Pescadillo Interacts with the Cadmium Response Element of the Human Heme Oxygenase-1 Promoter in Renal Epithelial Cells*

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Renal tubular cells elicit adaptive responses following exposure to nephrotoxins, such as cadmium. One response is the up-regulation of the 32-kDa redox-sensitive protein, heme oxygenase-1. Exposure of renal proximal tubular epithelial cells to 10 μM cadmium demonstrated induction (~20-fold) of heme oxygenase-1 mRNA and protein. Using a 4.5-kb human heme oxygenase-1 promoter construct, the importance of a previously identified cadmium response element (TGCTAGAT) in HeLa cells was verified in renal epithelial cells. Specific protein-DNA interaction with this sequence was demonstrated using nuclear extracts from cadmium-treated cells. Yeast one-hybrid screen of a human kidney cDNA library resulted in the identification of pescadillo, a unique nucleolar, developmental protein, as an interacting protein with the cadmium response element and was confirmed by chromatin immunoprecipitation in vivo and gel shift assays with purified glutathione S-transferase-pescadillo protein in vitro. The specificity of the DNA-protein interaction was verified by the absence of a binding complex when the core sequence of the cadmium response element was mutated or deleted. In addition, B23/nucleophosmin, another nucleolar protein, did not interact with the cadmium response sequence. Overexpression of pescadillo resulted in increased activity of the 4.5-kb human heme oxygenase-1 promoter construct but failed to activate this construct when the cadmium response sequence was mutated. The findings demonstrate the important and previously unrecognized role of pescadillo as a DNA-binding protein interacting specifically with the cadmium response element of the human heme oxygenase-1 gene.

Cadmium absorbed accumulates mainly in the kidney and liver and has a long biological half-life of 15 years (3). Following absorption, cadmium is bound to the apoprotein, metallothionein, and is filtered through the glomerulus into the urinary space, where it is endocytosed by the proximal tubule cells and degraded by the lysosomes, resulting in the release of cadmium. The intracellular release of cadmium is responsible for the generation of reactive oxygen species, glutathione depletion, lipid peroxidation, protein cross-linking, and DNA damage, culminating ultimately in oxidant-induced cell death (4–8).

Recent studies have demonstrated that under states of increased oxidant stress, heme oxygenase (HO)2-1 is induced as an adaptive and beneficial response. HO-1 is a 32-kDa microsomal enzyme responsible for catalyzing the conversion of heme to biliverdin, liberating equimolar amounts of carbon monoxide (CO) and iron (9). Biliverdin is converted to bilirubin by biliverdin reductase. There are two main isoforms of heme oxygenase, a highly inducible form, HO-1, and a constitutive form, HO-2 (9, 10). A third isoform, HO-3, closely related to HO-1 (11), has recently been shown to be a pseudogene (12). HO-1 is induced by several different stimuli, including heme, UV radiation, hydrogen peroxide, oxidized low density lipoprotein, nitric oxide and nitric oxide donors, growth factors, cytokines, shear stress, hyperoxia, hypoxia, glucose deprivation, and heavy metals (e.g. cadmium) (13).

The human HO-1 gene, located on chromosome 22q12, has five exons and spans ~14 kb (14). Previous studies have identified a potential cadmium response element (CDRE) (TGCTAGAT) in the 5’-flanking region of the human HO-1 gene in HeLa cells (15). However, the protein(s) interacting with the CDRE have not been determined. Using a yeast one-hybrid screen of a human kidney cDNA library, we have identified pescadillo as one of several candidate proteins interacting with the CDRE in renal epithelial cells. We focused our studies on pescadillo, given its localization in the nucleus/nucleolus and its abundance in organs, such as the kidney, liver, and testis (16), tissues that are particularly susceptible to cadmium-induced toxicity (3).

Pescadillo was originally identified in zebrafish (17) and has been shown to play important roles in normal embryonic devel-

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2 The abbreviations used are: HO, heme oxygenase; CDRE, cadmium response element; mCDRE, mutated CDRE; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GFP, green fluorescence protein; SRE, stress response element; BRCT, BRCA1 C-terminal; GST, glutathione S-transferase.
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opment, ribosome biogenesis, DNA replication, chromosome stability, and cell cycle progression (18–20). Mutations of the pescadillo gene result in severe defects in eye and head size, jaw formation, liver growth, and development of the gut and pancreas (17). Homologues for the pescadillo gene have been found in yeast (YPH1 and Nop7p) (21), mouse (Pes1) (16), and human (PES1) cells (18). The human pescadillo gene has been localized to chromosome 22q12.1 and codes for a 68-kDa protein (GenBank™ accession number NM_014303). Amino acid analysis reveals several nuclear localization signals, a SUMO-1 modification sequence, and the BRCA1 C-terminal (BRCT) domain (16, 18). The presence of a BRCT domain suggests the potential for interactions with other cellular, nuclear, and/or nucleolar proteins.

