Imaging Dynamic Redox Changes in Mammalian Cells with Green Fluorescent Protein Indicators*

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Changes in the redox equilibrium of cells influence a host of cell functions. Alterations in the redox equilibrium are precipitated by changing either the glutathione/glutathione-disulfide ratio (GSH/GSSG) and/or the reduced/oxidized thioredoxin ratio. Redox-sensitive green fluorescent proteins (GFP) allow real time visualization of the oxidation state of the indicator. Ratios of fluorescence from excitation at 400 and 490 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity. Because the indicator is genetically encoded, it can be targeted to specific proteins or organelles of interest and expressed in a wide variety of cells and organisms. We evaluated roGFP1 (GFP with mutations C48S, S147C, and Q204C) and roGFP2 (the same plus S65T) with physiologically or toxicologically relevant oxidants both *in vitro* and in living mammalian cells. Furthermore, we investigated the response of the redox probes under physiological redox changes during superoxide bursts in macrophage cells, hyperoxic and hypoxic conditions, and in responses to H_2O_2-stimulating agents, *e.g.* epidermal growth factor and lysophosphatidic acid.

Cells have elaborate homeostatic mechanisms to regulate the thiol-disulfide redox status of their internal compartments. Most thiol groups within the cytoplasm are normally reduced. Very few are present as disulfides. It has been speculated that the cytoplasm is reducing because many metabolic reactions evolved before oxygen became abundant in the atmosphere (1). Modest alterations in the thiol-disulfide equilibrium could have major consequences in the cell, including defective protein folding or enzyme activity (because many enzymes have a cysteine in their active site). When excess oxidation overwhelms the reductive capabilities of the cell, death results. Despite the

dangers of excessive oxidation, cells sometimes use redox adjustments as signaling events, such as in the activation of transcription factors (NF-κB and AP-1), caspases, protein tyrosine phosphatases, or GTPases (Ras). Thus, changes in the redox equilibrium influence a host of cell functions, including but not limited to growth, stress responses, differentiation, metabolism, cell cycle, communication, migration, gene transcription, ion channels, and immune responses (for reviews see Refs. 2–6). Alterations in the redox equilibrium are reflected in changes of the glutathione/glutathione-disulfide ratio (GSH/GSSG) and the reduced/oxidized thioredoxin ratio. Glutathione is found in high concentrations in cells (5–10 mM) and is considered to be the major player in maintaining intracellular redox equilibrium. Ratios of GSH to GSSG are reported to range from 100 to 300:1 (7, 8), but these measurements have been problematic because they require destruction of the tissue, during which great care must be taken not to allow further oxidation. The major source of error is the determination of GSSG concentration, because this species is at low abundance yet is measured only after complete removal of GSH to prevent oxidation. The spatial and temporal resolution of such destructive assays is very poor.

Redox-sensitive green fluorescent proteins (GFP)[1] described recently (9) allow real-time visualization of the oxidation state of the indicator. The indicators examined in this work are GFP mutants with two surface-exposed cysteine placed at positions 147 and 204 on adjacent β-strands close to the chromophore. Disulfide formation between the cysteine residues promotes protonation of the chromophore and increases the excitation spectrum peak near 400 nm at the expense of the peak near 490 nm. The ratios of fluorescence from excitation at 400 and 490 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity. Because the indicator is genetically encoded, it can be targeted to specific proteins or organelles of interest and expressed in a wide variety of cells and organisms. Here we evaluate roGFP1 (GFP with mutations C48S, S147C, and Q204C) and roGFP2 (the same plus S65T) with physiologically or toxicologically relevant oxidants both *in vitro* and in living mammalian cells. The probes expressed in cell cytoplasm responded as expected to a variety of oxidants. Although lower concentrations of hydrogen peroxide were required to oxidize

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[1] The abbreviations used are: GFP, green fluorescent protein; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BMES, bis(2-mercaptoethyl)sulfone; BSO, buthionine sulfoximine; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; DTT, dithiothreitol; DTT$_{ox}$, *trans*-4,5-dihydroxy-1,2-dithiane; roGFP, redox-sensitive GFP; EGF, epidermal growth factor; IMDM, Iscove's modified Dulbecco's medium; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; LPA, lysophosphatidic acid.
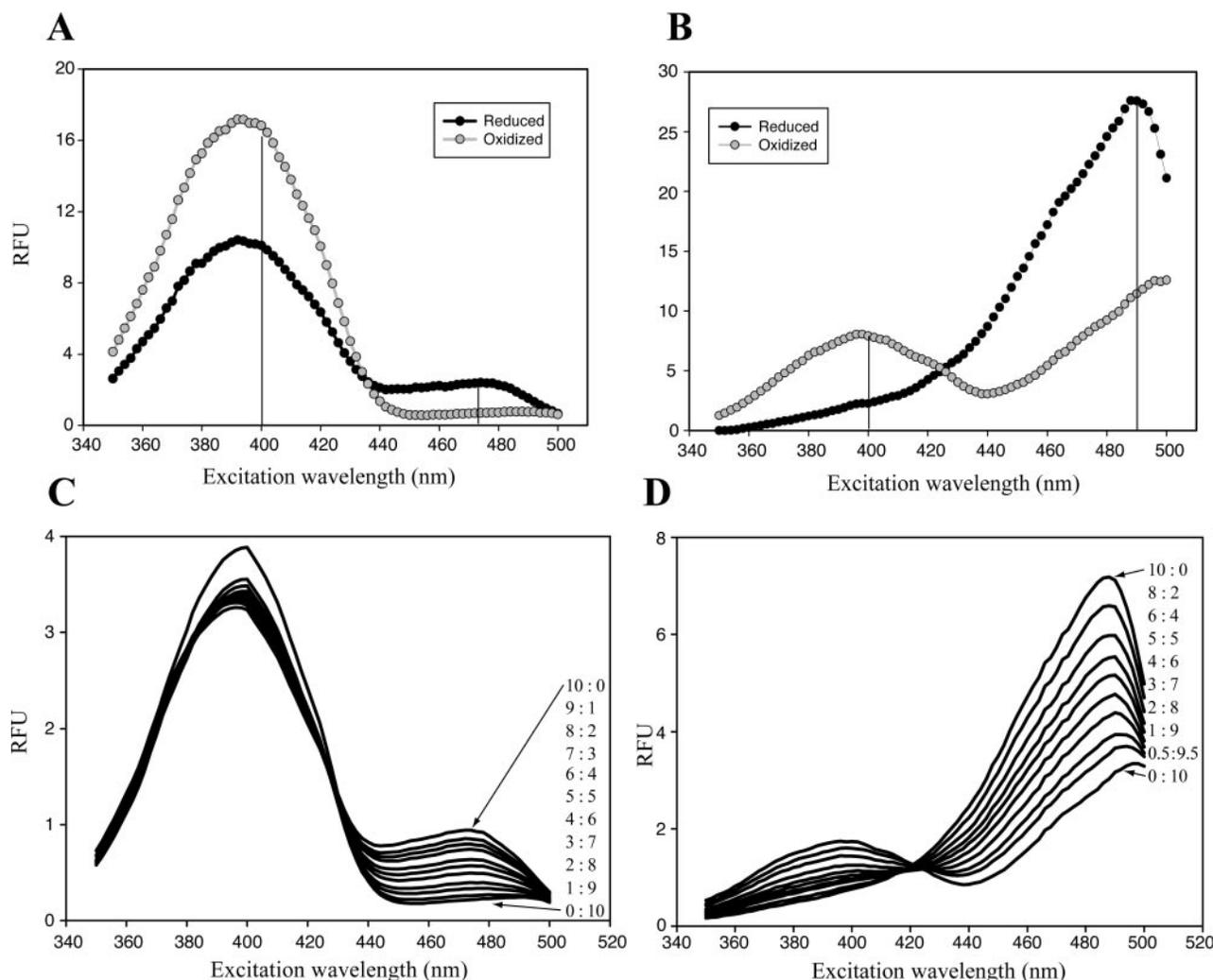
FIG. 1. **Excitation spectra and redox titration of roGFPs.** Excitation spectra for fully oxidized and reduced roGFP1 (*A*) and roGFP2 (*B*). Emission was monitored at 515 nm. *C*, titration of roGFP1 (1 μM) with dihydrolipoate + lipoate buffers (total 10 mM). *D*, titration of roGFP2 (1 μM) with reduced + oxidized BMES (total 10 mM). Conditions are as described under "Materials and Methods." Legends in *C* and *D* indicate the concentrations in millimolar of reduced:oxidized buffers, respectively. For clarity only the limiting spectra are connected by *arrows* to their buffer compositions.

cytosolic roGFPs than oxidize the same proteins *in vitro*, attempts to detect growth factor-stimulated production of hydrogen peroxide were not successful. However, the probes could detect superoxide generated during the oxidative burst of HL60 cells.

