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A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression

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Conour, J. E., W. V. Graham, and H. R. Gaskins. A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression. *Physiol Genomics* 18: 196–205, 2004. First published May 11, 2004; 10.1152/physiolgenomics.00058.2004.—The intracellular reduction-oxidation (redox) environment influences cell cycle progression; however, underlying mechanisms are poorly understood. To examine potential mechanisms, the intracellular redox environment was characterized per cell cycle phase in Chinese hamster ovary fibroblasts via flow cytometry by measuring reduced glutathione (GSH), reactive oxygen species (ROS), and DNA content with monochlorobimane, 2′,7′-dichlorohydrofluorescein diacetate (H_2DCFDA), and DRAQ5, respectively. GSH content was significantly greater in G_2/M compared with G_1 phase cells, whereas GSH was intermediate in S phase cells. ROS content was similar among phases. Together, these data demonstrate that G_2/M cells are more reduced than G_1 cells. Conventional approaches to define regulatory mechanisms are subjective in nature and focus on single proteins/pathways. Proteome databases provide a means to overcome these inherent limitations. Therefore, a novel bioinformatic approach was developed to exhaustively identify putative redox-regulated cell cycle proteins containing redox-sensitive protein motifs. Using the InterPro (**http://www.ebi.ac.uk/interpro/**) database, we categorized 536 redox-sensitive motifs as: *1*) active/functional-site cysteines, *2*) electron transport, *3*) heme, *4*) iron binding, *5*) zinc binding, *6*) metal binding (non-Fe/Zn), and *7*) disulfides. Comparing this list with 1,634 cell cycle-associated proteins from Swiss-Prot and SpTrEMBL (**http://us.expasy.org/sprot/**) revealed 92 candidate proteins. Three-fourths (69 of 92) of the candidate proteins function in the central cell cycle processes of transcription, nucleotide metabolism, (de)phosphorylation, and (de)ubiquitinylation. The majority of oxidant-sensitive candidate proteins (68.9%) function during G_2/M phase. As the G_2/M phase is more reduced than the G_1 phase, oxidant-sensitive proteins may be temporally regulated by oscillation of the intracellular redox environment. Combined with evidence of intracellular redox compartmentalization, we propose a spatiotemporal mechanism that functionally links an oscillating intracellular redox environment with cell cycle progression.

redox regulation; bioinformatics

THE INTRACELLULAR reduction-oxidation (redox) environment is constituted by reactive oxygen species (ROS) and intracellular antioxidants, primarily the thiol-containing tripeptide glutathione (Glu-Cys-Gly). ROS are highly reactive oxygen derivatives produced as byproducts of aerobic respiration in the electron transport chain of the mitochondrial inner membrane (42). Glutathione reacts with ROS to prevent oxidative damage to intracellular molecules (DNA, proteins, and lipids; 16, 60). Perturbations in the intracellular redox environment are reflected in the relative redox status of the oxidized (GSSG) vs. reduced (GSH) forms of glutathione. Thus cellular redox status is typically assessed by quantifying GSH and GSSG and calculating the half-cell redox potential (E_h, expressed in mV) of the GSSG/2GSH couple with the Nernst equation (4, 40, 45). In this way, intracellular E_h values ranging from −165 (oxidized) to −258 mV (reduced) have been measured in various cell types and cell states (24, 40).

A correlation between intracellular redox status and cell proliferation vs. differentiation has been observed for numerous cell types. Specifically, fibroblasts (20, 35), intestinal epithelial cells (24, 33), cardiomyocytes (38), and osteoclasts (53) are relatively reduced when proliferating and relatively oxidized once differentiated. Intracellular redox status also affects cell cycle progression (20, 31, 35, 48); however, underlying molecular mechanisms remain undefined. The numerous examples of redox modulation of transcription (41, 61), signal transduction (54), biosynthetic pathways (11), and post-translational modification (37) attest to the potential to which the intracellular redox environment may influence cell cycle progression. Indeed, several redox-sensitive proteins involved in cell cycle progression have been identified, including p53 (9, 15, 56), AP-1 (43, 44), NF-κB (19, 41), PKC (7, 13), and low-molecular-weight protein tyrosine phosphatases (10).