Here, we show that (i) cadmium stimulates human HO-1 gene expression in renal epithelial cells through specific interaction with the CdRE sequence; (ii) the developmental protein pescadillo associates with the CdRE in vivo and in vitro, and (iii) pescadillo regulates HO-1 promoter activity in renal epithelial cells. These findings demonstrate a novel transcriptional regulatory function for pescadillo and suggest that nucleolar proteins may play an important role in modulating the cellular response to stress.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture medium, serum, and supplements were obtained from Invitrogen. Hemin (iron protoporphyrin chloride) and cadmium chloride were obtained from Sigma. Restriction endonucleases and reagents for PCR, including synthetic oligonucleotides, were obtained from New England Bio-labs (Beverly, MA) and Invitrogen, respectively.

Cell Culture—HK-2 cells (ATCC), an immortalized human renal proximal tubular epithelial cell line from normal adult kidney, transduced with HPV-16 (22), were grown in keratinocyte serum-free medium with 5 ng/ml recombinant epidermal growth factor, 40 μg/ml bovine pituitary extract, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 μg/ml amphotericin B. HEK293 cells (ATCC), an immortalized human embryonic kidney epithelial cell line, transformed with adenovirus 5 DNA, were grown in minimal essential medium (Eagle) with 2 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Primary human renal proximal tubular cells (Clonetics, Walkersville, MD) were grown in renal epithelial basal medium supplemented with fetal calf serum (0.5%), insulin (5 μg/ml), transferrin (10 μg/ml), triiodothyronine (6.5 ng/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), and epidermal growth factor (10 ng/ml). All cells were maintained at 37 °C in a humidified incubator with 95% air and 5% carbon dioxide.

Plasmid Constructs and Transfections—The plasmid constructs, pHOGL3/4.5 and pHOGL3/4.0, containing −4.5 and −4.0 kb fragments, respectively, of the 5′-flanking region of the human HO-1 gene have been described previously (23, 24). The CdRE sequence (TGCTAGAT) in the pHOGL3/4.5 was mutated to TGCTGAT to generate pHOGL3/4.5/mCdRE using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The expression plasmids encoding pescadillo fused to the enhanced green fluorescent protein (GFP) or to a Myc epitope were created using a full-length human pescadillo cDNA inserted into the vector pEGFP-C3 (Clontech) or a Myc-tagged expression vector driven by the cytomegalovirus promoter, respectively, and have been described previously (18). A GST-pescadillo fusion protein was prepared by cloning the pescadillo gene into the BamHI-EcoRI site of the pGEX 4T-2 plasmid. GST fusion proteins were isolated according to the manufacturer’s protocol (Pierce).

All cells were plated in collagen-coated 10-cm dishes and transiently transfected at a confluence of ~85% using Lipofectamine 2000 (Invitrogen) with equimolar amounts of plasmid DNA using a batch transfection protocol (23, 25). Transfection was performed for 1.25 h, cells were rinsed one time with Hanks’ balanced salt solution, and fresh growth medium was added. After 5–7 h of recovery, each 10-cm dish was passaged into one collagen-coated 24-well tray. The transfected cells recuperated for 24 h prior to treatment with vehicle (phosphate-buffered saline) or cadmium (10 μM) for 16 h. Cell lysates were collected, and luciferase activity was measured according to the manufacturer’s instructions (Promega). To test the effects of pescadillo overexpression on HO-1 reporter activity, HEK293 cells were co-transfected with pHOGL3/4.5 (4 μg) with the GFP-pescadillo expression plasmid in 10-cm dishes using molar ratios of 1:0.03 (0.1 μg), 1:0.3 (1 μg), and 1:3 (10 μg), respectively. GFP alone was used as a control. Another group of HEK293 cells were co-transfected with pHOGL3/4.5/mCdRE and the GFP-pescadillo expression plasmid in a similar fashion using a batch transfection protocol.

