Proline oxidase is a p53-induced gene that can mediate apoptosis in lung carcinoma cells. Here, we provide evidence implicating a role for proline oxidase in renal carcinoma. We observed absent or reduced expression of proline oxidase in 8 of 12 primary renal cell carcinomas, with respect to their normal tissue counterparts. Two renal cell carcinomas, which displayed low or no expression of proline oxidase, expressed p53s that were less capable of inducing proline oxidase than p53 isolated from normal renal tissue. One of those tumor-derived p53s contained a double transition mutation at amino acid residues 125 (Ala to Thr) and 193 (Arg to His), and the other exhibited a single transition mutation at amino acid 149 (Ser to Phe). Forced up-regulation of proline oxidase induced the formation of reactive oxygen species and mediated apoptosis in the 786–0 renal carcinoma cell line. A proline oxidase antisense vector repressed p53-induced up-regulation of proline oxidase, release of cytochrome c from mitochondria, and apoptosis in 786–0 renal carcinoma cells. Taken together, these findings support a role for proline oxidase as a downstream effector in p53-mediated apoptosis. We hypothesize that its altered expression can contribute to the development of renal carcinomas. The presence of proline oxidase in mitochondria, a primary organelle that regulates apoptosis, places this molecule in a subcellular localization that can directly influence the apoptotic pathway and thus tumorigenesis.

The product of the p53 gene plays pivotal roles in several biological processes that are important in reducing the tumorigenic potential of cells. The protein mediates cell growth arrest by controlling cell cycle checkpoints (1), induces apoptosis (2), and can influence the differentiation state of cells (3). Many studies have implicated p53 in genomic stability, surveillance of DNA damage, and DNA repair, which are related to its control over cell growth (4–9).

A series of p53-induced genes has been identified by a serial analysis of gene expression in a colon carcinoma cell line; some of those genes are predicted to encode proteins that could generate or respond to oxidative stress (10). We performed a comparative gene expression analysis between p53-sensitive and -resistant cells, which also revealed the close association of redox enzymes with p53-mediated apoptosis (11). One of those redox enzymes, proline oxidase, and its product, pyrroline-5-carboxylate (P5C),1 exhibited the ability to inhibit cell growth and to induce marked apoptosis in a lung carcinoma cell line, implicating a role for the proline/P5C cycle in p53-mediated growth inhibition and apoptosis (11). Our work was supported by a later study showing that proline oxidase can generate reactive oxygen species (12), which can initiate apoptosis by directly acting on the mitochondrial permeability core complex and effecting the mitochondrial permeability transition (13–15). Here, we report that the expression of proline oxidase was absent or reduced in a significant number of renal cell carcinomas, which was associated with mutant p53 in two cases. A correlation of reduced proline oxidase expression with mutated p53 in two cases of renal carcinoma and the ability of proline oxidase to induce apoptosis in renal carcinoma cells functionally implicate the enzyme as a downstream player in p53-mediated apoptosis.

MATERIALS AND METHODS

Antibodies and Plasmids—The p53 monoclonal antibody, Bp53–12, was purchased from Santa Cruz Biotechnology, the M30 antibody specific for the caspase-cleaved form of cytokeratin 18 (16) from Roche Molecular Biochemicals, a monoclonal antibody for cytochrome c (C-8) from Santa Cruz Biotechnology, and an actin monoclonal antibody (N350) from Amersham Biosciences. A proline oxidase antibody (T338) was generated in rabbits against a synthetic peptide composed of amino acids 338 through 353 of the proline oxidase protein (TGQALEPLLSTQFQ). Kidney p53 cDNAs isolated by reverse-transcription PCR from primary kidney tissues were cloned into the pcDNA3.1 ECHO expression system (Invitrogen). The complete proline oxidase cDNA was cloned into the pAdtrack vector (17), which coexpresses GFP as a normalization control for transfection efficiency. The pAdtrack-proline oxidase vector was used to generate recombinant adenovirus using a simplified procedure as previously described (17). A proline oxidase antisense vector was constructed by subcloning the proline oxidase cDNA encoding amino acids 186–516 in the pEGFP/C2 expression vector (Clontech) in the antisense orientation. The proline oxidase and a wild type p53 recombinant adenovirus were amplified in 293 cells and virus-containing medium used to infect kidney cells.

