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Monitoring Cr Intermediates and Reactive Oxygen Species with Fluorescent Probes during Chromate Reduction

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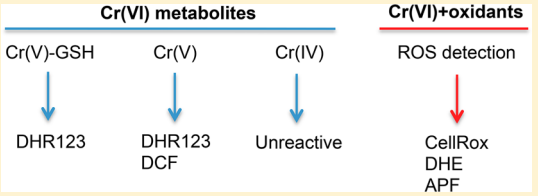
ABSTRACT: Cr(VI) genotoxicity is caused by products of its reductive metabolism inside the cells. Reactive oxygen species (ROS) and Cr(V,IV) intermediates are potential sources of oxidative damage by Cr(VI). Here, we investigated seven fluorescent probes for the detection of ROS and non-ROS oxidants in Cr(VI) reactions with its main reducers. We found that Cr(V)-skipping metabolism of Cr(VI) by ascorbate *in vitro* gave no responses with all tested dyes, indicating nonreactivity of Cr(IV) and absence of ROS. Cr(VI) reduction with glutathione (GSH) or Cys strongly enhanced the fluorescence of dichlorofluorescein (DCF) and dihydrorhodamine 123 (DHR123) but produced minimal fluorescence with dihydroethidium and no increases with aminophenylfluorescein and CellRox Green, Orange, and Red. Several tests showed that Cr(VI)-thiol reactions lacked ROS and that Cr(V) caused oxidation of DCF and DHR123. DCF reacted only with free Cr(V), whereas DHR123 detected both the free Cr(V) and Cr(V)-GSH complex. We estimated that Cr(VI)-GSH reactions generated approximately 75% Cr(V)-GSH and 25% free Cr(V), whereas Cys reactions appeared to produce only free Cr(V). DHR123 measurements in H460 cells showed that reduction of Cr(VI) was complete within 20 min postexposure, but it lasted at least 1 h without GSH. Cells with restored ascorbate levels exhibited no DCF or DHR123 oxidation by Cr(VI). Overall, our results demonstrated that Cr(VI) metabolism with its biological reducers lacked ROS and that DHR123 and DCF responses were indicators of total and free Cr(V), respectively. CellRox dyes, dihydroethidium and aminophenylfluorescein, are insensitive to Cr(V,IV) and can be used for monitoring ROS during coexposure to Cr(VI) and oxidants.



INTRODUCTION

Cr(VI) is a human respiratory carcinogen with documented inhalation exposures in numerous occupational groups.[1,2] Its presence at large toxic waste sites and in many drinking water supplies has also raised concerns about potential adverse health effects of environmental Cr(VI).[3−5] Chromate, the main form of Cr(VI) in physiological solutions, readily enters cells where it is reduced via direct (nonenzymatic) reactions with ascorbate (Asc) and small cellular thiols glutathione (GSH) and cysteine. The final product of chromate reduction is redox-inert Cr(III) that displays stable binding with proteins and other macromolecules, leading to its long-term intracellular retention. The reduction process also generates Cr(V) and Cr(IV) intermediates, a relative yield of which differs for Asc- and thiol-driven reactions. Reduction of Cr(VI) by Asc involves the initial transfer of two electrons, producing Cr(IV) and no Cr(V).[6,7] Only under the nonphysiological conditions of insufficient Asc for completion of Cr(VI) reduction, there was a detectable formation of Cr(V) resulting from secondary reactions of Cr(IV). GSH and Cys are one-electron reducers of Cr(VI), yielding Cr(V) as the first intermediate.[8−11] Asc is a dramatically faster reducer of Cr(VI) than thiols *in vitro*,[12,13] and it is responsible for the overwhelming majority of chromate metabolism in the lung, kidney, and liver.[14,15] In contrast to its low millimolar concentrations in cells *in vivo*,[16−18] Asc concentrations in cultured cells are in low micromolar range,[19−21] reflecting the absence of vitamin C in synthetic growth medium formulations and the addition of only 10−15% serum, which typically lost a majority of vitamin C during processing and storage. Restoration of physiological levels of Asc in cultured human cells has been found to alter DNA damage and cytotoxic responses induced by Cr(VI).[22−25]

The genotoxicity of Cr(VI) has been linked to the formation of Cr-DNA adducts[26,27] and DNA oxidation damage by reactive oxygen species (ROS) and Cr(V) intermediates.[28−30] ROS and Cr(V) are transient products, which makes them difficult to detect and estimate, particularly for environmentally relevant doses of Cr(VI). Widely employed tools for monitoring ROS formation in cultured cells are oxidant-sensitive fluorescent dyes. The use of specific probes can allow estimation of the general oxidative stress or the presence of specific ROS.[31,32] The two most popular dyes for the determination of the overall oxidative stress in cells are dihydrorhodamine 123 (DHR123) and dichlorofluorescein (DCF), which have also been used for the assessment of cellular ROS after Cr(VI) treatments. However, responses of these probes do not always reflect the presence of ROS in cells. For example, DCF fluorescence can be elevated by mitochondria-leaked cytochrome *C*.[32] The application of DCF and DHR123 for the detection of ROS produced by Cr(VI) is also potentially problematic in view of their susceptibility to oxidation by synthetic Cr(V) complexes.[33] It has been proposed that both Cr(V) and Cr(IV) can oxidize DCF and DHR123.[34] Thus, in light of the frequent use of both probes in Cr(VI) toxicology, it is important to identify a type of oxidant that reacts with DHR123 and DCF during

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Cr(VI) metabolism with biological reducers. Two related questions that also need to be addressed are (1) which Cr intermediates can cause oxidation of DHR123 and DCF and (2) what redox-sensitive probes are unreactive with Cr products and can be used for monitoring ROS in Cr(VI) reactions.

In this work, we examined the responses of seven redox-sensitive dyes during Cr(VI) metabolism. We found no significant ROS formation in Cr(VI) reactions with its main reducers and determined that Cr(V) species were responsible for the oxidation of DCF and DHR123. Dihydroethidium, aminophenylfluorescein, and three CellRox dyes were resistant to oxidation by Cr(V,IV), making them suitable for monitoring ROS in Cr(VI)/oxidant mixtures.

