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Cyclopentenone Prostaglandins with Dienone Structure Promote Cross-Linking of the Chemoresistance-Inducing Enzyme Glutathione Transferase P1-1

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ABSTRACT

Glutathione transferase P1-1 (GSTP1-1) plays crucial roles in cancer chemoprevention and chemoresistance and is a key target for anticancer drug development. Oxidative stress or inhibitor-induced GSTP1-1 oligomerization leads to the activation of stress cascades and apoptosis in various tumor cells. Therefore, bivalent glutathione transferase (GST) inhibitors with the potential to interact with GST dimers are been sought as pharmacological and/or therapeutic agents. Here we have characterized GSTP1-1 oligomerization in response to various endogenous and exogenous agents. Ethacrynic acid, a classic GSTP1-1 inhibitor, 4-hydroxy-nonenal, hydrogen peroxide, and diamide all induced reversible GSTP1-1 oligomerization in Jurkat leukemia cells through the formation of disulphide bonds involving Cys47 and/or Cys101, as suggested by reducing and nonreducing SDS-polyacrylamide gel electrophoresis analysis of cysteine to serine mutants. Remarkably, the electrophilic prostanoid 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) induced irreversible GSTP1-1 oligomerization, specifically involving Cys101, a residue present in the human but not in the murine enzyme. 15d-PGJ_2-induced GSTP1-1 cross-linking required the prostaglandin (PG) dienone structure and was associated with sustained c-Jun NH_2-terminal kinase activation and induction of apoptosis. It is noteworthy that 15d-PGJ_2 elicited GSTP1-1 cross-linking in vitro, a process that could be mimicked by other dienone cyclopentenone PG, such as Δ^{12}-PGJ_2, and by the bifunctional thiol reagent dibromobimane, suggesting that cyclopentenone PG may be directly involved in oligomer formation. Remarkably, Δ^{12}-PGJ_2-induced oligomeric species were clearly observed by electron microscopy showing dimensions compatible with GSTP1-1 tetramers. These results provide the first direct visualization of GSTP1-1 oligomeric species. Moreover, they offer novel strategies for the modulation of GSTP1-1 cellular functions, which could be exploited to overcome its role in cancer chemoresistance.

Introduction

Glutathione transferase (GST) enzymes are critical players in the cellular defense against the deleterious effects of oxidative stress and electrophilic compounds, including endogenous metabolites and various anticancer agents. Increased levels of several GST family members have been associated with improved cell survival in the presence of diverse insults. The GSTP1-1 isoform has been directly related to tumor cell resistance to chemotherapy and radiation (Su et al., 2003). Therefore, understanding the mechanisms of GST-mediated tumor cell survival and the development of inhibitors of these processes are areas of primary interest.

The various roles of GSTP1-1 in cell survival are supported by its multiple cellular functions. GSTP1-1 catalyzes the conjugation of various electrophilic compounds with the tripeptide glutathione (GSH), the adducts formed being substrates for cellular export by the multidrug transporter systems (Sibhatu et al., 2008). GSTP1-1 has also been found to promote the glutathionylation of cellular proteins (Townsend

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ABBREVIATIONS: GST, glutathione transferase; GSTP1-1, glutathione transferase isoform P1-1; JNK, c-Jun NH_2-terminal kinase; cyPG, cyclopentenone prostaglandin(s); 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; 15d-PGJ_2-B, 15d-PGJ_2-biotin; PGA_1-B, PGA_1-biotin; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; RMC, rat mesangial cell; PG, prostaglandin; DTT, dithiothreitol; PCR, polymerase chain reaction; 2D, two-dimensional; HNE, 4-hydroxy-2-nonenal.

et al., 2009), a process that can be considered a protective mechanism against irreversible oxidative damage and requires the catalytic activity of the enzyme. In contrast, the functions of GSTP1-1 in the sequestration of toxic compounds through covalent or noncovalent interaction, known as the "ligandin" role (Lu and Atkins, 2004), and in the binding of signaling proteins (Adler et al., 1999; Wu et al., 2006) are independent from GSTP1-1 catalytic activity. Of particular importance is the role of GSTP1-1 in the regulation of stress signaling pathways. In nonstressed cells GSTP1-1 has been found associated with various stress-activated kinases, including Traf-2 and JNK (Adler et al., 1999; Wu et al., 2006), thus maintaining them in an inactive state. Cell stress conditions, including oxidative stress and treatment with certain GSTP1-1 inhibitors, induce GSTP1-1 oligomerization and release of the kinases, leading to the activation of their signal transduction cascades and induction of apoptosis (Adler et al., 1999; Turella et al., 2005; Wu et al., 2006). Therefore, GSTP1-1 inhibitors and/or inducers of GSTP1-1 oligomerization are being sought as potential anticancer agents (Turella et al., 2005; Burg et al., 2006).

Cyclopentenone prostaglandins (cyPGs) are reactive lipidic mediators that arise by nonenzymatic dehydration of certain PG. cyPGs display varied biological effects, including antiproliferative and anti-inflammatory actions, for which a potential pharmacological use of these compounds has been put forward (Ianaro et al., 2003; Homem de Bittencourt et al., 2007). An important mechanism for cyPG action is the formation of covalent adducts with cellular proteins, resulting in altered protein function (Cernuda-Morollón et al., 2001; Pérez-Sala et al., 2003; Sánchez-Gómez et al., 2004). cyPGs possess an α,β-unsaturated carbonyl group in the cyclopentane ring, a structure that favors the formation of Michael adducts with thiol groups in proteins or in GSH. Among the proteins known to be targets for modification by addition of cyPG are transcription factors, such as activator protein-1 and nuclear factor-κB (Cernuda-Morollón et al., 2001; Pérez-Sala et al., 2003); proteins involved in the regulation of cellular redox status, such as thioredoxin and thioredoxin reductase (Shibata et al., 2003; Cassidy et al., 2006); and cytoskeletal proteins (Stamatakis et al., 2006; Aldini et al., 2007), among others.

cyPG and GSTP1-1 may interact at several levels (Kawamoto et al., 2000). cyPG are substrates for GSTP1-1 (Atsmon et al., 1990), and both conjugation with glutathione and sequestration by binding to the enzyme may lead to the attenuation of cyPG effects (Paumi et al., 2004). In turn, cyPG may inhibit GSTP1-1 activity, and both covalent and noncovalent mechanisms have been invoked for this effect (van Iersel et al., 1999; Paumi et al., 2004). We demonstrated recently the direct covalent modification of GSTP1-1 by the cyPG 15d-PGJ_2, both in vitro and in intact cells (Sánchez-Gómez et al., 2007). In addition, treatment with 15d-PGJ_2 elicited various oxidative modifications of GSTP1-1. 15d-PGJ_2-induced GSTP1-1 modifications were associated with an irreversible inhibition of GSTP1-1 enzymatic activity and with an impairment of its protective effect on cell survival (Sánchez-Gómez et al., 2007).

Because of the importance of GSTP1-1 oxidative modifications in catalytic activity, oligomerization, and signaling functions, here we have explored the effects of various oxidative stress-inducing agents and GSTP1-1 inhibitors on GSTP1-1 oligomerization status in intact cells. The results herein described unveil a novel interaction of 15d-PGJ_2, with GSTP1-1 leading to the irreversible oligomerization of the protein. Cross-linking and inactivation of GSTP1-1 by cyPG could be exploited as a novel strategy to overcome cancer cell resistance to chemotherapy.

Materials and Methods

Reagents. Human recombinant GSTP1-1 was from Sigma-Aldrich (St. Louis, MO) or from Alpha Diagnostics International Inc. (San Antonio, TX). 15d-PGJ_2-biotin (15d-PGJ_2-B; *N*-11-oxo-prosta-5*Z*,9,12*E*,14*E*-tetra-1-oyl-*N′*-biotinoyl-1,5-diaminopentane), PGA_1-biotin (PGA_1-B; *N*-9-oxo-15*S*-hydroxy-prosta-10,13*E*-dien-1-oyl-*N*-biotinoyl-1,6-diaminohexane), and other prostanoids used in this study were from Cayman Chemical (Ann Arbor, MI). Cell culture media and supplements, Lipofectamine 2000, dibromobimane, and Simply Blue Colloidal Coomassie stain were from Invitrogen (Barcelona, Spain). Annexin V-fluorescein isothiocyanate was from Bender Medsystems GmbH (Vienna, Austria). Neutravidin-agarose and the bicinchoninic acid kit for protein quantification were from Pierce Chemical (Rockford, IL). 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefablock SC) protease inhibitor was from Roche Applied Science (Mannheim, Germany).