MATERIALS AND METHODS

Aldrithiol, diamide, hydrogen peroxide, buthionine sulfoximine, 3-amino-1,2,4-triazole, apocynin (acetovanillone), and 4-(2-aminoethyl)-benzenesulfonyl fluoride were obtained from Aldrich. Oxidized and reduced lipoate, oxidized and reduced glutathione, menadione, diphenylene iodonium chloride, BCNU (1,3-bis-(2-chloroethyl)-1-nitrosourea) (carmustine), and apocynin were obtained from Sigma. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) and DTT were obtained from Calbiochem. Bis(2-mercaptoethyl)sulfone (BMES) was from USB Corp. Its cyclic disulfide was prepared by oxidation with a stoichiometric quantity of iodine (10) followed by recrystallization from hexane. HL60 cells were obtained from American Type Culture Collection.

In Vitro Studies—roGFPs were subcloned into pRSET$_B$ (Invitrogen) using BamHI and EcoRI restriction sites. The plasmid encodes a fusion protein of the insert and an N-terminal extension containing a (His)$_6$ tag, enabling purification by nickel affinity chromatography. The construct was expressed in the JM109 strain of *Escherichia coli*. Isolated protein was reduced daily by mixing concentrated protein (50–200 μM) with 10 mM DTT and diluting to the required concentration. When necessary, DTT was removed from the solution using Centri-spin 20 columns (Princeton Separations Inc.). Reactions were carried out in 125 mM KCl, 75 mM HEPES, and 1 mM EDTA, pH 7.3, which had been

degassed by repetitive evacuation and nitrogen bubbling. Excitation scans (350–500 nm, 2.5-nm bandwidth) were run in 100-μl volumes in 96-well plates (sealed when required) on a Safire spectrofluorometer (Tecan), collecting emission at 530 nm, 7.5-nm bandwidth.

Redox Titration Using Fluorescence Spectroscopy—Redox probes were titrated in degassed HEPES buffer containing 1 μM protein and 10 mM lipoate or BMES as redox buffers. Concentrations of oxidized and reduced forms were reciprocally varied from 0:10 to 10:0 mM in 1 mM increments. The eleven solutions comprising each titration series were incubated at 25 or 30 °C for 1 h while sealed under a nitrogen atmosphere before measuring fluorescence excitation spectra. The redox potential of the redox probes using BMES or lipoate as redox buffers were obtained by first applying the following formula to each of the incrementing concentrations in the titration: $Y = (R_n - R_{min})/(R_{max} - R_n)$, where R_n = ratio at a particular concentration of redox buffer, R_{min} = ratio in 10 mM reduced redox buffer, and R_{max} = ratio in 10 mM oxidized redox buffer. From a plot of log Y *versus* log([oxidized redox buffer]/[reduced redox buffer]), the buffer ratio (A) required to achieve 50% of the ratio change can be determined from the intercept where log $Y = 0$. K_{eq} was derived from log K_{eq} = log($F_{490 red}$/($F_{490 ox}$)) − log A. The redox potential (*E'*) was then calculated from the Nernst equation shown in Equation 1,

$$E' = E'_0 - (RT/nF)\ln K_{eq} \qquad \text{(Eq. 1)}$$

where E'_0 is the redox potential of the redox buffers (lipoate −290 mV (11); BMES −295 mV (see below)), R is the gas constant (8.313 J/mol/K), T is temperature (K), $n = 2$ is the number of electrons exchanged, and F is Faraday's constant (96490 J/mol/volt).
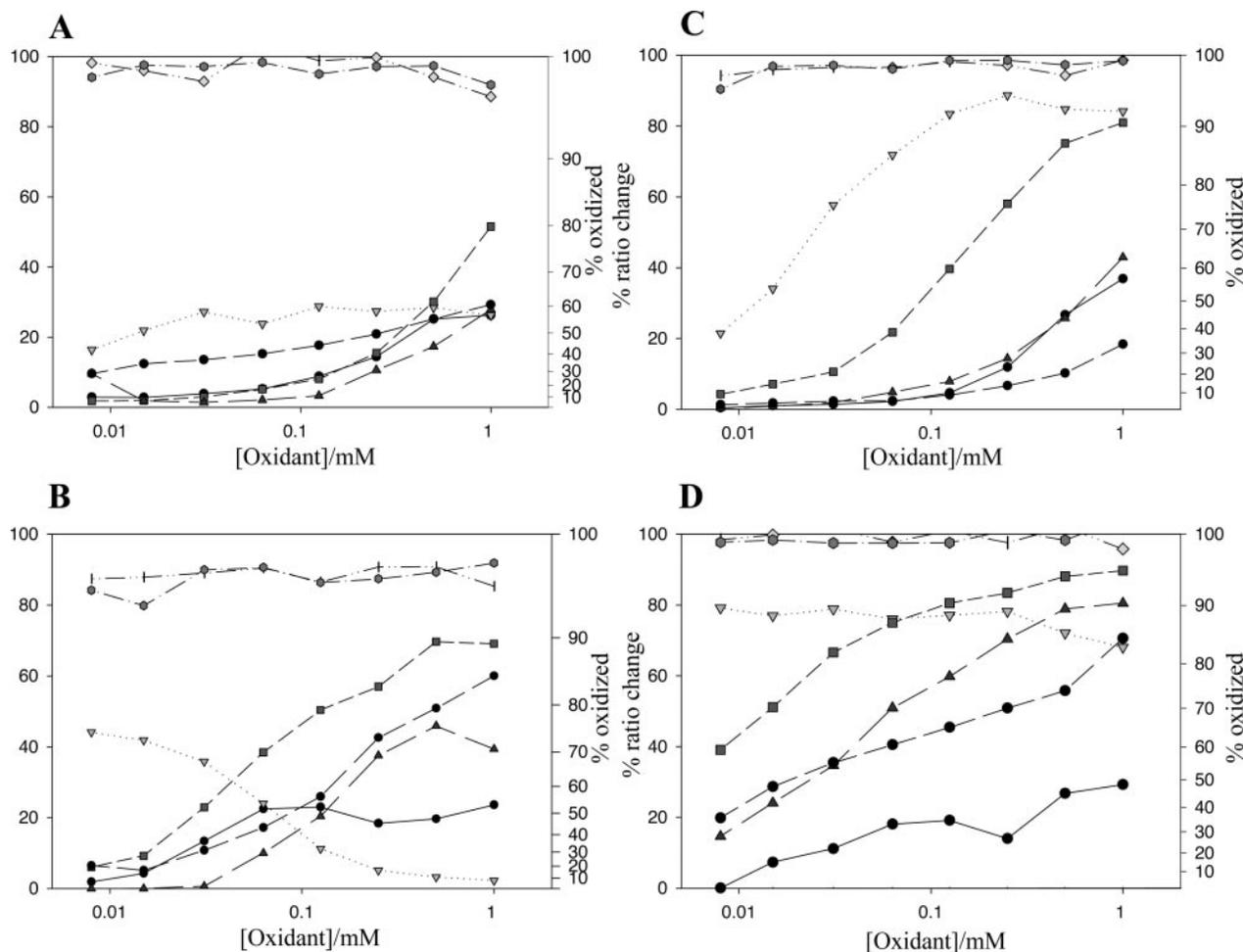
FIG. 2. **Oxidation of the two roGFPs by common oxidants.** *A*, roGFP1 following a 30-min incubation with oxidants. *B*, roGFP1 following 24-h exposure. *C*, roGFP2 following a 30-min incubation with oxidants. *D*, roGFP2 following 24-h exposure. Oxidation is expressed as a percentage of the fully reduced (0%) and fully oxidized (100%) proteins obtained under similar conditions. Excitation ratios were obtained from wavelengths of 400 and 472 nm for roGFP1 and 400 and 490 nm for roGFP2. *Symbols*: . . .▽. . . , menadione; - - -▦- - -, BMES; —◇—, aldrithiol; —▲—, GSSG; -·-○-·-, diamide; - -●- -, H_2O_2; —●—, DMNQ.

Redox Potential of BMES Using NMR Spectroscopy—Solution A initially containing 10 mM dihydrolipoate and 10 mM oxidized BMES and solution B initially containing 10 mM oxidized lipoate and 10 mM reduced BMES were separately prepared in deoxygenated sodium phosphate buffer (10 mM, pD 7.4) in D_2O. NMR spectra were recorded on a Varian 200-MHz spectrometer at 283 K. The spectra of solutions A and B taken following a 1-h incubation after preparation were essentially identical, showing that equilibrium had been attained. Peaks specific to each of the reagents both oxidized and reduced were found: oxidized lipoate (3.2 ppm); dihydrolipoate (2.65 ppm); oxidized BMES (3.85 ppm); and reduced BMES (3.4 ppm). To determine the equilibrium constant (K_{eq}) between lipoic acid and BMES, $K_{eq} = [BMES_{red}] \times [lipoate_{ox}]/([BMES_{ox}] \times [lipoate_{red}])$, we used the integrals under the peaks, $K_{eq} =$ (integral @ 3.4 ppm) $\times$ (integral @ 3.2 ppm)/((integral @ 3.85 ppm) $\times$ (integral @ 2.65 ppm)). Inserting the value $K_{eq} = 1.46$ into the Nernst equation (using a value of -290 mV for lipoate standard (11)), the redox potential of BMES in D_2O was determined to be -295 mV.