EXPERIMENTAL PROCEDURES

Materials. L-Ascorbic acid (99.9% pure), dehydro-L-(+)-ascorbic acid dimer (DHA), potassium chromate (K_2CrO_4, 99% pure), L-buthionine sulfoximine (BSO, ≥97% pure), L-glutathione (>98% pure), L-cysteine (>98% pure), nitric acid (>99.999% pure), 4-morpholinepropanesulfonic acid (MOPS), and salts were from Sigma-Aldrich (St. Louis, MO, USA). Chelex-100 and Bio-Gel P-30 columns were purchased from Bio-Rad (Hercules, CA). All dyes including dihydrorhodamine 123 (DHR123), 2′,7′-dichlorodihydrofluorescein diacetate (DCF-diacetate), dihydroethidium (DHE), aminophenyl-fluorescein (APF), CellRox Deep Red, CellRox Orange, CellRox Green, and 1,2-diamino-4,5-dimethoxybenzene dihydrochloride were from Molecular Probes (Eugene, OR, USA). Removal of adventitious metals was performed as described previously.[35]

Cell Culture. H460 human lung epithelial cells were propagated in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin. HF/SV human fibroblasts were grown in 90% (v/v) DMEM, 10% (v/v) fetal bovine serum, and antibiotics. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO_2. H460 cells were depleted of GSH by 24 h preincubation with 0.2 mM BSO.

Restoration of Asc Levels in Cells. H460 cells were incubated for 90 min with 1 mM DHA in Krebs-HEPES buffer [30 mM HEPES (pH 7.5), 130 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$] supplemented with 0.5 mM D-glucose. Stock solutions of 10 mM DHA were freshly prepared in the same buffer and kept on ice. Cellular Asc was determined by conjugation with 1,2-diamino-4,5-dimethoxybenzene dihydrochloride.[23] The volume of H460 cells was determined from forward scattering measurements by flow cytometry (FACSCalibur, BD Biosciences).

Cellular GSH and Cys. The amounts of cellular GSH and Cys were determined by HPLC as described earlier.[36] H460 cells were resuspended in cold 40 mM methanesulfonic acid and lysed by two cycles of freezing (−80 °C) and thawing (37 °C). After centrifugation at 12000*g* for 10 min at 4 °C, cell extracts were reacted with the thiol-specific dye monobromobimane. The fluorescent GSH and Cys monobromobimane conjugates were separated and quantified by HPLC.

Cr(VI) Reduction Measurements. A decrease in chromate absorbance at 372 nm was used to monitor the rates and the extent of Cr(VI) reduction. Equal volumes of prewarmed solutions of chromate and reducers were mixed in UV-transparent 96-well plates, and A_{372} readings were taken every 15 or 20 s. Plates were kept at 37 °C inside a microplate reader (the SpectraMax M5 microplate reader) for the duration of the reactions.

Cr(V)-GSH Preparation. $Na_4Cr(GSH)_4{\cdot}8H_2O$ was synthesized according to a published procedure.[37]

***In Vitro* Reactions with Redox-Sensitive Dyes.** Stock solutions of Asc, GSH Cys, and potassium chromate (or Cr(V)-GSH complex) were freshly made in deionized water and kept on ice. DCF was activated before usage by reacting 5 mM DCF-diacetate with 10 mM NaOH at room temperature for 30 min. Two master mixes were then made from these solutions. The first, made up in 100 mM MOPS (pH 7.0) and 200 mM NaCl, contained 2× the concentration of a reducer and 20 μM of a fluorescent dye. The second mix contained 2× the concentrations of chromate. The samples were prepared in a black-walled, clear-bottomed Costar 96-well plate. The reaction was initiated in the dark by mixing 100 μL of the reducer-dye mix with 100 μL of chromate in each well. Reactions with the Cr(V)-GSH complex contained 2 mM GSH. The plates were incubated at 37 °C in the dark inside the SpectraMax M5 plate reader. Excitation and emission wavelengths were as follows: 490/530 nm for DCF, 500/535 nm for DHR123, 395/580 nm for DHE, 490/515 nm for APF, 640/665 nm for CellRox Deep Red, 545/565 nm for CellRox Orange, and 485/530 nm for CellRox Green. Final fluorescence values were normalized for the amount of Cr(VI) reduction. Data in each Figure panel were obtained in parallel to avoid batch and autoxidative "aging" effects in stock solutions of dyes.

Fluorescence Measurements in Cells. H460 cells were seeded into black 96-well optical bottom cell culture plates (30000 cells/well) one day before treatments. Control, GSH-depleted and DHA-treated cells were preloaded with 10 μM DCF-diacetate or DHR123 in RPMI-1640 medium for 30 min. After a rinse with warm PBS, cells were incubated with the indicated concentrations of Cr(VI) for 1 h in serum-free RPMI-1640 medium. Cellular monolayers were rinsed once with warm PBS and then covered with a modified DPBS solution (Sigma-Aldrich, D4031) followed by measurements of fluorescence (DCF ex/em, 490/530 nm; DHR123 ex/em, 500/535 nm). Fluorescence was recorded using the SpectraMax M5 microplate reader.

Cellular Uptake of Cr(VI). Determination of total cellular Cr by graphite furnace atomic absorption spectroscopy (GF-AAS) was based on a previously described procedure.[36] H460 cells were seeded into 6-well plates at a density equivalent to seeding conditions for cellular fluorescence measurements. Cells were treated with Cr(VI) for 1 h on the following day. After removal of Cr-containing media, monolayers were rinsed twice with warm PBS followed by collection of cells by trypsinization in the presence of EDTA (Gibco 15400-054 Trypsin-EDTA solution). After two washes with cold PBS (5 min at 800*g* and 4 °C), cells were extracted with hot 5% (v/v) nitric acid followed by centrifugation at 10,000*g* for 10 min at 4 °C. Cr-containing supernatants were diluted to 2% (v/v) nitric acid prior to GF-AAS analyses (AAnalyst600 Atomic Absorption Spectrometer, Perkin-Elmer). Metal-extracted cellular pellets were washed twice with cold 5% (v/v) nitric acid (10,000*g* for 5 min at 4 °C) and then dissolved in 0.5 M NaOH at 37 °C for 30 min. The Cr amount of cellular samples was normalized per protein content.

Cytotoxicity. Cytotoxic effects of Cr(VI) treatments were assessed by the CellTiter-Glo luminescent assay (Promega, Madison, WI, USA). H460 cells were seeded into 96-well plates (30000 cells/well) and allowed to attach overnight prior to the addition of Cr(VI). Cytotoxicity was determined at 24 h after Cr removal.

Shuttle-Vector Mutagenesis. The formation of mutagenic DNA damage during the reduction of Cr(VI) with Cys was examined using the pSP189 vector containing *supF* as a target gene.[38] Reaction mixtures contained 25 mM MOPS (pH 7.0), 2 μg of pSP189 DNA, 2 mM Cys, and 0 or 100 μM chromate in a total volume of 50 μL. A parallel set of samples additionally included 2 mM EDTA. After 60 min of incubation at 37 °C, DNA was purified by passage through two Bio-Gel P-30 columns. The pSP189 plasmids were transfected into HF/SV cells, and their progeny were purified 48 h later using a plasmid isolation kit from Qiagen. Cell-recovered plasmids were electroporated into the *E. coli* MBL50, and the total number of transformants was scored on the agar plates containing 30 μg/mL ampicillin and 0.5 μg/mL chloramphenicol. The *supF* mutants were detected on plates containing 2 mg/mL L-arabinose and both antibiotics. Mutation frequency was calculated by dividing the number of colonies on ampicillin/arabinose-containing plates to the number of ampicillin-only resistant colonies.