Northern and Western Analysis—Total RNA was extracted using the Chomczynski and Sacchi method (26) and purified using Trizol. Total RNA was electrophoresed on a 1% agarose gel, electrotransferred to nylon membranes, and hybridized with a 32P-labeled human HO-1 cDNA probe. The membranes were stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe. To quantitate expression levels, autoradiographs were scanned on a Hewlett-Packard Scanjet 4C scanner using Deskscan II software. Experiments were adjusted for loading using glyceraldehyde-3-phosphate dehydrogenase quantification and then normalized and expressed as arbitrary units.

For Western blotting, samples were prepared in Laemmli buffer and then separated in a 10% SDS-polyacrylamide gel and transferred to a Hybrid-P polyvinylidene difluoride membrane. The membrane was then blocked in 5% nonfat dry milk, 1% bovine serum albumin solution for 1 h at room temperature, and the specified primary antibody was added (HO-1, 1:2000; pescadillo, 1:3000) to the blot and incubated at room temperature for 1 h. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution) was added after three washes and incubated at room temperature for 1 h, prior to exposure to enhanced chemiluminescence according to the manufacturer’s recommendation (Amersham Biosciences).

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were isolated from cells that were stimulated with vehicle (phosphate-buffered saline), hemin (5 μM), or cadmium (10 μM) for 2 and 4 h. Radiolabeled oligonucleotides (1 ng) (Table 1) containing the potential binding site sequence(s), determined
experimentally, were incubated with nuclear extracts (3–10 μg of protein) or purified column extracts (0.83–7 μg). Preincubation for 20 min at 4 °C in the presence of 1 mg of poly(dI–dC) (0.1 μg for column fractions) blocked nonspecific protein–DNA complex formation. The resulting protein–DNA complexes were analyzed by electrophoresis on a polyacrylamide gel followed by autoradiography. Competing oligonucleotides were used at 50- and 100-fold excess. Gel shift assays were also performed using the CdRE oligonucleotide with cadmium-stimulated nuclear extracts from HK-2 cells in the presence of a goat polyclonal anti-pescadillo antibody (1 μg) (ab19036) (Abcam, Cambridge, MA) or normal goat serum as control.

Yeast One-hybrid Screening—A yeast one-hybrid screening system for the identification of DNA-binding proteins was purchased from Clontech. Four tandem copies of the CdRE sequence (core sequence underlined) (5′-CGCGATTGCTAGATT-3′) were prepared with an EcoRI overhang for the 5′-end and a Sall site for the 3′-end, allowing directional cloning into the reporter plasmid pHsi. The recombinant plasmid was introduced into the genome of the yeast strain, Y4271. Following selection for stable transformants, the yeast strains mid was introduced into the genome of the yeast strain, Y4271. Cloning into the reporter plasmid pHsi. The recombinant plasmid was introduced into the genome of the yeast strain, Y4271.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed essentially as described (27) with some modifications. HK-2 cells were grown to ~80% confluence on 150-mm plates and induced with hemin (5 μM), cadmium (10 μM), or vehicle 4 h. 1 μg of a rabbit polyclonal affinity-purified anti-pescadillo antibody (A300-607A) (Bethyl Laboratories, Montgomery, TX) was added, and the samples were rotated at 4 °C overnight. An antibody control consisting of normal rabbit serum was also included. PCR products were electrophoresed in a 1.8% agarose gel and transferred to a Hybond XL membrane. The blots were then hybridized with a 32P-end-labeled nested oligonucleotide probe corresponding to the CdRE sequence.

Expression of GST Fusion Protein Constructs—GST; GST-pescadillo; deletions of GST-pescadillo, including the BRCT domain (ΔBRCT), the amino terminus (Δ1–272), and just the amino terminus (1–272); and GST-B23 constructs were transformed into Escherichia coli using electroporation. Cells were grown, and the fusion protein was expressed according to the manufacturer’s protocol (Pierce) with the following exceptions.