Superoxide Production by Xanthine Oxidase—Reactivity to superoxide was determined by incubating 1 μM roGFP with 25 milliunits of xanthine oxidase (which had been centrifuged to remove ammonium sulfate) and 50 μM xanthine in 150 μl of 50 mM Tris buffer, pH 7.4.

Imaging in Cells—roGFPs were expressed in HeLa, P388D1, or HL60 cells using modified pEFGP-N1 as expression vector and Lipofectin as transfection reagent. After 24–72 h of incubation at 37 °C in culture medium, the cells were washed twice with Hanks' balanced salt solution buffer. Cells were imaged on a Zeiss Axiovert microscope with a cooled CCD camera (Photometrics, Tucson, AZ) controlled by Metafluor 2.75 software (Universal Imaging, West Chester, PA). Dual-excitation ratio imaging used excitation filters 400DF15 and 480DF30 for roGFP1 and 400DF15 and 495DF10 for roGFP2 altered by a filter changer (Lambda 10-2, Sutter Instruments, San Rafael, CA). A

505DRLP dichroic mirror and an emission filter, 535DF25, were used for both probes. Fluorescence images were background-corrected by manual selection of background regions. Exposure time was 200–1000 ms, and images were taken every minute.

Fluorescence-activated Cell Sorter Analysis—Ratios for HL60 cells were obtained on a FACSVantage SE with DIVA option (BD Biosciences). Cells were excited using laser lines at 407 nm (50 milliwatts) and 488 nm (150 milliwatts). Emission filters were 510/21 for both excitation wavelengths. HL60 cells were grown in the presence of 1.3% Me_2SO in IMDM plus 10% fetal bovine serum, 1% penicillin-streptomycin for 5–7 days. The cell suspension (1×10^7 cells) was washed twice in IMDM without fetal bovine serum and resuspended in 1 ml of IMDM for electroporation. DNA (50–75 μg) was added to the solution and incubated at room temperature for 10 min. The cells were electroporated using a Cell-Porator electroporation system I (Invitrogen) using a 310-V pulse and 1180-microfarad capacitance. Cells were grown in IMDM (10% fetal bovine serum) for 2 days before oxidation experiments. Cells were washed and suspended in Hanks' balanced salt solution, and aliquots of cells were incubated in 50 μM PMA or 100 μM aldrithiol. For inhibition studies, cells were preincubated for 30 min in either 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or 200 μM apocynin.

Gene Cloning—Five mutations of amino acids neighboring Cys-147 and Cys-204 of roGFP1 and roGFP2 were incorporated using a QuikChange kit (Stratagene). They included N149K, A206K, F223K, N149K with A206K, and F223K with A206K. The mutated constructs in pRSET_B (Invitrogen) were expressed using JM109 bacteria and purified as described above. For expression in mammalian cells, the constructs were subcloned into pcDNA3 (Invitrogen) using BamHI and EcoRI restriction sites.

For membrane targeting, a sequence for myristoylation and palmi-

FIG. 3. **Oxidation upon addition of aldrithiol in HeLa cells expressing roGFP1.** Images were taken using an emission wavelength of 535 nm and both 400-nm and 480-nm excitation wavelengths. The ratios of light emitted from 400- and 480-nm excitation were obtained at 1-min intervals, and images of the ratios obtained are shown in pseudocolor calibrated by the color scale at the *far right*. Times of addition and concentrations of reactants are indicated by *arrows*.
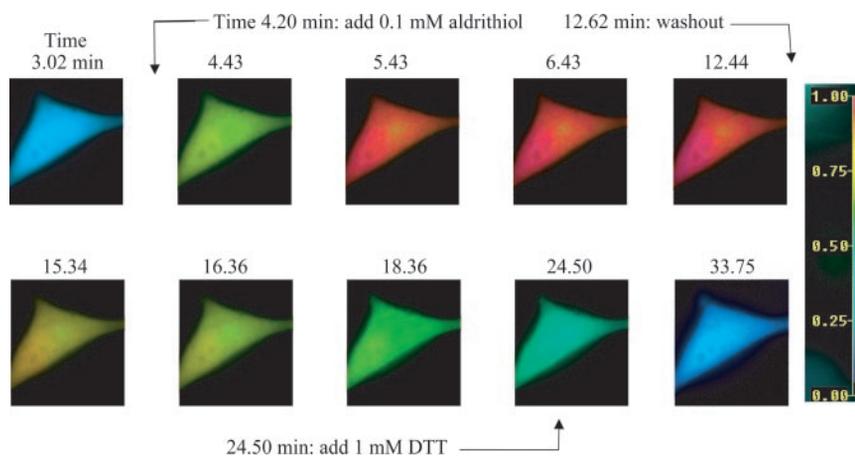
toylation was appended to the N terminus of roGFP1 and roGFP2. The signal sequence, MGCINSKRKDNLND, was derived from Lyn, a tyrosine kinase protein belonging to the Src family. The forward primer (5′-GCGGATCCTAAGCTTCGAGCCACCATGGGCTGCATCAACAGC-AAGCGCAAGGACAACCTGAACG-3′) and reverse primer (5′-GTTTC-AGGTTCAGGGGGAGGTGTGGGAGG-3′) were used to subclone the sequence into pEGFP-N1 (Clontech). For nuclear localization, the sequence PKKKRKVEDA was added to the C terminus of the redox probes using PCR to subclone the construct in pEGFP-N1. For mitochondrial localization, the redox probes were transferred to pECFP-mito (Clontech) using BamHI and NotI restriction to swap the cyan fluorescent protein and roGFP sequences.

RESULTS

Confirmation of Redox Potential—Preliminary experiments showed that roGFP1 and roGFP2 in the cytosol of mammalian cells were largely reduced, so it was important to characterize the behavior of reduced roGFP1 and roGFP2 *in vitro*. The proteins were expressed in *E. coli*, purified, and were freshly reduced each experimental day. Experiments were carried out in deoxygenated buffers unless stated otherwise (see "Materials and Methods"). Hanson *et al.* (9) measured the midpoint redox potentials (E'_0) of roGFP1 and roGFP2 to be −288 and −272 mV, respectively, using DTT ($E'_0 = -330$ mV) as the calibrating redox buffer. The large difference between E'_0 values for the roGFPs *versus* DTT means that roGFP is half-reduced with very small ratios of [DTT] to [DTT$_{ox}$]. It would be desirable to confirm the E'_0 values for roGFPs using redox buffers with E'_0s more closely matched to those of roGFPs. We chose dihydrolipoic acid (thioctic acid) and BMES because they form internal disulfides upon oxidation, similar to DTT and roGFPs. Therefore, roGFPs should react with these buffers with a 1:1 stoichiometry. Furthermore, both of these buffers are small molecules, which should cross cell membranes, possibly allowing *in situ* calibration of intracellular roGFPs. Titrations of the protein in redox buffers of increasing ratios of reduced to oxidized states were performed (Fig. 1) from which the midpoint potentials of the redox proteins were calculated. Initially, we found distinct redox potentials when using lipoate or BMES as standard solutions; however, the values for roGFP1 and roGFP2 differed by the identical values. Because E'_0 for BMES (−313 mV) (12) had only been determined in 1:1 methanol-water, we used NMR spectroscopy to determine the value for BMES in a purely aqueous solution relative to the dihydrolipoate/lipoate couple. When the resulting value $E'_0 = -295 \pm 1$ mV was used to determine E'_0 for the roGFPs, we obtained close agreement between the values obtained in the two standard buffers. These values, $E'_0 = -294$ and −287 mV for roGFP1 and roGFP2, respectively, are somewhat more negative than the redox potentials reported by Hanson *et al.* (9) based on DTT as standard, −288 and −272 mV, although the
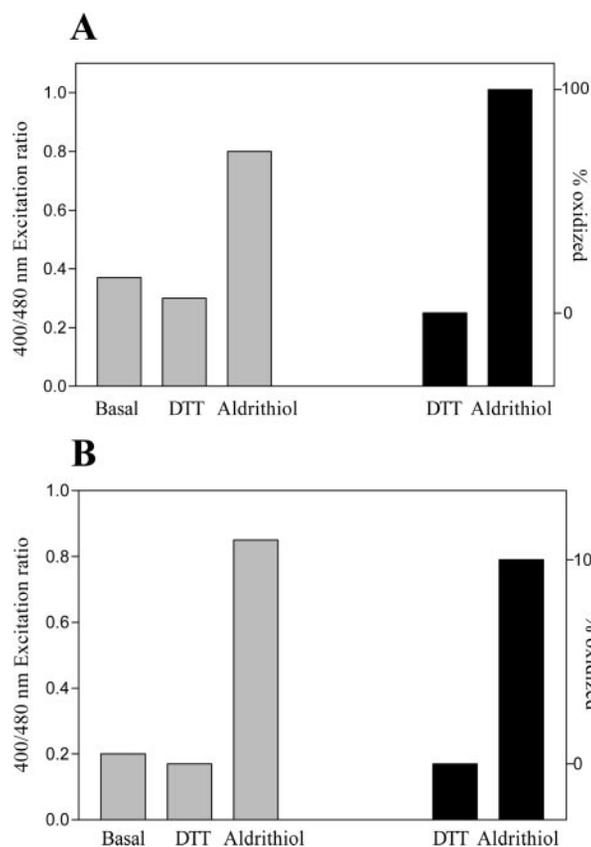
FIG. 4. **Dynamic range of roGFP ratios in cells *versus* protein microdroplets.** roGFP1 and roGFP2 were expressed in HeLa cells. Ratio of fluorescence obtained at 400/480 (roGFP1) or 400/495 (roGFP2) maximum and minimum ratio was observed for protein microdroplets under the same conditions. *Gray bars* represent protein expressed in HeLa cells. *Black bars* represent free protein. *A*, roGFP1. *B*, roGFP2.

