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Abstract approved:

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Aerobic organisms have evolved many sensory mechanisms that allow response to oxidants in the environment. One area of interest is the relationship between the activity of the tumor suppressor protein p53 and the redox state of thioredoxin. Human p53 activity is severely compromised in budding yeast lacking thioredoxin reductase. Evidence suggests p53 may similarly require an intact thioredoxin system in mammals. One explanation for thioredoxin reductase dependence is that p53 cysteines may form inhibitory oxidation products. To test this idea, each p53 cysteine was changed to serine, and the effect on p53 activity in wild-type and thioredoxin reductase-null yeast was determined. Basal activity of each allele was confirmed in p53-null H1299 cells. As expected, substitutions at zinc-coordinating cysteines C176, C238 or C242 resulted in p53 inactivation. Unexpectedly, substitution at cysteine C275 also inactivated p53, which is the first evidence for a non-zinc-coordinating cysteine being essential for p53 function. Substitutions at six positions (C124, C135, C141, C182, C229 and C277) neither

inactivated p53 nor relieved the requirement for thioredoxin reductase, either singly or in combination. The results suggest that p53 dependence on thioredoxin reductase either was an indirect effect or was due to oxidation of one or more of the four essential cysteines.

Another area of interest is the role of peroxiredoxins in oxidant response and signaling. Eukaryotic 2-Cys peroxiredoxins are sensitive to substrate inactivation at moderate H_2O_2 concentrations. The sensitivity of eukaryotic peroxiredoxins may have evolved as a mechanism to facilitate H_2O_2 signaling. For example, inactivation of protein tyrosine phosphatases by H_2O_2 may be necessary for efficient signaling by receptor tyrosine kinases, and peroxiredoxin inactivation may be necessary to prolong episodes of PTP inactivation. To test this model, we measured the oxidation state of peroxiredoxins and the phosphorylation state of EGF receptor in EGF- and peroxide-challenged A431 cells. Peroxide treatment sufficient to half-maximally inactivate Prx2, 3 and 6 did not increase EGFR tyrosine phosphorylation. EGF treatment sufficient to stimulate EGFR tyrosine phosphorylation levels had no effect on the oxidation state of Prx2, 3 or 6. Thus, global oxidation of cytosolic Prx was neither sufficient nor necessary for efficient EGFR phosphorylation.

Control of p53 Tumor Suppressor and Peroxiredoxin Activity Through Shifts in Cellular Redox Balance

by
Christopher S. Stoner

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Christopher S. Stoner, Author

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CONTRIBUTION OF AUTHORS

Christopher S. Stoner performed data collection. Dr. Gary F. Merrill and Christopher S. Stoner designed the experiments and co-authored Chapters 2 and 3.

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CHAPTER 1: INTRODUCTION

All organisms living in aerobic environments face the challenge of mitigating the toxicity of reactive oxygen species (ROS). Aerobes maintain an array of antioxidant enzymes to catabolize ROS and repair damage to nucleic acids, proteins and lipids that result from ROS exposure. It is now clear that aerobes have also developed sensitive signal transduction pathways for the detection of and response to extracellular ROS exposure. There is also evidence emerging that the cell itself produces ROS at low concentrations to serve as a second messenger molecule. Study of the protective signal transduction mechanisms may yield insight into the mechanics of ROS second messenger function.

Direct oxygen sensors

Prokaryotic aerobes have developed two distinct pathways for detection of and response to the ROS species superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). SoxR and SoxS proteins make up the superoxide response pathway. SoxR is a constitutively expressed homodimeric transcription factor that is activated upon superoxide exposure [Wu and Weiss, 1992; Nunoshiba et al., 1992]. Each homodimer contains a pair of [2Fe-2S] iron-sulfur centers [Wu et al., 1995; Hidalgo et al., 1995] that are maintained in the reduced state (Fe^{2+}- Fe^{2+}) under normal *in vivo* conditions [Ding et al., 1996]. Transcriptional activity of SoxR is only apparent once iron-sulfur centers are oxidized

(Fe^{3+}- Fe^{3+}), and is specific for the *soxS* promoter [Hidalgo and Demple, 1994]. SoxS protein is a constitutively active transcription factor responsible for induction of the *soxRS* regulon, encoding a variety of antioxidant and DNA repair enzymes including superoxide dismutase, flavodoxins and endonuclease IV [Amabile-Cuevas and Demple, 1991; Hidalgo and Demple, 1996; Storz and Imlay, 1999; Gaudu and Weiss, 2000].

Sensing of hydrogen peroxide stress in prokaryotes is mediated by OxyR transcription factor. Upon exposure to H_2O_2, OxyR stimulates transcription of a variety of genes encoding proteins with antioxidant functions [Hausladen et al., 1996; Altuvia et al., 1997]. Unlike the iron-sulfur center mechanism of SoxR, OxyR senses H_2O_2 through oxidation of a specific cysteine residue (Cys199) that is essential for OxyR transcriptional activity [Zheng et al., 1998]. Hydrogen peroxide oxidizes Cys199 to a cysteine sulfenic acid (-RSOH) that reacts with glutathione to form a mixed disulfide (-RSSG) [Kim et al., 2002].

The eukaryotic budding yeast *Saccharomyces cerevisiae* senses hydrogen peroxide via Yap1, a functional analog of bacterial OxyR. Yap1 (Yeast AP-1) was originally described as a bZip transcription factor homologous to human c-jun that recognizes the human AP-1 (c-fos/c-jun heterodimer) consensus DNA binding site [Moye-Rowley et al., 1989]. Yap1 is ubiquitously distributed in the yeast cell. Upon hydrogen peroxide challenge, the glutathione peroxidase Gpx3 Cys 36 residue becomes oxidized to sulfenic acid. The signal is transduced to Yap1 by disulfide formation between Gpx3 Cys 36 and Yap1 Cys 598. Yap1 Cys 303 resolves the

intermolecular disulfide, yielding an intramolecular disulfide between Yap1 residues Cys 598 and Cys 303. Intramolecular disulfide formation causes a conformational change that masks the Yap1 nuclear export sequence (NES). Yap1 subsequently accumulates in the nucleus, which increases target gene transcription [Delaunay et al., 2002]. Regulated genes include peroxiredoxins (*TSA1* and *AHP1*) [Lee et al., 1999a], thioredoxin 2 (*TRX2*) [Kuge and Jones, 1994], thioredoxin reductase 1 (*TRR1*) [Charizanis et al., 1999; Lee et al, 1999b], γ-glutamylcysteine synthetase (*GSH1*) [Wu and Moye-Rowley, 1994], glutathione peroxidase (*GPX2*) [Inoue et al., 1999] and glutathione reductase (*GLR1*) [Grant et al., 1996]. All of the products of these genes are required for efficient peroxide elimination. Thioredoxin terminates Yap1 signaling by reducing the Yap1 disulfide bond, thereby exposing the NES and eliminating excess Yap1 from the nucleus [Delaunay et al., 2002].

Redox-sensitive transcription factors

Mammalian cells contain several redox-sensitive transcription factors, including NF-κB, AP-1 and p53. The nuclear factor-kappa B (NF-κB) family consists of five polypeptides that form hetero- or homodimers: p50/p105, p52/p100, p65/RelA, RelB/RelB and c-Rel/c-Rel [Hayden and Ghosh, 2004]. NF-κB is kept in an inactive state by two distinct mechanisms. Cys 62 of the p50 subunit is prone to oxidation and must be in the reduced state for the protein to activate transcription [Nishi et al., 2002]. NF-κB is also sequestered in the cytoplasm by complex formation with members of the inhibitor of kappa B (IκB) family. IκB-binding activity is in turn

negatively regulated by IκB kinase (IKK). Amongst other modes of activation, oxidative stress stimulates NF-κB transcriptional activity at several points of regulation. Micromolar amounts of hydrogen peroxide stimulate activation of IKK, leading to phosphorylation of IκB Ser 32 and Ser 36 and subsequent IκB degradation [Gloire et al., 2006]. Liberated NF-κB is translocated to the nucleus, whereupon nuclear NF-κB transcriptional activity is stimulated by the 2-cysteine redox proteins thioredoxin and Ref-1. Upon stimulation of oxidative stress by UVB irradiation of cells, thioredoxin translocates to the nucleus, where it can influence the redox state and activity of translocated nuclear NF-κB [Hirota et al., 1999]. NF-κB activation by thioredoxin presumably occurs by reduction of p50 Cys 62, which is known to be accomplished by thioredoxin and is known to increase NF-κB DNA binding activity [Matthews et al., 1992]. Likewise, nuclear NF-κB p50 is activated via reduction of Cys 62 by the Ref-1 2-cysteine redox domain [Nishi et al., 2002]. NF-κB activation is associated with a wide variety of outcomes, from cell proliferation and invasive growth to inflammation and apoptosis. These varied effects are likely dependent on the context of cell type, mode of NF-κB activation, duration of activation and crosstalk between NF-κB-regulated and other processes.

The redox regulation of activator protein 1 (AP-1) shares many similar features with control of NF-κB activation. AP-1 comprises a class of homo- or heterodimeric transcription factors composed of Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra1 and Fra2), Maf or ATF polypeptides [Valko et al., 2006]. AP-1 is maintained in an inactive state in the cytoplasm by oxidation of several regulatory cysteine residues

[Klatt et al., 1999] and by dephosphorylation of several threonine and tyrosine residues. AP-1 is a target of the MAP kinases p38 and JNK (Jun N-terminal kinase). AP-1 activation by oxidants is affected by altering the balance between tyrosine kinase and tyrosine phosphatase activity. JNK and p38 are downstream effectors of the MAPKKK apoptosis signaling kinase 1 (ASK1) [Liu et al., 2000]. ASK1 is negatively regulated by complex formation with reduced thioredoxin [Saitoh et al., 1998]. Oxidized thioredoxin is unable to bind ASK1. Therefore, under oxidative stress, thioredoxin is no longer able to repress ASK1 activity. The resulting activation of the ASK1 MAP kinase cascade results in the activation of p38 and JNK, which in turn phosphorylate and activate AP1. Protein tyrosine phosphatase (PTP) activity downregulates AP-1, presumably by opposing the activity of activating kinases. However, PTPs are especially prone to oxidative attack on an essential cysteine by hydrogen peroxide [Denu and Tanner, 1998; Groen et al., 2005], thereby allowing higher phosphotyrosine levels to be achieved for longer periods of time at several levels of the signaling cascade. Once phosphorylated by p38 or JNK, AP-1 migrates to the nucleus. Similar to NF-κB activation, thioredoxin and Ref-1 activate DNA binding and transcriptional activity of AP-1 by reducing AP-1 regulatory cysteines [Abate et al., 1990; Xanthoudakis et al., 1992; Hirota et al., 1997; Wei et al., 2000]. AP-1 is also much like NF-κB in that AP-1 activation produces varied effects (both pro- and anti-apoptotic) that are based on the context of the signaling event.

p53 and redox state

A growing body of evidence implicates cellular redox balance as an important factor in the control of the tumor suppressor protein p53. The DNA binding domain of p53 contains nine highly conserved cysteine residues, of which three coordinate a zinc ion essential for sequence-specific DNA binding [Hainaut and Milner, 1993b; Cho et al., 1994; Rainwater et al., 1995]. Treatment with thiol reductants greatly increases *in vitro* p53 DNA binding activity, whereas oxidant treatment decreases binding [Hainaut and Milner, 1993b; Rainwater et al., 1995; Fotja et al., 1999; Ueno et al., 1999]. Treatment of cells with the zinc chelator pyrrolidine dithiocarbamate (PDTC) interferes with p53 nuclear translocation and the p53-mediated response to UV irradiation. Using 3-(maleimidoproprionyl)-biocytin (MPB) as a tag for detection of oxidized cysteine, 25 μM PDTC treatment was shown to increase p53 cysteine oxidation two- to three-fold over mock treatment [Wu and Momand, 1998]. Selenomethionine treatment of cells for 15 hours at 20 μM was shown by the same assay to fully reduce p53 cysteines [Seo et al., 2002]. Activity of p53 was simultaneously increased, as measured by increased levels of Ab1620 epitope exposure on westerns, two-fold increase of reporter gene activity and two-fold increase in cell survival post-UV irradiation. A p53 peptide containing only Cys 275 and Cys 277 was shown to be reduced fully by selenomethionine treatment. Addition of a dominant-negative form of Ref1 reversed all selenomethionine effects on p53 activity and cysteine oxidation state. It should be noted that the cysteine oxidation state assay employed by both Wu and Seo [Wu and Momand, 1998; Seo et al., 2002] is limited to detecting highly accessible surface cysteines, as cells are lysed in native

buffer and MPB is quite bulky (greater than 5 kDa). Therefore additional p53 cysteines may undergo redox reactions and remain undetected by this method.

Thioredoxin and Ref-1 enhance p53 DNA binding activity both *in vitro* and *in vivo* [Jayaraman et al., 1997; Gaiddon et al., 1999; Ueno et al., 1999]. Electrophilic prostaglandins inhibit thioredoxin reductase activity *in vivo,* likely due to the presence of an essential nucleophilic selenocysteine residue [Moos et al., 2003]. Only 15 proteins were attacked by prostaglandins, likely due to the rarity of selenocysteine incorporation into proteins. Prostaglandins do not directly attack p53, yet prostaglandin treatment of cells strongly inhibits p53 detection by conformation-specific antibody Ab1620, p53 activation of reporter gene expression and p53 induction of apoptosis [Moos et al., 2003]. A screen in *Schizosaccharomyces pombe* for mutations that suppress a p53-induced growth arrest revealed that recessive mutations at the *trr1* thioredoxin reductase locus relieve the p53-imposed block on growth [Casso and Beach, 1996]. No mutations at alternate loci were identified in the screen. Additional experiments demonstrated that the ability of p53 to transactivate a reporter gene is compromised in thioredoxin reductase-deficient *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [Casso and Beach, 1996; Pearson and Merrill, 1998]. Thioredoxin reductase deficiency has no effect on p53 protein levels [Casso and Beach, 1996; Pearson and Merrill, 1998] or nuclear localization [Casso and Beach, 1996]. Molecular oxygen is essential for thioredoxin reductase dependence in *S. pombe* [Casso and Beach, 1996]. Experiments with p53-LexA fusion protein showed that the p53 activation domain, when tethered to a DNA by a heterologous

DNA-binding protein, stimulates reporter gene transcription equally well in wild-type and thioredoxin reductase null budding yeast, which indicates that p53 dependence on thioredoxin reductase is not mediated through an effect on a downstream effector that is recruited to promoter regions by the p53 activation domain [Merrill et al., 1999]. Thioredoxin reductase deletion in budding yeast results in a higher thioredoxin disulfide:thioredoxin dithiol ratio. Thioredoxin reductase deletion also results in a higher GSSG:GSH ratio, presumably due to the glutathione system assuming some of the reductive duties of the thioredoxin system [Merwin et al., 2002]. However, inhibition of p53 activity in thioredoxin reductase null yeast is not due to oxidative stress imposed by the higher GSSG:GSH ratio, because p53 activity remains inhibited when the normal GSSG:GSH ratio is restored by over-expressing the glutathione reductase gene in thioredoxin reductase null yeast. Furthermore, p53 activity is not affected by deletion of the glutathione reductase gene, a condition that markedly increases the GSSG:GSH ratio. Thus, p53 is specifically dependent on the thioredoxin system, and is not sensitive to the general redox state of the cell, as defined by the GSSG:GSH ratio. It seemed likely, but remained unproven, that the thioredoxin system maintains p53 cysteines in a reduced and active state. Pursuant to this idea, work was undertaken to identify p53 cysteine residues involved in thioredoxin reductase-dependent redox regulation. The work is presented in Chapter 2 of the dissertation.

Peroxiredoxin response to oxidative stress

Eukaryotic 2-Cys peroxiredoxins (Prxs) are thioredoxin-dependent peroxidases that differ from their prokaryotic counterparts in that they are sensitive to substrate inactivation at moderate H_2O_2 concentrations. Inactivation is due to reaction of a sulfenic acid catalytic intermediate with a second molecule of H_2O_2 before it has time to react with a resolving cysteine to form a disulfide. It has been hypothesized that the sensitivity of eukaryotic Prxs evolved as a mechanism to facilitate H_2O_2 signaling. For example, inactivation of protein tyrosine phosphatases (PTPs) by H_2O_2 may be necessary for efficient signaling by receptor tyrosine kinases, and Prx inactivation may be necessary to prolong episodes of PTP inactivation following growth factor stimulation. To test this model, the oxidation state of Prxs and the phosphorylation state of EGF receptor in EGF- and peroxide-challenged A431 cells was measured. The work is presented in Chapter 3 of the dissertation.