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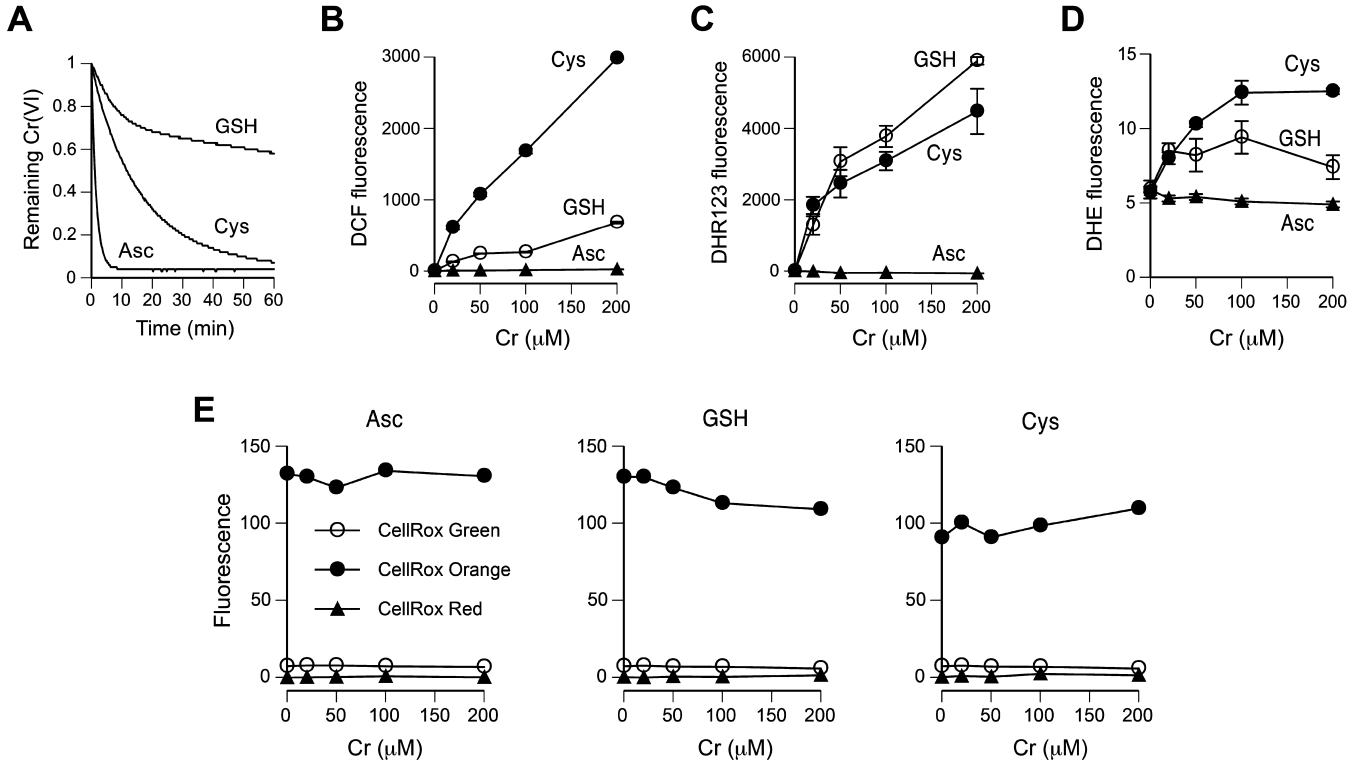

Figure 1. Oxidation of redox-sensitive dyes in reactions of Cr(VI) with its biological reducers. Fluorescence measurements were taken after 60 min incubations and adjusted for the extent of Cr(VI) reduction. (A) Kinetics of 100 μM Cr(VI) reduction at 37 °C in the presence of 2 mM Asc, Cys, or GSH. (B) DCF fluorescence and (C) DHR123 fluorescence in Cr(VI)-reducer reactions. Data are the means ± SD, n = 3. Where not visible, error bars were smaller than symbols. (D) DHE fluorescence in Cr(VI) reactions with 2 mM reducers. (E) Fluorescence of CellRox Green, CellRox Orange, and CellRox Red probes after incubation in Cr(VI)-2 mM reducer reactions for 60 min. Data are the means of triplicate measurements. Error bars are not shown for clarity.

RESULTS

Responses of General Redox-Sensitive Probes in Cr(VI) Reactions. In our initial studies, we examined the fluorescence of six oxidant-sensitive dyes during the reduction of Cr(VI) with its three main biological reducers (Asc, GSH, and Cys) under *in vitro* conditions with physiological ionic strength, temperature, and pH. Consistent with its principal role in Cr(VI) metabolism *in vivo*,[12,14,15] Asc exhibited a dramatically faster metabolism of Cr(VI) in comparison to that of GSH and Cys (Figure 1A). Reduction of Cr(VI) by 2 mM Asc was essentially complete after approximately 5 min, whereas even 60-min long incubations with the same concentrations of Cys or GSH contained unreduced Cr(VI) (7% for Cys and 58% for GSH). In subsequent experiments with redox probes, fluorescence values were normalized for the amount of Cr(VI) reduced in 60 min reactions. The use of prolonged incubations that are necessary for the completion of Cr(VI) reduction by 2 mM GSH were avoided due to the accumulation of autoxidation products. Cr(VI)-reducer reactions were first examined for the presence of oxidizing species using a general oxidant indicator DCF. We found major differences among Cr(VI) reduction reactions in their ability to generate oxidants that were detectable by DCF fluorescence (Figure 1B). Cys-mediated metabolism of Cr(VI) produced very high amounts of oxidants, whereas the yield of DCF-targeting reactants in GSH reactions was on average 4.5-times lower. Samples containing Asc showed background DCF fluorescence even at the highest Cr(VI) concentrations. Cr(VI) metabolism by Cys and GSH resulted in extensive oxidation of another general redox probe, DHR123 (Figure 1C). Asc-Cr(VI) reactions again tested negative for the presence of oxidants. Next, we studied responses of dihydroethidium (DHE), a dye that is commonly used for the detection of superoxide.[31] Cr(VI) reactions with Cys displayed only very modest increases in DHE fluorescence (maximally 2.5-fold over background), whereas GSH samples had even lower DHE responses (Figure 1D). No increase in DHE oxidation was detected in Asc reactions. The use of three other general redox indicators CellRox Green, CellRox Orange, and CellRox Red showed no significant changes in fluorescence above control values for any of the Cr(VI)-reducer reactions (Figure 1E).