Cell Culture and Treatments. T-cell leukemia Jurkat cells, lung cancer A549 cells, and primary rat mesangial cells (RMCs) (Stamatakis et al., 2006) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Breast carcinoma MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with serum and antibiotics as above. For treatments, cells, at 5×10^5 cells/ml were cultured in serum-free medium. Prostaglandins were added in dimethyl sulfoxide, and control cells received an equivalent amount of this solvent (0.1%, final concentration).

Redox Two-Dimensional Electrophoresis. This procedure was performed essentially as described previously (Cumming et al., 2004). Jurkat cells treated with the various agents were collected in phosphate-buffered saline containing 50 mM iodoacetamide. Cells were lysed in 20 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, 0.5% SDS, and 50 mM iodoacetamide containing protease inhibitors [2 μg/ml each of trypsin inhibitor leupeptin and aprotinin, and 1.3 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride]. Protein concentration of cell lysates was estimated with the bicinchoninic acid kit. Aliquots of cell lysates containing 10 μg of protein were analyzed by nonreducing gel electrophoresis in the first dimension. Cell lanes were then excised and subjected to a series of equilibration and washing steps consisting of the following: equilibration in sample buffer containing 100 mM DTT for 20 min; three 5-min washes with SDS running buffer; equilibration in sample buffer containing 100 mM iodoacetamide for 10 min; and three 5-min washes with SDS running buffer. For the second dimension, lanes processed as above were placed horizontally on top of reducing SDS-PAGE gels. Proteins were later transferred to Immobilon P membranes (Millipore, Billerica, MA) and processed for Western blot.

Western Blot. Western blot analysis was performed as described previously (Sánchez-Gómez et al., 2007). Antibodies and dilutions used were the following: at 1:1000, anti-GSTP1-1 (BD Biosciences, San Jose, CA), anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-AU5 (Babco, Berkeley, CA); at 1:500, anti-JNK and anti-p-JNK (Cell Signaling Technology, Danvers, MA).

Plasmids and Transfections. A plasmid containing the open reading frame of human GSTP1-1 was obtained from Origene (Rockville, MD) and subjected to mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) to create an EcoRI site. The open reading frame of GSTP1-1 was then obtained by digestion with EcoRI and NotI and cloned into the same sites of the plasmid pECFL-KZ-AU5. The C47S, C101S, and C169S mutants of GSTP1-1 were obtained by site-directed mutagenesis using the QuikChange II kit (Stratagene) and primers 5′-GGCTCACTCAAAGCCTCC**TCC**CTATACGGGCAGC-3′ for the C47S mutation, 5′-GGCGTGGAGGACCTCCGC**TCC**AAATACATCTCCC-3′ for the C101S mutation, and 5′- GCCCCTGGC**AGC**CTGGATGCGTTCCCC-3′ for the C169S mutation, along with the corresponding complementary reverse oligonucleotides (mutated nucleotides appear in bold). The C14S mutant was obtained by the overlap extension mutagenesis technique (Pogulis et al., 1996). In brief, in two separate PCRs, two overlapping fragments of the GSTP1-1 sequence were amplified using the following primers: 5′- CGAGGATCCATGCCGCCCTACACC-3′ and 5′-CAGCATGCGCAGGGCCGC**GCT**GCGGCC-3′ for the N-terminal fragment; and 5′-GGCCGC**AGC**GCGGCCCTGCGCATGCTG-3′ and 5′- GCAGCGAATTCTCACTGTTTCCCGTTGCC-3′ for the C-terminal fragment. The overlapping fragments obtained in these PCRs were subjected to a third PCR using the flanking primers, which contain EcoRI sites, to obtain the GSTP1-1 sequence containing the C14S mutation. The GSTP1-1 C14S product was then digested with EcoRI and cloned into the same site of the pECFL-KZ-AU5 plasmid.

Cells were transfected with Lipofectamine 2000 using 1 μg of DNA per 1.5×10^6 cells. After 6-h incubation, the transfection medium was diluted with RPMI plus 10% fetal bovine serum, and incubation was continued overnight. For cell treatments, the medium was changed to serum-free medium as described above.

Apoptosis Assay. To monitor the induction of apoptosis, cells treated with the indicated agents were collected and incubated at 200,000 cells/ml in 10 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$ in the presence of Annexin V-fluorescein isothiocyanate [1:80 (v/v)] for 15 min at 37°C in the dark and washed. Immediately before analysis, propidium iodide was added at 20 μg/ml final concentration. Cells were analyzed by flow cytometry on an Epics Coulter cytometer, as described previously (Pérez-Sala et al., 1998).

Immunoprecipitation and Pull-Down Assays. Immunoprecipitation of AU5-GSTP1-1 from transfected Jurkat cells lysates was achieved by using the Immunocatcher kit from Cytosignal (Irving, CA) following the manufacturer's instructions. Aliquots of lysates containing 100 μg of protein and 0.2 μl of anti-AU5 antibody were used per immunoprecipitation assay. Incorporation of 15d-PGJ_2-B into the immunoprecipitated protein was confirmed by Western blot and detection with HRP-Streptadivin. Pull-down on Neutravidin-agarose beads was performed as described previously (Sánchez-Gómez et al., 2007).

In Vitro Oligomerization Assays. GSTP1-1 was incubated at a final concentration of 2.5 μM in 20 mM Tris-HCl, pH 7.0, 45 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT, 0.25% glycerol in the presence of cyPG or dibromobimane at 10 μM (final concentration) or vehicle (dimethyl sulfoxide) for the indicated times at room temperature. Immediately afterward, iodoacetamide was added at 50 mM final concentration for 30 min. Mixtures were analyzed by SDS-PAGE on 15% polyacrylamide gels under reducing conditions. GST oligomers were detected by Coomassie staining of the gels or after Western blot by means of anti-GSTP1-1 antibody or with HRP-Streptavidin (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in the case that oligomerization was induced by biotinylated cyPG.

Electron Microscopy. A few microliters of the Δ^{12}-PGJ_2-treated or untreated GSTP1-1 samples was applied to carbon-coated grids after glow-discharge and negatively stained with 1% uranyl acetate. Grids were observed in a JEOL 1230 electron microscope operated at 100 kV, and micrographs were recorded at a nominal magnification of 50,000. Micrographs were digitized in a scanner to a final sampling of 2.1 Å/pixel (Konica Minolta, Tokyo, Japan). Images of individual molecules, 697 for untreated and 1351 for Δ^{12}-PGJ_2-treated GSTP1-1, were extracted from the micrographs, centered, low-passed filtered, and reference-free 2D averages obtained using methods and algorithms found in the platform EMAN (Ludtke et al., 1999).

Structural Models. The human GSTP1-1, for which a 1.9-Å resolution crystal structure is available (Protein Data Bank code 22GS), was used to obtain the molecular surface of the dimer. The distances between cysteine residues of interest were obtained using the program Swiss PDBViewer (http://spdbv.vital-it.ch/).

