Glutathionylation of Two Cysteine Residues in Paired Domain Regulates DNA Binding Activity of Pax-8*

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We reported that the first two cysteine residues out of three present in paired domain (PD), a DNA-binding domain, are responsible for redox regulation of Pax-8 DNA binding activity. We show that glutathionylation of these cysteines has a regulatory role in PD binding. Wild-type PD and its mutants with substitution of cysteine to serine were synthesized and named CCC, CSS, SCS, SSC, and SSS according to the positions of substituted cysteines. They were incubated in a buffer containing various ratios of GSH/GSSG and subjected to gel shift assay. Binding of CCC, CSS, and SCS was impaired with decreasing GSH/GSSG ratio, whereas that of SSC and SSS was not affected. Because [3H]glutathione was incorporated into CCC, CSS, and SCS, but not into SCC and SSS, the binding impairment was ascribed to glutathionylation of the redox-reactive cysteines. This oxidative inactivation of PD binding was reversed by a reductant dithiothreitol and by reductase factor (Ref)-1 in vitro. To explore the glutathionylation in cells, Chinese hamster ovary cells overexpressing CSS and SCS were labeled with [35S]cysteine in the presence of cycloheximide. Immunoprecipitation with an antibody against PD revealed that treatment of the cells with an oxidant diamide induced the [35S] incorporation into both mutants, suggesting the PD glutathionylation in cells. Since the two cysteine residues in PD are conserved in all Pax members, this novel posttranslational modification of PD would provide a new insight into molecular basis for modulation of Pax function.

Various cellular functions such as gene expression, signal transduction, and enzyme activity are modulated by intracellular redox potential (1). In the context of gene expression, it has been shown that the redox potential alters the DNA binding activity of a number of transcription factors, such as AP-1 and NF-κB, through oxidoreductive modification of thiols on the redox-reactive cysteine residues that mostly reside in the DNA-binding domain (2–6). It has also been shown that such modification is often catalyzed by intracellular redox enzymes such as redox factor-1 (Ref-1) and thioredoxin (5, 7, 8).

Since the two cysteine residues in PD are conserved in all Pax members, this novel posttranslational modification of PD would provide a new insight into molecular basis for modulation of Pax function.

The abbreviations used are: PD, paired domain; CHO, Chinese hamster ovary; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; NRS, normal rabbit serum.
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Glutathionylation was assessed by measuring radioactivities of trichloroacetic acid precipitates. Statistical analysis was performed by one-way analysis of variance followed by Student's t-test.

Metabolic Labeling and Immunoprecipitation—CSS and SCS cDNAs were cloned into mammalian expression vector pcDNA3.1/Myc-His (Invitrogen), and the plasmids were transiently transfected into CHO cells by using a Lipofectamine reagent (Invitrogen). After an 18-h incubation with the liposome-DNA solution, the reagent was replaced with a fresh culture medium. After an additional 24-h incubation, the medium was changed to Hanks’ balanced salt solution containing 10 μg/ml cycloheximide (Sigma). After a 60-min incubation, endogenous glutathione pool was labeled with 3.7 MBq of [35S]cytidine (39.8 TBq/mmol, 370 MBq/ml, PerkinElmer Life Sciences) for 60 min. Cycloheximide was used to facilitate its incorporation into glutathione. The cells were then treated with 1 mM diamide for 5 min and harvested for immunoprecipitation. As a parallel experiment, the transfected cells were labeled with [35S]methionine. The detailed procedure was reported previously (29).

In brief, the cells were preincubated in methionine-free Eagle's minimal essential medium for 2 h and then incubated in minimal essential medium containing [35S]methionine (Express [35S]-protein labeling mix, PerkinElmer Life Sciences) for 2 h. Immunoprecipitation was performed using anti-Myc antibody (Roche Diagnostics), normal rabbit serum (NRS), and GammaBind G beads (Amersham Biosciences). The precipitates were incubated for 5 min at 100 °C in a gel loading buffer with or without 10 mM DTT and then subjected to 15% SDS-polyacrylamide gel electrophoresis. After fixation, gels were incubated in an enhancer for fluorography (Amplify, Amersham Biosciences), dried, and exposed to the BAS 2000 system. To evaluate the efficiency of transfection, the whole cell lysates prepared from the transfected cells or their immunoprecipitates with anti-Myc antibody were subjected to Western blot analysis using the same antibody. Procedures for Western blot analysis were described previously (30). The proteins were visualized using enhanced chemiluminescence reagents (Pierce).