Testing for ROS in Cr(VI) Reactions. Although both DCF and DHR123 are known as general oxidative stress probes in cells, their ROS responsiveness in buffer solutions lacking peroxidases is limited to hydroxyl radicals.[31,32,39,40] Ethanol is a potent scavenger of hydroxyl radicals, but its addition at 100-fold molar excess over DCF had no effect on fluorescence of this dye in Cr(VI)-Cys and Cr(VI)-GSH reactions (Figure 2A,B). We next employed a highly reactive species sensor aminophenylfluorescein (APF) to test the presence of OH radicals in Cr(VI) reactions. None of our three reduction systems showed increases in APF fluorescence (Figure 2C), which taken together with the absence of ethanol effects on DCF fluorescence argues against the formation of hydroxyl radicals during Cr(VI) reduction.

ROS cause oxidation of DNA bases leading to mutations during replication. Thus, testing for the formation of mutagenic DNA damage can help determine the presence of genetically important ROS. We investigated the production of mutagenic

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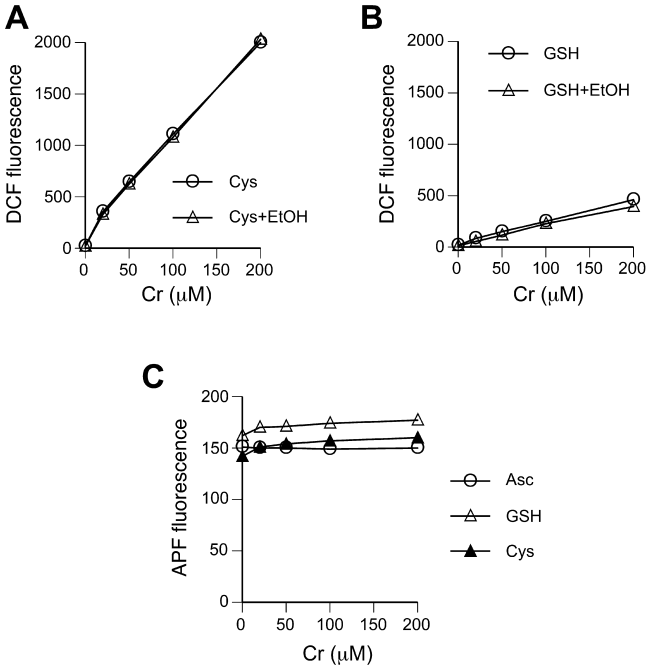

Figure 2. Testing for OH· radicals in Cr(VI)-reducer reactions. Samples were incubated for 60 min with 2 mM Cys or GSH. Fluorescence was normalized for the amount of reduced Cr(VI). (A) No effect of ethanol on DCF fluorescence in Cr(VI)-Cys and (B) Cr(VI)-GSH reactions (EtOH, 1 mM ethanol). Data are the means for three measurements. Error bars are not shown for clarity. (C) Fluorescence of the highly reactive species indicator APF. Shown are the means for three determinations.

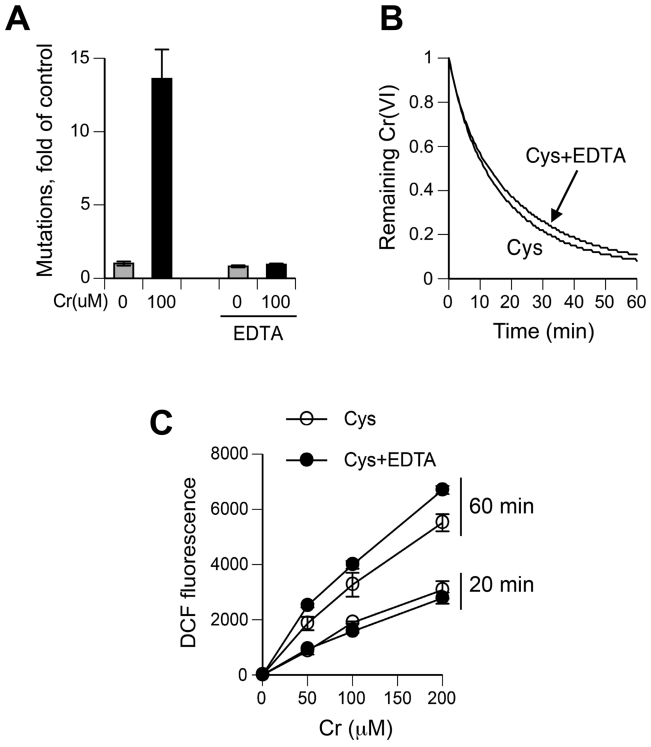

Figure 3. Cr(III) chelation by EDTA blocks the formation of mutagenic DNA damage by Cr(VI). (A) Mutagenic responses in pSP189 plasmids incubated in Cr(VI) reactions with 2 mM Cys in the absence or presence of 2 mM EDTA. Data are the means ± SD from two experiments. (B) Kinetics of Cr(VI) reduction by 2 mM Cys with and without 2 mM EDTA. (**C**) DCF fluorescence in Cr(VI)-Cys reactions lacking or containing 2 mM EDTA. Measurements were taken after 20 and 60 min incubations. Data are the means ± SD, n = 3. Where not visible, error bars were smaller than symbols.

lesions using the pSP189 shuttle-vector system. In this approach, the plasmid DNA was included in Cr(VI) reduction reactions, purified, and then transfected into human cells for replication and fixation of DNA damage as mutations. Replicated pSP189 molecules were isolated from cells and then electroporated into *E. coli* for scoring of mutations at the vector-encoded *supF* gene. In addition to a possible presence of ROS or other DNA-oxidizing species, reductive metabolism of Cr(VI) is known to produce mutagenic Cr-DNA adducts.[26,38] To reveal the presence of a potential adduct-independent mutagenic damage, we compared pSP189 mutagenesis in standard reactions and in samples containing EDTA, which chelates Cr(III) and completely abrogates the production of Cr-DNA adducts.[41] We found that reduction of 100 μM Cr(VI) with Cys resulted in robust mutagenic responses in pSP189 vectors, whereas no mutagenicity was observed when reactions contained Cr(III)-sequestering EDTA (Figure 3A). Control experiments showed that EDTA did not significantly change reduction kinetics and did not suppress the production of DCF-oxidizing species (Figure 3B,C). Overall, the non-responsiveness of four oxidant-sensitive probes (three CellRox dyes and APF), inability of ethanol to inhibit DCF oxidation, and the absence of Cr adduct-independent mutations all indicate that Cr(VI)-thiol reactions lacked a significant production of ROS. Thus, extensive fluorescence of DCF and DHR123 was caused by non-ROS oxidants.