**TABLE 1**

| Oligonucleotide | Nucleotide sequence |
|-----------------|---------------------|
| CdRE            | 5′-AAATCCGGCGATTTTCGAGATTTTGCTG-3′ |
| mCdRE           | 5′-AAATCCGGCGATTTTCGAGATTTTTGCTG-3′ |
| ΔCdRE           | 5′-AAAGGAAGCCCGGATTTTTTCGCTGATCACC-3′ |
| AP-1            | 5′-CGCTTGTGACTCGGGAGG-3′ |

**FIGURE 1.** Induction of HO-1 by cadmium (Cd) in human renal epithelial cells requires a CdRE. HK-2 cells were stimulated with phosphate-buffered saline (V, control) or 10 μM cadmium for 4 h (RNA) or 16 h (protein). A, total RNA was isolated, and Northern analysis was performed using a 32P-labeled human HO-1 cDNA probe. The blot was stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase probe to control for loading and transfer of RNA. B, total protein was isolated, and Western analysis was performed using an α-HO-1 antibody. The blot was reprobed for actin to control for loading and transfer of protein. C, HK-2 cells were transfected with luciferase reporter plasmids, pHOGL3/4.5, pHOGL3/4.5/mCdRE, or pHOGL3/4.0 containing a 5B11; Cell Signaling). The CdRE in the 4.5-kb fragment is indicated, and the mCdRE is shown in lowercase type. Cells were treated with vehicle or 10 μM cadmium, and luciferase activity was determined as described. XbaI (X) and PstI (P) restriction sites are shown. Results are mean ± S.E. and are derived from three independent experiments, performed each time with 12 replicates/condition.

Induction of the fusion protein was done with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside for 5 h. Isolation on a GST column was performed as directed by the manufacturer, except after loading the extract onto an equilibrated column, 1 column volume of buffer was run through, and the column was then capped and allowed to incubate with extract for 30 min at room temperature.

**In Vitro Translation of Myc-Bop1 and Pulldown with GST-Pescadillo**—Myc-tagged BOP1 (block of proliferation 1) protein was produced from the Cs2 + Myc tag plasmid by *in vitro* transcription-coupled translation using the TNT SP6 quick coupled transcription/translation system (Promega, Madison, WI) according to the manufacturer’s instructions. The rabbit reticulocyte lysates containing *in vitro* transcription-coupled translation product were subsequently employed in a GST pulldown assay using GST, GST-pescadillo, and deletion mutants, including pescadillo ΔBRCT, Δ1–272, and 1–272 bound to glutathione-Sepharose beads (GE Healthcare). After a 2-h incubation in EBC buffer (150 mM NaCl, 20 mM Tris, pH 7.8, and 0.5% Nonidet P-40), the beads were washed four times with the same buffer, and the protein complexes were released by boiling in 2× SDS-PAGE loading buffer. After resolving the protein complexes by SDS-PAGE, Myc-tagged BOP1 was detected by Western blot using anti-Myc tag antibody (1:5000; 5B11; Cell Signaling).
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**FIGURE 2.** EMSA showing DNA-protein interaction with the CdRE. A, oligonucleotides used for EMSAs are shown. The CdRE and StRE core sequences are underlined, and the mCdRE is shown in lowercase type. B, specificity of CdRE binding activity to cadmium-stimulated nuclear extracts. The CdRE oligonucleotide was incubated with vehicle-treated (lane 1), cadmium-treated (Cd; lanes 2 and 3), or hemin-treated (lane 4) nuclear extracts from human renal proximal tubular cells (HPTC). C, EMSA reaction using cadmium-stimulated nuclear extracts with the CdRE (lane 1) and 50 or 100× nonradioactive competitor. Lanes 2, 3, 6, and 7 demonstrate specific competition of binding activity, whereas lanes 4, 5, 8, and 9 show a lack of competition with the mCdRE oligonucleotide. The arrow indicates the specific DNA-protein complex.

