hand, this oxidized maspin form increased with H_2O_2 exposure gradually and in a dose-dependent manner (Fig. 4A) and could be detecting as a faint spot under physiological conditions, using two-dimensional sequential SDS-PAGE under non-reducing/non-reducing conditions or non-reducing/reducing condition combined with an overexposure technique of Western blot analysis (Fig. 4B).

Functional analysis of the oxidized maspin using the GSH bead pull-down assay. To investigate whether this oxidized modification changes the binding affinity of maspin to GST, we used the GSH bead pull-down assay according to a previous report (6). Unexpectedly, as shown in Fig. 5, intramolecular disulfide-bonded maspin could not be detected in the product bound to the endogenous GST, although two bands of maspin could be confirmed, suggesting that the oxidized maspin form lost or decreased its binding affinity to endogenous GST compared to the intact and smaller maspin isoforms.

Discussion

In this study, for the first time we have demonstrated the existence of a novel intramolecular disulfide-bonded maspin in MCF-10A cells under oxidative stress by electrophoretic methods. Additionally, it was indicated that this novel isoform generated by intramolecular disulfide-bonded linkage had different properties from the two native maspin isoforms, the 42 kDa intact form and the 36 kDa processed form, in terms of its binding affinity to endogenous GST.

Generally, it was suggested that multiple forms of serpin might contribute to the different functions of serpin under pathological conditions (16). Since maspin was identified as a tumor suppressive serpin in 1994 (1), there have been two reports on the post-translational modifications of maspin. Maspin was reported to be phosphorylated on a tyrosine moiety in normal mammary epithelia cells, which may be important for signal transduction (17). On the other hand, Smith *et al* (15) reported the biochemical properties of a smaller maspin isoform, generated by post-translational processing of the protein. Although the phosphorylated form of maspin could not be detected in our electrophoretic analyses, it is likely that the 36 kDa maspin band shown in this study corresponds to the smaller isoform of maspin described in Smith *et al* (15). Therefore, the oxidized form of maspin identified in this study is the third form of the post-translational modifications of maspin.

There are several reports on the oxidized modification of other serpins, including the oxidized α1-antitrypsin generated by the oxidation of methionines in native α1-antitrypsin (18). Remarkably, this oxidation changed the property of the native serpin molecule (19). However, to date, there is no report on the identification and function of oxidized serpins generated by intramolecular disulfide-bonded linkage. Therefore, the oxidized maspin form is likely to be unique among oxidized serpins. On the other hand, it is well-known that disulfide bonds serve two functions, stabilization of the native protein conformation and maintainance of protein integrity (20). Therefore, to clarify the biological significance of this oxidized modification, we postulated that oxidized maspin might act to reduce ROS generation by enhancing binding affinity to endogenous GST compared to native maspin, since the level of oxidized maspin was increased in accordance with oxidative stress, as shown in Fig. 4. Surprisingly, as shown in Fig. 5, we found that the oxidized maspin lost or decreased its binding affinity to endogenous GST, indicating that the intramolecular disulfide-bonded formation of maspin might have some distinct properties compared to the native maspin isoforms under oxidative stress. Although the precise function of this oxidized maspin remains elusive, this form might be involved to a great extent in the molecular functions of maspin, possibly by affecting the biochemical properties of maspin directly and its subcellular localization indirectly, which is related to its specific function such as apoptosis, cell migration and cell adhesion (13,21-23). Another possibility is that oxidized maspin through disulfide bond may protect the maspin protein against damages such as oxidative stress and aging, which may result in an increase in the half-life of the protein. In addition, it has been reported that aging could also influence the gene expression of maspin (24).

Interestingly, there were several reports on paradoxical maspin expression in malignant tissues such as non-small cell lung cancer, ovarian and pancreatic cancer (15,25-27). Specifically, maspin's expression pattern in pancreatic cancer (26,27) is different from that found in breast cancer (1). For example, Cao *et al* (27) demonstrated that overexpression of maspin in pancreatic ductal adenocarcinoma is associated with worse postoperative survival. However, the association of the molecular heterogeneity with aberrantly expressed maspin in various malignant tissues remains unknown. Thus, the new isoform of maspin that we identified in this report may serve as a new biomarker in some malignant tissues. We believe that it might be important to characterize the expression pattern of specific maspin isoforms in normal and various malignant tissues. It has been reported that squamous cell carcinoma antigen (SCCA), another tumor-associated serpin, displays different isoforms in normal and malignant squamous cell epithelial tissues (28).

In conclusion, we utilized non-reducing SDS-PAGE to identify a novel oxidized form of maspin, in addition to its intact and 36 kDa processed forms. The identification of the novel oxidized maspin form might not only serve as an important biomarker of cancer, but may also provide a clue for understanding the molecular mechanism of maspin's functions *in vivo*.

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