Oxidation of DCF and DHR123 by Cr(V) in Asc Reactions. Cys and GSH-mediated reductions of Cr(VI) proceed through the initial one-electron transfer generating Cr(V) as the first intermediate.[8−11] In contrast, reduction of Cr(VI) by several-fold molar excess of Asc involves a two-electron transfer producing Cr(IV).[6,7,42] Thus, large increases in DCF and DHR123 fluorescence in 2 mM thiols-based reactions but no responses with 2 mM Asc (Figure 1A,B) suggest that Cr(V) was probably responsible for the oxidation of both probes. To test whether the formation of Cr(V) in Asc reactions would also cause DCF and/or DHR123 fluorescence, we examined reactions with low ratios of Asc to Cr(VI). ESR studies have found a significant formation of Cr(V) under conditions of 1:1 and 1:2 molar ratios of Asc to Cr(VI).[6] Limited amounts of Asc in these reactions permitted the production of Cr(V) via comproportionation and disproportionation of the initially formed Cr(IV). In agreement with the requirement of 1.5 mol of Asc for the reduction of 1 mol of Cr(VI), we found incomplete reduction of 0.1 and 0.2 mM Cr(VI) by 0.1 mM Asc (Figure 4A). The amount of reduced Cr(VI) was 70% for 0.1 mM Cr(VI) and 39% for 0.2 mM Cr(VI). Although 0.1 mM Asc was theoretically sufficient to complete the reduction of 0.05 mM Cr(VI), approximately 11% of Cr(VI) remained at the end of 60 min incubations due to a progressive depletion of reduced Asc over the course of the reduction and the resulting decrease in the reaction rates. Unlike reductions with >10 molar excess of Asc (2 mM samples) that again gave no changes in DCF and DHR123 fluorescence, reactions containing 0.1 mM Asc and 0.1 or 0.2 mM Cr(VI) produced oxidants reacting with both dyes (Figure 4B,C). The observed pattern of fluorescence responses under conditions of Asc insufficiency for the completion of Cr(VI) reduction parallels the appearance of Cr(V) in ESR studies.[6]

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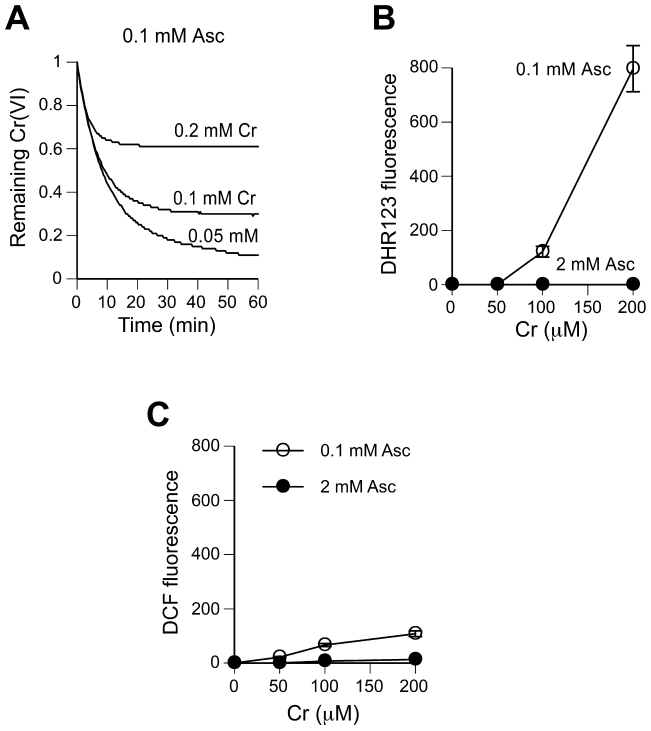

Figure 4. DCF and DHR123 fluorescence in Cr(VI) reactions with low Asc concentrations. Fluorescence was recorded after 60 min and adjusted for the amount of reduced Cr(VI). Data are the means ± SD, n = 3. (A) Kinetics of Cr(VI) reduction by 0.1 mM Asc. (B) DHR123 fluorescence and (C) DCF fluorescence in Cr(VI) reactions with 0.1 and 2 mM Asc.

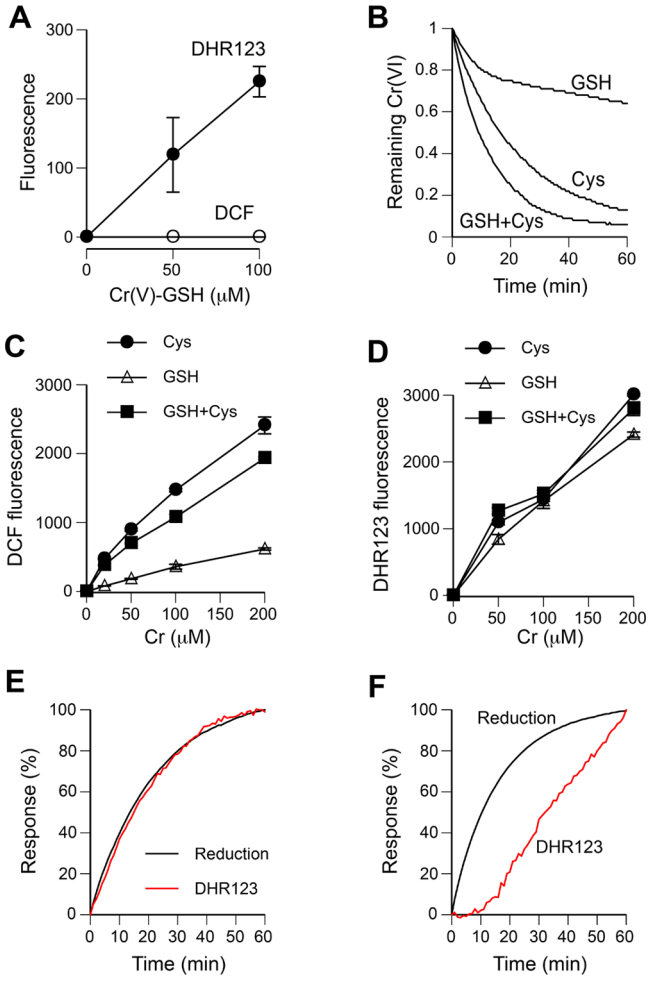

Figure 5. DCF and DHR123 fluorescence in reactions with Cr(V)-GSH and GSH/Cys mixtures. (A) Differential susceptibility of DCF and DHR123 to oxidation by Cr(V)-GSH complex. Background-subtracted fluorescence values recorded after 30 min of incubation are shown (means ± SD, n = 3). Fluorescence was not adjusted for the extent of Cr(V) to Cr(III) conversion. (B) Kinetics of Cr(VI) reduction by 2 mM GSH, 2 mM Cys, and a mixture containing both thiols. (C) DCF fluorescence and (D) DHR123 fluorescence in Cr(VI) reactions with 2 mM thiols alone and together. Means ± SD, n = 3. Error bars were smaller than symbols in most cases. Fluorescence in panels C and D was adjusted for the amount of Cr(VI) reduction at the end of 60 min incubations. (E) Time-course of DHR123 oxidation and Cr(VI) reduction in reactions containing 2 mM Cys and 100 μM Cr(VI). Data were normalized to the final readings at 60 min. (F) Time-course of DHR123 oxidation and Cr(VI) reduction in reactions containing 2 mM GSH and 100 μM Cr(VI). For both panels E and F, Cr(VI) absorbance was recorded every 15 s, and DHR123 fluorescence was measured every 1 min. Data are the means of triplicate measurements.