Immunocytochemical Analysis—CHO cells transfected with CSS- or SCS-expressing plasmid were subjected to immunocytochemical analysis. The procedures were described previously (30). Anti-Myc antibody and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) were used to visualize CSS and SCS proteins in cells. To identify the nucleus, the cells were simultaneously stained with Hoechst 33258 (Molecular Probes). The images were obtained using an Axioshot 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Oxidative Modification of Cys-45 or Cys-57 Impairs DNA Binding of PD—PD and its mutants that contain only one or no cysteine residue were synthesized as fusion proteins with His6/6×His. According to the previous report (25), it was also reported that glutathionylation of one cysteine residue in the DNA-binding domain of p50 NF-kB inhibits its binding (21).

In the present study, we investigated the possible involvement of glutathionylation in redox regulation of the DNA binding activity of Pax-8 PD in vitro and in vivo. We provide evidence that a decrease in the GSH/GSSG ratio induces glutathionylation of Cys-45 and Cys-57, resulting in loss of DNA binding.

MATERIALS AND METHODS

Plasmid Construction, in Vitro Translation, and Protein Purification—Cloning of rat Pax-8 cDNA and preparation of the mutant cDNA (Pax146), which encodes only 146 amino acids from N-terminal and thus harbors only the PD, were described previously (12, 15). Site-directed mutagenesis was also described previously (12, 15). The sequencing of the entire cDNAs verified the mutations. As shown in Fig. 1, wild-type and PD mutants were named CCC, CSS, SCS, SSC, and SSS, according to the positions of substituted cysteines. These cDNAs were ligated into pJEX2.3- MCS vector (Roche Applied Science). This vector contains six His codons at the 3′-end of the cloning sites. In vitro transcription and translation were performed using the Rapid Translation System (RTS 500 instrument, Roche Applied Science). The His-tagged PD proteins were then purified by using nickel chelate resin (His MicroBeads, Amersham Biosciences). After removing imidazole by Ultra Free C3-GC filter (molecular weight cut-off, 10,000, Millipore, Bedford, MA), the proteins were stored at −80 °C in the presence of 1 mM DTT. Protein contents were determined by Bradford method.

Electrophoretic Mobility Shift Assay (EMSA)—Procedures for EMSA were described previously (26). Oligo(C) containing a recognition site for Pax-8 in the C region of rat thyroglobulin promoter (10) was used as a probe. In vitro translation lysates or purified PD proteins were first treated with 3 mM DTT and then subjected to an Ultra Free C3-GC filter to remove excess DTT. Subsequent chemical oxidation was achieved by incubation with 1 mM diamide (Sigma). After the removal of excess diamide by the filtration, the samples were again incubated with 1 mM DTT. These lysates or purified PD proteins (final 50 pmol) were incubated with 50 μl labeled oligo(C) in EMSA reaction buffer without DTT. The reaction mixture without dye was subjected to polyacrylamide gel electrophoresis. The gel was dried and exposed to Fujix bioimage analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan). In some experiments, the reduced PD proteins were incubated for 60 min at 25 °C in EMSA reaction buffers containing various ratios of GSH/GSSG and then subjected to EMSA. Since two GSH molecules are oxidized into one GSSG, the total concentration of glutathione, GSH plus 2 GSSG, was kept at 3 mM. Therefore, GSH/GSSG ratios of 200, 20, 2, and 0.2 correspond to final GSH/GSSG concentrations (in mM) of 3.0/0.015, 1.5/0.75, and 0.3/1.35, respectively. GSH and GSSG were kept at 3 mM. Therefore, GSH/GSSG ratios of 200, 20, 2, and 0.2 were evaluated by measuring radioactivities of trichloroacetic acid precipitates. Statistical analysis was performed by one-way analysis of variance followed by Student’s t-test.