CHAPTER 2: EFFECT OF CYSTEINE REPLACEMENT ON P53 REDOX SENSITIVITY

Abstract

Reporter gene activation by human p53 is severely compromised in budding yeast lacking thioredoxin reductase. Indirect evidence suggests p53 may similarly require an intact thioredoxin system in mammals. One explanation for thioredoxin reductase dependence is that p53 cysteines may tend to form inhibitory oxidation products. To test this idea, each p53 cysteine was changed to serine either individually or combinatorially, and the effect of the substitution on p53 activity in wild-type and thioredoxin reductase-null yeast was determined. The effect of each substitution on basal p53 activity was confirmed in transfected p53-null human H1299 cells. As expected, substitutions at zinc-coordinating cysteines C176, C238 or C242 resulted in p53 inactivation. Unexpectedly, substitution at cysteine C275 also inactivated p53, which is the first evidence for a non-zinc-coordinating cysteine being essential for p53 function. Cysteine substitutions at six positions (C124, C135, C141, C182, C229 and C277) neither inactivated p53 nor relieved the requirement for thioredoxin reductase. Furthermore, no tested combination of cysteine substitutions relieved thioredoxin reductase dependence. The results suggest that p53 dependence on thioredoxin reductase either was an indirect effect or was due to oxidation of one or more of the four essential cysteines that could not be assayed for thioredoxin reductase dependence.

Introduction

Cellular redox balance is an important factor in the control of many transcription factors and cell cycle regulators. Several lines of evidence suggest redox control of the tumor suppressor protein p53. Detailed examination of the cysteine residues of p53 may yield insight into the possible redox-mediated control mechanism of this important protein.

Medical genetics and structural data point to potential cysteine residue targets of redox reactions. The *p53* gene is mutated in over one-half of all cancers, illustrating the critical importance of p53 in tumor surveillance. Of the 21,000 documented mutations of the *p53* gene in human cancer, over 90% consist of single point mutations, and fully 87% reside in the core DNA binding domain (IARC tp53 mutation database, R10, July 2005) [Oliver et al., 2002]. The DNA binding domain contains all ten of the cysteine residues of human p53, and all cysteines but Cys 229 are strongly conserved. Several cysteine codons exhibit above-average point mutation rates, including Cys135 (3.5-fold increase over expected rate), Cys 141 (2.8-fold), Cys 176 (6.3-fold), Cys 238 (3.4-fold), Cys 242 (3.6-fold), Cys 275 (2.9-fold and Cys 277 (1.7-fold) [Oliver et al., 2002]. Three of these residues - Cys 176, 238, 242– coordinate a zinc ion [Cho et al., 1994] that is requisite for sequence-specific DNA binding [Hainaut and Milner, 1993b; Rainwater et al., 1995]. Cysteines 275 and 277 reside in the loop of a sheet-loop-helix domain that makes DNA contact at specific sites, and Cys 135 and 141 reside in a helix-loop-helix domain adjacent to the Cys 275/277 loop [Cho et al., 1994].

Treatments known to influence cysteine residue chemistry alter p53 conformation and activity. Co-incubation with reductant or oxidant modulates site-specific DNA binding by p53 *in vitro*. Treatment with the reductant dithiothreitol (DTT) greatly enhances binding of purified p53 protein to specific DNA target sequence in electrophoretic mobility shift assays (EMSAs) [Hainaut and Milner, 1993b; Rainwater et al., 1995; Fotja et al., 1999; Ueno et al., 1999]. Conversely, diamide treatment abrogates sequence-specific DNA binding in EMSAs [Hainaut and Milner, 1993b; Fotja et al., 1999; Ueno et al., 1999]. Pretreatment of p53 with DTT followed by alkylation of free thiols with N-ethylmaleimide also abrogates DNA binding [Hupp et al., 1993; Rainwater et al., 1995]. Loss of a bound zinc cation - an essential cofactor for p53 sequence-specific DNA binding – also correlates with oxidant treatment [Hainaut and Milner, 1993b; Fotja et al., 1999]. Conformational change in p53, assessed using conformation-specific antibody, accompanies diamide treatment and zinc loss. However, the mechanism responsible for these conformational changes remains unknown.

Exposure to chelators causes oxidation of p53 and inhibits p53 DNA-binding activity. Exposure of cells to either the zinc chelator N,N'-tetrakispyrimidylethylenediamine (TPEN) [Verhaegh et al., 1998] or the copper chelator pyrrolidine dithiocarbamate (PDTC) [Verhaegh et al., 1997] results in p53 conformational change, oxidation and loss of activity. PDTC also oxidizes p53 and depresses p53 activity in vivo, and is able to counteract selenomethionine-induced reduction of p53 and stimulation of p53 activity [Seo et al., 2002]. The exact

mechanism for p53 inactivation in response to metal chelators is unknown. However, p53 has been shown to bind Cu^{1+} *in vitro* [Hainaut and Milner., 1993a; Hainuat et al., 1995], and thus PDTC may inactivate p53 by increasing intracellular concentrations of Cu^{1+} enough to displace p53-bound zinc. Intracellular cycling of Cu^{1+} to the Cu^{2+} form results in the generation of hydroxyl radical, which may contribute to p53 oxidation. TPEN offers no similar redox cycling mechanism for oxidation of p53.

The disulfide-reducing proteins Ref-1 and thioredoxin enhance p53 DNA binding *in vitro* and transcriptional activity *in vivo* [Jayaraman et al., 1997; Gaiddon et al., 1999; Ueno et al., 1999]. Ref-1 was identified as a factor from whole cell extracts that co-purifies with p53 and enhances p53 DNA binding *in vitro* [Jayaraman et al., 1997]. Ref-1 binds purified p53 *in vitro*, co-immunoprecipitates with p53 from cell extracts [Gaiddon et al., 1999] and stimulates p53 reporter gene transactivation in transfected cells [Jayaraman et al., 1997; Gaiddon et al., 1999]. Ref-1 was originally discovered as a reductive activator of the AP-1 Fos/Jun heterodimer [Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992]. Thioredoxin was shown to bind Ref-1 and enhance the ability of Ref-1 to activate AP-1 [Hirota et al., 1999; Wei et al., 2000]. Similarly, purified thioredoxin - alone and synergistically with Ref-1 - stimulates p53 DNA binding. Furthermore, thioredoxin overexpression in transfected cells - alone and synergistically with Ref-1 - stimulated p53 reporter gene transactivation [Ueno et al., 1999]. It must be noted that, while Ref-1 and thioredoxin are known as disulfide reducing proteins, it has not been shown that either protein increases p53 DNA binding or transactivation through p53 cysteine reduction.

Thioredoxin reductase augments p53 activity as well. Electrophilic prostaglandins inhibit thioredoxin reductase activity *in vivo,* likely due to the presence of an essential nucleophilic selenocysteine residue [Moos et al., 2003]. Only 15 proteins were attacked by prostaglandins, likely due to the rarity of selenocysteine incorporation into proteins. Prostaglandins do not directly attack p53, yet prostaglandin treatment strongly inhibits p53 detection by conformation-specific antibody Ab1620, p53 activation of reporter gene expression and p53 induction of apoptosis [Moos et al., 2003]. A screen in *Schizosaccharomyces pombe* for mutations that suppress a p53-induced growth arrest revealed that recessive mutations at the *trr1* thioredoxin reductase locus relieve the p53-imposed block on growth [Casso and Beach, 1996]. No mutations at alternate loci were identified in the screen. Additional experiments demonstrated that the ability of p53 to transactivate a reporter gene is compromised in thioredoxin reductase-deficient *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [Casso and Beach, 1996; Pearson and Merrill, 1998]. Thioredoxin reductase deficiency has no effect on p53 protein levels [Casso and Beach, 1996; Pearson and Merrill, 1998] or nuclear localization [Casso and Beach, 1996]. Molecular oxygen is essential for thioredoxin reductase dependence in *S. pombe* [Casso and Beach, 1996]. Experiments with p53-LexA fusion protein showed that the p53 activation domain, when tethered to a DNA by a heterologous DNA-binding protein, stimulates reporter gene transcription equally well in wild-type and thioredoxin reductase null budding yeast, which indicates that p53 dependence on thioredoxin reductase is not mediated through an effect on a downstream effector that

is recruited to promoter regions by the p53 activation domain [Merrill et al., 1999]. Thioredoxin reductase deletion in budding yeast results in a higher thioredoxin disulfide:thioredoxin dithiol ratio. Thioredoxin reductase deletion also results in a higher GSSG:GSH ratio, presumably due to the glutathione system assuming some of the reductive duties of the thioredoxin system [Merwin et al., 2002]. However, inhibition of p53 activity in thioredoxin reductase null yeast is not due to oxidative stress imposed by the higher GSSG:GSH ratio, because p53 activity remains inhibited when the normal GSSG:GSH ratio is restored by over-expressing the glutathione reductase gene in thioredoxin reductase null yeast. Furthermore, p53 activity is not affected by deletion of the glutathione reductase gene, a condition that markedly increases the GSSG:GSH ratio. Thus, p53 is specifically dependent on the thioredoxin system, and is not sensitive to the general redox sate of the cell, as defined by the GSSG:GSH ratio. It remains unclear whether thioredoxin directly affects the redox state of p53 or affects p53 activity by an alternative mechanism.

The link between thioredoxin reductase and p53 may be highly relevant to human tumor suppression. Human thioredoxin reductase is a selenoenzyme [Gladyshev et al., 1996], one of about twenty-five mammalian proteins that contain the unusual amino acid selenocysteine [Gladyshev and Hatfield, 1999; Kryukov et al., 2003]. Selenium compounds have been shown to induce thioredoxin reductase specific activity in numerous human cell lines [Berggren et al., 1996] and rodent tissues [Berggren et al., 1999], and to have strong cancer-preventive activity in animals [Bjorkhem-Bergman et al., 2005]. Clinical studies indicate p53 also has

cancer-preventive activity in humans [Clark, 1996]. Given the evidence that p53 is sensitive to oxidative inactivation *in vitro* and p53 transcriptional activity is maintained by the thioredoxin system and selenomethionine, it is tempting to speculate that the anti-cancer activity of dietary selenium supplements is due to increased thioredoxin reductase activity and resulting maintanence of p53 activity.

While much circumstantial evidence suggests that reduction of p53 cysteine residues by the reducing proteins Ref-1 and thioredoxin activates p53 DNA binding and transcription, alternative explanations for the effects that reducing proteins exert on p53 must be considered. Binding of Ref-1 or thioredoxin to p53 may alone be sufficient to enhance p53 DNA binding and transactivation. Additionally, many upstream activators for p53 are known, including many kinases and acetylases [Thiagalingham et al., 1993]. Ref-1 and thioredoxin may, at least in part, stimulate p53 activity through activation of upstream activators of p53.

If, however, control of p53 activity is indeed through p53 cysteine redox chemistry, then replacement of the specific redox-sensitive cysteine residues with non-oxidizable serines should produce an oxidation-resistant p53 protein, and thus similar reporter gene stimulation under reducing and oxidizing conditions. We therefore constructed a comprehensive set of single Cys-to-Ser replacement alleles of human p53 to dissect thioredoxin reductase dependence. Four replacements (C176S, C238S, C242S and C275S) resulted in inactive p53 protein. Inactivity of p53 C275S was surprising, as C275 is neither involved in zinc chelation nor has been previously reported as essential in the literature. The other six replacements (C124S, C135S,

C141S, C182S, C229S and C277S) retained at least partial activity, but in none of these cases did a single Cys-to-Ser replacement produce an oxidation-resistant p53 protein.

Materials and methods

Yeast strains and media

Saccharomyces cerevisiae strains MY320 (*TRR1*) and MY 321 (Δ*trr1*) were used for all experiments involving yeast. These strains were derived from W303 (*TRR1*) [Wallis et al., 1989] and MY301 (Δ*trr1*) [Pearson and Merrill, 1998] by integration of pRS305-p53RE-Z p53-responsive β–galactosidase reporter gene into the genomic LEU2 locus. pRS305-p53RE-Z was derived from pRS315-p53RE-Z [Thiagalingam et al., 1995]. Strains were grown in rich medium (YEPD) or defined medium (YNB plus required supplements) (DIFCO, Franklin Lakes, NJ). Media, lithium acetate method transformations and standard yeast procedures were as described [Rose et al., 1990].

PCR site-directed mutagenesis

The p53 gene was amplified by PCR using terminal oligonucleotides 5'-GTCCTCGAGTTCACCATGGAGGAGCCGCAGTCAGAT-3' (left terminus) and 5'-TTAGGATCCTCAGTCTGAGTCAGGCCCTTC-3' (right terminus). Mutation of Cys codons to Ser or Ala was achieved by PCR site-directed mutagenesis. Mutagenic primers were: C124S, 5'-TCTGTGACTAGCACGTACTCCC-3' (left) and 5'-GGAGTACGTGCTAGTCACAGAC-3' (right); C135S, 5'-AACATGTTTAGCCAACTGGCC-3' (left) and 5'-GGCCAGTTGGCTAAACATCTTG-3' (right); C141S, 5'-GCCAAGACCAGCCCTGTGCAGC-3' (left) and 5'-

CTGCACAGGGCTGGTCTTGGCC-3' (right); C176S, 5'-

GTGAGGCGCAGCCCCCACCAT-3' (left) and 5'-

ATGGTGGGGGCTGCGCCTCAC-3' (right); C182S, 5'-

CATGAGCGCAGCTCAGATAGC-3' (left) and 5'-

GCTATCTGAGCTGCGCTCATGG-3' (right); C229S, 5'-

GGCTCTGACAGTACCACCATC-3' (left) and 5'-

GATGGTGGTACTGTCAGAGCC-3' (right); C238S, 5'-

AACTACATGAGTAACAGTTCC-3' (left) and 5'-

GGAACTGTTACTCATGTAGTT-3' (right); C242S, 5'-

AACAGTTCCAGCATGGGCGGC-3' (left) and 5'-

GCCGCCCATGCTGGAACTGTT-3' (right); C275S, 5'-

GAGGTGCGTGTTAGTGCCTGTCCT-3' (left) and 5'-

CCCAGGACAGGCACTAACACGCAC-3' (right); C277S, 5'-

CGTGTTTGTGCCAGTCCTGGGAGA-3' (left) and 5'-

GTCTCTCCCAGGACTGGCACAAAC-3' (right); C275/277S, 5'-

GTGCGTGTTAGTGCCAGTCCTGGGAGA-3' (left) and 5'-

TCTCCCAGGACTGGCACTAACACGCACC-3' (right); C275A, 5'-

GAGGTGCGTGTTGCTGCCTGTCCTGGG-3' (left) and 5'-

CCCAGGACAGGCAGCAACACGCACCTC-3' (right); C124A, 5'-

GTCTGTGACTGCCACGTACTCCCCT-3' (left) and 5'-

GGAGTACGTGGCAGTCACAGACTTG-3' (right); C135/141A, 5'-

AAGATGTTTGCCCAACTGGCCAAGACCGCCCCTGTGCAGCTG-3' (left) and

5'-CTGCACAGGGGCGGTCTTGGCCAGTTGGGCAAACATCTTGTTG-3' (right). For each mutation, three PCR reactions were done. Using pBS-p53 as template, the left terminus and right mutagenic primers were used to generate the left subfragment, and the left mutagenic and right terminal primers were used to generate the right subfragment. The left terminus and right terminus primers and a fifty-fold dilution of gel-purified left and right subfragment PCR reactions were mixed and used in a third PCR reaction to generate a full-length fragment containing the mutation. Gel-purified full-length fragments were blunt end-ligated into the EcoRV site of pBlueScript (Stratagene, La Jolla, CA), screened for proper orientation, and subcloned using vector-derived EcoR1 and Sal1 sites into the yeast expression plasmid $p414^{GPD}$ [Mumberg et al., 1995]. Expression plasmids were purified using Plasmid Midi-Kits (Qiagen, Hilden, Germany). Each mutated allele was sequenced to confirm its structure. For alleles containing multiple mutations, either natural restrictions sites in the p53 coding region were used to make recombinants, or additional mutations were serially introduced by PCR site-directed mutagenesis, as described above. For mammalian cell studies, mutated p53 alleles were subcloned as EcoR1/Sal1 fragments into the mammalian expression plasmid pTL1, which expresses inserts from the constitutive cytomegalovirus promoter.