Thus, our positive findings in reactions with low Asc concentrations offer further support that Cr(V) is a principal cause of DCF and DHR123 oxidation during Cr(VI) metabolism by its biological reducers.

Different Reactivity of Cr(V)-GSH with DCF and DHR123. Both Cys and GSH reduce Cr(VI) via one-electron transfer generating Cr(V).[8−11] Similar amounts of oxidized DHR123 in Cys and GSH reactions (Figure 1C) are consistent with the same production of Cr(V). However, DCF fluorescence in GSH samples was much lower in comparison to that of Cys samples, suggesting a potential presence of different Cr(V) species. The main Cr(V) product in GSH reactions is a well-characterized Cr(V)-GSH complex,[37,43] whereas no Cr(V)-thiol complex has been detected in reactions with Cys.[11] To better understand the responses of both redox probes in GSH reactions, we prepared the Cr(V)-GSH complex and tested its reactivity toward DCF and DHR123. We found that DCF was completely resistant to oxidation by Cr(V)-GSH, whereas DHR123 showed a strongly increased fluorescence (Figure 5A). Next, we examined DCF and DHR123 responses in mixtures of Cys and GSH. The presence of two reducers resulted in a slightly larger than additive increase (1.14-fold synergism) in the initial rates of Cr(VI) reduction (Figure 5B), suggesting largely independently proceeding reactions for each thiol. However, the inclusion of Cys eliminated a slow reduction component that was always present in our GSH-containing reactions and observed earlier in different buffer systems.[36] Reduction-adjusted DCF fluorescence in GSH + Cys reactions was 3.7-times higher than that in GSH-alone samples and only about 20% lower than that in Cys-alone samples (Figure 5C). DHR123 fluorescence was equally strong for GSH, Cys, and GSH + Cys reactions (Figure 5D). Low DCF fluorescence in GSH-Cr(VI) reactions can be explained by the inability of the main Cr(V) product, Cr(V)-GSH complex, to oxidize this dye. The production of Cr(V)-GSH probably also accounted for a moderately lower DCF fluorescence in samples containing GSH + Cys versus Cys alone. A significant oxidation of DCF in GSH reactions despite the complete nonreactivity of Cr(V)-GSH suggests the presence of another, less abundant Cr(V) product with a reactivity similar to that of Cr(V) generated in Cys reactions.

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ESR studies have found that the kinetics of the Cr(V) signal followed that of the Cr(VI) reduction in Cys-driven reactions, whereas the initial Cr(V) accumulation was delayed relative to the loss of chromate absorbance in GSH reactions.[9] Consistent with a greater stability of Cr(V) complexed with GSH, the Cr(V) signal remained steady for the duration of the reduction of chromate by GSH but quickly disappeared in Cys reactions.[9] Since DHR123 appeared to be sensitive to the presence of various of Cr(V) forms (acting as a sensor of total CrV), we tested its responsiveness in time-course studies of Cr(VI) reduction by both thiols. We found that the kinetics of DHR123 oxidation and Cr(VI) reduction were essentially identical in Cys reactions (Figure 5E), indicating a rapid formation of Cr(V) and its fast reactivity with DHR123. In GSH reactions, oxidation of DHR123 initially lagged the disappearance of Cr(VI) and then showed a steady increase (Figure 5F). The observed linear buildup in DHR123 fluorescence after the initial lag is consistent with Cr(V) reaching a steady level at approximately 5 min in GSH reactions monitored by ESR.[9] However, the lag time in DHR123 fluorescence appeared longer than that for the Cr(V)-ESR signal, which probably reflects a slow reactivity of Cr(V)-GSH with the probe. Lack of DCF oxidation by Cr(V)-GSH supports the weak oxidation power of this product.

Responses in Asc-Thiol Mixtures and in Cr(VI)-Treated H460 Cells. To better understand the potential applications of DCF and DHR123 in the monitoring of Cr(VI) metabolism in human cells, we first examined the fluorescence of these dyes in mixtures of all three biological reducers at their physiological concentrations. Asc and GSH are typically present in mammalian cells in low millimolar concentrations,[18,44] whereas the cellular content of Cys is several times lower than that of GSH. H460 cells that we studied below contained 0.19 mM Cys. Therefore, we chose to test Cr(VI) reactions with 2 mM Asc, 2 mM GSH, and 0.2 mM Cys. As expected based on the preceding results, GSH/Cys mixtures caused the oxidation of both DHR123 and DCF (Figure 6A,B). Similar to the GSH-alone reactions (Figure 1), the overall fluorescence responses for DCF were much lower in comparison to those of DHR123, which reflected a dominant role of GSH in Cr(VI) reduction when it was present at 10-fold molar excess over Cys (Figure 6C). The addition of Asc to GSH-Cys mixtures completely abrogated the oxidation of both probes, which can be attributed to the overwhelming role of Asc in Cr(VI) metabolism due to its dramatically faster reduction rates (Figure 1A) and the absence of the Cr(V) intermediate under the employed toxicologically relevant ratio of Asc to Cr(VI). In the final series of *in vitro* experiments, we also examined the impact of Asc on the oxidation of DHE in thiol-containing Cr(VI) reactions. Cys- and to a lesser degree, GSH-containing reactions showed modest responses with this redox indicator (Figure 1D). As for DCF and DHR123, we found that the presence of Asc blocked DHE oxidation during Cr(VI) reduction by thiols (Figure 6D), suggesting that Cr(V) was also likely responsible for the fluorescence of this probe in Cys/GSH-containing reactions.