Results

15d-PGJ_2 Induces GSTP1-1 Cross-Linking, Whereas Oxidative Stress and Enzyme Inhibitors Elicit Reversible Oligomerization. The GST oligomerization state has been reported to be modulated both by cellular redox conditions and by GST inhibitors (Adler et al., 1999; Bernardini et al., 2000; Turella et al., 2005). Moreover, GST oligomerization has important consequences for signal transduction. Therefore we explored the induction of GSTP1-1 oligomerization by various oxidative stress-inducing agents, such as H_2O_2 and diamide (Fig. 1A). In addition, we used 4-hydroxy-2-nonenal (HNE), a reactive aldehyde generated in micromolar levels in vivo under inflammatory or oxidative stress conditions (Esterbauer et al., 1991) and the specific GST inhibitor ethacrynic acid (Fig. 1B). Jurkat cells, in which the expression of GSTP1-1 has been characterized previously (Sánchez-Gómez et al., 2007), were treated with the various agents for 16 h. To avoid disulfide exchange and protein aggregation during sample processing, lysates were obtained in the presence of 50 mM iodoacetamide according to the method of Cumming et al. (2004). All agents tested induced GSTP1-1 oligomerization, although with different patterns. H_2O_2 treatment gave rise to the appearance of an oligomer of approximately 75 kDa when explored under nonreducing conditions. Other agents, including diamide, HNE, and ethacrynic acid induced GSTP1-1 oligomers, with the major species migrating at approximately 50 kDa. In samples from ethacrynic acid and HNE-treated cells, levels of both GSTP1-1 and actin detected under nonreducing conditions were clearly lower, resulting from protein aggregation leading to the formation of large species that did not enter the gel (data not shown). In some exposures, the appearance of a GSTP1-1 band migrating faster than the monomer (at approximately 20 kDa), consistent with a GSTP1-1 monomer bearing an intramolecular disulfide bond, was also noted,

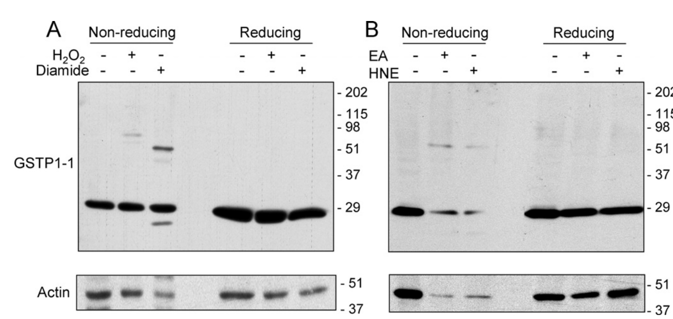

Fig. 1. GSTP1-1 oligomerization induced by oxidative stress conditions and GST inhibitors. A, Jurkat cells were treated with 8 mM H_2O_2 or 1 mM diamide for 10 min, after which the medium was replaced by fresh medium and the incubation was continued for 16 h. B, treatment with 50 μM ethacrynic acid (EA) or 50 μM HNE was carried out for 16 h. Cells were lysed in the presence of iodoacetamide, as detailed under *Materials and Methods*. Duplicate aliquots containing 15 μg of protein were subjected in parallel to electrophoresis under nonreducing or reducing conditions, as indicated, and levels of GSTP1-1 and actin were assessed by Western blot. Blots shown are representative from at least three experiments with similar results.

although its proportion showed interexperimental variability. Remarkably, in all cases, both GSTP1-1 oligomerization and the formation of oxidized monomeric species were fully reversible under reducing conditions.

We have reported previously that 15d-PGJ$_2$ can inhibit GST activity in vitro and in cells and that this inhibition is accompanied by various chemical modifications of GST, including the direct binding of 15d-PGJ$_2$ and oxidative modifications of cysteine residues (Sánchez-Gómez et al., 2007). Here, we have observed that treatment of Jurkat cells with 15d-PGJ$_2$ at 5 μM for 16 h led to the formation of several GSTP1-1-immunoreactive species that could be detected in nonreducing SDS-PAGE gels, including a band migrating at ~20 kDa, several oligomeric species between 50 and 75 kDa, and a smear at higher molecular mass (Fig. 2). These species were resolved under reducing conditions into two main bands: the GSTP1-1 monomer, and a prominent 75-kDa GSTP1-1 oligomer. To confirm the irreversible nature of this oligomer, we performed nonreducing followed by reducing SDS-PAGE (redox two-dimensional electrophoresis), according to methods described previously (Cumming et al., 2004). In this procedure, monomers and irreversible oligomers migrate in the diagonal line of the reducing gel, whereas proteins associated by disulfide bonds dissociate into their components and migrate to the left of the diagonal. Proteins containing internal disulfide bonds, which display increased electrophoretic mobility under nonreducing conditions, migrate to the right of this line. As shown in Fig. 2B, the 15d-PGJ$_2$-elicited 75-kDa GSTP1-1 oligomer remains in the diagonal line of the gel showing that oligomerization is not reversible under these conditions. The 20-kDa band observed under nonreducing conditions runs to the right of the diagonal line in the two-dimensional gels, thus showing that it corresponds to a GSTP1-1 monomer with an intramolecular disulfide bond. Similarly to other reported effects (Fionda et al., 2007), 15d-PGJ$_2$-induced oligomerization also occurred in the presence of 10% fetal calf serum (Fig. 2C), indicating that micromolar concentrations of the prostanoid could be effective in a physiological environment.

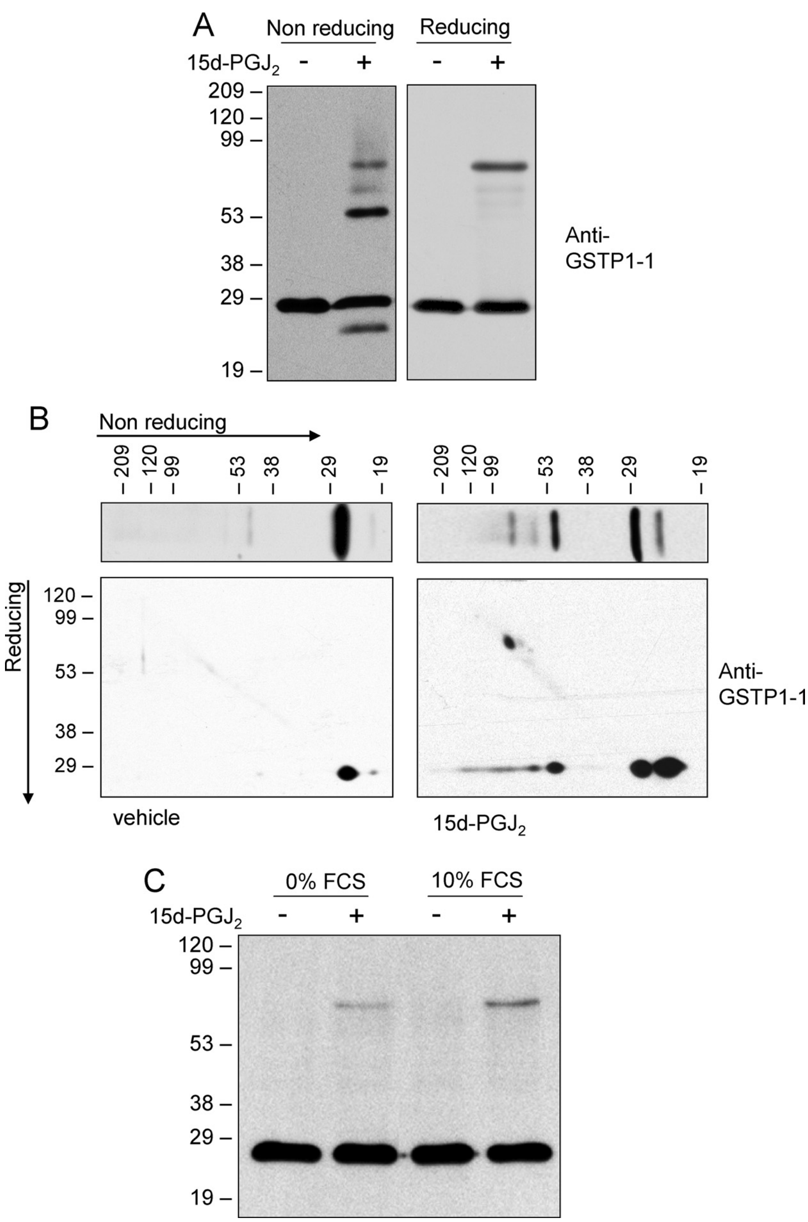

Fig. 2. 15d-PGJ$_2$ induces irreversible GSTP1-1 oligomerization in Jurkat cells. A, analysis of the redox status of GSTP1-1. Jurkat cells were treated in the presence of vehicle or 5 μM 15d-PGJ$_2$ for 16 h. Cell lysates were obtained in the presence of iodoacetamide and aliquots containing 15 μg of protein were analyzed by SDS-PAGE under nonreducing and reducing conditions. B, analysis of GSTP1-1 oligomerization by redox two-dimensional electrophoresis. Lysates from control or 15d-PGJ$_2$-treated Jurkat cells, containing 10 μg of protein, were analyzed under nonreducing conditions in the first dimension. Lanes from this gel were excised and subsequently equilibrated in reducing and alkylating solutions. For the second dimension, the lanes were placed over the second gel, as indicated, and electrophoresis was performed under reducing conditions. GSTP1-1 was detected by Western blot. C, Jurkat cells were treated with 15d-PGJ$_2$ in the absence or presence of 10% fetal calf serum, as indicated and the oligomeric state of GSTP1-1 was assessed as in A. Results shown are representative of at least three experiments with similar results.