SDS-PAGE and immunoblotting

Yeast were disrupted using a reciprocating bead beater (BioSpec, Bartlesville, OK) and 0.6 mm glass beads (Sigma, St. Louis, MO). Protein concentration was

determined by RC-DC assay (Bio-Rad, Hercules, CA). Precast 10% acrylamide SDS-PAGE Tris-Cl gels (Bio-Rad) were used. After transfer to nitrocellulose (Bio-Rad) membranes using a Mini TransBlot instrument (Bio-Rad) at 200 mA overnight (approximately 2.5 amp-hours), total transferred protein was detected by SYPRO Ruby staining and a Molecular Imager FX Pro Plus scanner (Bio-Rad). All subsequent immunodetection steps were done in tris-buffered saline plus 0.1% vol/vol Tween 20 (TBST). Briefly, membranes were blocked for 1 h in TBST containing 5% non-fat dry milk, followed by a 1-h incubation with 1° antibody (1: 5,000 dilution of DO-1 mouse monoclonal anti-p53 IgG) (Santa Cruz Biotechnology, Santa Cruz, CA), and a 1h incubation with 2° antibody (1:10,000 dilution of goat anti-mouse IgG:HRP) (BioRad). Antibody binding was detected by ECL chemiluminescence (Amersham-Pharmacia, Piscataway, NJ). Quantitation of p53 signal was done using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

yeast β–galactosidase assay

The activity of p53 in yeast was measured by β–galactosidase assay [Kippert, 1995]. Briefly, cells were grown to 1 x 10^7 cells per ml density in YNB shaking culture as measured by spectroscopic absorbance at A_{600}. Aliquots (200 µl) were removed from each sample and frozen at –80°C. Aliquots were then treated with 400 µl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 0.2% sarkosyl, 50 mM β–mercaptoethanol, pH 7.0) and thawed in a 30°C waterbath for 45 min. Each aliquot then received 150 µl Z-buffer plus 4 mg / ml ortho-nitrophenyl-β-D-galactopyranoside

(ONPG) and further incubation for 20 min. ONPG is colorless, but is cleaved by β–galactosidase to a yellow product detectable by spectroscopic absorbance at A_{420}. Reactions were stopped by addition of 400 µl 1.5 M Na_2CO_3. Normalized β–galactosidase activity was calculated by dividing the β–galactosidase activity for each individual sample (A_{420} value) by the cell density of the sample (A_{600} value). To facilitate comparison between alleles and strains, the mean and standard deviation for each group of normalized β–galactosidase activities was divided by the mean normalized β–galactosidase activity exhibited by wild-type (MY320) cells transformed with wild-type p53.

Isolation of yeast nuclei

Yeast nuclei were isolated by the lyticase spheroplasting method [Rose et al., 1990]. Briefly, yeast were collected by centrifugation, rinsed in wash buffer (50 mM potassium phosphate, pH 7.6, 10 mM $MgCl_2$, 30 mM DTT, 0.5 mM PMSF and 1 M sorbitol), and resuspended in lysis buffer (25 mM potassium phosphate, 25 mM sodium succinate, pH 7.6, 10 mM $MgCl_2$, 30 mM DTT, 0.5 mM PMSF and 1 M sorbitol). Lyticase (Sigma) was added to a final concentration of 0.1 mg/ml and, after a 30- min incubation at 37°C, spheroplasts were collected by centrifugation at 1500 x g, resuspended in lysis buffer without lyticase and disrupted by 10 strokes with a Dounce homogenizer. Nuclei were collected by centrifugation at 1100 x g, resuspended in gradient buffer (50% Percoll, 40 mM PIPES, pH 6.8, 10 mM MgC l_2, 0.5 mM PMSF and 0.5% Triton X-100) and centrifuged at 21,000 rpm for 45 min

using a Beckman Type 30 rotor. Banded nuclei were removed from tubes, collected at 1100 x g, and washed once in gradient buffer without Percoll.

Human cell transfections

Human lung carcinoma line H1299 [Giaccone et al., 1992], which is homozygous for a p53 null mutation, was used. Cells were cultured in 50% Dulbecco's modified Eagle's medium and 50% Hamm's F12 medium (F/D), containing 10% bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin. For transfection, 5 x 10^5 cells were dispensed to 1.5-cm wells of 12-well plates and allowed to adhere for 24 hours. Cells were washed free of serum and antibiotic with F/D, and co-transfected using lipofectamine (Stratagene) with 0.5 µg of transfection control plasmid pCMV: β -gal (Invitrogen, Carlsbad, CA), 0.5 µg of p53-responsive luciferase reporter gene plasmid p53-luc (Stratagene), and 0.5 µg of a pTL1 plasmid expressing a p53 allele from the SV40 promoter. Culture medium was adjusted to 10% serum at 24 h post transfection. Cells were harvested at 48 h post transfection. Luciferase and β–galactosidase activities were determined using the Luciferase Assay System (Promega, Madison, WI) and ONPG, respectively, using Promega protocols. A liquid scintillation counter, with coincidence correction disabled, was used to quantify luminescence. The luciferase activity of each sample was normalized to its β–galactosidase activity, and the mean and standard deviation for each group of normalized luciferase activities was divided by the mean normalized luciferase activity exhibited by cells transfected with wild-type p53.

Results

Effect of Cys-to-Ser mutations on p53 activity

A model is proposed in which one or more p53 cysteines engage in intramolecular or intermolecular disulfide bonds that result in p53 inactivation (Fig. 1, top two panels). Under reducing conditions, disulfide formation is antagonized and active p53 predominates. Under oxidizing conditions, or in a cell lacking thioredoxin reductase, disulfide bond formation is favored and inactive p53 predominates. If indeed p53 contains oxidation-prone cysteines, then changing those cysteines to structurally similar but non-oxidizable serines should make the protein resistant to oxidative inactivation (Fig. 1, lower two panels).

To identify putative oxidation-prone p53 cysteines, a series of alleles was produced in which each Cys codon was individually changed to Ser via PCR site-directed mutagenesis. Each allele was expressed in wild-type (MY320) and Δ*trr1* (MY321) yeast that contained an integrated p53-dependent LacZ reporter gene (p53RE-Z). The ability of each p53 allele to stimulate reporter gene expression was determined by measuring β–galactosidase activity (Fig. 2). For native p53, the ratio of p53 activity in wild-type versus Δ*trr1* yeast was 12.5, and a similar ratio was obtained for all mutated alleles that retained activity. Four p53 alleles were completely inactive in stimulating reporter gene activity. Three of the inactive alleles corresponded to replacements of zinc-coordinating residues Cys 176, 238 and 242 [Cho et al., 1994], the murine homologs of which were known previously to be essential for p53 activity [Rainwater et al., 1995]. Unexpectedly, replacement of Cys 275 with Ser also completely

abrogated activity. The absence of activity for the C176S, C238S, C242S and C275S alleles precluded assessment of thioredoxin dependence in these cases. For the six alleles that retained activity, it was evident that no single Cys-to-Ser replacement relieved the dependence of p53 on thioredoxin reductase.

It was shown previously that reduced p53-dependent reporter expression in Δ*trr1* yeast transformed with wild-type p53 was not due to a reduction in p53 protein levels, but rather was due to a reduction in specific activity [Pearson and Merrill, 1998; Casso and Beach, 1996]. To confirm that reduced reporter expression in Δ*trr1* yeast transformed with mutated p53 alleles was similarly due to a reduction in p53 specific activity, immunoblot assays were used to measure p53 protein levels (Fig. 3A, upper panel). To correct for variation in loading between samples, a prominent 80-kD protein in each lane was visualized by SYPRO Ruby blot staining (Fig. 3A, lower panel) and used to normalize p53 protein signals (Fig. 3B). As shown in Fig. 3A and 3B, some of the p53 point mutations markedly affected protein levels, but importantly, the Δ*trr1* deletion mutation had no consistent inhibitory effect on p53 protein levels. For most p53 alleles, p53 protein levels in Δ*trr1* transformants were equal to or slightly greater than in wild-type transformants. Only for the C238S and C242S alleles were p53 protein levels lower in Δ*trr1* transformants than in wild-type transformants (2.9-fold and 2.1-fold, respectively). However, these two mutations resulted in complete loss of p53 activity in both wild-type and Δ*trr1* cells. Fig. 3C shows p53 reporter gene activity normalized to p53 protein levels. The normalized results showed that the Δ*trr1* mutation had a 14-fold inhibitory effect on the specific

activity of wild-type p53, and between an 11-fold effect (C124S allele) and 52-fold effect (C141S allele) on the specific activity of mutated p53. Therefore, reduced p53 reporter gene activity in Δ*trr1* yeast versus wild-type yeast was due to a reduction of p53 specific activity rather than a reduction of p53 protein levels.

Effect of combinatorial Cys-to-Ser mutations on p53 activity

One explanation for continued thioredoxin reductase dependence of p53 alleles carrying single Cys-to-Ser mutations was that more than a single cysteine or pair of cysteines was prone to oxidation. To test this idea, a series of p53 alleles was constructed comprising all pair-wise combinations of Cys-to-Ser mutations, excepting the essential residues C176, C238, C242 and C275. In addition, alleles containing three replacements (C124/135/141S and C182/229/277S) or six replacements (C124/135/141/182/229/277S) were also produced. All combinatorial alleles were tested for reporter gene transactivation in wild-type and Δ*trr1* yeast (Fig. 4). All combinatorial alleles that retained p53 activity (C124/141S, C124/182S, C124/229S, C124/277S, C135/182S, C135/C277S, C141/182S, C141/229S, C141/277S, C182/229S, C182/277S, C229/277S, and C182/229/277S) remained thioredoxin reductase dependent (Fig. 4). As no pairwise combination of C124S, C141S, C182S, C229S and C277S mutations was sufficient to relieve dependence on thioredoxin reductase, we concluded that putative oxidations solely involving these five cysteines were not the basis for thioredoxin reductase dependence. In addition, the pairwise combinations involving the C135S mutation and either the C182S or C277S mutations

were active and regulated. We therefore concluded that putative oxidations involving solely Cys 135 and either Cys 182 or Cys 277 were not the basis for thioredoxin reductase dependence. However, several combinations (C124/135S, C135/141S, C135/229S, C124/135/141S and C124/135/141/182/229/277S) resulted in severe impairment of reporter gene activation, and precluded assessment of thioredoxin reductase dependence. A common feature of the inactive combinatorial alleles was the C135S mutation, which by it self gave a 20-fold decrease in activity (Fig. 2), and in combination with C124S, C141S or C229S exhibited near-complete elimination of activity (Fig. 4). We therefore could not exclude the possibility that putative oxidations involving Cys 135 and either Cys 121, Cys 141 or Cys 229 were the basis for thioredoxin reductase dependence based on this data.

As C124, C135 and C141 reside in a buried helix-loop-helix motif, it was possible that serine replacement at these positions was deleterious to p53 activity due to destabilization of native structure. If so, then alanine replacement would be more compatible with proper packing and might preserve activity. We therefore constructed C135/141A, C124/135/141A and C135/141A-C124/182/229/277S alleles and tested them for activity (Fig. 5). In wild-type yeast, reporter gene activation by the C135/141A allele was 14% that of wild-type p53 levels. Importantly, the C135/141A allele remained redox sensitive, exhibiting ten-fold less reporter gene expression in thioredoxin reductase null yeast. In wild-type yeast, reporter gene activation by the C124/135/141A allele was only 2% that of the wild-type allele. Furthermore, activity in thioredoxin reductase null yeast was barely detectable above spectroscopic

background, which made it difficult to quantitate thioredoxin reductase dependence of the allele. Nevertheless, at least qualitatively, C124/135/141A p53 appeared to remain redox sensitive. An allele containing C135/141A and C124/182/229/277S (labeled C135/141A-Hex) exhibited zero activity and could therefore not be assessed for thioredoxin dependence. Thus, with respect to the six non-essential cysteine residues, there are few candidates remaining for oxidative control of p53, namely (A) oxidations at both C135 and C229, or (B) three or more oxidations spread across the upstream and downstream triplet groups of cysteine residues.

Nuclear localization of p53 in yeast

Thioredoxin reductase gene deletion produces a wide variety of gene expression changes [Carmel-Harel et al., 2001], including reduction of messages for several nuclear import proteins (karyopherins, importins). Therefore, differential import of p53 into the nucleus in *TRR1* and Δ*trr1* yeast was investigated as a possible explanation for the observed differences in p53 activity. To test this idea, wild-type and Δ*trr1* yeast transformed with native p53 were spheroplasted, and isolated nuclei were assayed for p53 protein by immunoblotting (Fig. 6). Nuclear levels of p53 protein in Δ*trr1* yeast were 79% that of wild-type yeast ($p = 0.033$). The 21% reduction in nuclear p53 levels was only a small fraction of the 1400% reduction in p53 activity levels. Hence, we concluded that p53 mislocalization was not responsible for the majority of the regulation observed.

Analysis of essential cysteine residue C275

The inactivating effect of the C275S mutation was unexpected. The equivalent of Cys 275 in murine p53 (Cys 272) was not found to be essential for p53 activity in a previous study that used transfected mammalian cells [Rainwater et al., 1995]. Therefore, several confirmatory experiments were performed. Although the structure of all mutated p53 alleles had been confirmed by sequencing, it was possible that an alteration elsewhere in the plasmid expressing the C275S allele resulted in the observed phenotype. To eliminate this possibility, the C275S allele was independently reconstructed and shown to give an identical phenotype. In protein folding and crystallography studies, alanine rather than serine is often used when replacing cysteines, with the idea that a small hydrophobic methyl group is less likely to disrupt protein structure than a highly polar hydroxyl group. Thus, we constructed a p53 allele in which Cys 275 was replaced with Ala. As shown in Fig. 7, the Cys-to-Ala mutation had the same inactivating effect on p53 activity as the Cys-to-Ser mutation. Finally, replacement of Cys 275 with either Ser or Ala was shown to inactivate p53 reporter gene transactivation in human cells (see below). We therefore concluded that Cys 275 was absolutely essential for p53 activity.

The inactivating effect of the C275 replacement was intriguing because C275 and C277 were within disulfide bonding distances of each other and were part of a sheet-loop-helix motif that makes contact with DNA. It was possible that interactions between these vicinal residues were involved in regulating p53 activity. For example, activation of the yeast transcription factor Yap1 during oxidative stress is known to

involve transient formation of an intermolecular disulfide between Yap1 residue Cys 598 and Gpx3 residue Cys 36, which is subsequently resolved by reaction with a second Yap1 residue – Cys 303. The resulting intramolecular Cys 595-Cys 303 disulfide results in Yap1 activation [Delaunay et al., 2002]. Similarly, p53 residue Cys 277 may tend to form an inactivating disulfide with another polypeptide or thiol compound in the cell and may need to be resolved by Cys 275. To investigate this possibility, a C275S/277S double mutation was constructed. If Cys 275 was essential because it resolved an intermolecular disulfide involving Cys 277, mutation of Cys 277 to Ser should relieve the requirement for Cys 275. In contrast to this expectation, the doubly mutated C275/277S allele, like the singly mutated C275S allele, was completely inactive in stimulating reporter gene expression (Fig. 8). The failure of the C277S mutation to suppress the C275S mutation suggested that the essential role of Cys 275 was not resolution of a putative disulfide involving Cys 277.

Effect of Cys-to-Ser replacements on p53 activity in human cells

The ability of native p53, each single Cys-to-Ser replacement allele, the C275/277S double replacement allele and the C124/135/141/182/229/277S hextuple replacement allele (HEX C-S) to transactivate reporter gene expression was assessed in co-transfected human H1299 cells that lack an endogenous p53 gene (Fig. 9). The relative levels of reporter gene transactivation exhibited by the mutated p53 alleles in H1299 cells were generally similar to that previously observed in yeast, with the exceptions of C182S and C229S, which exhibited essentially wild-type activity in the

H1299 system. As in yeast, mutation of the three zinc-coordinating cysteines resulted in complete loss of activity in H1299 cells, as did the C275S and C275/277S mutations. The latter results confirmed the finding from yeast that Cys 275 was essential for p53 activity.

The possibility that Cys 275 was essential for discriminating between specific DNA binding sites was considered. As the reporter construct used in all of the above analyses was based on the PG repeat binding site [Kern et al., 1991; 1992; Thiagalingam et al., 1995], it was possible that Cys 275 was required only for binding to that specific sequence. This would not be unprecedented, as mutation of p53 Cys 277 was previously shown to alter the affinity with which p53 binds different p53 target sequences [Chene, 1999; Buzek et al., 2002]. We therefore transfected H1299 cells with either the wild-type or C275S p53 allele, along with p53-responsive luciferase reporter constructs containing the p53 binding sites from the MDM2, cyclin G, 14-3-3 and Pig3 genes, as well as the PG-based p53-luc reporter (Stratagene) used previously. In no case did the C275S allele stimulate reporter activity above the empty vector control (Fig. 10). Therefore, Cys 275 likely was universally essential for site-specific DNA binding across all binding site sequences.