Cell Culture, Transfection, Western Blotting, and Proline Oxidase Enzyme Assays—The 786–0 renal carcinoma and the H1299 nonsmall cell lung carcinoma cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. To conduct transfections of renal and lung carcinoma cells, a 75-cm² flask of confluent cells was incubated for 2 h at 37 °C in 3 ml of Optimem medium (Invitrogen) containing 4 μg of plasmid DNA and 3 μl of LipofectAMINE-2000 (Invitrogen). The transfection medium was then replaced with Dulbecco’s modified Eagle’s medium and the cells incubated for 24–36 h to allow expression of the transfected gene. To normalize for transfection efficiency, pAdtrack, which expresses green fluorescence protein, was included in the transfection assay where appropriate. Western blotting was performed by using the SuperSignal immunodetection system (Pierce Chemical), as previously described (18). Proline oxidase activity was detected by the

1 The abbreviations used are: P5C, pyrroline-5-carboxylate; GFP, green fluorescence protein; RT, reverse transcription; DIC, 2,6-dichloroindophenol; POX, proline oxidase.

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reduction of the colorimetric indicator 2,6-dichloroindophenol (an electron acceptor) as a result of oxidation of proline in extracts of isolated mitochondria, as previously described (19). Mitochondria were isolated by homogenization of cells in a 0.25 M sucrose buffer (20). After microcentrifugation of whole-cell lysates, the mitochondria-enriched pellet was washed twice in sucrose buffer.

**Apopotosis Assays—**Flow cytometry to quantify apoptosis was performed on ethanol-fixed and permeabilized cells that were stained with propidium iodide according to a previously published protocol (11). Apoptotic cells were quantitated in the subG1 DNA content portion of the flow cytometric scans. Reactive oxygen species were detected by the oxidation of dihydroethidium in the cytoplasm of viable cells. The oxidized compound then migrated to the nucleus as ethidium bromide and emitted a red-orange fluorescence under ultraviolet light when bound to DNA (21). Early apoptosis was detected by immunohistochemistry using a monoclonal antibody (M50) specific for the caspase-cleaved form of cytocheratin 18 (16, 22), according to the manufacturer’s specifications (Roche Molecular Biochemicals). Apoptotic cells were also identified by morphological criteria that included cell blebbing, fragmented and shrunken nuclei, and apoptotic bodies.

**Tumor cDNA Blotting—**Paired normal/tumor cDNA blot arrays were purchased from Clontech. cDNA was normalized by rehybridizing the blot with a ubiquitin housekeeping cDNA probe (Fig. 1, upper panel). The tumor array blot was rehybridized with an ubiquitin cDNA probe for normalization of cDNA (lower panel). Twelve different renal cell carcinomas (4–12) were analyzed.

**RESULTS**

**Proline Oxidase Expression Is Absent or Reduced in Many Renal Cell Carcinomas—** A commercial array containing cDNA amplified from paired normal and tumor mRNA from 12 patients presenting with renal carcinoma was hybridized with a 32P-labeled proline oxidase cDNA probe (Fig. 1, upper panel). The cDNA was normalized by rehybridizing the blot with a ubiquitin housekeeping cDNA probe (Fig. 1, lower panel). The cDNA spotted on the membrane has been shown to correlate closely with the expression of a number of different known mRNAs in the tissue (Clontech). As shown in Fig. 1, five kidney tumors (POX panel, T row, lanes 3, 4, 5, 7, 10) appeared to exhibit little or no expression of proline oxidase mRNA as compared with the matched normal tissue (N row), even though similar amounts of normal and tumor cDNA were present on the blot as shown by rehybridization of the blot with a ubiquitin housekeeping cDNA probe (Fig. 1, Ubiq.). When taking the ubiquitin normalization blot into consideration, it appeared that several other renal cell carcinomas exhibited reduced expression of proline oxidase mRNA (POX panel, T row, lanes 1, 8, 9). Reduced or absent proline oxidase expression thus occurred in two-thirds of the renal cell carcinomas that we examined. This tumor array data suggested that altered expression of proline oxidase is frequently associated with renal cell carcinomas. However, to validate the differential expression of proline oxidase between normal and tumor tissues that we observed in the tumor array cDNA blot, the cDNA derived from kidney 4 and kidney 7 normal and tumor tissues were subjected to PCR. As shown in Fig. 2, the expected 270-bp-size proline oxidase product was generated from normal kidney tissue cDNA (N lanes) cDNA, whereas little or no product was derived from tumor tissue cDNA (T lanes), confirming the array data showing that kidney tumors 4 and 7 lacked apparent expression of proline oxidase. In both renal cell carcinomas 4 and 7, the reduced expression of proline oxidase was very specific, because transcripts for p53, p21waf1/cip1, and Bax were expressed at similar levels in both normal and tumor tissues, relative to the ribosomal S9 protein that was used as a normalization control. From an analysis of the classification and staging of these renal carcinomas, it appeared that reduced expression of proline oxidase was more prevalent in clear cell-type renal cell carcinomas at stages II to IV (Table I; tumors 1, 7, 8, 9, 10). However, two clear cell-type carcinomas did display
normal levels of proline oxidase (Table I; tumors 6, 11). No differences in expression of proline oxidase were detected by the tumor array blotting method in three prostate carcinomas relative to their normal tissue counterparts (data not shown).