15d-PGJ_2-elicited GSTP1-1 oligomerization occurred in other cell types (Fig. 3). In RMCs and A549 lung cancer cells, the characteristic 75-kDa oligomer was observed after treatment with 10 μM 15d-PGJ_2. MCF-7 breast cancer cells do not express GSTP1-1. Therefore, to explore GSTP1-1 oligomer formation in these cells, we transfected them with a GSTP1-1 expression plasmid. Treatment of MCF-7 with 10 μM 15d-PGJ_2 for 16 h did not induce GSTP1-1 oligomerization, and a short incubation with high concentration of 15d-PGJ_2 was necessary to elicit the appearance of the oligomer.

Role of GSTP1-1 Cysteine Residues in Oligomerization Induced by Oxidative Stress and GSTP1-1 Inhibitors. The results shown above suggest that oligomeric GSTP1-1 species induced by various treatments including H_2O_2, diamide, HNE, or ethacrynic acid were due to the formation of disulfide bonds. Therefore, we assessed the nature of the cysteine residues involved in the formation of oligomers elicited by these agents through transient transfection of AU5-tagged GSTP1-1 wild-type and cysteine to serine mutant constructs and detection of the oligomers formed by Western blot with anti-AU5 antibody (Fig. 4, A and B). The pattern of H_2O_2-induced oligomers changed remarkably by mutation of GSTP1-1 cysteine residues. In the case of C47S mutant, the formation of the 75-kDa oligomer was clearly increased (Fig. 4A). In contrast, the C101S mutant was largely resistant to H_2O_2-induced oligomerization. In the case of diamide-induced oligomers, mutation of either Cys47 or Cys101 reduced, although it did not abolish oligomerization. All oligomeric species formed by treatment with these agents were completely reversible under reducing conditions. Treatment with ethacrynic acid elicited the appearance of multiple GSTP1-1-containing high molecular mass species that could be detected under nonreducing conditions (Fig. 4B). The most prominent of these species appeared as a doublet at approximately 50 kDa. Mutation of Cys47 led to the disappearance of the lower component of this doublet and the increase in the intensity of a 75-kDa signal. On the other hand, mutation of Cys101 led to the disappearance of the upper component of the doublet. Thus, Cys101 is not essential for ethacrynic acid-induced GST oligomerization, but it may be important for the conformation of the oligomer formed. Ethacrynic acid-induced oligomerization of GSTP1-1 constructs was also fully reversible under reducing conditions, indicating that the oligomeric species are linked by disulfide bonds.

Cys101 of GSTP1-1 Is Critical for 15d-PGJ_2-Induced Oligomerization. Because we have shown the irreversible nature of 15d-PGJ_2-induced GSTP1-1 oligomerization, we studied the potential involvement of cysteine residues only under reducing conditions. As shown in Fig. 5A, mutation of Cys47 increased the proportion of oligomer formed upon treatment of Jurkat cells with 15d-PGJ_2 with respect to the

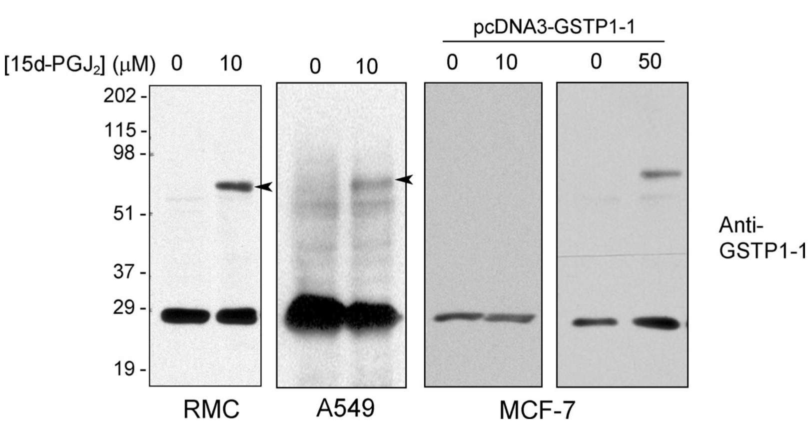

Fig. 3. Cell-type dependence of GSTP1-1 oligomerization. RMC or A549 lung cancer cells were treated with 10 μM 15d-PGJ_2 for 16 h, and GSTP1-1 oligomers (arrowheads) were detected as above. MCF-7 cells were transfected with pcDNA3-GSTP1-1 and, after 24 h, the cells were treated with 10 μM 15d-PGJ_2 for 16 h or with 50 μM 15d-PGJ_2 for 2 h, as indicated. After treatment, cells were lysed and processed in the presence iodoacetamide. Blots shown are representative of three experiments with similar results.

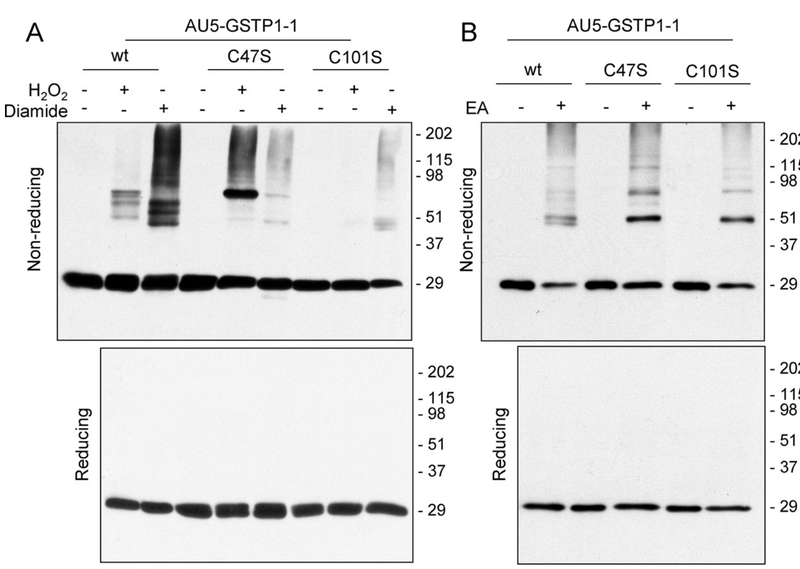

Fig. 4. Involvement of cysteine residues in GSTP1-1 oligomerization induced by oxidative stress and GSTP1-1 inhibitors. Jurkat cells transfected with the indicated plasmids coding for the wild-type or cysteine to serine mutants of GSTP1-1 with an N-terminal AU5 tag were treated with H_2O_2 or diamide (A), or ethacrynic acid (EA) (B), as in Fig. 1. Cell lysates were obtained in the presence of iodoacetamide and analyzed by SDS-PAGE under nonreducing or reducing conditions, as indicated. Oligomerization of the transfected GSTP1-1 wild type (wt) and mutants was monitored by Western blot with anti-AU5 antibody.

wild-type construct. In contrast, mutation of Cys101 completely abolished oligomerization. Mutation of Cys14 or CysC169 did not block GSTP1-1 oligomerization, although it tended to reduce the proportion of oligomer formed. Thus, according to these results, Cys101 is the only residue essential for 15d-PGJ_2-induced GSTP1-1 cross-linking. We showed previously that a biotinylated 15d-PGJ_2 analog (15d-PGJ_2-B) directly bound to endogenous GSTP1-1 in cells (Sánchez-Gómez et al., 2007). To assess the site of modification of GSTP1-1 in cells, we transfected Jurkat cells with AU5-tagged GSTP1-1 constructs. Immunoprecipitation with anti-AU5 antibody of lysates from cells incubated with 15d-PGJ_2-B clearly showed the incorporation of biotin into the immunoprecipitated protein (Fig. 5B). Remarkably, a C47S mutant showed increased incorporation of 15d-PGJ_2-B, whereas modification was totally abolished in the C101S mutant. Thus, Cys101 is also essential for covalent binding of 15d-PGJ_2 to GSTP1-1. It is noteworthy that 15d-PGJ_2-B also elicited GSTP1-1 oligomerization in cells (Fig. 5C). Moreover, pull-down of lysates from 15d-PGJ_2-B-treated cells on Neutravidin beads resulted in retention of both monomeric and oligomeric forms of GSTP1-1, indicating that both species contain bound biotinylated PG (Fig. 5D). No GSTP1-1 was retained on avidin when lysates from cells treated with non-biotinylated 15d-PGJ_2 were used (data not shown).