Discussion

Activity of single cysteine replacements under non-stressed conditions

The most direct comparison of this work to previously published work is the analysis of murine p53 Cys-to-Ser replacement alleles conducted by Rainwater in $p53^{(-/-)}$ NCI-H358 cells [Rainwater et al., 1995]. We confirmed their finding that the zinc-binding cysteine residues C173, C253 and C239 (homologous to human C176, C238 and C242) were absolutely essential for p53 activity. Murine alleles C121S, C138S, C179S and C274S (homologous to human alleles C124S, C141S, C182S and C275S) retained essentially wild-type activity, while murine alleles C132S and C272S (homologous to human alleles C135S and C275S) had about 50% of wild-type activity. The activity of several human p53 alleles differed from their murine counterparts. Human C141S and C277S exhibited about 40% of wild-type activity, in contrast to the essentially wild-type activity of its murine homologs. In addition, human allele C135S was far less active than its murine homolog. Finally, human allele C275S was completely inactive, a finding quite dissimilar from its murine counterpart.

The reason for these activity differences between human and murine p53 Cys-to-Ser replacement alleles is unclear. The reporter construct employed in each study was based on the PG p53 recognition sequence, and thus, it is unlikely that the different effects of the same Cys-to-Ser replacements on human and mouse p53 activity is due to the type of reporter gene used. It is more likely that different effects of the same replacements is due to structural differences between human and mouse

p53. Although both proteins have high sequence homology overall, and very high homology in the DNA binding domain, the sequences do contain two non-conservative substitutions in loop structures adjacent to Cys 124 through Cys 135 and Cys 141. Human p53 Ala 129 aligns to Pro 126 in the murine homolog, and human Pro 153 aligns to murine Ala 150. The crystal structures of the DNA binding domains of human p53 (Protein Data Bank file 1TUP) [Cho et al., 1994] and murine p53 (1HU8) [Zhao et al., 2001] are nearly superimposable in the regions in question. While the crystal structures are remarkably similar, the non-conservative Pro-to-Ala substitutions could considerably alter tertiary structure dynamics, thereby allowing varied interaction of the S2 and S3 helices with the surrounding environment and possibly altering reporter gene affinity.

Thioredoxin reductase-dependence of p53 activity

Previous work established that p53 activity is dependent on thioredoxin reductase status in yeast [Casso and Beach, 1996; Pearson and Merrill, 1998]. In *Saccharomyces cerevisiae*, it was shown that the activation domain of p53 functions equally well in wild-type and thioredoxin reductase null yeast when tethered to DNA by a heterologous DNA-binding protein, indicating that thioredoxin reductase deletion is not affecting a process downstream of DNA binding, such as co-activator recruitment, chromatin modification or general transcription factor activity [Merrill et al., 1999]. Also in *Saccharomyces cerevisiae*, it was established that p53 activity was not sensitive to changes in glutathione redox status and was, instead, specifically

correlated with changes in thioredoxin redox status [Merwin et al., 2002]. Collectively, the previously published yeast results suggested that an intact thioredoxin system is required to maintain p53 in an active state under aerobic conditions.

If loss of p53 activity in thioredoxin reductase null yeast was due to oxidation of reactive cysteines, then replacement of the oxidation-prone cysteines with non-oxidizable residues should have prevented oxidative inactivation. However, no single replacement of cysteine with serine relieved the dependence of p53 on thioredoxin reductase. Four of the ten replacement alleles (C176S, C238S, C242S and C275S) exhibited no activity in either yeast strain. No conclusion as to the involvement of these residues in redox regulation can be made in the absence of measurable activity.

The possibility remained that oxidation of two or more of these nonessential cysteine residues was responsible for thioredoxin reductase dependence. Therefore, a comprehensive set of alleles containing combinations of the six replacements that retained measurable activity was produced and screened for thioredoxin reductase dependence. For combinatorial alleles exclusively containing C124S, C141S, C177S, C182 and C229S, no combination of Cys-to-Ser replacements relieved thioredoxin reductase dependence. Combinatorial alleles containing C135S had insufficient activity to confidently interpret the data. It was possible that the inhibition of p53 activity observed when Cys 135 was replaced with Ser was due to aberrant protein folding. Residues Cys 135, as well as Cys 121 and Cys 141, are interior cysteines, and their replacement with hydrophilic serine residues might disrupt the local fold of the

polypeptide. We therefore made a second set of combinatorial alleles in which these cysteines were replaced with the hydrophobic amino acid alanine. Of the Cys-to-Ala alleles analyzed, C135/141A and C124/135/141A were active and remained thioredoxin dependent, while the C135/141A-C124/182/229/277S allele was inactive and could not be assayed for thioredoxin reductase dependence. Thus, oxidation of Cys 135 and Cys 229 or oxidation of three or more non-essential cysteines remained as a possible basis for thioredoxin dependence.

Inactivation of p53 by the C275S or C275A mutation was intriguing, as this residue is not essential in mouse p53 [Rainwater 1995]. Cys 275 is located within disulfide bonding distance of Cys 277, a surface cysteine known to make contact with DNA and be responsible for a measure of binding site sequence discrimination [Cho et al., 1994; Chene, 1999; Buzek et al., 2002]. We investigated the possibility that Cys 277 might tend to form transient inter-molecular disulfides with other thiol-containing molecules, and that Cys 275 is essential because it serves as aYap1-like resolving cysteine for a Cys 277 mixed disulfide. We tested this idea by determining the activity of an allele containing a C275/277S double mutation. Introduction of the C277S mutation did not suppress the inactivating effect of the C275S mutation. Intermolecular disulfide trapping involving Cys 277 thus does not account for the inactivity of the C275S and C275A alleles.

If p53 dependence on thioredoxin reductase is due to the existence of oxidation-prone cysteines on p53 that are maintained in the reduced state by the thioredoxin system, we have narrowed the field of cysteines potentially involved.

Clearly, individually mutating C124, C135, C141, C182, C229 and C277 to non-oxidizable residues does not relieve the thioredoxin reductase requirement. Also, combinatorial mutations exclusively involving C124, C141, C182, C229 and C277 can rule out these combinations of residues as playing any role in thioredoxin reductase dependence. Because of the low activity of combinatorial mutations involving C135, we have not ruled out the possibility that C135 and C229 are both oxidation-prone, or that C135 and two or more other residues are oxidation-prone. Similarly, the absence of an activity when C176, C238, C242 are C275 are mutated prevents us from assessing whether these residues are oxidation-prone. Finally, we must consider the possibility that p53 dependence on thioredoxin reductase is not due to the existence of oxidation-prone cysteines within the p53 polypeptide, but rather is due to oxidative inactivation of an upstream protein required for p53 activity.

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Fig. 1. A model for p53 dependence on thioredoxin reductase. In wild-type cells containing thioredoxin reductase, redox-sensitive p53 cysteines remain reduced and p53 activity is preserved (upper left). In Δ*trr1* cells lacking thioredoxin reductase (and perhaps also in wild-type cells under oxidative stress), redox-sensitive p53 cysteines form inhibitory disulfides and p53 activity is compromised (upper right). Replacement of redox-sensitive cysteines with serine prevents disulfide formation and maintains p53 activity in the absence of thioredoxin reductase (lower right).

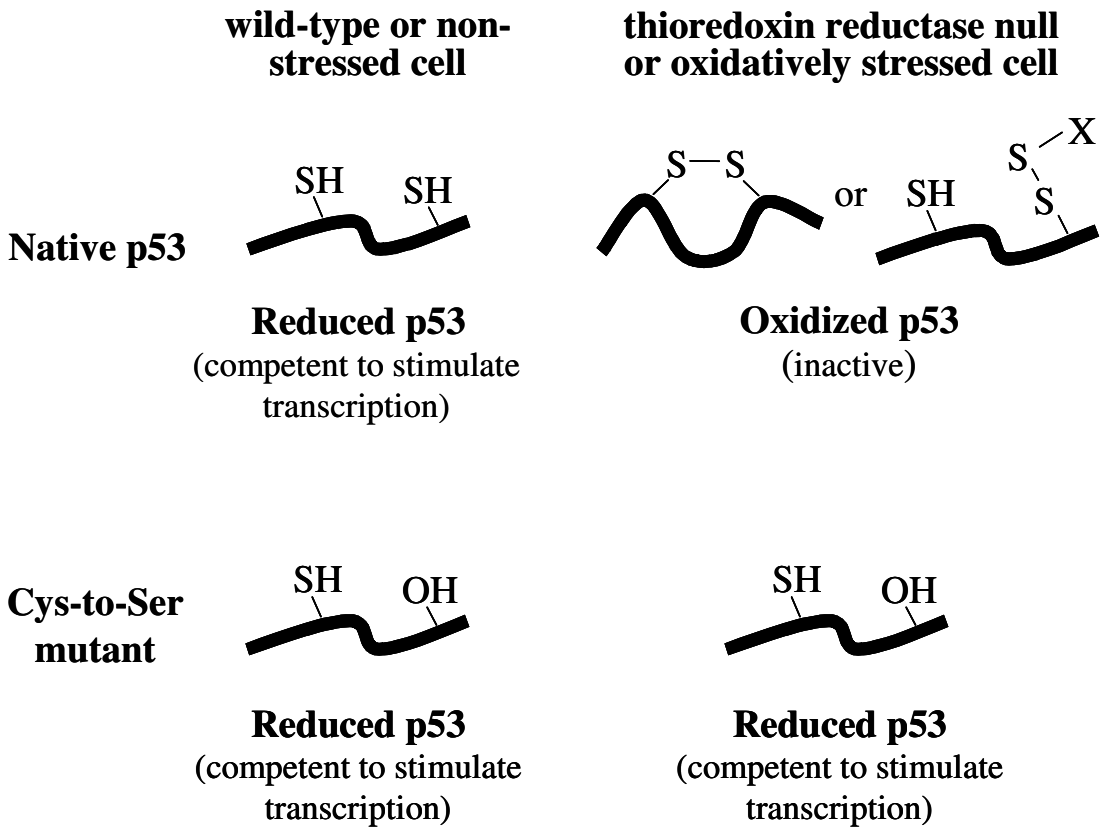

Fig. 1. A model for p53 dependence on thioredoxin reductase.

Fig. 2. Activity and thioredoxin reductase-dependence of p53 alleles carrying single Cys-to-Ser mutations. Wild-type (*TRR1*) and thioredoxin-null (Δ*trr1*) yeast carrying an integrated p53-dependent LacZ reporter gene were transformed with single-copy plasmids expressing the indicated p53 allele, and three independent transformants were assayed for β-galactosidase activity. Bars represent activity levels (mean ± S.D.), normalized to the level in wild-type yeast expressing native p53.

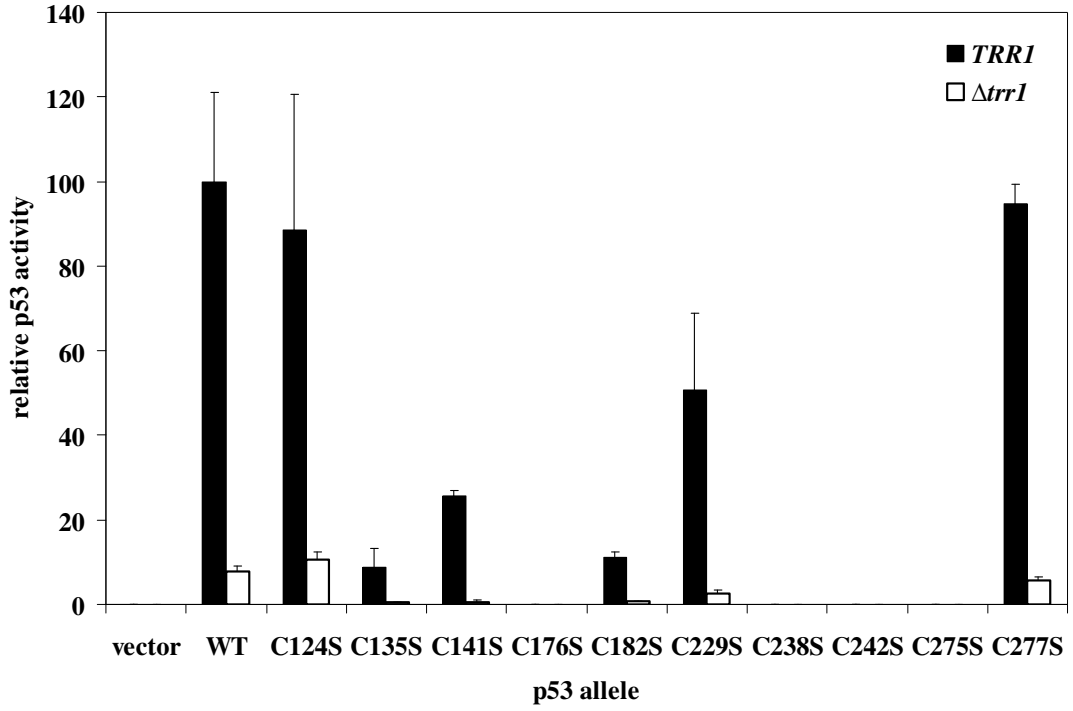

Fig. 2. Activity and thioredoxin reductase-dependence of p53 alleles carrying single Cys-to-Ser mutations.

Fig. 3. Immunoblot analysis of p53 protein levels in yeast transformed with mutated p53 alleles and specific activity of each protein in transactivating reporter gene expression. (A, upper panel) Twenty micrograms of lysate protein from representative transformants was resolved by SDS-PAGE and p53 protein was detected by immunoblotting using DO-1 antibody. (A, lower panel) An 80-kDa protein, detected by SYPRO Ruby staining prior to immunostaining, was used as a loading control. (B) The relative amount of p53 protein in each sample was calculated by dividing the p53 band intensity by the 80-kDa protein band intensity, and dividing the resulting ratio by the ratio observed in wild-type yeast expressing native p53. (C) The specific activity of p53 in stimulating reporter gene expression was calculated by dividing the β-galactosidase levels shown in Fig. 2 by the p53 protein levels determined in Fig. 3B. Bars represent mean ± S.D. for three transformants.

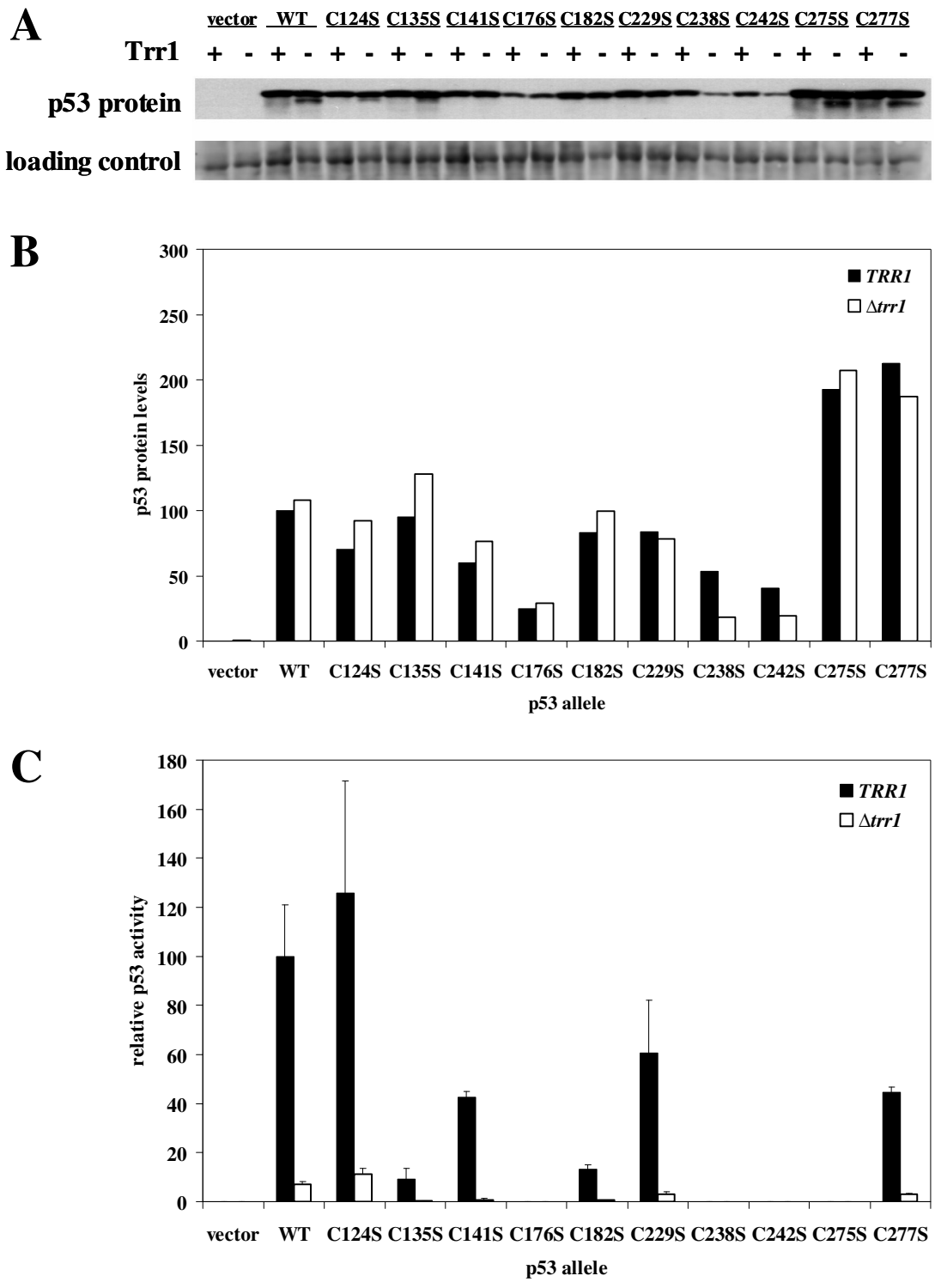

Fig. 3. Immunoblot analysis of p53 protein levels in yeast transformed with mutated p53 alleles and specific activity of each protein in transactivating reporter gene expression.