**Up-regulation of Proline Oxidase Induces Apoptosis in Renal Carcinoma Cells**—To determine the biological function of proline oxidase in renal cell carcinoma, the 786–0 renal carcinoma cell line was transfected with the proline oxidase expression vector (pAdtrack-Pox-1) to up-regulate proline oxidase. Proline oxidase expression was determined by RT-PCR, Western blotting, and a colorimetric assay to detect oxidation of proline in cell lysates. As shown in Fig. 3A, lane 3, little or no expression of proline oxidase mRNA was detected in 786–0 cells transfected with the pAdtrack-GFP expression vector, whereas considerable levels of proline oxidase transcripts were detected in cells transfected with the pAdtrack-Pox-1 vector (Fig. 3A, lane 4). Considerable induction of proline oxidase protein was observed in 786–0 cells 24 h after transfection with the pAdtrack-Pox vector (Fig. 3C, lane 3) or infection with two different isolates of recombinant proline oxidase adenovirus (lanes 1 and 2) or GFP adenovirus (lane 4) or transfected with the pAdtrack-POX vector (lane 3). Proteins were extracted 24 h after infection or transfection and were immunoblotted for proline oxidase expression by using the T338 proline oxidase peptide antibody. GFP- and proline oxidase (POX)-transfected lysates were also immunoblotted for actin as a protein loading control.

**Forced expression of proline oxidase in renal carcinoma cells.** Proline oxidase cDNA was cloned into the pAdtrack adenovirus expression vector and transfected into 786–0 renal carcinoma cells. Expression of proline oxidase was determined by RT-PCR (A), by an enzyme assay to detect proline-specific oxidation (B), and by Western blotting using a rabbit antibody that was generated against a proline oxidase peptide (C). A, total RNA was isolated from 786–0 cells transfected with pAdtrack-GFP (lane 3) or pAdtrack-proline oxidase (lane 4) expression vector and was subjected to RT-PCR using a combination of glyceraldehyde-3-phosphate dehydrogenase and proline oxidase primers. Lanes 1 and 2, control assays of pAdtrack-POX-transfected 786–0 cells using only glyceraldehyde-3-phosphate dehydrogenase or proline oxidase primers, respectively. Lanes 5 and 6, RT-PCR assays of kidney 4 normal and tumor tissues, respectively, as additional controls for proline oxidase expression. B, proline oxidase-specific reduction of DIC in lysates of 786–0 cells 24 h after transfection with pAdtrack-POX. The reduction of DIC was reflected in the corresponding decrease in absorbance of the reduced DIC at 600 nm. Values obtained from proline oxidase-transfected cell lysates were subtracted from those determined from GFP-transfected lysates to detect proline oxidase-specific activity. C, forced expression of proline oxidase protein in 786–0 cells. Whole-cell lysates were prepared from 786–0 cells infected with two different isolates of recombinant proline oxidase (POX) adenovirus (lanes 1 and 2) or GFP adenovirus (lane 4) or transfected with the pAdtrack-POX vector (lane 3). Proteins were extracted 24 h after infection or transfection and were immunoblotted for proline oxidase expression by using the T338 proline oxidase peptide antibody. GFP- and proline oxidase (POX)-transfected lysates were also immunoblotted for actin as a protein loading control.

Forced expression of p53 in 786–0 cells by infection with the pcDNA3.1-wild type p53 expression vector or with the recombinant p53 adenovirus induced apoptosis (Fig. 6A) and up-regulated the proline oxidase protein (Fig. 4A, mitochondria, lane 3). Expression of p53 was easily observed by Western blotting in 786–0 cells infected with p53 adenovirus, in contrast to the apparent absence of p53 protein in GFP-infected cells (Fig. 4A, whole-cell extract, lower left panel). Essentially all of the induced proline oxidase protein was observed in the mitochondria (Fig. 4A, mitochondria, lane 3) with little or no enzyme located in the cytoplasm (cytoplasm panel, lane 3). Forced up-regulation of proline oxidase by transfection with pAdtrack-POX resulted in significant increases in apoptosis in 786–0 cells, as indicated by the increases in the subG1 population in flow cytometric scans relative to those in GFP-transfected cells (Fig. 4B).