The Dienone Structure of cyPG Is Required for GSTP1-1 Irreversible Oligomerization. As we have shown above, 15d-PGJ_2-induced GSTP1-1 oligomers are stable under strong reducing conditions (i.e., 700 mM β-mercaptoethanol or 100 mM DTT). This indicates that oligomerization is unlikely to be due exclusively to disulfide bond formation. 15d-PGJ_2 is a cyPG with dienone structure; that is, it possesses two electrophilic carbons that are able to react simultaneously with two thiol groups (Atsmon et al., 1990; Pérez-Sala et al., 2003). To explore the potential role of this mechanism in GSTP1-1 oligomerization we used a battery of prostanoids with single enone or dienone structure (Fig. 6A). The cyPG Δ^{12}-PGJ_2, which possesses a dienone structure, effectively induced the formation of a 75-kDa GSTP1-1 oligomer, which was stable under reducing conditions and accounted for 50% of the immunodetected GSTP1-1 protein after a 16-h treatment. In contrast, PGA_1, a cyPG with single enone structure and 9,10-dihydro-PGJ_2, an analog of 15d-PGJ_2, which lacks the endocyclic double bond but possesses one electrophilic carbon, did not induce the formation of oligomeric species (Fig. 6B). Δ^{12}-PGJ_2-induced GSTP1-1 oligomers were analyzed by redox two-dimensional electrophoresis (Fig. 6C). This analysis confirmed the stability of the 75-kDa oligomer formed, whereas other oligomeric species with lower electrophoretic mobility observed under non-reducing conditions were clearly not stable, and thus, their formation probably involved disulfide bonding. Likewise, the intramolecular disulfide bond of the fast migrating GST monomer was also reduced under these conditions.

GSTP1-1 Oligomerization Is Associated with Activation of the JNK Cascade. GSTP1-1 oligomerization under oxidative stress conditions or in the presence of GSTP1-1 inhibitors has been reported to lead to the activation of the JNK cascade as a result of the loss of its inhibitory interaction with various stress kinases, such as Traf-2 or JNK itself (Adler et al., 1999; Wu et al., 2006). To explore whether 15d-PGJ_2-induced GSTP1-1 oligomerization was associated with JNK activation in Jurkat cells, we studied the temporal course of both phenomena (Fig. 7A). The formation of the 75-kDa oligomer could be detected as early as 15 min after 15d-PGJ_2 addition. It is noteworthy that the GSTP1-1 oligomer clearly accumulated over time in cells. JNK activation followed a parallel temporal course resulting in a sustained activation throughout the duration of the treatment with 15d-PGJ_2. Phosphorylation of c-Jun, as detected with an anti-P-c-Jun antibody, was slightly delayed, weak levels of P-c-Jun being detected after 1-h treatment (data not shown) and a clear increase after 2 h (Fig. 7B). It is noteworthy that although serum deprivation induced c-Jun levels, c-Jun

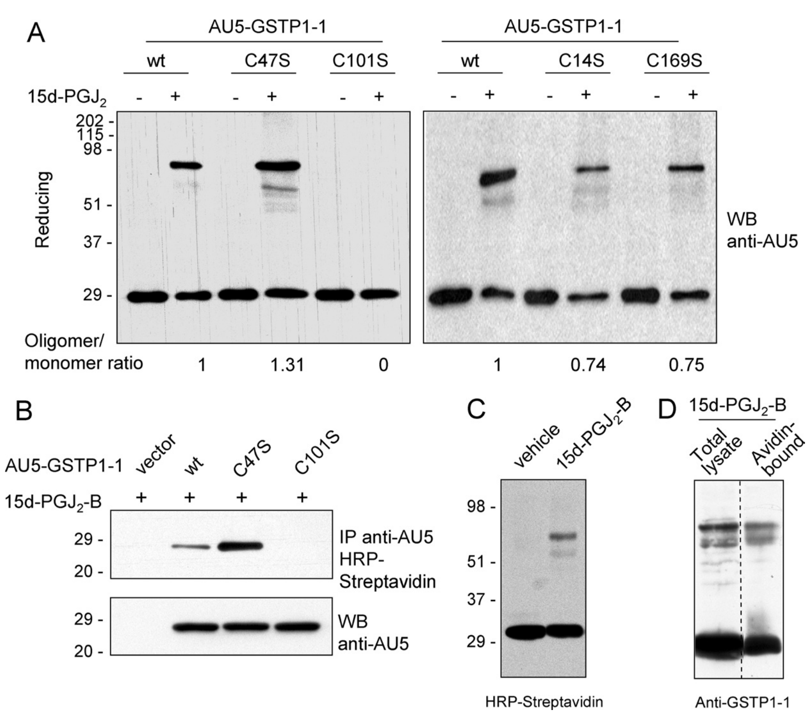

Fig. 5. Role of Cys101 in 15d-PGJ_2-induced GSTP1-1 oligomerization and modification. A, Jurkat cells transfected with plasmids coding for AU5-GSTP1-1 wild-type or cysteine to serine mutants were treated with 15d-PGJ_2 for 16 h, and total cell lysates were obtained in the presence of iodoacetamide. Detection of monomeric and oligomeric AU5-GSTP1-1 species was achieved by Western blot with anti-AU5 antibody. The ratio between levels of oligomeric and monomeric species for every construct is given below the corresponding lanes in arbitrary units referred to the values obtained for wild-type constructs. Results are average values of at least three determinations. B, transfected cells were treated with 15d-PGJ_2-B for 2 h, lysed, and subjected to immunoprecipitation with anti-AU5 antibodies. The incorporation of 15d-PGJ_2-B was revealed by the biotin signal associated with the immunoprecipitated protein as detected with HRP-streptavidin and ECL. Levels of AU5-GSTP1-1 wild-type (wt) and mutants were assessed by Western blot with anti-AU5 antibody. C, Jurkat cells were incubated with 15d-PGJ_2-B for 16 h and oligomerization was detected by Western blot. D, cell lysates from 15d-PGJ_2-B-treated cells were subjected to pull-down on neutravidin-agarose beads and the presence of GST in the avidin-bound fraction was assessed by Western blot. Dotted line shows where lanes from the same gel have been cropped. Blots shown are representative from three assays with similar results.

phosphorylation was detected only in cells treated with 15d-PGJ_2. Sustained activation of JNK is known to lead to apoptosis. Consistent with this, 15d-PGJ_2 treatment selectively induced a reduction in the proportion of viable cells (Fig. 7C, quadrant a) and an increase in the proportion of early apoptotic cells, characterized by positive annexin V in the absence of propidium iodide staining (Fig. 7C, quadrant b). Other cell populations, including late apoptotic (annexin V and propidium iodide positive, quadrant c) and necrotic cells (annexin V-negative and propidium iodide positive, quadrant d) did not change significantly after 15d-PGJ_2 treatment. In addition, treatment with PGA_1 did not increase the proportion of apoptotic cells significantly (data not shown). Thus, treatment of Jurkat cells with 15d-PGJ_2 leads to GSTP1-1 oligomerization, correlating with JNK activation and induction of apoptosis.