| Protein Function | Table 2 | Protein  | Function |
|------------------|---------|----------|----------|
| Acyl-CoA dehydrogenase | Catalyzes the initial step of the mitochondrial fatty acid β-oxidation pathway. | Acyl-CoA dehydrogenase | Catalyzes the initial step of the mitochondrial fatty acid β-oxidation pathway. |
| ADP-ATP carrier protein | Catalyzes the exchange of adenine nucleotides across the mitochondrial membrane. | ADP-ATP carrier protein | Catalyzes the exchange of adenine nucleotides across the mitochondrial membrane. |
| Aldolase B | Metabolism of exogenous fructose. | Aldolase B | Metabolism of exogenous fructose. |
| Aminopeptidase N | Zinc-dependent peptidase that catalyzes removal of N-terminal residues from peptides. | Aminopeptidase N | Zinc-dependent peptidase that catalyzes removal of N-terminal residues from peptides. |
| ATP synthase | Synthesis of ATP from ADP. | ATP synthase | Synthesis of ATP from ADP. |
| Capping protein (actin filament) | “Caps” actin filaments, preventing loss of actin subunits. | Capping protein (actin filament) | “Caps” actin filaments, preventing loss of actin subunits. |
| Chloride intracellular channel protein 1 | Nuclear localized; controls movement of calcium across nuclear membrane. | Chloride intracellular channel protein 1 | Nuclear localized; controls movement of calcium across nuclear membrane. |
| Elongation factor 1 α1 | Transcription factor; false positive due to artificial binding site created in CdRE repetitive sequence. | Elongation factor 1 α1 | Transcription factor; false positive due to artificial binding site created in CdRE repetitive sequence. |
| Enoyl coenzyme A hydratase 1 | Isomerization of 3-trans,5-cis-dienoyl-CoA to 2-trans,4-trans-dienoyl-CoA. | Enoyl coenzyme A hydratase 1 | Isomerization of 3-trans,5-cis-dienoyl-CoA to 2-trans,4-trans-dienoyl-CoA. |
| Haplotype mitochondrion | Mitochondrial polymorphism of alleles. | Haplotype mitochondrion | Mitochondrial polymorphism of alleles. |
| Hypothetical protein H17 | Predicted protein. | Hypothetical protein H17 | Predicted protein. |
| Mitogen-activated protein kinase kinase | Direct activation of the stress-activated protein kinase and extracellular signal-regulated protein kinase pathways by an inducible mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase kinase 3 derivative. | Mitogen-activated protein kinase kinase | Direct activation of the stress-activated protein kinase and extracellular signal-regulated protein kinase pathways by an inducible mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase kinase 3 derivative. |
| NADH dehydrogenase | First complex in electron transport chain; transfers electrons from NADH to coenzyme Q. | NADH dehydrogenase | First complex in electron transport chain; transfers electrons from NADH to coenzyme Q. |
| Pescadillo | Nuclear/nucleolar localized factor; used in ribosome biogenesis and DNA replication. | Pescadillo | Nuclear/nucleolar localized factor; used in ribosome biogenesis and DNA replication. |
| YSC-like 1 | Potential protein chaperone (contains Src homology 3 domain). | YSC-like 1 | Potential protein chaperone (contains Src homology 3 domain). |

**Statistical Analysis**—Results were derived from at least 2–3 independent experiments. Data are expressed as mean ± S.E. For the luciferase data, results were derived from three independent experiments with 12 replicates for each condition. Analyses were performed using analysis of variance and the Student-Newman-Keuls test. All results are considered significant at p < 0.05.

**RESULTS**

**Cadmium Induces HO-1 mRNA and Protein through a CdRE in Human Renal Epithelial Cells**—HK2 cells were stimulated with vehicle (control) or cadmium (10 μM) for either 4 h for Northern analysis or 16 h for Western analysis, respectively. HO-1 mRNA (Fig. 1A) and protein (Fig. 1B) were markedly increased following exposure to cadmium. In order to define the regulatory region for cadmium responsiveness in the human HO-1 promoter, luciferase reporter constructs containing either a −4.5-kb fragment, a −4.0-kb fragment, or a −4.5-kb fragment with a mutation in the CdRE were transiently transfected into HK-2 cells. As shown in Fig. 1C, cadmium (10 μM) resulted in a 4-fold increase in reporter activity of the 4.5-kb human HO-1 promoter, whereas mutation of the CdRE in the 4.5-kb promoter or a 4.0-kb promoter fragment with a deletion of the CdRE significantly reduced basal and cadmium-dependent reporter activation. These results demonstrate that the region located between −4.5 and −4.0 kb, in particular the CdRE, was necessary for cadmium-mediated induction of HO-1 gene expression in renal epithelial cells.