ordering of roGFP1 as more reducing than roGFP2 is preserved.

Oxidation of Redox Probes in Vitro—Because roGFPs were largely reduced inside cells until membrane-permeant oxidants were added, we characterized the *in vitro* dose-response relationships of roGFPs to those same oxidants. roGFPs are easily oxidized upon storage *in vitro*, so they were freshly reduced prior to most experiments and characterized in degassed buffers and sealed reactions. Fig. 2 shows the extent of roGFP oxidation by varying concentrations of oxidants after 30 min and 24 h of reaction. Aldrithiol (2,2-dipyridyl disulfide) and diamide (1,1-azobis(*N*,*N*-dimethylformamide)) were very reactive and completely oxidized roGFPs in <30 min at all of the
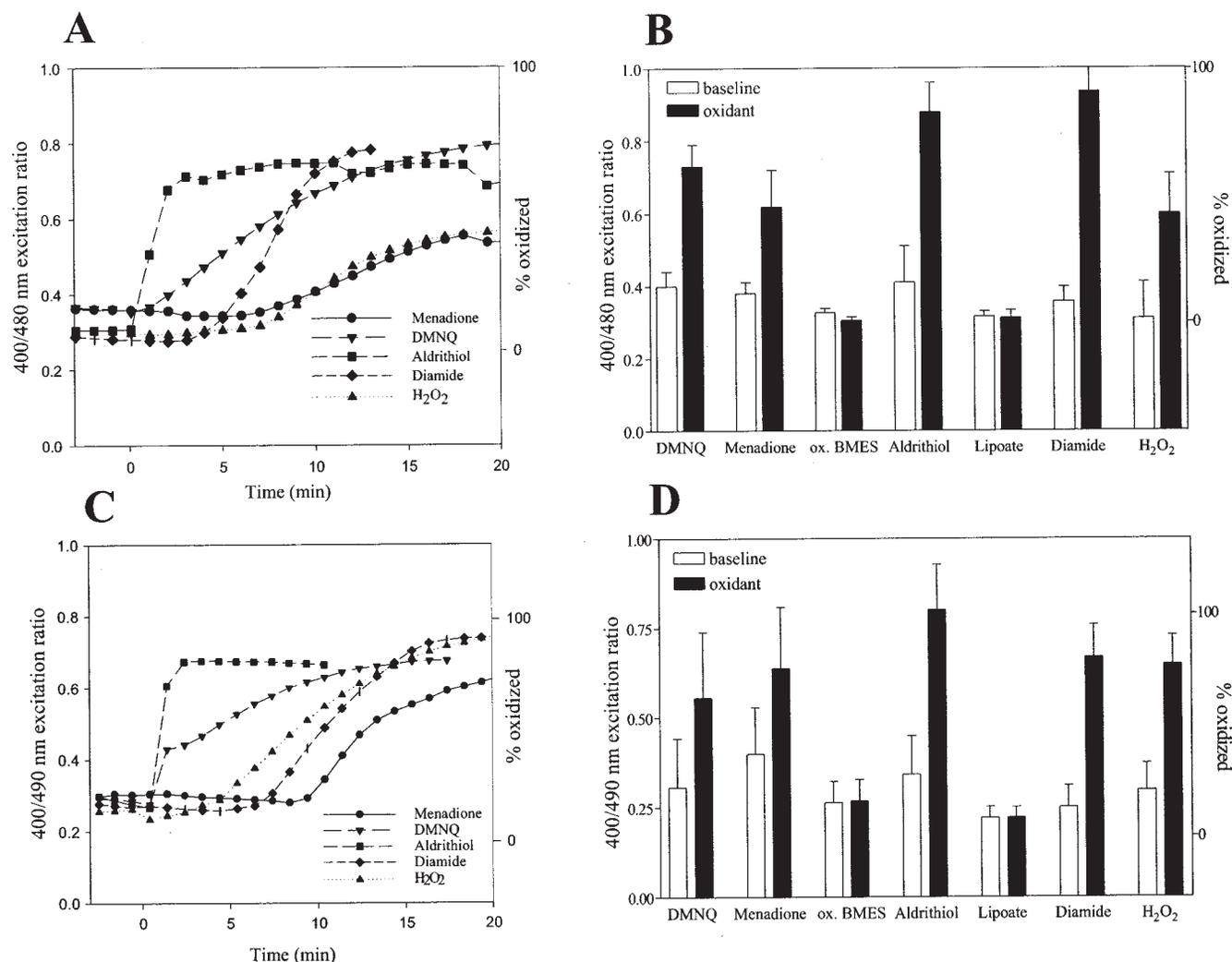
FIG. 5. **Oxidation of roGFPs expressed in HeLa cells upon stimulation with exogenous oxidants.** Each oxidant was added to cells at a concentration of 100 μM. *A*, time course of oxidation of roGFP1. Oxidants were added at time zero, an average of three or more experiments. *B*, oxidation of roGFP1-averaged results from multiple experiments. *Open bars*, ratio observed before addition; *solid bars*, ratio observed when oxidation had reached a plateau. *C*, time course of oxidation for roGFP2. *D*, oxidation of roGFP2 averaged results from multiple experiments.

concentrations tested (0.008–1 mM). Two other disulfides, GSSG and the oxidized form of BMES, required longer reaction times and higher concentrations to oxidize roGFPs, even partially. Hydrogen peroxide was not very effective *in vitro* until given at nearly millimolar concentrations for >30 min. We also tested two naphthoquinones commonly used to stimulate redox cycling in live cells. Menadione, 2-methyl-1,4-naphthoquinone, caused an initial increase in emission ratio consistent with oxidation, but prolonged incubations gave anomalous ratios and large decreases in fluorescence emission at all of the excitation wavelengths. Mass spectral analysis of the proteins after menadione exposure revealed the addition of 344 Da, consistent with the addition of 2 molecules of menadione to the proteins. Arylation of cysteines by menadione has been reported previously (13, 14). GSH reacts with menadione to give the thioether as well as hydrogen peroxide and superoxide (15). DMNQ (16), reported to elicit redox cycling without arylating thiols, did not cause fluorescence quenching but only partially oxidized roGFPs even after 24 h. Surprisingly, a given oxidant concentration and exposure time generally caused more oxidation of roGFP2 than those of roGFP1, even though roGFP1 is thermodynamically a stronger reductant (*i.e.* has a more negative E'_0) than roGFP2.

Oxidation of Redox Probes in HeLa Cells—When roGFPs were transiently expressed in HeLa cells, expression was ob-

served throughout the cytosol and nucleus and ratios were comparable for all of the areas of the cell. Oxidants such as aldrithiol increased the excitation ratio (400/480 nm for roGFP1, 400/495 nm for roGFP2) over a few minutes in a spatially uniform manner (Fig. 3). Upon washing out the oxidant, the cells slowly reduced the roGFPs. Administering DTT usually accelerated this reaction. To calibrate these ratios, we attempted to use saturating doses of membrane-permeant reductants and oxidants to establish minimum and maximum ratios *in situ*. However, we generally could not achieve ratios as extreme as those from fully reduced or oxidized protein in microdroplets of buffer observed with the same imaging system (Fig. 4). Based on the *in vitro* ratios, roGFP1 in unperturbed HeLa cells was 16% oxidized, implying a basal redox potential of −315 mV. After the addition of 4 mM DTT or 0.5 mM aldrithiol (concentrations determined to cause maximal effects), the extent of roGFP1 oxidation changed to 6.5 and 72%, respectively. Unperturbed cells maintained roGFP2 5% oxidized, after which DTT reduced the ratio to 0% and aldrithiol oxidized the protein to a slightly greater ratio than obtainable in droplets of buffer on the microscope stage. Thus, roGFP2 reports a basal redox potential of −325 mV, reasonably consistent with the value seen by roGFP1.