As a first step toward understanding the link between the intracellular redox environment and cell cycle progression, we examined changes in the intracellular redox environment associated with cell cycle progression in untreated, asynchronously growing Chinese hamster ovary (CHO) fibroblasts. Identification of redox-sensitive amino acids or redox-sensitive structures within proteins, such as AP-1 (1, 25) or low-molecular-weight protein tyrosine phosphatases (10), may be used to predict cell cycle proteins that provide a regulatory link between the intracellular redox environment and cell division. Accordingly, a subsequent bioinformatic approach, which takes advantage of extensive proteome and protein motif databases, was implemented to exhaustively identify candidate redox-regulated proteins that may provide a mechanistic link between the intracellular redox environment and control of cell cycle progression.

MATERIALS AND METHODS

Cell culture. CHO fibroblasts were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with a final glucose concentration of 25 mM, 0.1 mM nonessential amino acids (GIBCO; Invitrogen, Grand Island, NY), 44 mM sodium bicarbonate, 15 mM HEPES, 10% fetal bovine serum (FBS), streptomycin (10,000 U/ml), penicillin (10,000 U/ml), and Fungizone (250 μg/ml) in T-75 cell

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culture flasks (Costar, Cambridge, MA). The cells were maintained at 37°C in 5% CO_2.

Analysis of the intracellular redox environment per cell cycle phase. The intracellular redox environment was examined per cell cycle phase in live CHO cells using the fluorescent dyes monochlorobimane (mBCl; Molecular Probes, Eugene, OR; 17) for GSH and 2′,7′-dichlorohydrofluorescein diacetate (H_2DCFDA; Molecular Probes; 62) for ROS by flow cytometric analysis. Cell cycle phases were distinguished by the addition of the anthraquinone derivative DRAQ5 (Biostatus, Shepshed, UK), which fluoresces in the deep red spectrum and has a high affinity for DNA (49). mBCl is a membrane-permeant probe that fluoresces in the UV spectrum upon reacting with GSH in a reaction catalyzed by the enzyme glutathione-*S*-transferase (17). Due to the enzymatic catalysis of mBCl-GSH adduct formation, mBCl has greater specificity for GSH compared with other thiol-specific probes such as monobromobimane, which reacts freely with both GSH and intracellular protein thiols (17). Once the membrane-permeant H_2DCFDA enters a cell, its acetate moieties are cleaved by intracellular esterases resulting in an impermeable H_2DCF form. Subsequent oxidation of H_2DCF produces the fluorescent 2′,7′-dichlorofluorescein, which can be detected in the green spectrum.

Cells grown to confluence were subcultured into 6-well plates (Costar) at a density of 4.0×10^5 cells per well and incubated in normal medium overnight at 37°C. Parallel experiments were seeded concurrently from the same culture for ROS and GSH analysis. For GSH measurement, cells were washed with Hanks' balanced salt solution (HBSS; GIBCO) and treated with 1 ml of warm HBSS containing 80 μM mBCl and 15 μM DRAQ5 for 30 min at 37°C. The cells were subsequently trypsinized with 0.5 ml of 1× trypsin/EDTA in HBSS and resuspended with 0.5 ml HBSS, aliquoted into 1.5-ml microcentrifuge tubes, and stored on ice until further analysis. For ROS measurement, the cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; GIBCO) and treated with 1 ml of warm PBS containing 10 μM H_2DCFDA and 15 μM DRAQ5 for 30 min at 37°C. The cells were then trypsinized, resuspended in 0.5 ml PBS, and stored in 1.5-ml microcentrifuge tubes on ice until further analysis.

Flow cytometric analysis. Intracellular fluorescence of CHO cells stained for DNA, GSH, and ROS content was measured by flow cytometry with a MoFlo MLS high-speed flow cytometry instrument (Cytomation, Fort Collins, CO) equipped with a Coherent Innova 90C laser for excitation at 488 nm, a Coherent Innova 70C Spectrum for excitation at 647 nm, and a Coherent I90 for excitation at 351 nm. All three lasers operated at 100 mW. DRAQ5, mBCl, and H_2DCFDA fluorescence levels were detected using 675/30, 530/40, and 485/25 band-pass filters, respectively. Flow cytometric data were analyzed using Summit V3.1 software (Cytomation). Initially, cells were gated by scatter properties to exclude cellular debris. Peak forward scatter was plotted against pulse width to exclude doublets. Separate bar regions were used to gate separate cell cycle phases on DNA curves to determine the fluorescence intensity of GSH and ROS probes in separate histograms. A minimum of 10,000 gated events were analyzed per sample.