**Specific Cadmium-inducible DNA-Protein Interaction in Human Renal Epithelial Cells**—Using oligonucleotides containing the CdRE region as well as the adjacent stress response element (StRE) sequence (Fig. 2A), nuclear extracts from vehicle, hemin, or cadmium-treated human renal proximal tubular cells were tested in an EMSA. As shown in Fig. 2B, a cadmium-inducible DNA-protein interaction was observed at 2 and 4 h following stimulation. Nuclear extracts from control and hemin-treated human renal proximal tubular cells did not demonstrate significant binding activity. Competition with a 50- and 100-fold excess of the CdRE oligonucleotide resulted in attenuation of the cadmium-inducible DNA-protein complex (lanes 2 and 3), whereas competition with an oligonucleotide...
containing a 2-bp mutation in the core CdRE sequence (mCdRE) failed to compete out the DNA-protein interaction (lanes 4 and 5) (Fig. 2C). An oligonucleotide containing both the CdRE and the adjacent StRE was able to compete out the DNA-protein complex (lanes 6 and 7); however, mutation of the CdRE in this oligonucleotide did not significantly affect DNA-protein binding (Fig. 2C, lanes 8 and 9). These results suggest that the CdRE and not the StRE is required for cadmium-inducible DNA-protein interaction in human renal epithelial cells.

**Pescadillo Interacts with the CdRE in Human Renal Epithelial Cells**—To identify the protein binding to the CdRE, a yeast one-hybrid screen using four tandem repeats of the CdRE with a 12-bp intervening spacer sequence was used to screen a human kidney cDNA library. A total of 7×10⁶ colonies were screened for growth on histidine-free plates, and 19 colonies were judged to be potential positives based on growth in the presence of 45 mM 3-amino-1,2,4-triazole. Plasmid DNAs isolated from these colonies were sequenced, and the corresponding proteins were identified from the GenBank™ data base (Table 2). Of the 15 clones that were sequenced, two DNA-associated proteins, elongation factor 1α and pescadillo, appeared to be likely candidates for further evaluation. Elongation factor 1α was a false positive, since it is known to bind to a site (GCGGAT) that was artificially created due to the intervening spacer sequence in the tandem repeats of the CdRE used as the bait. To examine if the nuclear localized protein, pescadillo, associated with the CdRE in vivo, a ChIP assay was performed on control, hemin, and cadmium-stimulated HK-2 cells. The primers and the probe used for the ChIP assay are indicated in Fig. 3 (top). As shown in Fig. 3, pescadillo associated with the CdRE region of the human HO-1 promoter in cadmium-induced HK-2 cells but not in control or hemin-stimulated cells.

To further confirm the pescadillo-CdRE interaction, a GST-pescadillo fusion protein or GST alone was utilized. A Western blot confirming the identity of the GST-pescadillo fusion protein is shown in Fig. 4A. An EMSA was performed using the CdRE, which demonstrated a shift (asterisk) with GST-pescadillo but not with GST alone (Fig. 4B). A concentration-dependent increase in DNA-protein binding was observed when increasing amounts of the purified GST-Pes protein (1, 3, 5, and 7 μg) were used in the EMSA reaction with the CdRE oligonucleotide (Fig. 4C, top). The bottom of Fig. 4C shows a Coomassie-stained gel loaded with 1 (lane 1), 3 (lane 2), 5 (lane 3), and 7 μg of GST-Pes protein and 1 μg of bovine serum albumin (lane 5). Densitometric
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To further confirm the specificity of the GST-Pes DNA binding, the CdRE oligonucleotide was incubated with GST-B23/nucleosomin, another abundant nucleolar protein. No significant DNA-protein complex was observed (Fig. 4D, lane 3). Further confirmation for the specificity of the CdRE-Pes interaction was demonstrated by the absence of binding activity to a mutated CdRE (mCdRE) (Fig. 4D, lane 4) or an AP-1 sequence (Fig. 4D, lane 5) or when an oligonucleotide lacking the CdRE core sequence was utilized (ΔCdRE) (Fig. 4D, lane 6). These in vitro results provide evidence of a specific interaction between pescadillo and the CdRE.

To further confirm the specificity of the DNA-protein interaction, gel shift experiments using nuclear extracts from cadmium-treated HK-2 cells and the CdRE in the presence of an anti-pescadillo antibody were also performed. As shown in Fig. 5A, a significant loss of DNA binding activity was observed when the DNA-protein complex was incubated with anti-pescadillo antibody (Fig. 5A, lane 2, arrow), compared with normal goat serum, which was used as a control (Fig. 5A, lane 3). The band intensity of the higher complex also appeared to be increased with the anti-pescadillo antibody, compared with lanes 1 and 3.