Cells transiently expressing roGFPs were treated with 0.1 mM of the oxidants tested *in vitro* with the exception of GSSG,
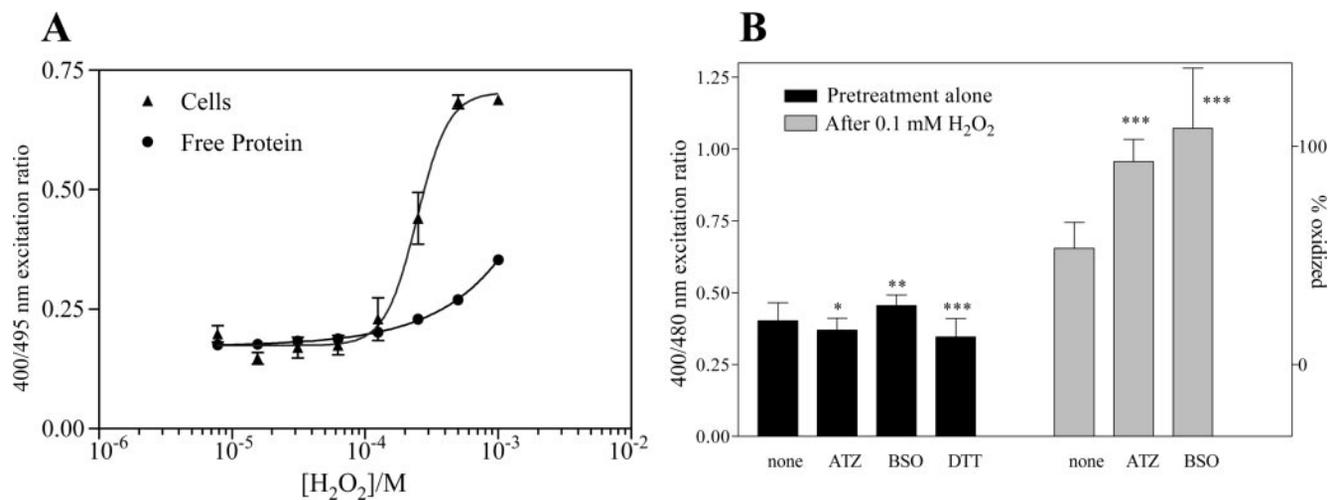
FIG. 6. **Reactivity of roGFPs to hydrogen peroxide in HeLa cells.** *A*, effective concentration for hydrogen peroxide induced oxidation in a HeLa cell line stably expressing roGFP2, and *in vitro*, the EC_{50} value obtained in HeLa cells was 213 ± 65 μM ($n = 3$). *B*, altering the GSH:GSSG ratio in the cell by pretreating cells with BSO (100 μM for 2 h) or aminotriazole (50 mM for 10 min) had small but significant effects on basal ratios, but those obtained after hydrogen peroxide treatment (100 μM) were greater (***, $p < 0.005$; **, $p < 0.05$; * $p < 0.5$) in HeLa cells transiently expressing roGFP1.

which does not cross cell membranes. Similar results were observed for both roGFP1 and roGFP2 (Fig. 5). The rank order of oxidant efficacies, aldrithiol, diamide > H_2O_2, menadione, DMNQ > oxidized BMES, and lipoate was similar to that *in vitro*, but 0.1 mM hydrogen peroxide or the quinones produced robust increases in ratio in cells within 10 min, whereas even 1 mM concentrations were not capable of full oxidation *in vitro*. Although oxidized BMES and lipoate were ineffective at 0.1 mM, at 5 mM they did cause considerable oxidation. However, this maximal effect was still somewhat less than that produced by 0.1 mM aldrithiol, so we concluded that redox buffers made from BMES and lipoate would probably not clamp intracellular redox potentials robustly enough to serve as standards for *in situ* calibration.

To determine the roles of glutathione and catalase in maintaining cellular redox status under basal and H_2O_2-challenged conditions, cells were pretreated with buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, or aminotriazole, a catalase inhibitor (Fig. 6). GSH depletion by BSO resulted in a small but significant ($p < 0.05$) increase in the resting excitation ratio of the probe and an even greater extent of oxidation due to (100 μM) hydrogen peroxide ($p < 0.005$). Pretreatment with BSO for 2, 5, or 24 h showed similar results. Pre-incubation with aminotriazole did not significantly change the basal excitation ratio (*i.e.* had no effect on the basal oxidation state) but was almost as effective as BSO at enhancing the response to 100 μM hydrogen peroxide. Identification of the reductase responsible for reducing the roGFPs in the cell was inconclusive, but reduction is most likely enzyme-dependent because it is observed immediately after removal of oxidant when there is little glutathione available for reduction. Unfortunately, the inhibitors of the NADPH reductases are selective rather than specific. We observed that inhibition of reduction following a 1-h preincubation with 100 μM cisplatin (thioredoxin reductase inhibitor (17)) and 200 μM 5-methoxyindole-2-carboxylic acid (dihydrolipoamide dehydrogenase inhibitor (18)) both prevented cells from reducing roGFPs after brief exposure to aldrithiol (Fig. 7). BCNU (carmustine) interacts with the roGFPs directly and could not be used for inhibition studies.

A possible explanation for H_2O_2 and the quinones being much more potent in cells than *in vitro* is that in cells they can generate reactive oxygen species (ROS) such as superoxide, O_2^-, which might react more rapidly with the roGFPs. The superoxide generated *in vitro* by reaction of xanthine with
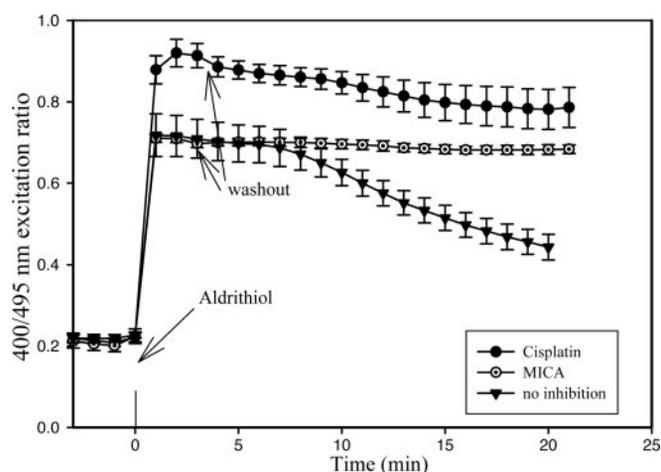
FIG. 7. **Inhibition of cellular reduction of roGFP2.** The reduction normally observed following oxidation with 100 μM aldrithiol was inhibited following a 1-h incubation with 200 μM 5-methoxyindole-2-carboxylic acid (*MICA*) or a 45-min incubation with 100 μM cisplatin.

xanthine oxidase was able to oxidize roGFPs to a modest extent (Fig. 8) but was greater than air oxidation observed in the control. However, the identical superoxide-generating system placed outside roGFP-expressing cells caused a much larger and faster response, even though superoxide would have to diffuse across the plasma membrane to reach the roGFP. Therefore, the effect of superoxide itself seems to be amplified inside cells. We considered the possibility that hydroxyl radicals generated from H_2O_2 by Fenton chemistry might be the kinetically reactive oxidants, but the addition of Fe(II) to H_2O_2 and roGFPs *in vitro* destroyed the protein fluorescence rather than accelerate the normal change in emission ratio. Another hypothesis might be that glutathione peroxidase or related enzymes either catalyze H_2O_2 reaction with roGFPs or generate enough GSSG to oxidize the roGFPs by thiol-disulfide exchange. However, commercially available glutathione peroxidase failed to accelerate H_2O_2 reaction *in vitro* with roGFPs with or without added GSH. Therefore, the basis for the enhanced H_2O_2 response in cells remains unclear.