Statistics. Results from flow cytometric analyses are the mean values of four separate experiments (n = 3–4 per experiment). One-way ANOVA statistical tests were used to compare phase-specific data. When a significant main effect was observed, differences between groups were determined using t-tests. Values of $P < 0.05$ were considered statistically significant.

Identification of protein redox motifs. A list of putative redox-regulated protein motifs (redox motif) was assembled using the InterPro (IPR) protein signature database (**http://www.ebi.ac.uk/interpro/**), an integrated database of protein domains, families, and functional sites (3). The redox motif list was constructed using the Sequence Retrieval System (SRS) web server at EBI (**http://srs.ebi.ac.uk/**) as follows. Initially, a broad, text-based search was performed for all entries associated with the key words reduction, oxidation, redox, electron transfer, metal binding, heme, cysteine, and disulfide. Next, motifs identified from the key word search were manually examined to establish their redox sensitivity in relation either to their structure, function, or cofactor association (e.g., metal binding) before being considered redox motifs. The coverage of this approach is limited by the extent to which redox is associated with protein motif annotation. For example, p53 has been demonstrated to be redox regulated (9, 15) and also has an associated IPR accession number (IPR008967). However, this motif does not establish a connection with redox regulation and so was not included in the list.

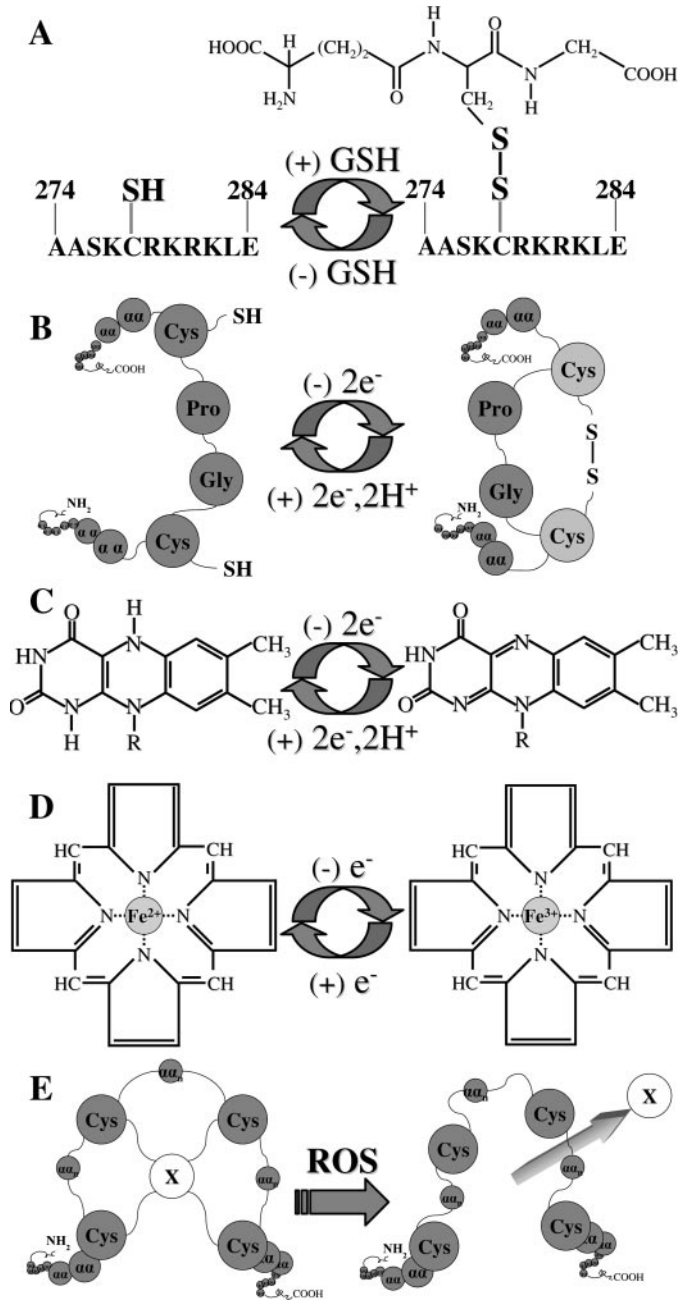

Fig. 1. Representational diagram of redox-regulated protein motifs. *A*: active/binding site cysteine coupled with glutathione. *B*: disulfide bond formation within a thioredoxin domain. *C*: the electron transporting ring system of a flavin under reduced and oxidized conditions. *D*: oxidized and reduced forms of a heme moiety. *E*: general structure of a metal-binding domain where "X" can be different metals including Zn^{2+}.

Query results consisting of IPR accession numbers and corresponding descriptions for each entry were downloaded and formatted using Excel (Microsoft, Redmond, WA). The results (redox motifs) were categorized into the following categories based on specific structural motifs or relevant redox chemistry: active/functional-site cysteines (AC), disulfides (SS), electron transport, heme (H), iron binding (FeB), metal binding (MeB, exclusive of zinc and iron), and zinc binding (ZnB; Fig. 1). These categories were chosen based on prior evidence (redox-sensitive amino acid sequence or redox cofactor binding ability) linking each with redox modulation of protein function (2, 18, 25, 55, 58, 63).