Fig. 4. Activity and thioredoxin reductase-dependence of p53 alleles with combinatorial Cys-to-Ser mutations. Wild-type (*TRR1*) and thioredoxin-null (Δ*trr1*) yeast carrying an integrated p53-dependent LacZ reporter gene were transformed with single-copy plasmids expressing indicated p53 allele, and three independent transformants were assayed for β-galactosidase activity. Bars represent activity levels (mean ± S.D.), normalized to the level in wild-type yeast expressing native p53.

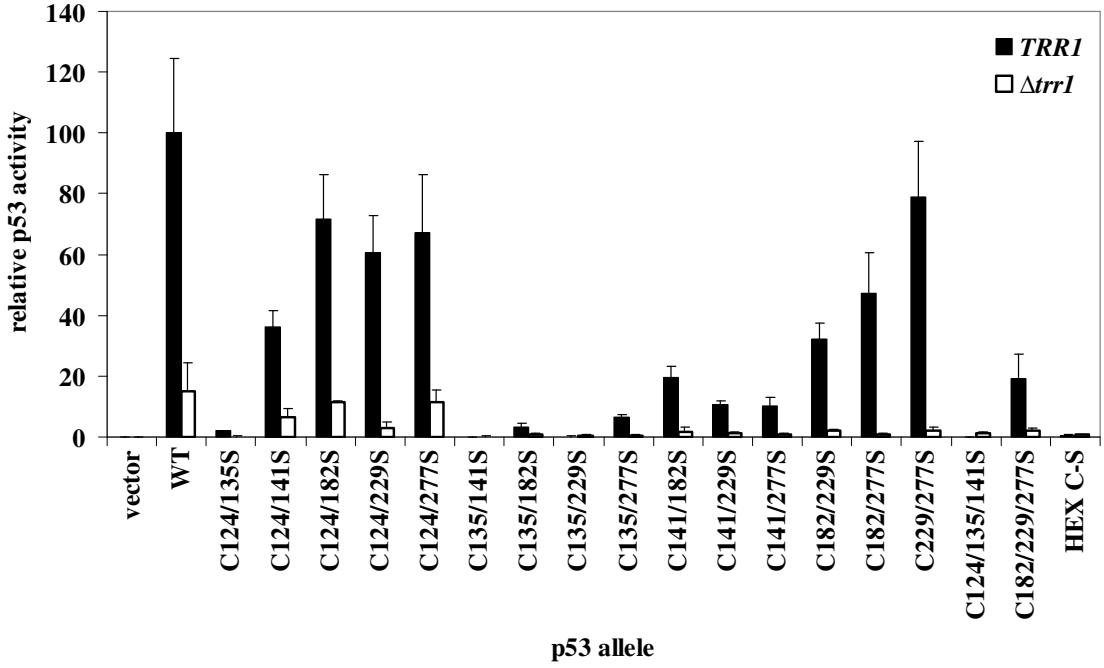

Fig. 4. Activity and thioredoxin reductase-dependence of p53 alleles carrying multiple Cys-to-Ser mutations.

Fig. 5. Activity and thioredoxin reductase-dependence of p53 alleles carrying combinatorial Cys-to-Ala mutations. Wild-type (*TRR1*) and thioredoxin-null (Δ*trr1*) yeast carrying an integrated p53-dependent LacZ reporter gene were transformed with single-copy plasmids expressing indicated p53 allele, and three independent transformants were assayed for β-galactosidase activity. Bars represent activity levels (mean ± S.D.), normalized to the level in wild-type yeast expressing native p53. (A) Reporter gene transactivation by complete set of p53 alleles. (B) Reporter gene transactivation by weak p53 alleles, plotted using an expanded y-axis scale to better show relative activity in wild-type and Δ*trr1* yeast.

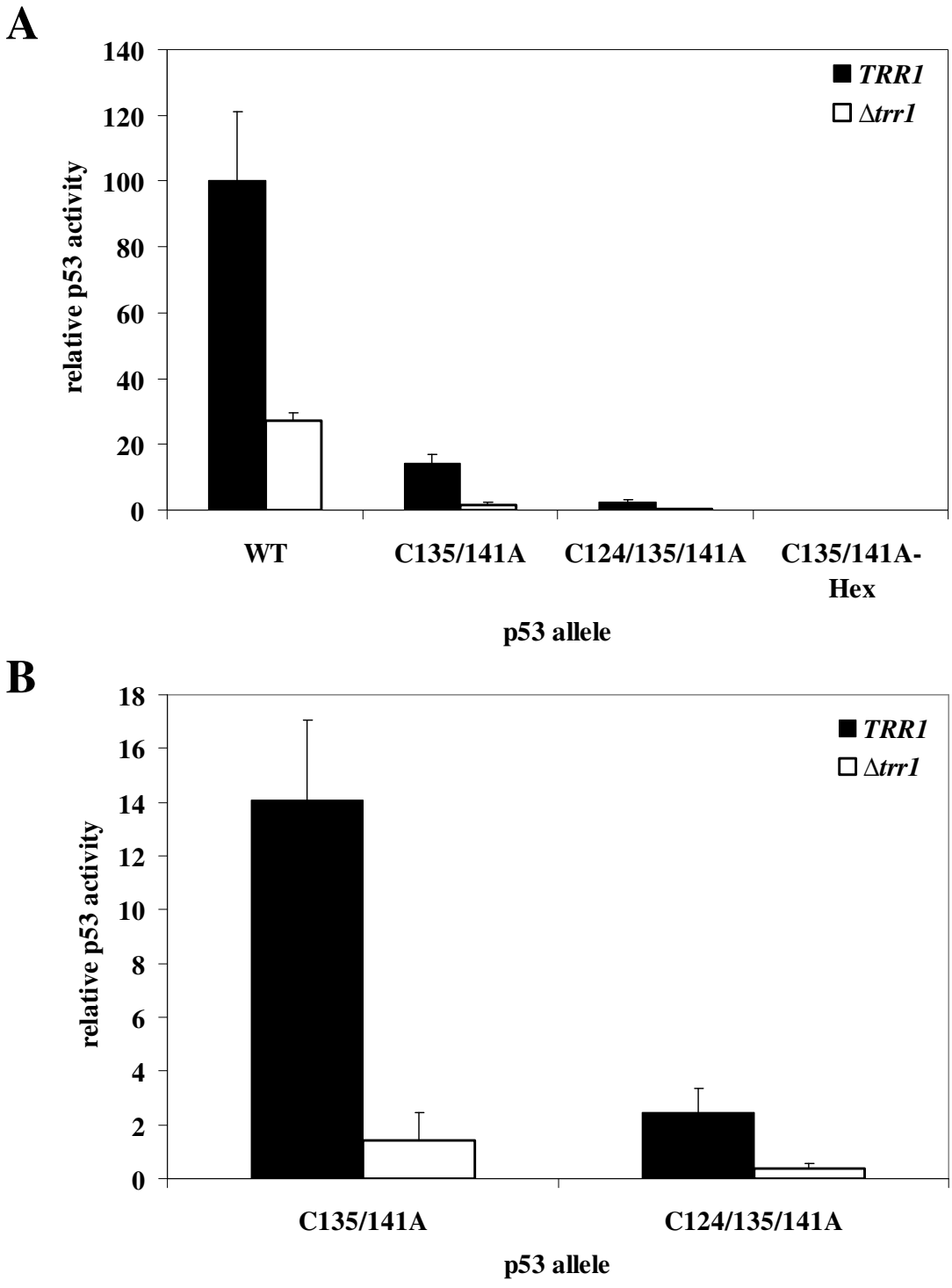

Fig. 5. Activity and thioredoxin reductase-dependence of p53 alleles carrying combinatorial Cys-to-Ala mutations.

Fig. 6. Nuclear levels of p53 in wild-type and Δ*trr1* yeast transformants. Twenty micrograms of nuclear lystate protein from triplicate samples of wild-type *(TRR1)* and mutant (Δ*trr1*) yeast transformed with wild-type p53 allele were resolved by SDS-PAGE, and p53 was detected by immunoblotting using DO-1 antibody. Numbers below each set of lanes show densitometric intensity of p53 bands (mean ± S.D.).

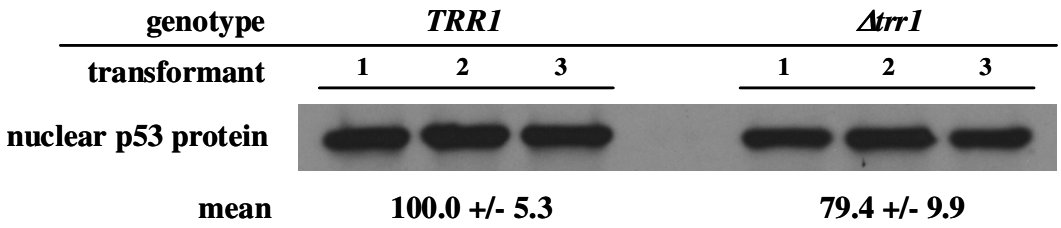

Fig. 6. Nuclear levels of p53 in wild-type and Δ*trr1* yeast transformants.

Fig. 7. Activity and thioredoxin reductase-dependence of p53 allele carrying Cys-to-Ala rather than Cys-to-Ser mutation at residue 275. Wild-type (*TRR1*) and mutant (Δ*trr1*) yeast carrying an integrated p53-dependent reporter gene were transformed with indicated p53 alleles, and β-galactosidase activity in three independent transformants was determined. Bars represent activity levels (mean ± S.D.), normalized to the level in wild-type yeast expressing native p53.

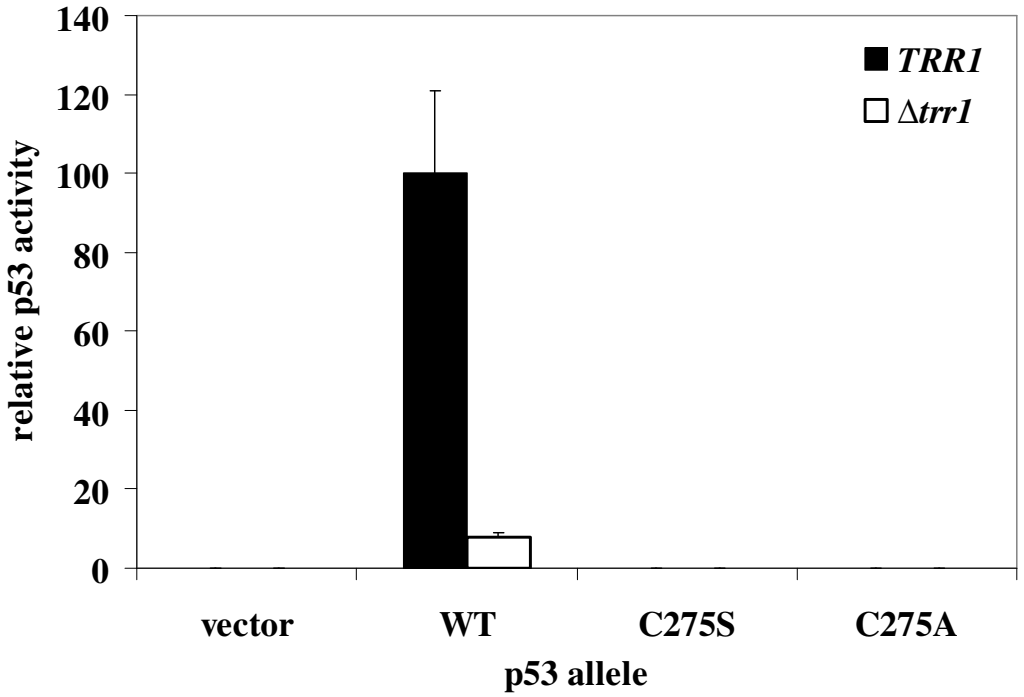

Fig. 7. Activity and thioredoxin reductase-dependence of p53 allele carrying Cys-to-Ala rather than Cys-to-Ser mutation at essential residue 275.

Fig. 8. Activity and thioredoxin reductase-dependence of p53 allele carrying a potentially compensatory C277S mutation in addition to a C275S mutation. Wild-type (*TRR1*) and mutant (Δ*trr1*) yeast carrying an integrated p53-dependent reporter gene were transformed with indicated p53 alleles, and β-galactosidase activity in three independent transformants was determined. Bars represent activity levels (mean ± S.D.), normalized to the level in wild-type yeast expressing native p53.

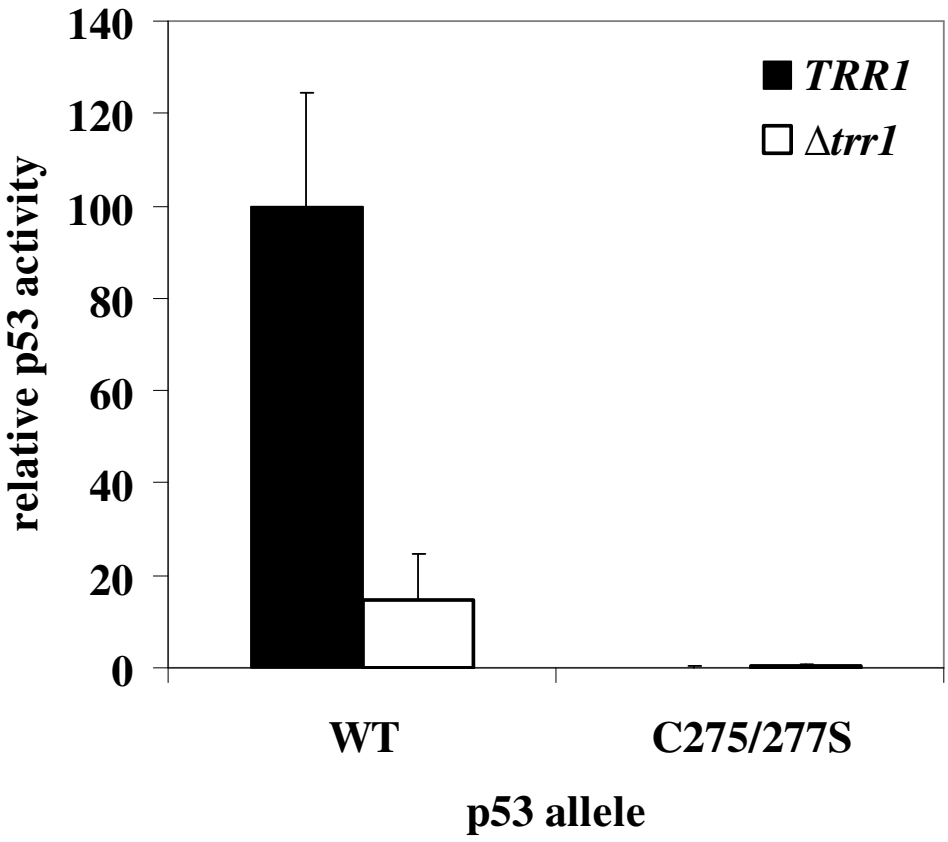

Fig. 8. Activity and thioredoxin reductase-dependence of p53 allele carrying a potentially compensatory C277S mutation in addition to a C275S mutation.

Fig. 9. Effect of p53 Cys-to-Ser mutations on reporter gene transactivation in co-transfected human cells. Human H1299 cells, which lack an endogenous p53 gene, were co-transfected with a pTL1-effector plasmid expressing the indicated p53 allele, a p53-dependent luciferase reporter gene (p53-luc), and a CMV promoter-driven LacZ transfection control plasmid (pCMV-β-gal). Luciferase activity and β-galactosidase activity in three independent transfectants was determined. (A) Luciferase activity (mean ± S.D.), normalized to luciferase activity in cells transfected with native p53. (B) β-galactosidase activity (mean ± S.D.). (C) Luciferase activity (mean ± S.D.), normalized to β-galactosidase activity.