Redox Responses during Physiological Stimulation—Can roGFPs detect redox changes under physiological conditions and not just direct nonphysiological oxidants and reductants as
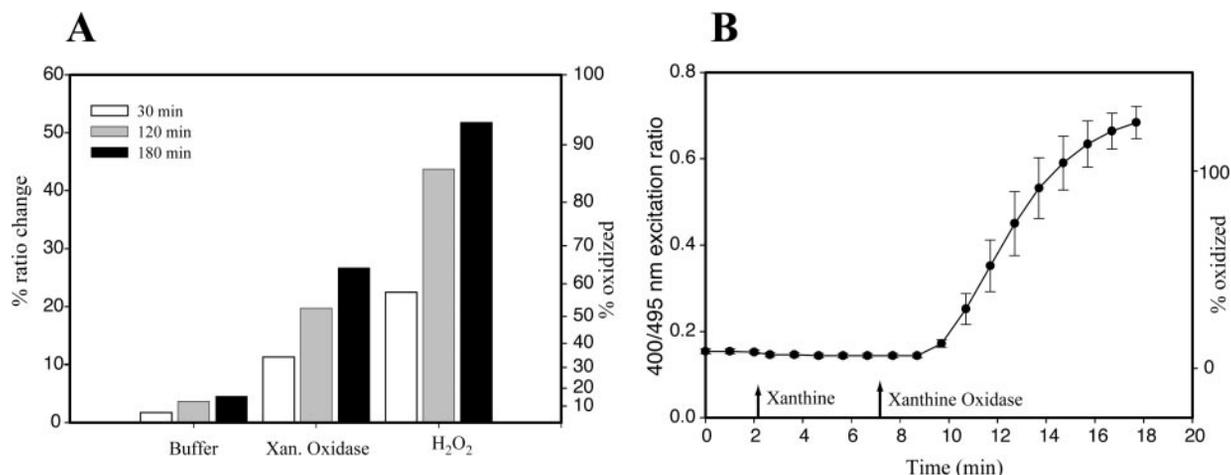
FIG. 8. **Superoxide-induced oxidation of roGFP2.** *A*, oxidation of roGFP2 (0.75 μM) following incubation with xanthine oxidase (50 milliunits) and xanthine (50 μM) under aerobic conditions or co-incubated with 750 μM H_2O_2. *B*, oxidation of roGFP2 expressed in HeLa cells following extracellular production of superoxide by xanthine oxidase (36 milliunits) and xanthine (200 μM). roGFP1 gave similar results.

tested above? The simplest challenge was to vary the partial pressure of O_2 from 0 to 1 atm, corresponding to pO_2 values ranging from anoxic to hyperoxic. Media bubbled with pure N_2 or O_2 were passed over roGFP-expressing HeLa, HEK293, PC12, or P388D1 cells in a closed perfusion chamber, but neither treatment caused any detectable change in the excitation ratios compared with air-saturated medium, even when the cells had been pretreated with BSO (reducing GSH levels) or aminotriazole (inhibiting catalase). Thus, cytosolic roGFPs (and presumably the intracellular redox potential) seem to be very well buffered against simple changes in pO_2, in contrast to their sensitivity to H_2O_2, superoxide, and quinones.

One of the most dramatic oxidative events in mammalian cells is the oxidative burst in immune cells, which plays a major role in destroying pathogens (4). Therefore, we expressed roGFPs in HL60 cells induced to differentiate into monocytes by incubation in 1.3% Me_2SO for 7 days (19) and then stimulated with PMA to activate protein kinase C. Observation of time courses by microscopy was difficult because of poor transfection efficiency by electroporation (30%), incomplete transformation by Me_2SO, and nonadherence of the cells. However, we were able to obtain the ratios by flow cytometry (FACSVantage SE, BD Biosciences). Upon application of 20 μM PMA, the roGFPs indicated substantial oxidation within 10–15 min (Fig. 9). Diphenyleneiodonium could not be used to inhibit NADPH oxidase (20), because preincubation with this compound prevented oxidation of the roGFPs by aldrithiol. This effect was not observed *in vitro*. Inhibition of the PMA-induced oxidation was observed with AEBSF (21) and apocynin (22), two other inhibitors of NADPH oxidase.

Localization of roGFP2 to Plasma Membranes—Endogenous production of H_2O_2 has also been implicated in growth factor responses such as in NR6 cells treated with epidermal growth factor (EGF) (23) or HeLa cells stimulated with lysophosphatidic acid (LPA) (24). The concentrations of H_2O_2 and other ROS attained during such signaling are still unknown but presumably much less than those used to kill pathogens during oxidative bursts. We were unable to detect any oxidation of the probe following the addition of EGF to NR6 cells expressing EGF receptor or LPA to HeLa cells. As a positive control, flow cytometry with dichlorodihydrofluorescein diacetate (data not shown) verified the previously reported increase in oxidation of this traditional probe to the dichlorofluorescein product (23). Presumably, the dye is more sensitive than the proteins because oxidation of the dye is irreversible so that it qualitatively integrates even significant localized and transient production
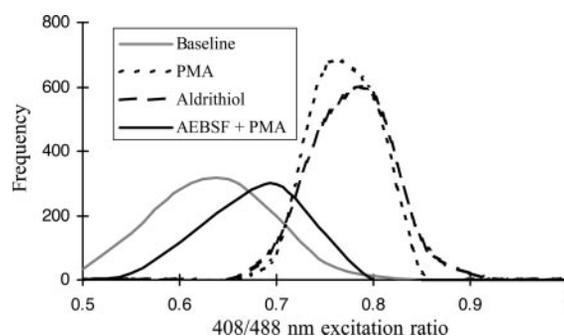
FIG. 9. **Detection of superoxide burst in differentiated HL60 cells.** roGFP2 F223K/A206K was expressed in HL60 cells differentiated with 1.3% Me_2SO for 5 days. Histograms of excitation ratios are shown for unstimulated cells (*gray line*), cells stimulated with 50 μM PMA (*dotted line*), and cells treated with 100 μM aldrithiol (*dashed line*) and preincubated with AEBSF (500 μM) followed by 50 μM PMA (*solid line*). Similar results (not shown) were obtained with roGFP1.

of ROS. By contrast, roGFPs are reversible indicators that should track the overall redox equilibrium of the cell, which may well be constant.

One possible explanation for the different responses of the roGFP *versus* dichlorofluorescein to growth factors was that the dye was integrating localized increases in oxidant potential, whereas the overall redox potential of the entire cell, recorded using roGFPs, remained constant. Therefore, we tested whether redox responses were highly localized near the growth factor receptors on the plasma membrane (Fig. 10). We targeted roGFP2 to the plasma membrane using a N-terminal myristoylation and palmitoylation signal from Lyn, a tyrosine kinase protein belonging to the Src family. Following transfection into NR6 and HeLa cells, the redox probe was indeed localized to the plasma membrane. Responses to aldrithiol and to DTT were noticeably faster than those of untargeted roGFP2. However, the membrane-targeted protein remained unreactive to stimulation by EGF or LPA. Targeting the roGFPs to the mitochondria and the nucleus did not result in any significant difference in activity to roGFP expressed in the cytosol.