Analysis of PD Glutathionylation—A part of tritium-labeled glutathione ([3H]GSH, 1924 GBq/mmol, 37 MBq/ml, PerkinElmer Life Sciences) was diluted at 1:10 with 33 mM solution of unlabeled GSH to make 30 mM stock solution of [3H]GSH. According to the previous report (28), another part of [3H]GSH was oxidized by hydrogen peroxide to prepare a 30 mM stock solution of [3H]GSSG. Then the purified CCC, CSS, SSS, and SSS proteins (each 10 pmol/tube) were incubated for 60 min at 37 °C in EMSA reaction buffer containing the labeled GSH/GSSG with the ratio of 200 or 0.2. Incorporation of [3H]gluta-
tion did not affect the binding of SSC and SSS (lanes 11 and 14). These results demonstrate that oxidative modification of the thiol of a single cysteine residue, Cys-45 or Cys-57, leads to impaired binding of PD. Of note, this modification is reversible because subsequent reduction of the oxidized proteins by DTT entirely restored the binding (lanes 3, 6, 9, 12, and 15). Specific PD/oligo(C) complexes are indicated by closed arrowhead.

**Fig. 2.** Oxidation of either Cys-45 or Cys-57 by diamide abolishes PD DNA binding. The in vitro translation products of CCC, CSS, SCS, SSC, and SSS were reduced by 3 mM DTT. After removal of excess DTT by the filtration, they were subjected to EMSA using oligo(C) as a probe. Chemical oxidation was performed by the incubation of these products with 1 mM diamide (lanes 2, 5, 8, 11, and 14). Subsequent reduction was achieved by the incubation of the oxidized products with 3 mM DTT after removal of excess diamide by the filtration (lanes 3, 6, 9, 12, and 15). Specific PD/oligo(C) complexes are indicated by closed arrowhead.

**Fig. 3.** DNA binding of CCC, CSS, and SCS is sensitive to GSH/GSSG ratio. The purified and reduced CCC, CSS, SCS, SSC, and SSS were incubated for 60 min in EMSA reaction buffer containing different ratios of GSH/GSSG, 200, 20, 2, and 0.2. In some experiments, the samples were subsequently incubated with 1 mM DTT. These samples were subjected to EMSA using oligo(C) as a probe.

**Fig. 4.** Glutathionylation is incorporated into CCC, CSS, and SCS. The purified CCC, CSS, SCS, SSC, and SSS proteins (10 pmol of each) were incubated for 60 min at 37 °C in EMSA reaction buffer containing [3H]GSH/GSSG with the ratios of 200 and 0.2. Incorporation of [3H]glutathione was assessed by measuring the radioactivities of trichloroacetic acid precipitates. The experiment was performed in triplicate. The mean radioactivity of SSS was set as a background and was subtracted from those of CCC, CSS, SCS, and SSC. The values are expressed as mean ± S.D. (n = 3), *p < 0.01 versus the values at 200 of GSH/GSSG ratio. Similar results were obtained from a separate experiment.

**Fig. 5.** Ref-1 is capable of reducing glutathionylated CSS and SCS. CSS or SCS protein (final concentration, 30 nM) was incubated for 60 min in EMSA reaction buffer containing [3H]GSH/GSSG with the ratios of 200 and 0.2. The samples were subsequently incubated with 30 μM reduced Ref-1 or heat-denatured (dn) Ref-1. These samples were subjected to EMSA using oligo(C) as a probe.
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FIG. 6. Possible glutathionylation of CSS and SCS in cells under oxidative stress. CHO cells were transiently transfected with the plasmids expressing Myc-tagged CSS and SCS and with their parent plasmid pcDNA3.1/Myc-His (pcDNA). A, after transfection for 42 h, whole cell lysates (lanes 2–4) and their immunoprecipitates (IP) with anti-Myc antibody (Ab) (lanes 6, 8, and 10) and with NRS (lanes 5, 7, and 9) were subjected to Western blot (WB) analysis using anti-Myc antibody. Lane 1 shows the positions of molecular mass markers for 30 and 20 kDa. The closed arrowhead indicates the positions of Myc-tagged CSS and SCS. The asterisk indicates the bands for immunoglobulin light chain. B, after transfection for 42 h, the medium was changed to Hanks’ balanced salt solution containing 10 μg/ml cycloheximide. After a 60-min incubation, the endogenous glutathione pool was labeled with [35S]cysteine for 60 min (lanes 3–13). The cells were then treated with 1 mM diamide for 5 min. Immunoprecipitation was performed using anti-Myc antibody or NRS. The precipitates were subjected to polyacrylamide gel electrophoresis in reducing (DTT) and non-reducing (DTT-) conditions. As a parallel experiment, the transfected cells were labeled with [35S]methionine (lanes 1 and 2). The gel image analyzed by the BAS 2000 system is shown. The closed arrowhead indicates the positions of Myc-tagged CSS and SCS. Lane 14 indicates the positions of molecular mass markers (MWM) for 20 and 14 kDa.