To identify the regions of pescadillo that are necessary for the CdRE-binding activity, EMSA was performed using ABRCT, Δ1–272, and 1–272 GST-pescadillo expressed fusion proteins. Fig. 5B shows that the full-length GST-pescadillo was required for DNA binding activity (lane 3), since deletion of the BRCT domain (lane 4) or amino acids 1–272 (lane 5) or a truncated pescadillo protein (amino acids 1–272) (lane 6) did not demonstrate DNA-protein interactions. A Coomassie-stained gel confirming expression and isolation of the various GST-pescadillo deletions is shown in Fig. 5C. To demonstrate that the deletion constructs maintain functional protein-protein interactions, GST pulldown experiments were performed using in vitro translated Myc-tagged Bop1 (a conserved nucleolar protein involved in ribosomal RNA processing and ribosome assembly and a pescadillo-binding protein) (28) incubated with wild-type GST-pescadillo or the various deletion constructs. The N-terminal deletion (Δ1–272) did not bind Bop1, but the other constructs showed significant protein-protein interaction comparable with the wild-type pescadillo, suggesting that these constructs maintained functional integrity (Fig. 5D). These results suggest that multiple domains are required for pescadillo to bind to the CdRE.

Pescadillo Regulates Cadmium-inducible HO-1 Promoter Activity—To determine the role of pescadillo in activating HO-1 promoter activity, increasing amounts of a GFP-pescadillo expression vector were co-transfected with the 4.5-kb human HO-1 promoter-reporter construct in HEK293 cells. As shown in Fig. 6, a dose-dependent increase in HO-1 reporter gene activity was observed with overexpression of GFP-pescadillo in both control and cadmium-stimulated cells compared with cells transfected with GFP alone. However, overexpression of GFP-pescadillo in cells transfected with the 4.5-kb HO-1 promoter construct containing a mutated CdRE (pHOGL3/4.5/mCdRE) failed to show any significant increase in reporter activity.

DISCUSSION

The results of the present study provide evidence for a unique DNA-protein interaction involving the CdRE of the

![FIGURE 5. Multiple domains are required for DNA-protein interaction of the CdRE with pescadillo. A, EMSA performed using the CdRE as a probe with cadmium-stimulated nuclear extracts (NE) (lane 1) in the presence of a goat polyclonal anti-pescadillo antibody (1 μg) (Abcam) (lane 2) or normal goat serum (NGS) (lane 3). The arrow indicates the specific DNA-protein complex. B, EMSA performed using the CdRE as a probe with GST alone (lane 1), GST-PesWT (lane 2), GST-PesABRCT (lane 3), GST-PesΔ1–272 (lane 4), or GST-Pes1–272 (lane 5). The asterisk indicates the complex formed by the interaction of purified GST-pescadillo protein with the CdRE probe. C, Coomassie-stained gel showing the indicated purified wild-type and mutated GST-pescadillo fusion proteins. D, Western blot analysis showing pescadillo-Bop1 interaction. In vitro translated Myc-tagged Bop1 was employed in a GST pulldown assay using GST, GST-pescadillo, and deletion mutants, including pescadillo ABRCT, Δ1–272, and 1–272 bound to glutathione-Sepharose beads. Protein complexes were released by boiling in 2 × SDS-PAGE loading buffer. After resolving the protein complexes by SDS-PAGE, Myc-tagged Bop1 was detected by Western blot using an anti-Myc antibody as described under "Experimental Procedures."](image-url)
human HO-1 gene with pescadillo, a nucleolar localized protein, in renal epithelial cells. Several findings in this work support this novel observation, showing that pescadillo binds directly to a specific DNA sequence and regulates gene transcription. The identification of pescadillo as a CdRE-binding protein was first observed on a yeast one-hybrid screening of a human kidney cDNA library and confirmed by ChIP and gel shift assays. More importantly, the specificity for the CdRE-pescadillo interaction was verified by several findings. (i) A significant association between the CdRE region and pescadillo was observed only in cadmium-treated cells and not in unstimulated (control) or hemin-treated cells by ChIP assays. (ii) A gel shift assay using increasing concentrations of GST-Pes showed a dose-dependent increase in DNA-protein binding, whereas GST alone did not show any binding. (iii) Mutation or deletion of the core CdRE sequence eliminated DNA-protein binding. (iv) The presence of an anti-pescadillo antibody decreased DNA-protein binding. (v) Deletion of the BRCT domain and amino terminus of pescadillo eliminated DNA-protein binding. (vi) No binding was observed with an AP-1 (StRE) sequence, which was located immediately downstream of the CdRE in the human HO-1 promoter. (vii) Gel shift assays performed with another abundant nucleolar protein, B23, and the CdRE did not reveal a discernible DNA-protein complex. Furthermore, the CdRE-pescadillo interaction was functionally significant, since overexpression of pescadillo was capable of activating an HO-1 promoter construct containing the CdRE, but not when the CdRE was mutated.