Increasing the Sensitivity of roGFPs to H_2O_2—Responses of the current roGFPs generally take minutes or longer, which may be too slow to detect some signaling events. Redox kinetics of thiols are often limited by the need to deprotonate the thiol before it can oxidize. Placing positive charges near cysteine residues increases sensitivity to oxidation by H_2O_2 (25), pre-
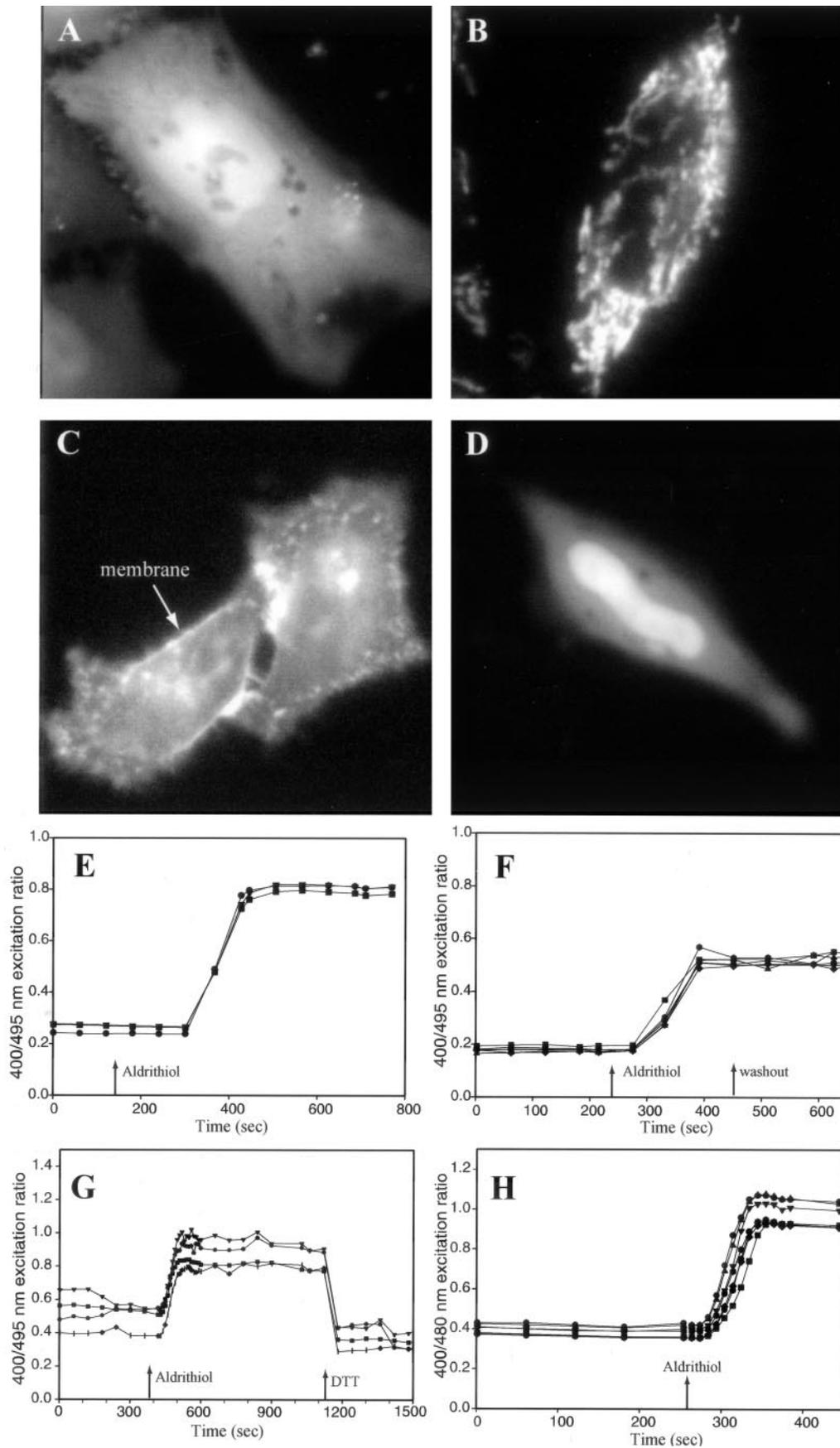
FIG. 10. **Targeting of the redox probes.** Fluorescence of roGFP2 (495-nm emission) targeted to cytosol (*A*), mitochondria (*B*), plasma membrane (*C*), or fluorescence (*D*) of roGFP1 (400-nm emission) targeted to the nucleus. The constructs did not reveal significant differences in roGFP2 reactivity to aldrithiol (100 μM) in the cytosol (*A* and *E*) and mitochondria (*B* and *F*), at the plasma membrane (*C* and *G*), or roGFP1 in the nucleus (*D* and *H*). Each trace within *panels E–H* indicates a separate cell.
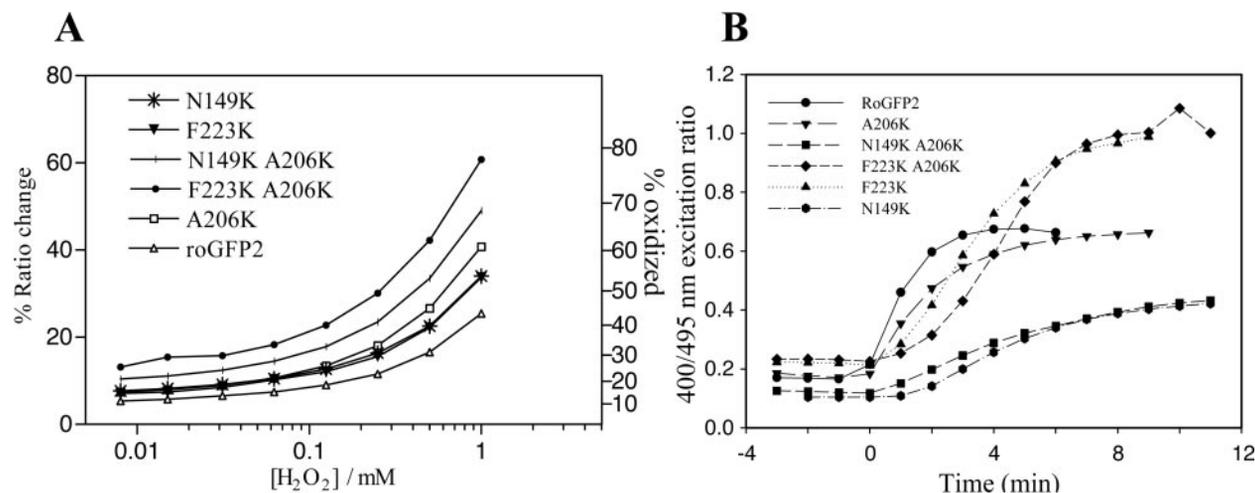
FIG. 11. **Responses of lysine mutants of roGFP2.** *A*, reactivity of lysine mutants to H_2O_2 *in vitro* after 1 h. *B*, time course for response of roGFP2 lysine mutants in HeLa cells to 100 μM H_2O_2 added at time 0.

sumably by facilitating deprotonation. Therefore, we designed a series of roGFP variants with outward-facing lysine residues placed in close proximity to the key cysteine residues. Asn-149, Ala-206, and Phe-223 were individually replaced with lysine. Two double mutants were also prepared in which both positions 149 and 223 or positions 206 and 223 were replaced with lysine. Mutated proteins were isolated and tested *in vitro*. All exhibited greater sensitivity to oxidation by H_2O_2 (Fig. 11*A*). Five of the mutated proteins were transfected into HeLa cells, but only F223K and F223K/A206K showed a larger response to H_2O_2 than those of the parent roGFP2 (Fig. 11*B*). These two mutants were tested for responses to EGF stimulation, but oxidation of the mutated roGFPs was still not detected.

DISCUSSION

GFP-based probes of redox potential offer many advantages over previous methods for assessing redox status. Genetic encoding means that the probes can be introduced into any cell or organism that can express recombinant cDNA, that the proteins can be targeted to specific subcellular locations or tissue distributions, and that reagent distribution costs are minimized. Continuous nondestructive monitoring of redox potential with a reversible indicator is far easier and higher in spatial and temporal resolution than traditional discontinuous sampling using destructive assays of thiol and disulfide contents. A similar redox probe based on yellow fluorescent protein (YFP) has been reported previously (26), but this probe gives only an intensity change, not a ratiometric response. In each case, disulfide formation favors protonation of the chromophore, which typically quenches YFPs completely but shifts excitation maxima of GFPs to ~400 nm as observed here. Ratiometric output is valuable not only for all of the usual reasons of indifference to variable expression levels, such as cell thickness, lamp intensities, detector sensitivities, and photobleaching, but also to distinguish genuine redox changes from artifacts such as arylation of the probe as observed here with menadione and roGFP1. By the standards of other GFP-based indicators, roGFPs give fairly large changes in ratio from maximally reduced to maximally oxidized, up to 6-fold *in vitro* and up to ~3-fold in cytosol of viable mammalian cells.

Quantitative calibration of redox potentials reported by roGFPs posed some unexpected problems. We felt that roGFPs should be re-titrated in redox buffers whose midpoint potential E'_0 was a better match for the proteins than that of DTT. Eventually, we succeeded with dihydrolipoate and bis(mercaptoethyl)sulfone, although the E'_0 of the latter had to be redetermined in a fully aqueous medium. The resulting estimates

of $E'_0 = -294$ and -287 mV for roGFP1 and roGFP2, respectively, are only slightly more negative than the independently measured values of Hanson *et al.* (9) based on DTT, -288 and -272 mV, respectively (9). For future work, we suggest consensus average values of -291 and -280 mV for roGFP1 and roGFP2, respectively (9). Thus, the roGFPs require significantly stronger reducing conditions than rxYFP whose E'_0 was reported to be -261 mV (26). Østergaard *et al.* (26) reported that rxYFP was 50% oxidized when expressed in bacteria.

Oxidation of the probes by a panel of common oxidants was studied *in vitro* to determine relative oxidation rates before examination *in vivo*. Strong oxidants such as aldrithiol and diamide fully oxidized the probes in minutes. Hydrogen peroxide was also found to be a weak oxidant. Even 1 mM concentrations did not fully oxidize the protein even after a 24-h incubation. Prolonged exposure to menadione caused a reduction in fluorescence when excited at either peak, and it was found to arylate the cysteine thiols of the redox probes as it has been found to do with other proteins (13). However, the dimethoxy analogue DMNQ caused only modest oxidation after 24 h *in vitro*.

When expressed in the cytoplasm of HeLa cells, roGFP1 and roGFP2 were either 84 or 95% reduced, implying a basal redox potential of -315 and -325 mV, respectively, at pH 7.0. The apparent redox potential of the mitochondrial matrix is even more negative, approximately -360 mV (9), because the pH of that compartment is almost 8. These values measured with roGFPs suggest that the cytosol and mitochondria are much more reducing than predicted from measurements of intracellular GSH and GSSG (-200 to -240 mV) (27, 28). The discrepancy persists even during glucose deprivation, which has been reported to make cells less reducing. Therefore, we suspect that the various redox couples in the cell are not in equilibrium with each other. Instead, the NADPH, dihydrolipoamide, and thioredoxin redox couples may have to be poised substantially more negatively than the GSH/GSSG redox pair in order to drive substantial net flow of electrons to GSSG. Although reduced roGFPs react with GSSG *in vitro*, albeit slowly (Fig. 2), oxidized roGFPs are difficult or impossible to reduce with GSH *in vitro*. This unreactivity is probably because the E'_0 values are so far apart that the slightest contamination of GSH with GSSG is enough to make the reaction thermodynamically unfavorable at millimolar concentrations of GSH. roGFPs do react readily and reversibly with more strongly reducing couples such as dihydrolipoate/lipoate, so they will primarily reflect the potential of the most strongly reducing redox buffers in the cell.