Proline oxidase has been shown to induce the formation of
Fig. 4. **Up-regulation of proline oxidase induces apoptosis and reactive oxygen species in 786–0 renal carcinoma cells.**

**A,** p53-mediated induction of proline oxidase in 786–0 renal carcinoma cells. 786–0 cells were mock-infected (lanes 1) or infected with GFP (lanes 2) or p53 recombinant adenovirus (lanes 3). Mitochondria were isolated 24 h after transfection, and cytoplasmic proteins in the postmitochondrial supernatant were acetone-precipitated. Proteins in mitochondrial and cytoplasmic pellets were solubilized in SDS buffer and analyzed for proline oxidase expression in Western blots by using the T338 proline oxidase antibody. Whole-cell extracts were also prepared by lysing 786–0 cells infected with recombinant GFP and p53 adenoviruses in SDS gel-loading buffer. The extracts were immunoblotted for proline oxidase (POX), p53, or actin (as a protein loading control).

**B,** 786–0 cells were transfected with pAdtrack-GFP or pAdtrack-proline oxidase. 24 h later, apoptotic cells were quantitated by flow cytometry of ethanol-fixed and propidium iodide-stained cells. Generation of reactive oxygen species in GFP-only and proline oxidase-transfected 786–0 cells. Cells were infected with GFP or proline oxidase recombinant adenovirus for 12 h and then incubated with dihydroethidium, which when oxidized by reactive oxygen binds to DNA as ethidium, emitting an orange-red fluorescence under ultraviolet light. Generation of reactive oxygen was then quantitated by flow cytometry of GFP and ethidium fluorescence. Proline oxidase-induced cells showed considerably more ethidium-staining than GFP-only cells, indicating the increased generation of reactive oxygen species.

**C,** a dihydroethidium-stained proline oxidase-transfected cell (24 h after transfection) showing nuclear condensation and fragmentation that is characteristic of the later stage of apoptosis.
reactive oxygen species (12). Dihydroethidium localizes in the cytoplasm and, when oxidized by reactive oxygen, binds DNA and emits an orange-red fluorescence under UV light. As shown in Fig. 4C, proline oxidase-transfected 786–0 cells treated with dihydroethidium showed considerably more orange-red nuclear fluorescence than GFP-only transfected cells, indicating that proline oxidase induced the generation of reactive oxygen species. Fig. 4C shows the condensed and fragmented nuclear morphology characteristic of the latter stages of apoptosis in 786–0 cells.

Caspase-mediated cleavage of cytokeratin 18 is an event associated with the early stages of apoptosis (22). An antibody (M30) specific for an epitope that is uncovered only in caspase-cleaved forms of cytokeratin-18 in proline oxidase-transfected 786–0 cells. The left panels show only the green fluorescence emitted by GFP, and the right panels show only the orange-red fluorescence emitted by the phycoerythrin secondary antibody, using the appropriate filters. Numerous cells transfected with proline oxidase exhibited orange-red staining, indicating activation of caspase activity and cleavage of cytokeratin 18. B, little or no M30 staining was observed in GFP-transfected 786–0 cells (right panel).

A direct role for proline oxidase in p53-mediated apoptosis was suggested from proline oxidase antisense expression studies. Forced up-regulation of wild type p53 in 786–0 cells by transfection with the pcDNA3.1-wtp53 expression vector induced considerable apoptosis 36 h after transfection (48%) (Fig. 6A). Cotransfection of these cells with wild type p53 and a proline oxidase antisense vector resulted in down-regulation of the amount of proline oxidase induced in 786–0 cells (lane 2) relative to cells cotransfected with p53 and GFP (lane 1). 786–0 cells were transfected and subjected to Western blotting by using the T338 antibody as described under “Materials and Methods.” C, release of cytochrome c from mitochondria by p53 (lane 2) is suppressed by the proline oxidase antisense (lane 3). Lane 1, a GFP-transfected control. Postmitochondrial supernatant proteins were acetone-precipitated and subjected to Western blotting using the C-8 cytochrome c monoclonal antibody. The cytochrome c blot was stripped and reprobed with an actin monoclonal antibody to normalize for protein loading.