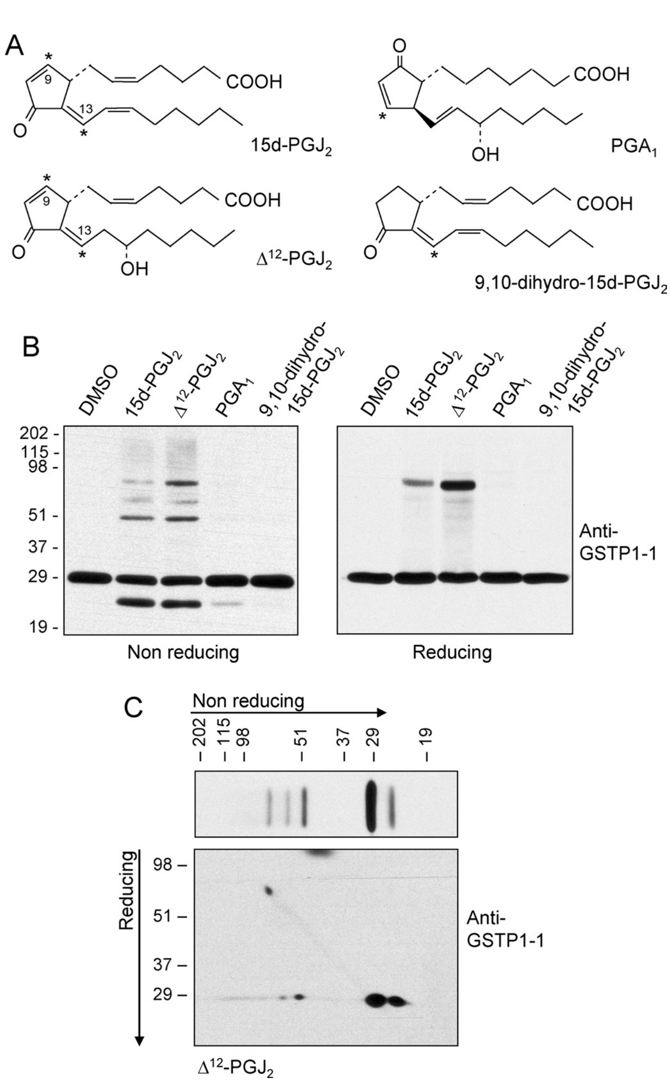

Fig. 6. Effect of various prostanoids on GSTP1-1 oligomerization. A, structure of the prostanoids used in these assays. Compounds on the left possess dienone structure, whereas those on the right are single enone compounds. B, Jurkat cells were treated with the indicated compounds at 5 μM for 16 h, after which cells were lysed in the presence of iodoacetamide and lysates were analyzed by SDS-PAGE under nonreducing and reducing conditions. GSTP1-1 monomeric and oligomeric forms were detected with an anti-GSTP1-1 antibody. C, lysates from Jurkat cells treated with Δ^{12}-PGJ_2 as above were analyzed by redox two-dimensional electrophoresis as in Fig. 2B and GSTP1-1 was detected by Western blot. Results are representative of at least three assays.

15d-PGJ_2 Induces GSTP1-1 Oligomerization In Vitro. The observation that the presence of two electrophilic carbons in cyPG is required to induce stable GST oligomerization in cells suggests that dienone cyPG may directly act as cross-linking agents by reacting with thiol groups from different GSTP1-1 monomers. To obtain insight into the structural aspects of 15d-PGJ_2-induced GSTP1-1 oligomerization, we explored this phenomenon in vitro. Treatment of recombinant GSTP1-1 with 15d-PGJ_2 elicited the appearance of a distinct 75-kDa oligomer of recombinant GSTP1-1 that was resistant under reducing conditions (Fig. 8A). A detectable amount of dimer (approximately 50 kDa) was present in both control and 15d-PGJ_2-treated GSTP1-1 under these conditions. The presence of 15d-PGJ_2 in the 75-kDa oligomer was demonstrated by the use of the biotinylated analog, which was detected by HRP-conjugated streptavidin and ECL (Fig, 8B, arrowheads). It is noteworthy that incubation of GSTP1-1 in the presence of an excess of GSSG or a nonmetabolizable analog of GSH such as *S*-(4-nitrobenzyl)-glutathione clearly reduced both the incorporation of 15d-PGJ_2-B into the GST monomer and the formation of oligomers. This indicates that GSH analogs reduce 15d-PGJ_2-GSTP1-1 interaction either by inducing conformational changes or by steric hindrance. Given the importance of Cys101 in GSTP1-1 oligomerization in cells, we explored the position of these residues in the GSTP1-1 molecule. In the structure of the GST dimer Cys101 residues from either monomer occupy a central position in the interior of a cleft formed at the subunit interface (Fig. 8C). The distance between the two residues is estimated as 6.25 Å, which is within the range of the distance that can be cross-linked by one prostaglandin molecule (Fig. 8D). Other dienone cyPGs, including 15d-PGJ_2-B and Δ^{12}-PGJ_2 also induced the formation of the 75-kDa GSTP1-1 oligomer. In the view of these data, we explored the ability of dibromobimane, a bifunctional agent with a cross-linking distance of approximately 6 Å, to elicit GSTP1-1 oligomerization. It is noteworthy that dibromobimane, widely used to perform cysteine mapping in proteins, was able to induce several oligomeric species of GTSP1-1 in vitro, including a clear oligomer at 75 kDa (Fig. 8E, arrowhead). In contrast, the single enone cyPG PGA_1 did not induce GSTP1-1 oligomerization in vitro, as evidenced by use of its biotinylated analog (PGA_1-B) compared with 15d-PGJ_2-B (Fig. 8F).

Analysis of GSTP1-1 Oligomeric Species by Electron Microscopy. Because Δ^{12}-PGJ_2 seemed to be the most effective compound inducing GSTP1-1 oligomerization, we used Δ^{12}-PGJ_2-treated GSTP1-1 in subsequent experiments to characterize the general shape and dimensions of GSTP1-1 oligomeric forms. Samples of GSTP1-1 before and after treatment with Δ^{12}-PGJ_2 were applied to a carbon support film and observed in an electron microscope after negative staining to increase the contrast. Single molecules were easily spotted in the electron microscopy fields as white densities over a noisy background. Control GSTP1-1 molecules appeared at first sight as relatively homogeneous square-shaped molecules (Fig. 9A). On the other hand, the molecules detected after treatment with Δ^{12}-PGJ_2 were found to be highly heterogeneous in shape and size (data not shown). Within this heterogeneity detected after treatment, most images revealed significant larger dimensions than the same protein before the treatment (Fig. 9C). To improve the signal-to-noise ratio of this structural data, micrographs for each sample were taken and digitized, and the images corresponding to single molecules were extracted using image-processing techniques. Single-molecule images were aligned and classified using reference-free methods to generate a collection of 2D averages for GSTP1-1 before (Fig. 9B) and after treatment (Fig. 9D). Averaging increased the signal-to-noise ratio, thus permitting a better interpretation of the structural details revealed by our data (Fig. 8E). The dimensions of the averages of GSTP1-1 were compatible with 2D projections of a dimeric GSTP1-1 as revealed by the comparison between the electron microscopy images, whose dimensions are measured based on the magnification of the microscope, and the published atomic structures (Fig. 9E, left). On the other hand, most averages after treatment corresponded to molecules with one dimension in the range of dimeric GSTP1-1 and the other dimension closer to double that length. This later result could be compatible with a tetramer molecule interacting in such a way that only one of the dimensions of the new oligomeric form doubles in the complex.