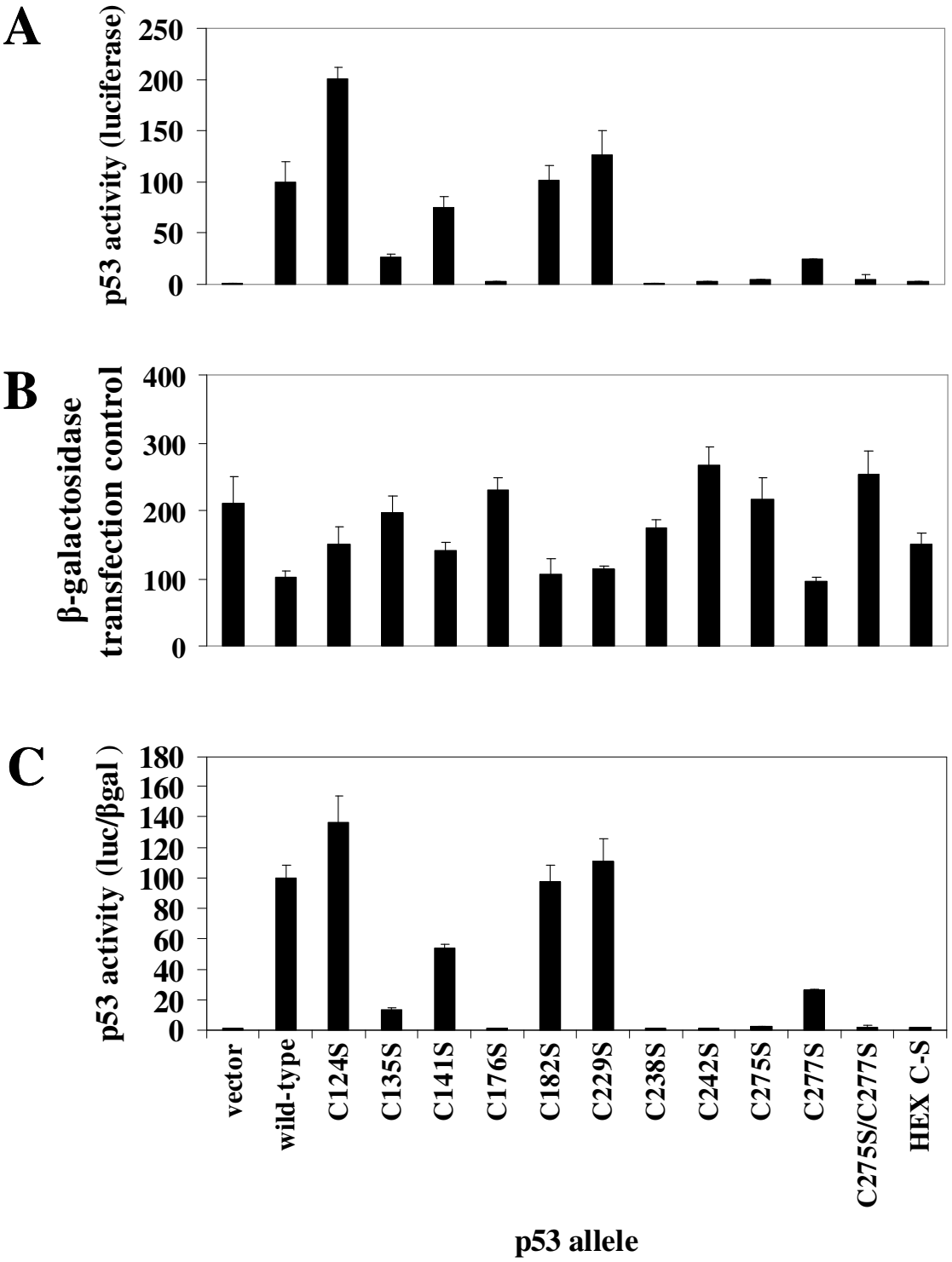

Fig. 9. Effect of p53 Cys-to-Ser mutations on reporter gene transactivation in co-transfected human cells.

Fig. 10. Effect of wild-type and C275S p53 alleles on transactivation of reporter genes carrying p53 response elements from different p53 target genes. H1299 cells were co-transfected with the pCMV-ß-gal transfection control plasmid; an effector plasmid containing no insert, wild-type p53 allele or C275S p53 allele (vector, WT or C275S, respectively); and a luciferase reporter gene plasmid containing p53 response elements from either the human PG genomic fragment or the p53 target genes MDM2, cyclin G, 14-3-3 or PIG3 (p53-luc, MDM2-luc, cyclin G-luc, 14-3-3-luc and PIG3-luc, respectively). Luciferase activity was normalized to β-galactosidase activity for three independent transfectant populations. Bars show normalized luciferase reporter gene activity (mean ± S.D.), relative to reporter gene activity in cells transfected with p53-luc reporter gene and expressing wild-type p53.

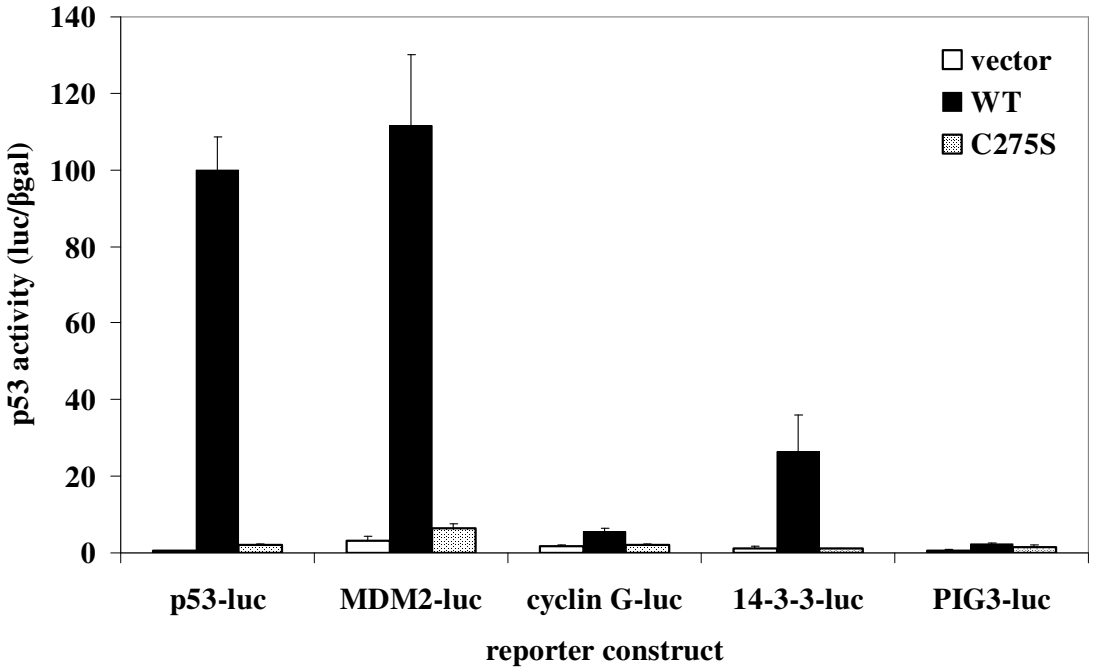

Fig. 10. Effect of wild-type and C275S p53 alleles on transactivation of reporter genes carrying p53 response elements from different p53 target genes.

CHAPTER 3: RESPONSE OF PEROXIREDOXINS TO RECEPTOR TYROSINE KINASE-INDUCED HYDROGEN PEROXIDE BURSTS

Abstract

Eukaryotic 2-Cys peroxiredoxins (Prxs) are thioredoxin-dependent peroxidases that differ from their prokaryotic counterparts in that they are sensitive to substrate inactivation at moderate H_2O_2 concentrations. Inactivation is due to reaction of a sulfenic acid catalytic intermediate with a second molecule of H_2O_2 before it has time to react with a resolving cysteine to form a disulfide. It has been hypothesized that the sensitivity of eukaryotic Prxs evolved as a mechanism to facilitate H_2O_2 signaling. For example, inactivation of protein tyrosine phosphatases (PTPs) by H_2O_2 may be necessary for efficient signaling by receptor tyrosine kinases, and Prx inactivation may be necessary to prolong episodes of PTP inactivation following growth factor stimulation. To test this model, we measured the oxidation state of Prxs and the phosphorylation state of EGF receptor in EGF- and peroxide-challenged A431 cells. Peroxide treatment sufficient to half-maximally inactivate Prx2, 3 and 6 did not significantly increase EGFR tyrosine phosphorylation. EGF treatment sufficient to significantly stimulate EGFR tyrosine phosphorylation levels had no observable effect on the oxidation state of Prx2, 3 or 6. Thus, global oxidation of cytosolic Prx was neither sufficient nor necessary for efficient EGFR phosphorylation.

Introduction

Receptor tyrosine kinases (RTKs) are cell surface receptors characterized by a shared organization consisting of extracellular ligand binding, transmembrane and intracellular tyrosine kinase domains [Schlessinger, 2000; Hubbard and Miller, 2007]. Members of the RTK family include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and insulin-like growth factor receptor (IGFR). Growth factor binding is thought to stimulate RTK dimerization, an event critical for downstream signaling [Heldin, 1995]. However, more recent evidence suggests RTKs exist in equilibrium between the inactive monomeric form (or α,β-heterodimer in the case of insulin receptor) and a ligand binding-competent dimeric form (or heterotetramer in the case of insulin receptor [Moriki et al., 2001]. The intracellular tyrosine kinase domain is activated upon sequential binding of two molar equivalents of growth factor, resulting in the autophosphorylation of several RTK tyrosine residues [Teramura et al., 2006]. Src-homology 2 (SH2) [Pawson et al., 2001] and phosphotyrosine binding (PTB) [Margolis, 1999] domains of several downstream effector proteins involved in growth factor signal transduction are recruited to phosphorylated receptor. Recruited signal transduction proteins include the Ras-activating GTP-exchange protein Sos, phospholipase C, and PI-3-kinase [Schlessinger, 2000]. Growth factor signaling is antagonized by both the action of protein tyrosine phosphatases (PTPs) [Ostman and

Bohmer, 2001] and receptor degradation following endocytosis [Ullrich and Schlessinger, 1990].

Hydrogen peroxide (H_2O_2), once thought to be purely a damaging reactive oxygen species (ROS), is now considered an important signal transduction molecule produced in response to growth factor and cytokine stimulation [Thannickal and Fanburg, 2000]. High concentrations of H_2O_2 (>1 mM) can activate RTK signaling without accompanying growth factor treatment [Chen et al., 2001; Sato et al., 2003; Gamou and Shimizu, 1995]. EGF treatment of A431 epidermoid cells elicits a burst of reactive oxygen species (ROS) that approximately doubles the normal intracellular H_2O_2 load as measured by dichlorofluorescein fluorescence [Bae et al., 1996], and is associated with increased EGFR phosphorylation [Bae et al., 1996; DeYulia et al., 2005b]. Catalase pretreatment reverses both the increased dichlorofluorescein fluorescence and associated increased EGFR stimulation, which suggests that H_2O_2 is the relevant ROS that accumulates during signaling [Bae et al., 1996]. PTP1B oxidation and loss of phosphatase activity is coincident with EGF-associated ROS bursts [Bae et al., 1996; DeYulia et al., 2005b]. Platelet-derived growth factor (PDGF) elicits a strong burst of ROS production in vascular smooth muscle cells and causes catalase-reversible phosphorylation of PDGFR [Sundaresan et al., 1995]. Interleukin and tumor necrosis factor alpha (TNFα) also elicit ROS bursts [Meier et al., 1989] and enhanced tyrosine phosphorylation of target proteins [Kang et al., 2004]. Treatment with diphenyleneiodonium, a flavinoid inhibitor, attenuates c-fos transcription following bFGF and TNFα exposure, thereby implicating a flavin-

containing enzyme such as NAD(P)H oxidase (NOX) in ROS formation [Lo and Cruz, 1995]. Spermine is converted to spermidine in TNFα-treated lung epithelial cells and is associated with ROS production [Babbar and Casero, 2006]. RNA interference of the spermine oxidase SMO/PAOh1 abrogated ROS production in response to TNFα. Alternatively, the extracellular domains of RTKs and cytokine receptors are known to possess an intrinsic ability to produce H_2O_2 [DeYulia et al., 2005a and 2005b], a trait in common with immunoglobulins [Wentworth et al., 2000]. Purified EGFR-EGF complex produces H_2O_2 and inactivates co-incubated PTP1B [DeYulia et al., 2005b]. Differences in the peroxide-generating mechanism may account for cell-, time- and context-specific outcomes of H_2O_2 production. For example, the intracellular concentration of H_2O_2 increases 10-20 nM within 10 minutes of transforming growth factor beta 1 (TGFβ1) exposure [Ohba et al., 1994]. Activated growth factor and cytokine receptors intrinsically produce H_2O_2 in excess of 20 nM [DeYulia et al., 2005a and 2005b]. A much stronger burst of H_2O_2 is elicited at around 16 hours after TGFβ1 exposure and is NADH-dependent [Thannikal and Fanburg, 1995; Thannikal et al., 1998].

Protein tyrosine phosphatases (PTPs) are especially prone to oxidative inactivation. PTP1, VHR and LAR PTPs exhibit rapid inactivation *in vitro* at H_2O_2 concentrations as low as 45 μM [Denu and Tanner, 1998]. Reactivity of PTPs varies directly with their catalytic efficiency, with high efficiency PTPs exhibiting oxidation at H_2O_2 concentrations as low as 31 μM [Groen et al., 2005]. The high sensitivity of PTPs to oxidative attack by H_2O_2 is due to the presence of a low pKa cysteine residue

in the PTP active site (pKa = 5.6) [Denu and Tanner, 1998]. Due to the low pK of this residue the thiolate form ($-RS^-$) predominates under normal intracellular conditions. Thus, this cysteine residue is particularly susceptible to electrophilic attack, including attack by H_2O_2. Reaction of cysteine thiolate with H_2O_2 yields cysteine sulfenic acid (-RSOH), which attacks the peptide bond of the following amino acid, thereby forming a sulfenylamide [Salmeen et al., 2003; van Montfort et al., 2003]. PTP activity is restored upon reduction of the sulfenylamide to the free thiolate by the thioredoxin system [Lee et al., 1998].

Protein tyrosine phosphatases are known regulators of RTK function. Deletion of certain PTPs augments RTK tyrosine phosphorylation and signal transduction, whereas overexpression conversely reduces RTK activation. EGF stimulation, known to elicit H_2O_2 formation [Bae et al., 1996], oxidizes PTP1B [Lee et al., 1998] and depresses overall tyrosine phosphatase activity [deYulia and Carcamo, 2005b]. Likewise, EGF and PDGF treatment stimulate oxidation of PTEN [Kwon et al., 2004], and insulin provokes oxidation of PTP1B and TC45 PTPs in human hepatoma cells [Meng et al., 2004]. In each case, oxidation of PTPs corresponds with increased RTK signal transduction. PTPs bind RTKs via SH2 domains that recognize specific receptor phosphotyrosines. Growth factor-stimulated recruitment of PTPs to RTK is abrogated in transfected cells overexpressing SH2-domain peptides, and abrogation of PTP binding is associated with increased resistance of PTP to oxidation [Meng et al., 2002]. Conversely, bolus application of H_2O_2 (>250 μM) oxidizes PTPs *in vivo* and allows binding of PTP to RTK in the absence of growth factor stimulation [Hao et al.,

2006]. Therefore, PTP binding to RTK seems to make the PTP catalytic cysteine especially prone to H_2O_2 attack. Once inactivated, PTP likely resides on the RTK longer than activated PTP would, thereby helping to shield RTK phosphotyrosines from active PTP.

Hydrogen peroxide levels in the cells are determined by the rates of H_2O_2 production and turnover. Enzymes capable of decomposing H_2O_2 include the peroxiredoxins (Prxs), glutathione peroxidases (Gpxs), and catalase. Catalase is peroxisomal, and thus growth factor-induced peroxide would need to diffuse far from its site of production to be eliminated via this route. Gpxs are cytosolic and have high catalytic efficiencies, and thus are potential scavengers of growth factor-induced peroxides. However, Prxs are present at much higher levels than Gpxs, suggesting that Prx activity may be the primary determinant of H_2O_2 turnover rates in the cytosol [Chae et al., 1999]. Consistent with this idea, Prx2 levels were shown to be inversely correlated to H_2O_2 levels, PDGFR phosphorylation, phospholipase C activation, proliferation and migration in mouse embryo fibroblasts and NIH 3T3 cells [Choi et al., 2005]. Furthermore, overexpression of Prx2 was shown to protect the phosphatase PTEN from oxidation, while dominant-negative inhibition of Prx2 had the opposite effect [Kwon et al., 2004]. Taken together, the evidence suggests that Prxs likely are the primary enzymes responsible for decomposition of RTK-stimulated H_2O_2.