On the basis of the equally robust responsiveness of DHR123 to the formation of Cr(V) in reactions with Cys and GSH, we decided to test this dye for the monitoring of Cr(VI) metabolism in H460 human lung epithelial cells. To assess the role of GSH, cells were treated with the glutathione synthase inhibitor BSO for 24 h, which lowered cellular GSH concentrations by >100-fold, from 2.9 ± 0.2 mM to 26 ± 11

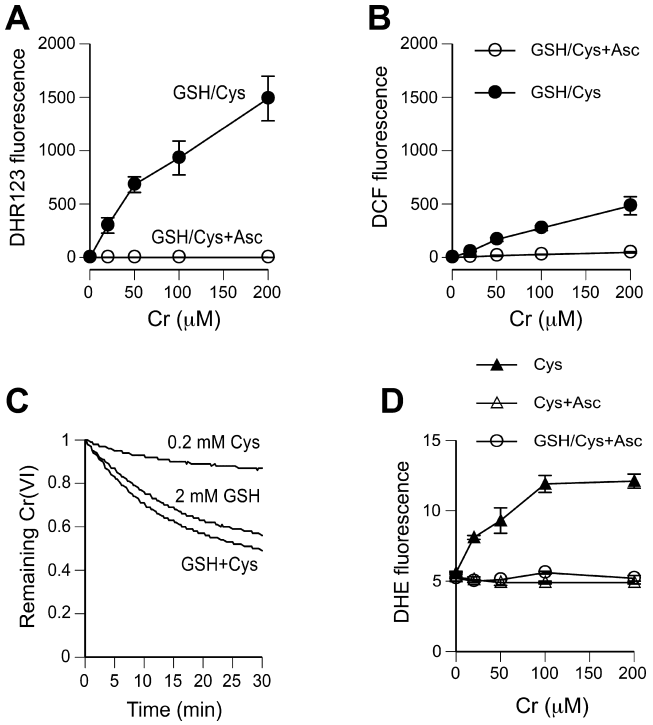

Figure 6. Responses of DHR123, DCF, and DHE in Cr(VI) reactions with Asc-thiols mixtures. Fluorescence was recorded after 30 min incubations and adjusted for the amount of Cr(VI) reduction. Shown are the means ± SD, n = 3. (A) DHR123 fluorescence and (B) DCF fluorescence in reactions containing 2 mM GSH and 0.2 mM Cys (GSH/Cys label) or both thiols plus 2 mM Asc (GSH/Cys + Asc label). (C) Kinetics of Cr(VI) reduction by 0.2 mM Cys, 2 mM GSH, and their mixture. (D) DHE fluorescence in Cr(VI) reactions with 2 mM Cys (Cys label), 2 mM Cys and 2 mM Asc (Cys + Asc label), or 2 mM GSH, Cys, and Asc (GSH/Cys + Asc label).

μM. Time-course measurements showed a moderate increase in DHR123 fluorescence up to approximately 20 min after a 1-h long treatment of control H460 cells with 20 μM Cr(VI) (Figure 7A). This dose of Cr(VI) corresponded to IC_{50} for cell viability at 24 h postexposure. In GSH-depleted cells, the amount of oxidized DHR123 was lower at the early time points but continued to rise up to 60 min after Cr(VI) removal, indicating slower reduction rates of Cr(VI) in the absence of the predominant cellular thiol. We determined that control and GSH-depleted cells contained only 2 μM Asc, which excludes its significant contribution to Cr(VI) reduction. When cellular concentrations of Asc were raised to 0.7 mM by preincubation with 1 mM dehydroascorbic acid, we found no changes in either DHR123 or DCF fluorescence at any time point after 30 μM Cr(VI) treatment (Figure 7B). No significant changes in the fluorescence of both dyes were also detected in Asc-restored cells treated with three other doses of Cr(VI) (10, 20, and 40 μM, data not shown). Control and Asc-supplemented cells showed essentially the same accumulation of Cr(VI) (Figure 7C), indicating that a different spectrum of Cr metabolites was responsible for the lack of oxidized DHR123 in the presence of Asc. The unchanged fluorescence of both redox probes in Asc-normalized cells is in agreement with the observed absence of DHR123- or DCF-oxidizing species during *in vitro* reduction of Cr(VI) with an excess of Asc. In human A549 cells, preloading with ~80 μM Asc stimulated DCF and DHR123 fluorescence by Cr(VI),[34] which is consistent with the

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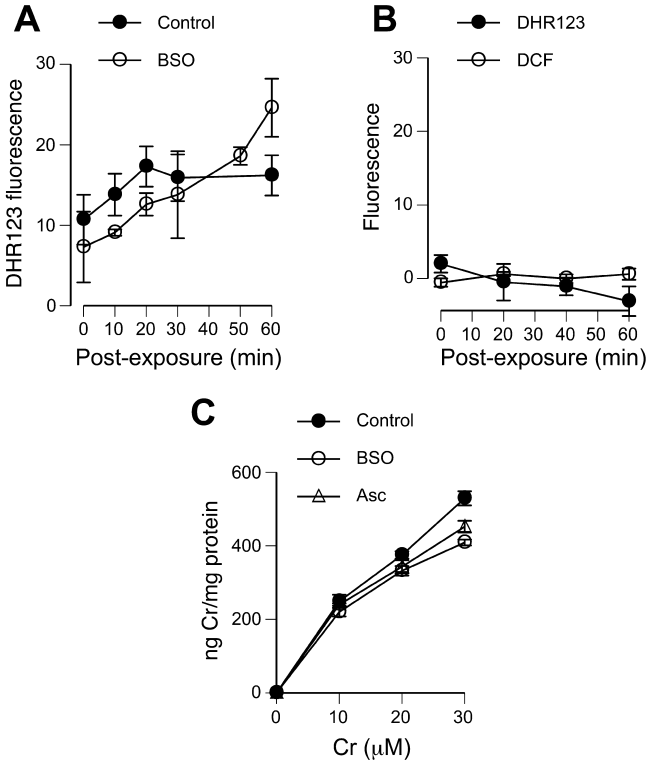

Figure 7. DHR123 and DCF fluorescence in Cr(VI)-treated H460 cells. Cells were preloaded with 10 μM dye for 30 min prior to a 1-h long treatment with 20 μM Cr(VI). Data are the means ± SD (n = 3). (A) Background-subtracted DHR123 fluorescence at different times after Cr removal. BSO cells were treated with 0.2 mM BSO for 24 h to deplete GSH. (B) Background-subtracted DCF and DHR123 fluorescence in Asc-restored cells treated with 30 μM Cr(VI). (C) Cr(VI) uptake by control and Asc-restored cells.

formation of Cr(V) and oxidation of both dyes in our low-Asc reactions (100 μM Asc).