Discussion

GSTP1-1 plays a key role in the control of cell redox homeostasis both in physiological and in pathophysiological conditions, such as cancer and degenerative processes. This has spurred the study of this protein as a drug target, and several compounds targeting GSTP1-1 functions are being developed as potential anticancer agents (Tew, 2007). We have reported that the cyPG 15d-PGJ_2 potently inhibited GSTP1-1 activity in vitro and in Jurkat cells (Sánchez-Gómez et al., 2007). Here, we show that this compound selectively binds to Cys101 of human GSTP1-1 in cells and induces the formation of oligomeric species, which are stable under strong reducing and denaturing conditions, thus providing the first example of irreversible oligomerization of GSTP1-1 in vitro and in cells. In contrast, other agents, including ethacrynic acid, HNE, and diamide, were found to induce reversible GSTP1-1 oligomerization, which could involve other cysteines of the protein. Therefore, the interaction of 15d-PGJ_2, and other dienone cyPG, with this protein displays unique features, and given its stability, it may represent an advantage for the inhibition of GSTP1-1 functions in tumor cells.

Besides its catalytic role in the detoxification of electrophilic agents and in the modulation of protein glutathionylation, GSTP1-1 prosurvival functions depend on its oligomeric state. GSTP1-1 has been typically described to form homodimers in cells, but it can also exist in complex with other proteins involved in redox regulation (Manevich et al., 2004) or cell signaling (Adler et al., 1999; Wu et al., 2006), thus playing a role in the modulation of stress-induced signaling cascades. Homo-oligomerization of GSTP1-1 under stress conditions leads to its dissociation from stress kinases such as JNK or Traf-2, thus releasing them from an inhibitory interaction and favoring the induction of apoptosis (Turella et al., 2005; Wu et al., 2006). Consistent with these observations, we found that 15d-PGJ_2 induced apoptosis in Jurkat cells in correlation with GSTP1-1 oligomerization and sustained activation of the JNK cascade. This implies that

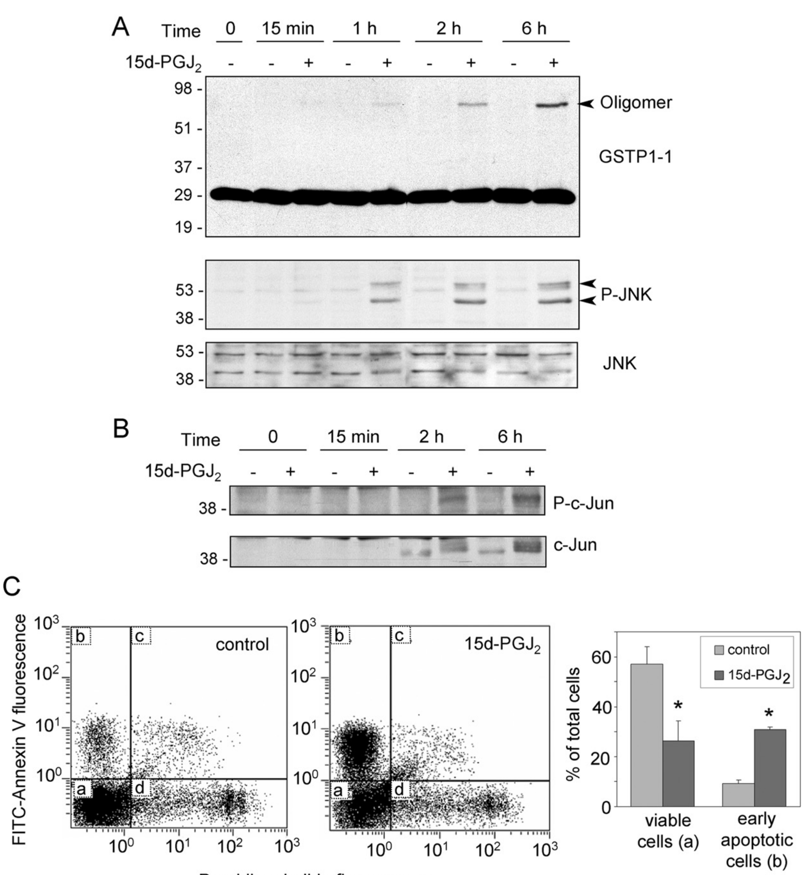

Fig. 7. Effect of 15d-PGJ_2 on the temporal course of GSTP1-1 oligomerization and JNK activation. A, Jurkat cells were treated with 5 μM 15d-PGJ_2 for the indicated times. Cell lysates obtained in the presence of iodoacetamide were analyzed by SDS-PAGE under reducing conditions. The levels of GSTP1-1 oligomer and of P-JNK were assessed by Western blot with the indicated antibodies. Total JNK is shown as control. B, Jurkat cells were treated with 15d-PGJ_2 as above and levels of P-c-Jun and total c-Jun were detected by Western blot. C, Jurkat cells were treated with 5 μM 15d-PGJ_2 for 6 h, after which cells were collected and analyzed by flow cytometry as described under *Materials and Methods*. The percentage of cells in every quadrant was calculated: a, viable cells; b, early apoptotic cells; c, late apoptotic cells; and d, necrotic cells. Graph shows the average values ± S.E.M. of the percentage of viable cells and early apoptotic cells from control and 15d-PGJ_2-treated-cells from four independent experiments. *, $p < 0.05$ by Student's t test.

15d-PGJ$_2$ may block survival circuits elicited by GSTP1-1, which are dependent on either catalytic or noncatalytic mechanisms. In this context, failure of As_2O_3 to induce apoptosis in promyelocytic leukemia cells has been found to correlate with lack of GSTP1-1 oligomerization in the resistant clones (Bernardini et al., 2006), possibly due to the antioxidant defenses of these cells. It is remarkable that we noted that MCF-7 breast cancer cells were less susceptible to dienone cyPG-induced GSTP1-1 oligomerization and cell toxicity than Jurkat cells. A number of factors could contribute to this lack of effect. MCF-7 expressed high levels of other GST isoforms, such as GST-ζ. Moreover, 15d-PGJ$_2$ has been reported to induce various cytoprotective effects in this cell type, including the expression of heme oxygenase-1 and of multidrug resistance protein 1 (Song et al., 2009). In preliminary assays, we have observed that MCF-7 cells are also more resistant than Jurkat cells to the effects of diamide, which supports the hypothesis that they possess more efficient mechanisms for cell defense against oxidants (B. Díez-Dacal, unpublished results). In light of these observations, it would be interesting to explore whether monitoring GSTP1-1 oligomerization could provide an indication of drug resistance. In this line, our work outlines the conditions for the direct visualization of GSTP1-1 oligomeric species by electron microscopy, a technique that could prove valuable for obtaining structural information with limited amounts of protein.

One striking feature of the interaction of 15d-PGJ$_2$ with GSTP1-1 is the nature of the cysteine residue involved. Human GSTP1-1 possesses four cysteine residues, of which, Cys47, which displays a low pK_a value, is the most reactive (Jenkinson et al., 2009). In fact, Cys47 has been shown to be modified by a wide variety of compounds of diverse chemical nature. Cys47 has been long known to be the site of modification of GSTP1-1 by the classic inhibitor ethacrynic acid. Other α,β-unsaturated carbonyl aldehydes and ketones including acrolein, HNE, and curcumin, as well as the skin sensitizer p-phenylenediamine, also react with Cys47 (van Iersel et al., 1997; Jenkinson et al., 2009). Remarkably, our results show that mutation of Cys47 in GSTP1-1 favors modification of GST by 15d-PGJ$_2$-B and 15d-PGJ$_2$-induced GSTP1-1 oligomerization. This suggests that Cys47 may be involved in interactions that shield the modification of Cys101. In fact, the formation of an intramolecular disulfide bond involving Cys47 and Cys101 under conditions of oxidative stress has been proposed (Ricci et al., 1991). In this