used above. Immunoprecipitation and Western blot analysis demonstrated that the substantial amounts of CSS and SCS proteins could be precipitated and detected with anti-Myc antibody (lanes 8 and 10) but not with NRS (lanes 7 and 9). No protein was detected in the lysates from the pcDNA-transfected cells (lanes 5 and 6). These results led us to the following metabolic labeling experiment.

CHO cells were transfected with the plasmid expressing CSS or SCS, and the glutathione pool was labeled with [35S]cysteine in the presence of cycloheximide. The cells were then exposed to oxidative stress by diamide. As a parallel experiment, the transfected cells were labeled with [35S]methionine. As shown in Fig. 6B, when the cells were labeled with [35S]methionine, CSS and SCS proteins with a molecular mass of 17 kDa were precipitated with anti-Myc antibody (lanes 1 and 2), whereas no corresponding protein was detected with NRS (data not shown). Also, no corresponding protein was precipitated with anti-Myc antibody in a non-reducing condition, when the pcDNA-transfected cells were labeled with [35S]cysteine, and then treated with diamide (lane 3). In contrast, 17-kDa protein was detected with anti-Myc antibody in a non-reducing condition, when the CSS-expressing cells were labeled with [35S]cysteine, and then treated with diamide (lane 7). However, the corresponding protein disappeared, when the precipitate was reduced by DTT (lane 8) or in the absence of diamide treatment (lane 4). Also, no protein was detected by the immunoprecipitation with NRS (lanes 5 and 6). The similar results were obtained by the experiment with SCS (lanes 9–13). Together, these results strongly suggest that CSS and SCS form a complex with [35S]glutathione via reversible disulfide bond in cells under oxidative stress with diamide, although [35S] incorporation into the mutants may imply not only the incorporation of [35S]glutathione but also that of [35S]cysteine itself (S-thiolation).

We next examined subcellular localization of Myc-tagged CSS 42 h after transfection when the above metabolic labeling experiments were conducted. Fig. 7, A and C, show the staining of CSS- and pcDNA-transfected CHO cells with anti-Myc antibody, respectively. Fig. 7, B and D, are the nuclear staining with Hoechst 33258. It was clearly shown that Myc-tagged CSS was dominantly present in the nucleus; two nuclei out of four were stained with anti-Myc antibody (Fig. 7, A and B). The two non-stained nuclei would represent the absence of CSS-expressing plasmid in the cells. When the cells were transfected with pcDNA, no remarkable staining with anti-Myc antibody was detected in nucleus and cytoplasm (C and D). The similar results were obtained by the experiment with SCS (data not shown). These observations are consistent with the previous report showing that the endogenous Pax-8 is detected in the nucleus of dog thyrocytes (31) and with the report demonstrating the presence of nuclear translocation signal in PD (32). These results indicate that glutathionylation of Pax PD may occur in the nucleus.