Human cells express six peroxiredoxin proteins (Prx1, NCBI accession NP_859048; Prx2, NP_005800; Prx3, NP_054817; Prx4, Q13162; Prx5, P30044; Prx6, NP_004896) [Fujii and Ikeda, 2002; Rhee et al., 2005]. Prx1, 2, 3 and 4 are

active as head-to-tail dimers, while Prx5 and 6 are active as monomers. Prx1-5 are 2-Cys peroxiredoxins, meaning they possess two highly conserved cysteine residues. The N-terminal cysteine, referred to as the peroxidatic cysteine, initiates the catalytic cycle by reacting with H_2O_2 to form a sulfenic acid intermediate. All known dimeric Prx peroxidatic cysteines reside adjacent to an arginine guanidine group, which lowers the pKa of the cysteine residue from the normal ~8.5 to generally below 7.0 [Wood et al, 2003a]. For example, the peroxidatic cysteines of yeast Tsa1 and Tsa1 Prxs exhibit pKa values of 5.4 and 6.3, respectively [Ogusucu et al., 2007]. The C-terminal cysteine, referred to as the resolving cysteine, reacts with the sulfenic acid to form a disulfide, which is subsequently reduced by thioredoxin to regenerate the initial state.

While prokaryotic 2-Cys peroxiredoxins complete the full catalytic cycle under a wide range of substrate concentrations, eukaryotic 2-Cys peroxiredoxins are sensitive to inactivation at moderate substrate concentrations (Ki = 8 μM, A. Karplus, personal communication). Sensitivity is due to attack of the sulfenic intermediate by a second H_2O_2 molecule before it has a chance to form the disulfide. Such attack yields sulfinic acid (-RSO_2H) [Mitsumoto et al., 2001; Rabilloud et al., 2002; Yang et al., 2002; Chevallet et al, 2003]. The sulfinic acid "overoxidized" form of Prx is not able to be reduced by thiol electron donors, and, as such, is catalytically inactive until repaired. Restoration of the free thiolate form of Prx is accomplished by either sulfiredoxin [Chang et al., 2004a] or sestrin 2 [Budanov et al., 2004]. Both pathways are relatively slow. Consequently, once Prxs are overoxidized, there is a lag time during which the cell has lowered defenses against oxidative insult. Upon

overoxidation, Prx2 dimers assemble into a dodecameric form that exhibits protein chaperone activity, thereby providing some protection from peroxide-induced damage [Moon et al., 2005]. The gain of chaperone activity notwithstanding, it is paradoxical that 2-Cys peroxiredoxins are susceptible to inactivation by their substrate. One possible explanation for this phenomenon is the "floodgate" model [Wood et al., 2003b], which postulates that eukaryotes evolved H_2O_2-sensitive 2-Cys peroxiredoxins to facilitate oxidation of downstream targets such as protein tyrosine phosphatases and redox-sensitive transcription factors. In this scenario, RTK activation by growth factor binding initiates tyrosine autophosphorylation and a burst of H_2O_2 production. The H_2O_2 burst is functionally important because it inactivates protein tyrosine phosphatases and thereby stabilizes phosphotyrosines on the receptor and on downstream substrates. However, the H_2O_2 burst would be extremely short-lived if there was not a mechanism to defeat the highly abundant cytosolic 2-Cys peroxiredoxins. The sensitivity of eukaryotic peroxiredoxins to substrate inactivation may thus have evolved to perpetuate H_2O_2 levels once a critical threshold appropriate for signaling has been exceeded. We therefore hypothesize that inactivating peroxiredoxins is important for RTK signaling.

If floodgate regulation of RTK signaling is in fact occurring, overoxidized peroxiredoxins should accumulate shortly after growth factor exposure. Sulfinic acid contributes one extra negative charge to peroxiredoxin. The resultant change in pI of the protein is detectable by two-dimensional gel electrophoresis [Mitsumoto et al., 2001; Rabilloud et al., 2002; Yang et al., 2002; Chevallet et al., 2003]. Therefore, in

order to test the floodgate model, A431 cells (which overexpress EGFR and are hyper-responsive to EGF stimulation) were monitored for Prx overoxidation by 2-D electrophoresis during growth factor stimulation or treatment with H_2O_2.

Materials and methods

Cell lines and media

Human A431 cells [Giard et al., 1973] were used throughout. Cells were cultured in 50% Ham's F12 medium / 50% Dulbecco's modified Eagle's medium (F/D), containing 10% calf serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Life Technologies, Gaithersburg, MD). Cultures were incubated under 5% CO_2 atmosphere at 37°C.

Treatment with peroxides and EGF

Cultures on 10-cm plates that were 80% confluent were washed twice with 4 ml F/D and incubated for an additional 24 h in 10 ml F/D to deplete them of serum. Cells were then given 4 ml F/D containing H_2O_2 (100 μM or 500 μM) and/or EGF (100 ng/ml or 500 ng/ml) and incubated at 37°C until harvested.

Cell lysis and blocking of sulfhydryls

Cells were lysed in denaturing lysis buffer (6 M guanidine hydrochloride, 100 mM tris, pH 8.2) containing 5 mM iodoacetamide (Sigma, St. Louis, MO). Lysates were incubated at 37°C for 15 min, and clarified by centrifugation at 10,000 x g for 10 min. Supernatants were adjusted to 10 mM dithiothreitol and incubated at 37°C for 30 min. Liberated thiols were then blocked by addition of iodoacetamide to 30 mM followed by incubation at 37°C for 15 min.

Protein purification

Protein was separated from contaminants by organic extraction, whereby protein moves into the organic phase. Two volumes TE-saturated phenol, pH 8.0 (Mallinckrodt, Phillipsburg, NJ) and 0.5 volumes chloroform (Sigma) were sequentially added to one volume IAM-alkylated cell lysate. Samples were mixed vigorously by vortexing after each solvent was added. Phases were separated by centrifugation at 10,000 x g for 10 min. The lower, protein-containing organic phase was collected and back-extracted three times with one volume of fresh denaturing lysis buffer, with phase separation by centrifugation at each extraction. Protein was precipitated from the organic phase by addition of seven volumes cold 0.1 M ammonium acetate in 100% methanol, incubation for one hour at –20°C, and centrifugation at 10,000 x g for 10 min. The protein pellet was washed sequentially with one volume 0.1 M ammonium acetate in 100% methanol, 80% acetone and 70% ethanol. Protein was terminally resuspended in a solution of 7.8 M urea, 2.2 M thiourea (Sigma) that had been freshly deionized by using mixed-resin ion-exchange beads (Fisher, Waltham, MA). Protein concentration was determined by RC-DC protein assay (Bio-Rad, Hercules, CA).

Isoelectric focusing and 2-D electrophoresis

Samples containing 500 µg extract protein were adjusted to 7 M urea, 2 M thiourea, 4% CHAPS (Sigma), 1X Bio-Rad polyampholyte (appropriate match to pH gradient used) and 0.0002% bromophenol blue (Sigma) in 185 µl total volume. Bio-

Rad immobilized pH gradient (IPG) strips were passively rehydrated overnight with samples. Hydrated strips were focused for 30,000 V-hr in a Bio-Rad isoelectric focusing apparatus. Focused strips were given 5-min incubations in three changes of 6 M urea, 1% SDS, 20% glycerol, 50 mM Tris, pH 6.8 (Sigma), placed on precast 8-16% acrylamide Tris-HCl gels (Bio-Rad) and sealed with low-melt 2D sealing agarose (Bio-Rad). After electrophoresis at 50 V for 4 hours, gels were fixed and stained with SYPRO Ruby protein stain (Bio-Rad). Protein spots were detected on a Molecular Imager FX Pro Plus imager (Bio-Rad).

Mass spectrometry

Spots of interest on 2D gels were excised and identified by mass spectrometry. Excised protein spots were washed extensively with 50% / 50% acetonitrile / 50 mM ammonium bicarbonate, followed by dehydration in 100% acetonitrile. Trypsin digestion was accomplished by addition of 20 µl 0.0125 µg/µl trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate, followed by incubation on ice for 45 minutes. Supernatant was removed, followed by addition of 20 µl 25 mM ammonium bicarbonate and incubation at 37°C for 6 hours. Peptide fragment-containing supernatant was removed and saved. Peptides were further extracted by two 25 µl additions of 50% / 50% acetonitrile / 50 mM ammonium bicarbonate and one 25 µl addition of 100% acetonitrile, all of which were pooled with the original extract. Peptides were concentrated by vacuum centrifugation, resuspended in 5 µl 0.1% trifluoroacetic acid and desalted by Zip-Tip C18 tips (Millipore, Billerica, MA).

MALDI-TOF/TOF was performed on an ABI 4700 Proteomics Analyzer mass spectrometer (Foster City, CA) by the Oregon State University mass spectrometry core laboratory.

Determination of EGFR phosphotyrosine levels

IAM-alkylated and phenol-extracted protein lysate was prepared from triplicate groups of H_2O_2- and/or EGF-treated A431 cells as described above. After determination of protein concentration by Bio-Rad RC-DC assay, 50 µg lysate was adjusted to 7 M urea, 2 M thiourea and 1X SDS-PAGE loading buffer. Samples were resolved on a Bio-Rad Criterion pre-cast 8-16% Tris-HCl gel, and then transferred to Bio-Rad Trans-blot nitrocellulose using a Bio-Rad Mini Transblot apparatus at 200 mA overnight (approximately 2.5 Amp-hr). Nitrocellulose was blocked for 1 hr in Tris-buffered saline, 0.1% (vol/vol) Tween 20 (TBST) containing 5% non-fat dry milk. The phosphorylation level of the 170-kDa putative EGFR protein was monitored by incubation for 1 hr with mouse anti-phosphotyrosine primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 in TBST, followed by incubation for 1 hr with HRP-conjugated goat anti-mouse secondary antibody (Bio-Rad) diluted 1:5000 in TBST. Tubulin level was monitored by incubation for 1 hr with mouse anti-tubulin primary antibody (Sigma) diluted 1:5000 in TBST, followed by incubation for 1 hr with HRP-conjugated goat anti-mouse secondary antibody (Bio-Rad) diluted 1:5000 in TBST. Secondary antibody was detected by ECL chemiluminescence (Amersham-Pharmacia, Piscataway, NJ). Quantitation of

phosphotyrosine and tubulin signals was performed using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

Oxidative inactivation of 2-Cys peroxiredoxins has previously been demonstrated in numerous cells treated with exogenous H_2O_2 and other peroxides. We investigated whether peroxiredoxins undergo oxidative inactivation in response to the H_2O_2 produced during growth factor signaling. To test this idea, we used epidermoid cell line A431. These cells express extremely high levels of EGF receptor and consequently respond strongly to EGF stimulation. A431 cells were either mock treated or challenged with EGF or H_2O_2. Two-dimensional gel electrophoresis was used to monitor Prx2, 3 and 6 proteins. Reduced and sulfinic acid overoxidized isomers of Prx2, 3 and 6 were resolved by isoelectric point (pI) differences. Oxidation of Prx2, 3 and 6 was defined as a decrease in basic spot intensity and a concomitant increase in acidic spot intensity. Identity of proteins was confirmed by trypsin digestion of excised gel spots, followed by MALDI-TOF/TOF (Table 1). Prx2 and Prx3 were found at the exact molecular weight and pI expected from their unmodified primary sequences. Prx6, however, was found to be approximately 4 kDa higher molecular weight and resolved approximately 1.3 pH units more basic than expected pI. The position of Prx6 was in accordance with the previous findings of Chevallet [Chevallet et al., 2003]. Prx2, 3 and 6 were predominantly in the reduced state in mock-treated cells (Fig. 11A). In accordance with previous findings [Rabilloud et al., 2002], Prx2, 3 and 6 shifted from the reduced to oxidized forms upon treatment with 100 μM H_2O_2 for ten minutes (Fig. 11B). A431 cells were also treated with 100 ng/ml EGF for 3, 6, 10 or 20 minutes (Fig. 11C-F). In contrast to the results obtained with

H_2O_2 treatment, at no time point did EGF stimulate detectable levels of peroxiredoxin oxidation.

In order to confirm the findings of the first experiment, three additional replicates each of the mock, 100 μM H_2O_2 and 100 ng/ml EGF treatments were produced and resolved by two-dimensional gel electrophoresis. Spot intensity for the reduced and oxidized forms of Prx2, 3 and 6 was gathered. The percent amount of the oxidized form of each protein was calculated for all gels (Fig. 12). Hydrogen peroxide treatment achieved 50% oxidation of all three peroxiredoxins. However, EGF treatment did not result in the oxidation of any peroxiredoxin protein monitored. The results suggested peroxiredoxin overoxidation was not essential for EGFR-mediated signaling.

One explanation for the failure of EGF to induce Prx oxidation was that the EGF treatment regime employed was not sufficient to stimulate downstream signaling. EGFR, a 170 kDa protein, is the predominant phosphotyrosine phosphoprotein in A431 cells [Bae et al, 1996]. To confirm that our EGFR treatment regime was sufficient to stimulate receptor tyrosine kinase activity, A431 cell lysates were monitored for increased EGFR phosphotyrosine levels in response to EGF stimulation. Cells were treated with EGF or hydrogen peroxide, either alone or in combination. The phosphotyrosine level of the prominent ~170 kDa protein band corresponding to the molecular weight of EGFR was monitored via immunodetection by anti-phosphotyrosine antibody (Fig. 13A, upper panel). Tubulin signal was used as a loading control (Fig. 13A, lower panel). Signal density was measured for each, and

normalized data was obtained by dividing phosphotyrosine level by tubulin level (Fig. 13B). Phosphotyrosine levels in the EGFR band increased 3-4 fold upon addition of 100 ng/ml or 500 ng/ml EGF. Conversely, H_2O_2 treatment gave no increase in phosphotyrosine levels. The data therefore show that A431 cells were competent in the EGF response. Additionally, the combined EGF and H_2O_2 treatments exhibited a trend toward increasing EGFR phosphotyrosine levels as compared to EGF-only treatments, as expected from previously published findings [Gamou and Shimizu, 1995; Choi et al., 2005]. However, the combined EGF and H_2O_2 treatments did not affect a significant change in EGFR phosphotyrosine levels versus EGF-only treatments.

Discussion

The above data show that oxidation of Prx2, 3 and 6 did not occur in EGF-treated A431 cells and yet EGF efficiently induced EGF receptor autophosphorylation. Therefore, global oxidation of cytosolic Prx was neither sufficient nor necessary for efficient EGFR phosphorylation. We have not ruled out the possibility that a small, localized pool of Prx undergoes oxidation in EGF-treated cells and that this oxidation is important for EGFR autophosphorylation and signaling. Such Prx subpopulations could exist, either through subcellular compartmentalization or complex formation with RTKs and/or associated adaptor proteins. However, we estimate that such a privileged population of Prx must be less than 20% of the total Prx present in the cells, as we saw no oxidation in response to EGF and the standard deviation for each measurement was approximately +/- 10%.

If oxidative inactivation of peroxiredoxins is not required for efficient growth factor signaling, then the paradox of sensitivity of 2-Cys peroxiredoxins to substrate inactivation remains. The solution may be found in the relationship of Prx to thioredoxin. Peroxiredoxins require electrons from thioredoxin to regenerate the reduced initial state of the Prx catalytic cycle. Under moderate oxidative stress, it may be beneficial to conserve reduced thioredoxin rather than reduce peroxide. Preservation of reduced thioredoxin may be important either for enzymatic reactions, such as ribonucleotide reduction, or regulatory reactions such as transcription factor control or kinase inhibition. Several transcription factors are affected by thioredoxin redox status, including AP-1 [Hirota et al., 1997], NFκB [Hirota et al., 1999] and p53

[Casso and Beach, 1996; Pearson and Merrill, 1998]. ASK1, a MAPKKK that mediates JNK and p38 pro-apoptotic MAPK activation [Liu et al., 2000], is negatively regulated by complex formation with reduced thioredoxin but not oxidized thioredoxin [Saitoh et al., 1998]. At moderate peroxide concentrations, unabated flux through the highly abundant cytosolic peroxiredoxins might quickly exhaust the reduced thioredoxin pool and trigger ASK1-mediated apoptosis. Inactivation of peroxiredoxins at moderate substrate concentrations may thus represent a mechanism for conserving reduced thioredoxin and preventing ASK1-mediated apoptosis.

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Table 1: Identification of Prx2, 3 and 6 spots by MALDI mass spectrometry.