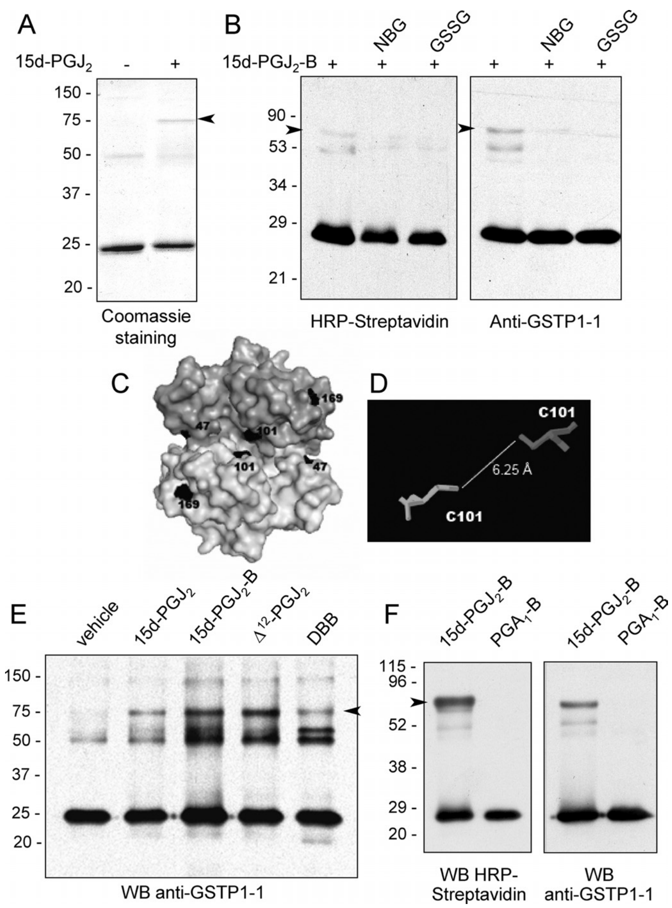

Fig. 8. Oligomerization of GSTP1-1 in vitro. A, recombinant human GSTP1-1 was incubated for 4 h at room temperature in the absence or presence of 10 μM 15d-PGJ$_2$ under the conditions detailed in the experimental section. B, GSTP1-1 was incubated with vehicle or 15d-PGJ$_2$-B in the presence of 2 mM GSSG or S-(4-nitrobenzyl)glutathione (NBG) for 1 h at room temperature. Incubation mixtures were analyzed by SDS and Western blot with anti-GSTP1-1 antibody or detection of biotin incorporated into GSTP1-1 by incubation with HRP-streptavidin and ECL. C, molecular surface of the human GSTP-1-1 (Protein Data Bank code22GS) prepared using the PyMol program. D, distances among Cys101 residues in the GSTP1-1 dimer were calculated using Swiss PDBViewer. E, GSTP1-1 was incubated as in A in the presence of the indicated compounds and analyzed by Western blot. F, GSTP1-1 was incubated for 1 h in the presence of 15d-PGJ$_2$-B or PGA$_1$-B, and incubation mixtures were analyzed by Western blot with HRP-Streptavidin or anti-GSTP1-1 antibody, as indicated. Results are representative of four assays with similar results.

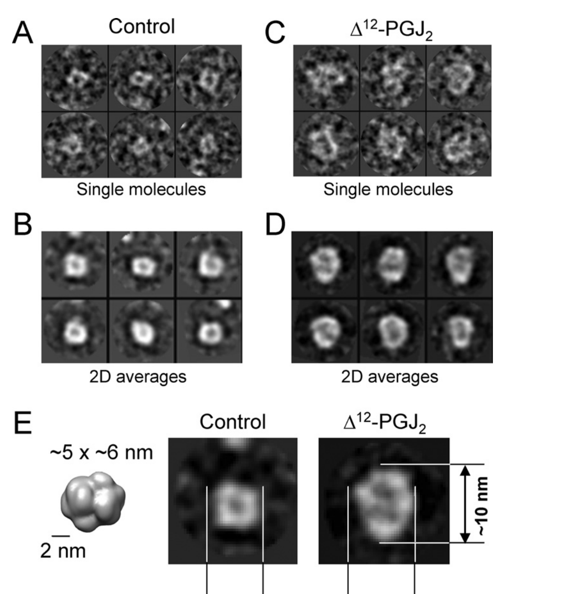

Fig. 9. Electron microscopy of control and Δ^{12}-PGJ$_2$-treated GSTP1-1. A, selected raw electron microscopy images of GSTP1-1 (control). The "square-shaped" molecule at the center of each image corresponds to a 2D-projection image of the molecule. B, selected reference-free 2D averages obtained after image processing of the whole data set of control GSTP1-1 molecules. C, some selected raw images of the GSTP1-1 preparation after treatment with Δ^{12}-PGJ$_2$. D, selected reference-free averages of the GSTP1-1 preparation after treatment. E, dimensions of the averages obtained after image processing of the electron microscopy images, based on the magnification of the microscope. Left, a view of the atomic structure of GSTP1-1 (Protein Data Bank ID 18GS) after low-pass filtering to a resolution similar to that of the electron microscopy images. Scale bar, 2 nm based on the atomic structure.

setting, the absence of Cys47, as in the C47S mutant, could favor the availability of Cys101 for modification by 15d-PGJ_2. Cys101 seems to be less reactive, and selective modification of this residue has not been reported. Our results show that Cys101 is essential for 15d-PGJ_2 interaction and is also important for H_2O_2-induced oligomerization. This may have important implications, because a wide number of studies on the involvement of GSTP1-1 in cancer susceptibility or chemoresistance have been performed in murine models. Because murine GSTP1-1 lacks Cys101, some of the results available may need to be reconsidered. Our observations also highlight the importance of using human(ized) models when studying GSTP1-1-mediated cancer chemoresistance.

An interesting question is the structural basis for the stability of the GSTP1-1 oligomer. Formation of the stable GSTP1-1 oligomer required the dienone structure of cyPG. Because dienone cyPG may react simultaneously with two nucleophilic residues (Pérez-Sala et al., 2003), this suggests that oligomer formation may involve cross-linking of GSTP1-1 through residues present in different monomers. Cys101 of the two monomers constituting the functional GSTP1-1 dimer are within the distance that can be cross-linked by dienone cyPG (~6 Å). Indeed, the compound dibromobimane, which may cross-link cysteine residues within 3 to 6 Å distance (Sinz and Wang, 2001), forms an oligomer similar to that observed after treatment with dienone cyPG. However, all the available data, the apparent molecular mass of the complex (75 kDa), the size as estimated from gel filtration analysis (70–80 kDa; F. J. Sánchez-Gómez, unpublished data) and the size estimation from the electron microscopy images, are compatible with the formation of a trimer or a compact tetramer. Formation of a covalently bound trimer or tetramer would require the involvement of another residue in addition to Cys101. Our results indicate that although Cys101 is essential, Cys14 and/or Cys169 may contribute to oligomer formation in the wild-type protein. This does not exclude the possibility that other nucleophilic residue(s) may be involved in dienone cyPG-induced cross-linking. In this context, the covalent modification of a histidine residue in human serum albumin by Δ^{12}-PGJ_2 has been reported recently (Yamaguchi et al., 2010), providing additional cross-linking possibilities that remain unexplored to date. Alternatively, it could be hypothesized that the oligomer observed may arise from a highly stable interaction between two GSTP1-1 dimers that could be elicited by the conformational change induced by cross-linking of Cys101. In addition, because cyPG induce oxidative stress in various cell types, including Jurkat cells (Nencioni et al., 2003), the possibility also exists that, in cells, other products derived from lipid peroxidation participate in GSTP1-1 cross-linking. These possibilities would be the subject of future studies.

Treatment with cyPG may elicit important antitumoral effects through various mechanisms (Ciucci et al., 2006). Moreover, endogenous generation of cyPG has been invoked as a mechanism of drug action. In particular, treatment of leukemia cells with bezafribrate and medroxyprogesterone acetate has been reported to increase synthesis and reduce metabolism of PGD_2, leading to increased levels of 15d-PGJ_2, recapitulating the antiproliferative actions of exogenously added 15d-PGJ_2 (Khanim et al., 2009). Bifunctional derivatives of GST inhibitors have been developed to selectively target different GST isoforms as a mechanism for antitumoral action (Maeda et al., 2006). Because Cys101 is not present in most GST isoforms, future work will aim at establishing whether 15d-PGJ_2-induced oligomerization displays isoform selectivity. In summary, the results presented in our study unveil unique features of the interaction of cyPG with the important cellular defense protein GSTP1-1. These observations may open new avenues for the design of therapeutic strategies aimed at overcoming cancer chemoresistance.

Acknowledgments

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