The molecular mechanism of how pescadillo regulates gene expression is not entirely known. Most nucleolar proteins, including pescadillo, are involved in ribosome biogenesis and assembly, DNA synthesis, cell proliferation, and transformation (16, 18, 19, 29–31). Pescadillo has been shown to interact with a few other proteins in various organisms. A zinc-binding, dual specificity phosphatase (YVH1p) found in malaria parasites, with an ortholog in humans, interacts with pescadillo (32). Pescadillo also interacts with BOP1, causing nucleolar localization, suggesting that nuclear localization may be due to a protein-protein interaction (28). This is consistent with our observation that mutations or deletion of the BRCT domain completely exclude pescadillo from the nucleolus, although it is retained in the nucleoplasm.³ Pescadillo also associates with insulin receptor substrate-1 and upstream binding factor-1, supporting a potential role in transcriptional gene regulation (33).

Whereas our results demonstrate that pescadillo binds directly to the CdRE, it may also indirectly regulate HO-1 gene expression. It is possible that changes in chromatin and accessibility of transcription factors to active sites on chromatin may be modulated by pescadillo. A recent study has shown that pescadillo induces large scale chromatin unfolding, the activity of which maps to the BRCT domain of pescadillo (34). Pescadillo also interacts with factors that positively affect RNA polymerase I activity (21). Furthermore, compartmentalization and translocation of pescadillo from the nucleolus to the nucleoplasm and subsequent binding to specific DNA sequences, such as the CdRE, may provide another potential level of regulation. In this regard, we have observed that upon exposure of HeLa cells to UV radiation, pescadillo trafficking occurs from the nucleolus to the nucleoplasm.³

Cadmium is known to modulate a wide array of intracellular signaling cascades, including various transcription factors, such as AP-1, MTF-1, HIF-1α, NF-κB, USF, and Nrf2 (2, 35). Cadmium can also indirectly mediate changes in target gene expression by effects on protein kinases that lead to gene induction through phosphorylation of transcription factors (2, 39). Examination of the promoter regions of other cadmium-inducible genes, such as metallothionein, c-fos, c-jun, and HSP70, reveal that only the c-fos promoter has a sequence with homology to the CdRE at ~3.987 kb from the transcriptional start site. Ishikawa et al. (36) have reported the induction of several unidentified nuclear proteins that interact with a heat shock element in the promoter of the c-fos gene in response to cadmium. Previous studies have identified a metal-responsive sequence (TGCRNC) in the metallothionein gene promoter that is also responsive to cadmium. At least two known transcription factors, MTF-1 and MBF-1, have been shown to bind to the metal response element of the murine metallothionein promoter (37, 38).

We have previously identified that an internal enhancer in conjunction with a ~4.5 kb promoter region is required for maximal heme and cadmium-mediated HO-1 induction in renal epithelial cells (24). In addition, multiple regulatory sequences in the ~4.5 to ~4.0 kb region of the human HO-1 promoter, immediately downstream and upstream of the CdRE, that bind to the AP-1 family of transcription factors as

³ T. Uo, J. T. Ho, Y. Kinoshita, and R. S. Morrison, unpublished observations.
well as a proximal E box sequence that binds to USF-1 and USF-2 regulate HO-1 gene expression in response to hemin and cadmium. Further studies to determine the interaction of pescadillo with these other regulatory factors for cadmium-inducible HO-1 gene expression would be of interest.

The physiological significance of cadmium-mediated HO-1 gene induction is relevant to cadmium-induced nephrotoxicity. Studies have demonstrated that the induction of HO-1 represents an adaptive and beneficial response to oxidant injury in multiple organ systems, including the kidney (40, 41). In this regard, cadmium-mediated HO-1 induction in the renal tubular cells may serve a protective response. Cadmium is known to cause gonadal toxicity (3), and pescadillo also localizes to the gonads in addition to the kidney (16). It is interesting to note that pescadillo localizes to tissues that are susceptible to cadmium-induced toxicity, including the kidney, testis, and liver.

In summary, our findings demonstrate a novel transcriptional regulatory function for pescadillo in cadmium-inducible HO-1 gene expression and suggest that nucleolar proteins may play an important role in modulating the cellular response to stress.

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