Cell cycle-associated proteins. Proteins involved in cell cycle progression were queried for putative redox motifs. To limit the query, only eukaryotic proteomes exclusive of the kingdom Viridiplantae were included. The Swiss-Prot and SpTrEMBL protein databases were used as the sources for assembling the cell cycle protein list. The Swiss-Prot database is a curated protein database with a low level of redundancy and extensive integration with other databases including IPR (5). The SpTrEMBL database is also integrated with IPR and serves as a database for proteins that are eligible for addition to the Swiss-Prot database upon further validation. Using the EBI SRS Web server, we performed a text-based search of the Swiss-Prot and SpTrEMBL databases for the key words proliferation, mitosis, cell division, cell cycle, replication, or cytokinesis, to select proteins either directly or purportedly involved functionally in cell cycle progression. Composite query results consisting of protein name, species, Swiss-Prot accession number, and IPR accession number(s) were compiled for each protein.

Identification of putative redox-regulated proteins. To identify putative redox-regulated cell cycle-associated proteins, the cell cycle protein list was searched for entries annotated with IPR accession numbers included in the redox motif list. Positive hits (proteins with redox motifs) were compiled into candidate protein lists. Each candidate protein was checked to eliminate "false positives," i.e., to exclude proteins having an IPR accession number included in the redox motif list but lacking amino acids conferring redox sensitivity. For example, the tumor susceptibility protein 101 (Swiss-Prot no. Q99816) is assigned IPR000608, which denotes a ubiquitin conjugating enzyme AC motif; however, this protein lacks the catalytic cysteine conferring redox sensitivity. Candidate proteins were categorized according to cell cycle phase as well as function. Literature queries were performed on all candidate proteins to determine whether they had previously been demonstrated to be redox regulated.

RESULTS

Intracellular redox environment per cell cycle phase. G_1, S, and G_2/M phase CHO cells were identified by DNA content determined by relative fluorescence intensity after staining with the membrane-permeant anthraquinone derivative DRAQ5, which fluoresces maximally in the red spectrum (Fig. 2*A*). Dual staining of cells with DRAQ5 and the cell-permeant fluorescent dye mBCl allowed analysis of reduced glutathione levels (GSH) per cell cycle phase (Fig. 2*B*). GSH-mBCl adduct mean fluorescence intensity (MFI) was significantly greater in cells in G_2/M phase compared with those in G_1 phase. The GSH-mBCl MFI of cells in S phase did not differ significantly from either G_1 or G_2/M phase cells but was numerically intermediate (Fig. 2*B*). Oxidative activity per cell cycle phase was determined by staining CHO cells with the ROS-specific probe H_2DCFDA. The relative MFI of H_2DCFDA did not differ between G_1, S, or G_2/M phase cells (Fig. 2*C*). The ratio of the percent change in MFI of GSH (Fig. 2*D*) and ROS (Fig. 2*E*) content per phase normalized to G_1 phase values (Fig. 2*F*)