DISCUSSION

Our previous studies defined Cys-45 and Cys-57 in PD as redox-reactive cysteine residues that are responsible for the reversible oxidative inactivation of Pax-8 binding (15). The present study expanded this finding. Using CSS and SCS mutants, it was clearly demonstrated that oxidation of either one
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*Fig. 7. Pax PD CSS mutant is localized in the nucleus.** CHO cells were transfected with the plasmid expressing Myc-tagged CSS or with the parent plasmid pcDNA3.1/Myc-His (pcDNA). After transfection for 42 h, the cells were subjected to immunocytochemical analysis using anti-Myc antibody and Alexa Fluar 488-conjugated anti-rabbit IgG as the first and second antibodies, respectively. The nuclei were simultaneously stained with Hoechst 33258. The images obtained by fluorescence microscopy are shown. Scale bars indicate 10 μm.

The cysteine residues of the cysteine residues, we investigated GSH/GSSG-dependent glutathionylation using purified PD mutants. A decrease in the GSH/GSSG ratio induced the inhibition of CSS and SCS binding, whereas SSC and SSS binding was not affected (Fig. 3). In addition, [3H]glutathione was only incorporated into CSS and SCS (Fig. 4). These results strongly indicated that GSH/GSSG-dependent glutathionylation of Cys-45 and Cys-57 induces oxidative inactivation of PD. Since the corresponding cysteine residues in Pax-6 and *Drosophila* paired (prd) are known to contact with the DNA backbone (33, 34), glutathionylation may directly interfere with the DNA contact of PD. Accordingly, it was reported that the mutation of Cys-57 in human Pax-8, which corresponds to rat Cys-57, to tyrosine abolished DNA binding and led to congenital hypothyroidism (35), also indicating that the large side group of tyrosine may interfere with DNA binding. Note that the glutathionylation-dependent inhibition of PD binding was reversed by DTT, indicating the reversible control of PD by glutathione (Fig. 3). Furthermore, it was suggested that glutathionylation of CSS and SCS occurs in cells in response to treatment with 1 mM diamide for 5 min (Fig. 6). Since it was reported that treatment of retina pigment epithelial cells with 0.5 mM diamide for 5 min induced a marked decrease in the GSH/GSSG ratio to less than 1 (36), the glutathionylation of CSS and SCS in cells may occur in a GSH/GSSG-dependent manner as it does *in vitro*.

Recently, several studies demonstrated that a change in intracellular redox potential influences thyroid cell function. Our previous study showed that the oxidative stress induced by redox-active copper attenuates thyroperoxidase and Pax-8 expression and stimulates cell proliferation (37). Lonigro et al. (38) demonstrated that a decline in intracellular GSH level by treatments with 1 mM diamide for 5 min (Fig. 6). Since it was reported that treatment of retina pigment epithelial cells with 0.5 mM diamide for 5 min induced a marked decrease in the GSH/GSSG ratio to less than 1 (36), the glutathionylation of PD present in the assay, without the reduction of the oxidized Ref-1.

In conclusion, the cysteine residues corresponding to Cys-45 and Cys-57 and the surrounding amino acid sequences are well conserved in all Pax family members (9). This may imply that the thiols of the conserved cysteine residues are highly reactive, as are those of Cys-45 and Cys-57 in Pax-8 PD. Thus, the glutathionylation, a novel posttranslational modification for PD, would provide a new insight into the molecular basis for the modulation of Pax function.

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18. Reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical are generated in cells as a result of normal aerobic metabolism as well as oxidative stress. Thyroid follicular cells are unique in that they produce hydrogen peroxide for thyroid hormone synthesis by a thyroid-specific system, and its generation is increased by thyrotropin (41). Therefore, one of the plausible roles of glutathionylation of Pax-8 PD may be the protection of Cys-45 and Cys-57 against oxidative damage by reactive oxygen species. On the other hand, we and others demonstrate that thyrotropin stimulates expression of various anti-oxidative, reducing enzymes such as Ref-1, thioredoxin, and peroxiredoxin. Indeed, this study showed that Ref-1 can enzymatically reduce the glutathionylated PD and restore its binding (Fig. 5). Therefore, the present findings of oxidative inactivation of Pax-8 by glutathionylation and its reversal by Ref-1 may provide a molecular basis for thyrotropin-dependent activation of Pax-8 binding.
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