Protein name (other names)	mass (kDa) / obs. mass	Theoretical pI / obs. pI (reduced)	Theoretical pI / obs. pI (oxidized)	trypsin fragments MALDI detection	fragment m/z
Prx2 (Prdx2, cTPxI)	23.5 / 23	5.66 / 5.6	5.44 / 5.4	QVTINDLPVGR	1210.6669
				EGGLGPLNIPLLADVTR	1733.9675
				KEGGLGPLNIPLLADVTR	1862.0625
Prx3, isoform b (Prdx3, mTPx, AOP1, SP-22)	25.8 / 26	7.04 / 6.9	6.65 / 6.4	HLSVNDLPVGR	1206.6716
				GLFIIDPNGVIK	1285.7432
				DYGVLLEGSGLALR	1462.7855
Prx6 (Prdx6, Horf6)	25.0 / 29	6.00 / 7.3	5.73 / 6.8	NFDEILR	906.4801
				LPFPIIDDR	1085.6108
				VVFVFGPDKK	1135.6543
				FHDFLGDSWGILFSHPR	2029.9616
				PGGLLLGDVAPNFEANTTVGR	2097.0691

All known names for each protein are listed. Mass and pI are listed as expected value / observed value pairs.

Fig. 11: Reduced and oxidized forms of peroxiredoxins Prx2, 3 and 6 in H_2O_2-and EGF-treated A431 cells. Five hundred micrograms of lysate protein were analyzed by 2-D gel electrophoresis. (A) Mock-treated cells. (B) Cells exposed to 100 μM H_2O_2 for 10 min. (C-F) Cells treated with 100 ng/ml EGF for 3, 6, 10 or 20 min. Mock, 100 μM H_2O_2 and 100 ng/ml EGF treatments for 10 min were performed four times, each producing similar results.

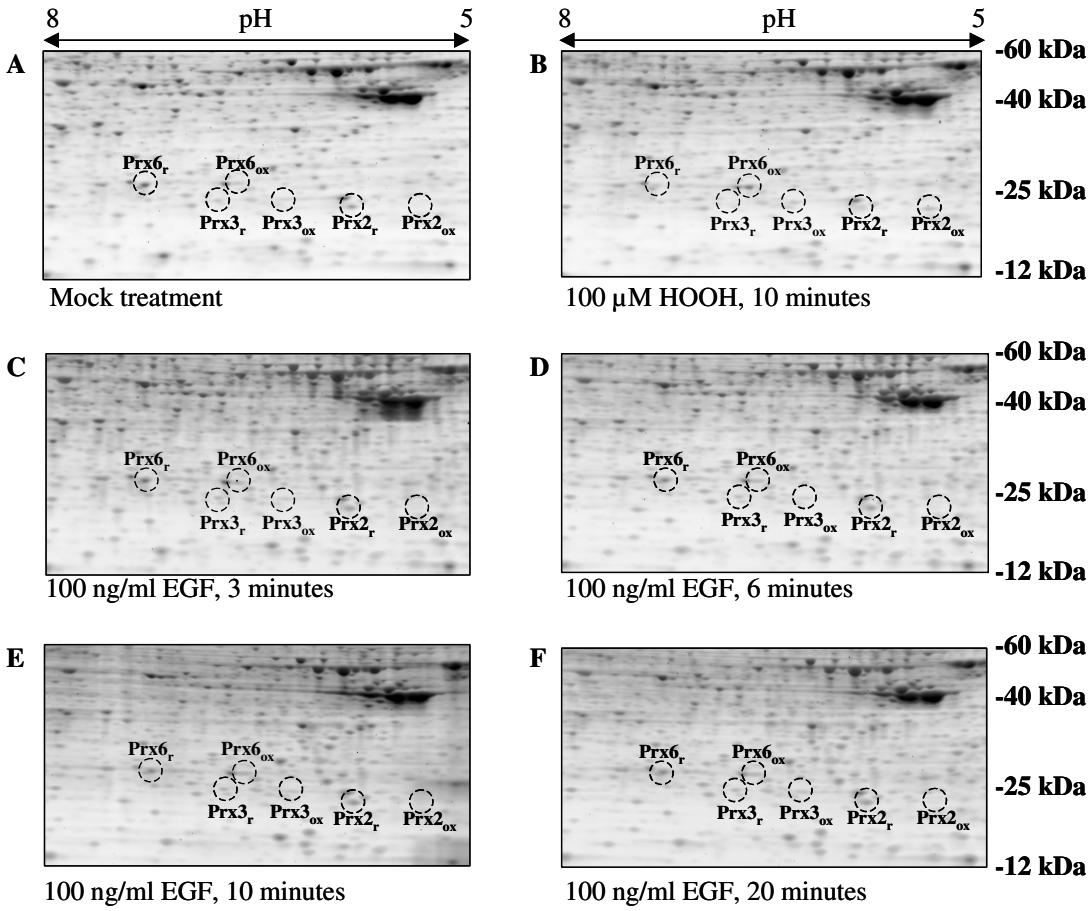

Fig. 11: Reduced and oxidized forms of peroxiredoxins Prx2, 3 and 6 in H_2O_2-and EGF-treated A431 cells.

Fig. 12: Analysis of oxidation state of peroxiredoxins Prx2, 3 and 6 in H_2O_2-and EGF-treated A431 cells. Five hundred micrograms of lysate protein were analyzed by 2-D gel electrophoresis, with four independent samples per treatment group

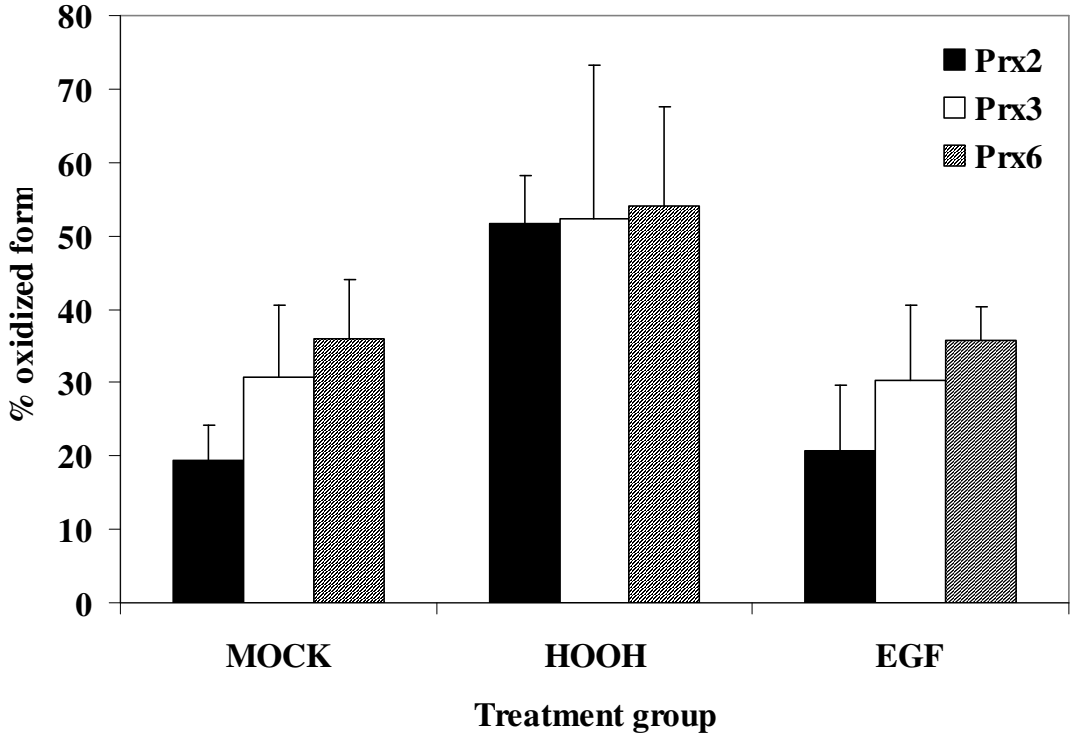

Fig. 12: Analysis of oxidation state of peroxiredoxins Prx2, 3 and 6 in H_2O_2-and EGF-treated A431 cells.

Fig. 13: Immunoblot analysis of EGFR phosphotyrosine levels in H_2O_2- and EGF-treated A431 cells. Fifty micrograms of lysate protein per sample was analyzed. (A, upper panel) EGFR phosphotyrosine levels, determined by immunodetection with anti-phosphotyrosine IgG. (A, lower panel) Tubulin levels, determined by immunodetection with anti-tubulin IgG. (B) EGFR phosphotyrosine levels normalized to tubulin levels. Mock treatment mean was arbitrarily adjusted to 100%. All other mean data points were adjusted accordingly.

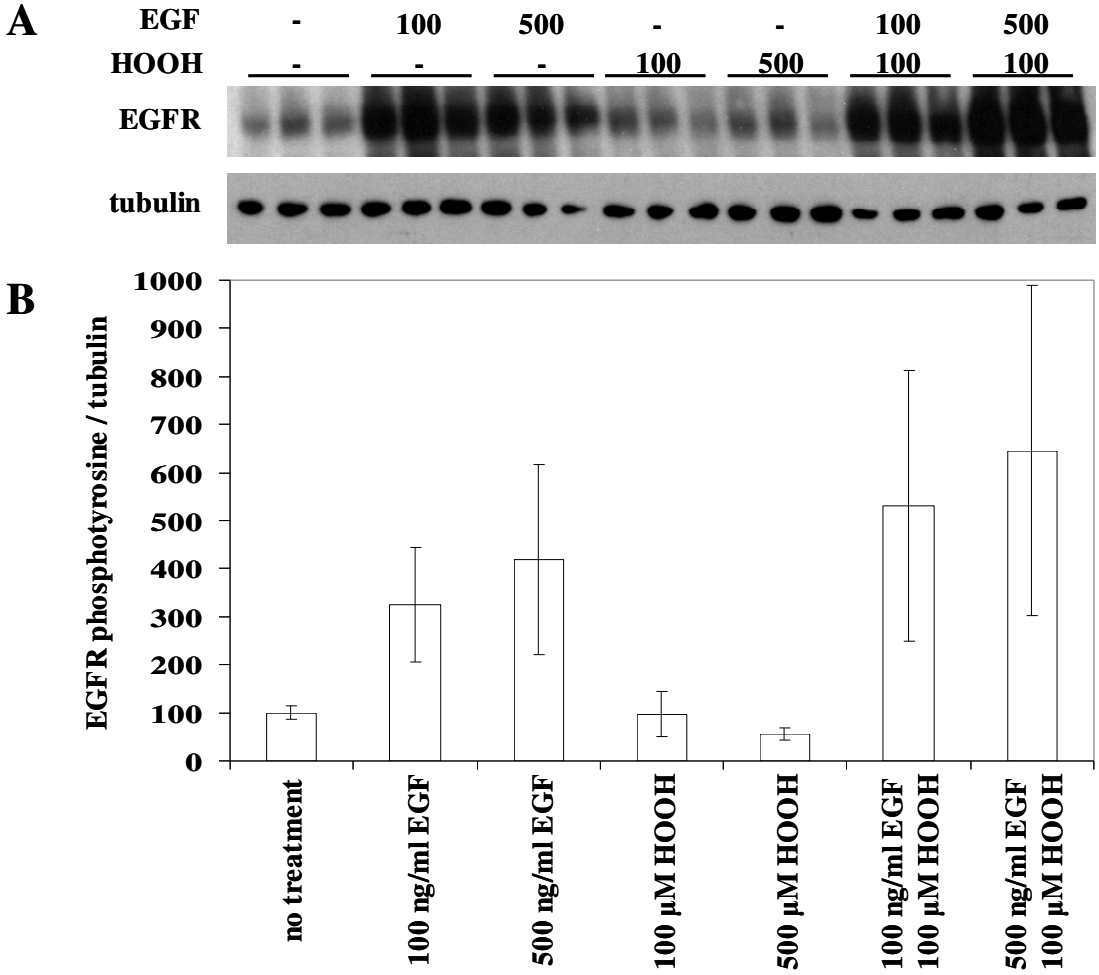

Fig. 13: Immunoblot analysis of EGFR phosphotyrosine levels in H_2O_2- and EGF-treated A431 cells.

CHAPTER 4: CONCLUSION

The work presented in Chapter 2 of the dissertation demonstrated that no cysteine replacement allele of p53 - either alone or in combination with other replacements - relieved dependence on thioredoxin reductase. However, several replacements completely inactivated p53 and precluded analysis. Therefore, limited explanations remain for continued thioredoxin reductase dependence of p53 activity. One or more of the essential cysteines may be regulated. Multiple non-essential cysteines may be regulated as well. Alternatively, regulation of p53 by thioredoxin reductase may be separate from oxidation of p53 cysteine residues. An example of such a mechanism would be regulation by thioredoxin reductase of an upstream regulator of p53 activity.

The work presented in Chapter 3 of the dissertation demonstrated that inactivation by hydrogen peroxide of peroxiredoxins 2, 3 and 6 was neither necessary nor sufficient for efficient epidermal growth factor receptor autophosphorylation. While the data show that a peroxiredoxin floodgate mechanism does not occur on a cellular scale, such a mechanism may operate within subcellular compartments or protein complexes containing growth factor receptor kinases.

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APPENDIX A: BIOCHEMICAL APPROACHES TO MONITOR P53 CYSTEINE RESIDUE REDOX STATE

The genetic approach for monitoring essential cysteine residue thioredoxin reductase dependence was practically exhausted, as demonstrated in Chapter 2 of this dissertation. A biochemical assay was required to monitor the redox state of essential p53 cysteine residues. Many attempts were made in the course of this research to monitor p53 cysteine redox state by biochemical means. This appendix provides an overview of these attempts.

An ideal assay would include the ability to both separate and quantitate each cysteine redox isomer of p53 in order to monitor the change in redox species between conditions. A protein mobility shift assay designed to ascertain cysteine redox state [Bersani et al., 2002; Takahasi and Hirose, 1990] was attempted. Briefly, reduced thiols were alkylated with iodoacetate, which bears a -1 charge. After removal of excess iodoacetic acid, disulfides and cysteine sulfenic acids were reduced with DTT, followed by alkylation of newly liberated thiols with uncharged iodoacetamide. All reactions were performed in denaturing buffer in order to alkylate all cysteine residues. Unfortunately, resolution of the individual charge isomers of p53 by urea-PAGE was not successful. It was eventually discovered that sample purification by phenol:chloroform extraction (see Chapter 3, *Materials and methods*) greatly improved charge isomer resolution. However, band resolution was still poor. In addition, reproducibility of the isomer band patterns was low. To overcome the isomer resolution problem, alkylated p53 was resolved on a slab isoelectric focusing gel. Individual charge isomers of p53 resolved into extremely crisp bands. However,

the number of bands generated greatly exceeded the expected number based solely on combinations of thiols and disulfides. It was evident that extensive post-translational modification of p53 by charged adducts occurred in yeast, as is known to occur in mammals.

Other methods were subsequently attempted, based on tagging oxidized cysteine residues followed by immunoprecipitation and gel purification of p53. Such assays are inherently limited to providing the change in overall cysteine oxidation, rather than the change in number of oxidations as is possible with the charge-based method. In brief, cells were lysed in the presence of a cysteine alkylant (N-ethylmaleimide or iodoacetamide) in order to block free thiols and arrest electron transfer amongst cysteine residues. Free alkylant was removed, followed by reduction with dithiothreitol (DTT). Free reductant was removed, followed by treatment with either 3-(maleimidoproprionyl)-biocytin (MPB) or 5-iodoacetamidofluorescein (IAF). Free tag was removed, followed by immunoprecipitation of p53. SDS-PAGE was then performed. MPB tag was detected by western analysis using streptavidin:HRP, while IAF was detected on SDS-PAGE gels using a Bio-Rad Molecular Imager FX Pro Plus fluorescence scanner. Due to technical issues, in no case was reliable p53 oxidation state data obtained. Assays performed in native buffer relied on dialysis or Biogel P6 spin columns to remove excess alkylant, reductant and tag. Dialysis, while thorough, likely allowed reoxidation of p53 once DTT was removed, thereby preventing tagging. Gel filtration spin columns, on the other hand, were not efficient at removing excess reactants. Contaminating reactants most certainly altered the

redox state data. Even more problematic was that the heavy chain of anti-p53 IgG became tagged during immunoprecipitation. As the heavy chain co-migrates with p53 on SDS-PAGE, any p53 redox data was obscured by uniformly tagged IgG heavy chain. Performing all protein chemistry steps in 8 M urea avoided problems with excess reactants, as protein was simply precipitated and washed between steps. However, we did not find a condition that allowed renaturation of p53 after tagging. Immunoprecipitation was therefore impossible since p53 could not be returned to native buffer in a soluble form.