A proline oxidase antisense vector suppressed p53-mediated apoptosis of 786–0 renal carcinoma cells. A, 786–0 cells were transfected with GFP, p53, or p33 and a proline oxidase antisense expression vector (p53+POX antisense)). After 36 h, cells were harvested, fixed, and prepared for flow cytometry. Apoptotic cells were quantitated in the subG1 region of the flow cytometric scan. B, cotransfection of p53 and POX(antisense) expression vectors resulted in down-regulation of the amount of proline oxidase induced in 786–0 cells (lane 2) relative to cells cotransfected with p53 and GFP (lane 1). 786–0 cells were transfected and subjected to Western blotting by using the T338 antibody as described under “Materials and Methods.” C, release of cytochrome c from mitochondria by p53 (lane 2) is suppressed by the proline oxidase antisense (lane 3). Lane 1, a GFP-transfected control. Postmitochondrial supernatant proteins were acetone-precipitated and subjected to Western blotting using the C-8 cytochrome c monoclonal antibody. The cytochrome c blot was stripped and reprobed with an actin monoclonal antibody to normalize for protein loading.

Two renal cell carcinomas expressed mutant p53s that were less efficient in transactivating the proline oxidase (POX) gene than a wild type p53 derived from normal kidney tissue. A, p53 cDNAs were cloned from normal renal tissue and renal carcinomas 4 and 7 into the ECHO expression system. Vectors were transfected into the p53-null H1299 nonsmall cell lung carcinoma cell line for 24 h. Proline oxidase (POX) and p53 expression were investigated in transfected cells by Western blot using T338 peptide antibody and Bp53–12 monoclonal antibody, respectively. Lane 1, GFP-transfected; lane 2, normal kidney p53; lane 3, renal carcinoma 4 p53; lane 4, renal carcinoma 7 p53. B, RT-PCR analysis of the expression of p53 and proline oxidase (POX, lanes 5–8) and ribosomal protein S9 (lanes 1–4) as a normalization control. Lanes 1 and 2, GFP-transfected; lanes 2 and 6, normal kidney p53; lanes 3 and 7, tumor kidney 4 p53; lanes 4 and 8, tumor kidney 7 p53. Transcript expression for p53 and proline oxidase (B) correlated well with that observed for the respective proteins (A).
compare lane 2 with lane 1). Moreover, the proline oxidase anti-sense vector prevented p53-mediated release of cytochrome c from mitochondria (Fig. 6C, compare lane 3 with lane 2).

Reduced or Absent Expression of Proline Oxidase in Two Renal Cell Carcinomas Is Associated with Mutated p53—Our published work (11) and data presented here indicate that proline oxidase is a proapoptotic p53-induced gene whose expression is frequently reduced or absent in renal cell carcinomas. We asked whether the altered expression of proline oxidase in kidney tumors might be associated with mutant p53 proteins. To determine the functionality of kidney tumor-derived p53, we cloned the p53 cDNAs from both normal and tumor tissue of kidneys 4 and 7. These p53 cDNAs were cloned into the ECHO expression system to determine whether they were capable of inducing proline oxidase in p53-null H1299 lung carcinoma cells. As shown in Fig. 7A, Western blotting demonstrated that p53 cDNAs isolated from normal and tumor kidney tissues were efficiently expressed in H1299 cells (lanes 2–4). Reverse-transcription PCR revealed that p53 isolated from normal kidney 4 dramatically induced the expression of proline oxidase in H1299 cells (Fig. 7B, lower level, compare lane 6 with lane 5). Up-regulation of the p53s from tumors 4 and 7 also resulted in the induction of proline oxidase transcription, albeit to considerably lower levels than that induced by the normal kidney p53 (Fig. 7B, compare lanes 7 and 8 with lane 6). Western blotting showed that the levels of expression of proline oxidase protein correlated closely with the expression of mRNA detected by RT-PCR (Fig. 7A, lanes 2–4). Because both p53 protein and mRNA levels were approximately equivalent between the normal and tumor p53-transfected cells and proline oxidase mRNA and protein were differentially up-regulated by normal and tumor-derived p53s, we surmised that the tumor-derived p53s were less efficient at transactivating the proline oxidase gene. Sequencing of the p53 cDNAs was thus performed in an attempt to derive a structural explanation for the differential induction of proline oxidase between these normal and tumor-derived p53s. The p53 isolated from renal cell carcinoma 4 contained a double transition mutation at amino acid residues 125 (Ala to Thr) and 193 (Arg to His), and that cloned from tumor 7 exhibited a single transition mutation at acid residues 125 (Ala to Thr) and 193 (Arg to His), and that.

In summary, our data implicate a role for altered expression of proline oxidase in renal cell tumorigenesis. The data also demonstrate a direct role for proline oxidase in p53-mediated apoptosis of renal carcinoma cells. We have also observed alterations in proline oxidase expression in primary lung carcinomas, lung carcinoma cell lines, and a primary stomach cancer, suggesting a role for this redox enzyme in other types of cancers as well.

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Proline Oxidase Induces Apoptosis in Tumor Cells, and Its Expression Is Frequently Absent or Reduced in Renal Carcinomas

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