Application of exogenous membrane-permeant oxidants to cells initiated a change in ratio of the roGFP probes. Although the extent of the ratio and the time required to reach a plateau differed among the oxidants, the reactivity closely followed that seen in experiments *in vitro*. The major exception was hydrogen peroxide, which was found to react at 100 μM concentrations in the cells, whereas millimolar concentrations were required *in vitro*. Thus, we wished to determine whether the redox probes would be sensitive to direct oxidation by other ROS agents such as superoxide and we found that xanthine oxidase + xanthine (as an external source for superoxide (29)) caused oxidation of the roGFPs both extracellularly and intracellularly.

Although air slowly oxidizes unprotected reduced roGFPs *in vitro*, shifting from air to 100% O_2 failed to change the resting ratio of roGFPs expressed in a number of cell lines including HeLa, HEK293, NR6, PC12, and P388D1. This may not simply be due to insensitivity of the probe because dihydrorhodamine 123 has also been found to be unresponsive to hyperoxic conditions (30). A 20-min exposure of cells to anoxia also did not affect the resting ratio. The ratio remained unchanged even under glucose-free media or serum starvation conditions known to sensitize cells to oxidative damage. It appears then that the cells possess a strong mechanism for preventing oxidation in the cytoplasm, which was not overcome in the duration of our experiments.

Phagocytes produce ROS such as superoxide as a primary response to bacterial invasion. These cells contain high quantities of NADPH oxidase, a complex capable of producing superoxide as a consequence of its enzymatic oxidation of NADPH to NADP. Phorbol myristic acid stimulates superoxide production in macrophages through the activation of protein kinase C. We used a human cell line HL60 prestimulated to differentiate to monocytes with Me_2SO cells (31). Stimulation of the human cell line HL60 prestimulated to differentiate to monocytes transfected with roGFPs with PMA caused an increase in the fluorescence ratio consistent with oxidation of the protein. The lysine replacement roGFP2 mutant F223K/A206K yielded the largest response to PMA treatment in accordance with its highest sensitivity to oxidation. When cells were pretreated with an inhibitor for NADPH oxidase, diphenylene iodonium, the oxidation induced by PMA was eradicated. We were concerned that diphenylene iodonium may inactivate the protein, because the addition of aldrithiol (which should directly oxidize the protein) after treatment with diphenylene iodonium did not induce oxidation. However, other inhibitors of NADPH oxidase such as apocynin and AEBSF, which act by different reaction mechanisms, also precluded the PMA-induced oxidation.

Many studies have argued that ROS may act as a second messenger in signaling by growth factors such as platelet-derived growth factor, EGF, and TNF and that H_2O_2 induces NF-κB (32) and AP-1 (33). It has been long recognized that high levels of ROS trigger apoptosis and necrosis. A more recent theory argues that differential GSH levels may discriminate between H_2O_2 acting as an oxidative stressor or a second messenger in growth factor regulation (34). Using roGFPs, we were unable to demonstrate any response of the redox probes to two growth factor stimuli reported to generate cellular H_2O_2: EGF in NR6 cells or LPA in HeLa cells. Targeting the probe to the plasma membrane or mutating the roGFPs to enhance sensi-

tivity to H_2O_2 failed to uncover sensitivity to EGF in NR6 cells or by LPA in HeLa cells, even though the modified probes still reacted to exogenous oxidants. Meanwhile, we verified in parallel experiments that the classic ROS probe 2′,7′-dichlorodihydrofluorescein diacetate in NR6 cells responded to EGF, although 2′,7′-dichlorodihydrofluorescein diacetate has been reported to be vulnerable to autocatalytic oxidation or other artifacts (35, 36). Therefore, we doubt that the above growth factors cause significant acute global perturbations in thiol-disulfide redox potential. If there is a chemically significant change in thiol-disulfide redox status, it would probably have to be highly compartmentalized.

REFERENCES

1. Krauth-Siegel, R. L., Jockers-Scherubl, M. C., Becker, K., and Schirmer, R. H. (1989) *Biochem. Soc. Trans.* **17,** 315–317
2. Forman, H. J., and Torres, M. (2002) *Am. J. Respir. Crit. Care Med.* **166,** S4–S8
3. Forman, H. J., and Torres, M. (2001) *Mol. Aspects Med.* **22,** 189–216
4. Droge, W. (2002) *Physiol. Rev.* **82,** 47–95
5. Yanping, L., and David, D. G. (2002) *Clin. Exp. Pharmacol. Physiol.* **29,** 305–311
6. Rojkind, M., Dominguez-Rosales, J. A., Nieto, N., and Greenwel, P. (2002) *Cell Mol. Life Sci.* **59,** 1872–1891
7. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502
8. Liu, S., Ansari, N. H., Wang, C., Wang, L., and Srivastava, S. K. (1996) *Curr. Eye Res.* **15,** 726–732
9. Hanson, G. T., Aggeler, R., Oglesbee, D., Capaldi, R. A., Tsien, R. Y., and Remington, S. J. (2004) *J. Biol. Chem.* **279,** 13044–13053
10. Goodrow, M. H., and Musker, W. K. (1981) *Synthesis* 457–459
11. Lamoureux, G. V., and Whitesides, G. M. (1993) *J. Org. Chem.* **58,** 633–641
12. Lees, W. J., Singh, R., and Whitesides, G. M. (1991) *J. Org. Chem.* **56,** 7328–7331
13. Di Monte, D., Ross, D., Bellomo, G., Eklow, L., and Orrenius, S. (1984) *Arch. Biochem. Biophys.* **235,** 334–342
14. Mirabelli, F., Salis, A., Perotti, M., Taddei, F., Bellomo, G., and Orrenius, S. (1988) *Biochem. Pharmacol.* **37,** 3423–3427
15. Ross, D., Thor, H., Orrenius, S., and Moldeus, P. (1985) *Chem. Biol. Interact.* **55,** 177–184
16. Gant, T. W., Ramakrishna, D. N., Mason, R. P., and Cohen, G. M. (1988) *Chem. Biol. Interact.* **65,** 157–173
17. Arner, E. S., Nakamura, H., Sasada, T., Yodoi, J., Holmgren, A., and Spyrou, G. (2001) *Free Radic. Biol. Med.* **31,** 1170–1178
18. Haramaki, N., Han, D., Handelman, G. J., Tritschler, H. J., and Packer, L. (1997) *Free Radic. Biol. Med.* **22,** 535–542
19. Collins, S. J. (1987) *Blood* **70,** 1233–1244
20. Hampton, M. B., and Winterbourn, C. C. (1995) *Free Radic. Biol. Med.* **18,** 633–639
21. Diatchuk, V., Lotan, O., Koshkin, V., Wikstroem, P., and Pick, E. (1997) *J. Biol. Chem.* **272,** 13292–13301
22. Van den, W. E., Beukelman, C. J., Van den Berg, A. J., Kroes, B. H., Labadie, R. P., and Van Dijk, H. (2001) *Eur. J. Pharmacol.* **433,** 225–230
23. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J. Biol. Chem.* **272,** 217–221
24. Sekharam, M., Cunnick, J. M., and Wu, J. (2000) *Biochem. J.* **346,** 751–758
25. Kim, J. R., Yoon, H. W., Kwon, K. S., Lee, S. R., and Rhee, S. G. (2000) *Anal. Biochem.* **283,** 214–221
26. Østergaard, H., Henriksen, A., Hansen, F. G., and Winther, J. R. (2001) *EMBO J.* **20,** 5853–5862
27. Hwang, C., Lodish, H. F., and Sinskey, A. J. (1995) *Methods Enzymol.* **251,** 212–221
28. Schafer, F. Q., and Buettner, G. R. (2001) *Free Radic. Biol. Med.* **30,** 1191–1212
29. Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V. (1974) *J. Biol. Chem.* **249,** 4363–4382
30. Royall, J. A., and Ischiropoulos, H. (1993) *Arch. Biochem. Biophys.* **302,** 348–355
31. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1979) *J. Exp. Med.* **149,** 969–974
32. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258
33. Karin, M., and Shaulian, E. (2001) *IUBMB Life* **52,** 17–24
34. De Bleser, P. J., Xu, G., Rombouts, K., Rogiers, V., and Geerts, A. (1999) *J. Biol. Chem.* **274,** 33881–33887
35. Rota, C., Fann, Y. C., and Mason, R. P. (1999) *J. Biol. Chem.* **274,** 28161–28168
36. Liochev, S. I., and Fridovich, I. (1998) *Free Radic. Biol. Med.* **25,** 926–928