DISCUSSION

Non-ROS Origin of Cr(VI) Reduction-Generated Oxidants. We found that among seven tested oxidant-sensitive dyes, only DCF and DHR123 showed strongly increased fluorescence (up to 100-fold over background) in reactions containing Cr(VI) and its biological reducers GSH and Cys. Fluorescence of the superoxide probe DHE was also elevated in these reactions, although the overall magnitude was very modest, with a maximal 2.5-fold increase for the highest Cr(VI) doses reduced with Cys. Several experimental observations strongly indicate that redox probes responded to oxidants other than ROS. These include the lack of fluorescence increases for three other general ROS indicators and the absence of oxidant-mediated mutagenic damage in pSP189 plasmids. In our *in vitro* conditions that lacked peroxidases, only highly reactive OH radicals but not other ROS can cause DCF and DHR123 oxidation.[31,39,40] The nonresponsiveness of APF which primarily reacts with OH radicals[45] and the absence of any effect by the radical scavenger ethanol further argue against OH radicals as the cause of DCF and DHR123 fluorescence in Cr(VI)-thiol reactions. Extensive oxidation of DCF and DHR123 by synthetic Cr(V) complexes has also been found to be ROS-independent.[33]

Cr(V) and Oxidation of Redox Probes. The absence of ROS points to Cr(V) and/or Cr(IV) intermediates as potential oxidants of redox-sensitive probes. We found no DCF or DHR123 (or DHE) oxidation in Cr(VI) reactions with a more than 10-fold excess of the two-electron reducer ascorbate, which lacked a detectable formation of Cr(V).[6,7,42] These findings strongly indicated that Cr(IV) did not react with the redox probes. Under the conditions permitting Cr(V) formation due to insufficient amounts of Asc for Cr(VI) reduction, we detected the oxidation of both DCF and DHR123. GSH and Cys are one-electron reducers of Cr(VI), producing abundant amounts of Cr(V).[8−11] In agreement with Cr(V) being the principal oxidant, both Cys and GSH reactions with Cr(VI) caused large increases in DCF and DHR123 fluorescence. Cys- and GSH-based reactions showed similarly high responses with DHR123, which is consistent with the expected comparable formation of total Cr(V) by both reducers. In the case of the DCF probe, its oxidation in GSH samples was about 4-times lower relative to Cys, pointing to a different reactivity of the main Cr(V) species produced by these two thiols. Our experiments with a preformed Cr(V)-GSH complex showed that it was capable of oxidizing DHR123 but had no reactivity toward DCF. Thus, elevated DCF fluorescence in GSH-Cr(VI) reactions indicated the presence of a Cr(V) product other than Cr(V)-GSH.

A commonly invoked reduction mechanism by GSH involves the initial formation of the Cr(VI)-thiolate complex followed by binding of a second GSH molecule leading to one-electron transfer and the production of the Cr(V)-GSH complex.[8] A similar reduction process has also been described for Cys.[11] However, whereas a relatively stable Cr(V)-GSH complex has been readily isolated from *in vitro* reactions[37,43] and detected in Cr(VI)-treated human A549 cells,[34] efforts to identify the Cr(V)-Cys complex have proven unsuccessful.[11] Thus, probably because of instability of Cys binding, it appears that all Cr(V) in Cys reactions is reducer-unbound (free) Cr(V). Studies on the mechanism of Cr(VI) reduction by GSH have been performed with the supraphysiological concentrations of this reducer, which would favor coordination and reduction of the Cr(VI)-thiolate complex by a second GSH molecule. At physiological concentrations, the rate of ternary complex formation is expected to be greatly diminished, which allows a fraction of the initially formed Cr(VI)-GSH thiolates to undergo reduction yielding free Cr(V) and the thyil radical. Taken together, the presented mechanistic considerations of Cr(VI) reduction and our findings on the average 4.1-fold difference between DCF and DHR112 responses indicate that Cr(VI) metabolism by a physiological 2 mM GSH concentration generated approximately 75% Cr(V) in the form of Cr(V)-GSH, which is reactive with DHR123 but not DCF, and 25% free Cr(V), which is reactive with both DHR123 and DCF. Lower yields and instability of free Cr(V) complicate its detection by ESR spectroscopy at biologically relevant conditions, although ESR studies of some Cr(VI)-GSH reactions have previously detected a Cr(V) product with a different g-value than that of Cr(V)-GSH.[46−48] Only a single Cr(V) form has been observed in Cr(VI) reactions with Cys. Similar to our results with Cr(V)-GSH, the Cr(V)-mannitol complex was able to oxidize DHR123 but not DCF,[33] pointing to a general trend for the nonreactivity of DCF with Cr(V) stabilized by biological ligands.

Cr(V) species are weak oxidants,[26,28,35] and the variable responses of the tested probes in Cr(VI) reactions can be related to a different susceptibility of target groups to oxidation reactions that are required for the formation of fluorescent

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products. DCF and DHR123 are converted into their fluorescent forms via two sequential one-electron oxidation reactions.[31] Both dyes are initially oxidized via the abstraction of the hydrogen atom at the 9′-position, whose location at the central carbon of a triphenylmethane makes it vulnerable to weakly oxidizing species such as Cr(V). A conversion of APF into fluorescein requires o-dearylation, which is initiated by the abstraction of one electron from the nitrogen atom, and only strongly oxidizing species such as the OH radical have a sufficient reactivity for this reaction.[45] A similar argument about the requirement for a higher oxidizing power than that provided by Cr(V) to cause oxidation of an aromatic amine moiety can be made to explain a very weak responsiveness of DHE in Cr(VI) reactions. CellRox dyes are proprietary probes with unknown chemical structures, which makes it difficult to consider chemical reasons for their insensitivity to the detection of Cr(V).

Implications for Monitoring Different Oxidants during Cr(VI) Metabolism. Our findings extend the earlier observations with synthetic Cr complexes[33] that intermediate Cr forms, which we showed here to be Cr(V) in biological systems, can oxidize DCF and DHR123. Thus, these two popular dyes are inappropriate for ROS detection in Cr(VI)-treated cells. The concern about the non-ROS origins of DCF/DHR123 oxidants is particularly important for standard cell cultures in which Asc deficiency leads to one-electron reduction of Cr(VI) by thiols. Although Cr(VI) treatments of human cells with restored Asc levels did not induce a detectable production of oxidants in this work or biologically significant DNA oxidation,[25] coexposure with other redox-active toxicants or phagocytosis of chromate-containing particles can result in elevated ROS through chemical or biological mechanisms, respectively. The complete nonresponsiveness of three CellRox dyes and APF and minimal responses of DHE to Cr intermediates make these probes suitable for ROS monitoring in Cr(VI)-exposed cells. A summary of the reactivity of different dyes and their applicability to the detection of specific oxidants in Cr(VI) reactions is shown in Figure 8.

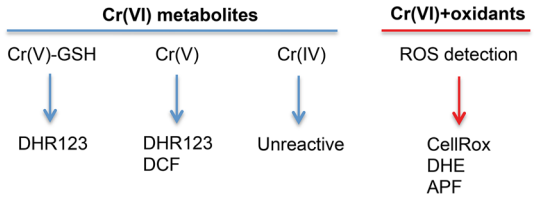

Figure 8. Detection of reactive intermediates during Cr(VI) metabolism using fluorescent probes.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

APF, aminophenylfluorescein; Asc, ascorbate; BSO, buthionine sulfoximine; DCF, dichlorodihydrofluorescein; DHA, dehydroascorbic acid; DHE, dihydroethidium; DHR123, dihydrorhodamine 123; GSH, glutathione; MOPS, 4-morpholinepropanesulfonic acid

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