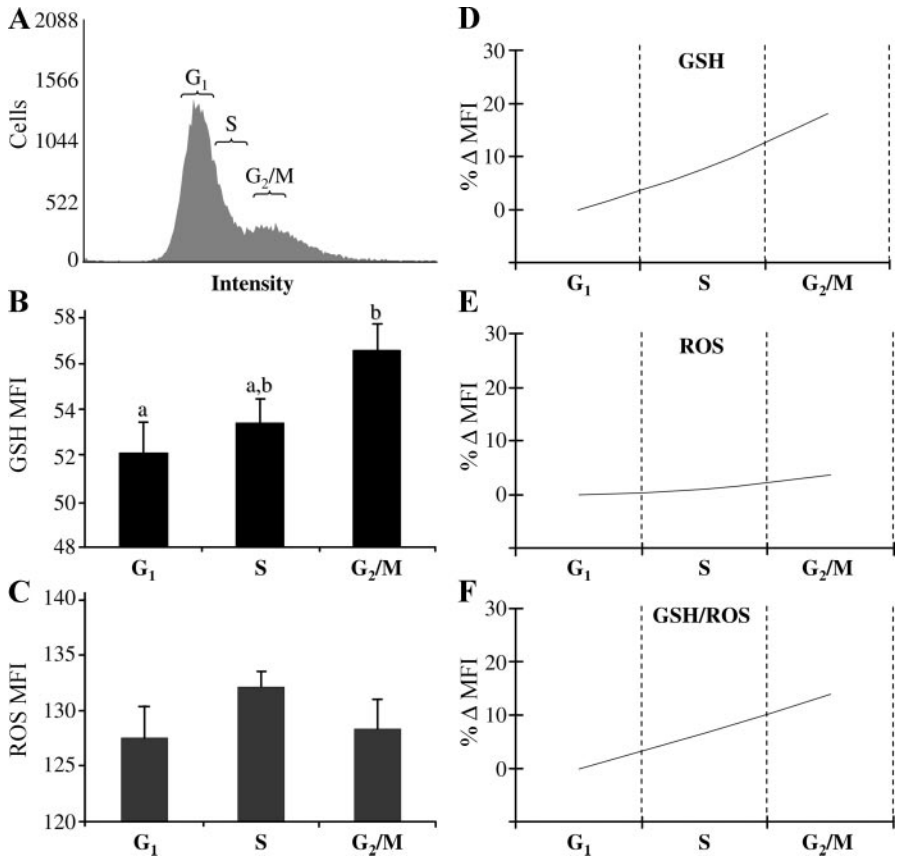

Fig. 2. An oscillating intracellular redox environment parallels cell cycle progression. *A*: DNA cell cycle curve of Chinese hamster ovary (CHO) cells stained with the DNA dye DRAQ5. *B*: representative chart of GSH content per cell cycle phase. Intracellular reduced glutathione (GSH) content was assessed per cell cycle phase in CHO cells with the fluorescent dye, monochlorobimane (mBCl). GSH-mBCl adduct mean fluorescence intensity (MFI) was determined by flow cytometric analysis. Significant differences ($P < 0.05$) between phases within experiments are indicated by different letters. *C*: representative chart of oxidative activity per cell cycle phase. Intracellular oxidative activity was assessed per cell cycle phase by measuring reactive oxygen species (ROS) in CHO cells stained with H_2DCFDA by flow cytometric analysis. No significant differences were observed between phases. Data are presented as means ± SE. These data are representational of 4 separate experiments ($n = 3–4$ per experiment). *D*: percent change in GSH MFI per cell cycle phase normalized to G_1 values. *E*: percent change in ROS MFI per cell cycle phase normalized to G_1 values. *F*: ratio of the percent change in MFI of GSH and ROS data per cell cycle phase.

Table 1. *Candidate cell cycle protein-associated redox motifs*

Functional Category	IPR Accession No.	Description
AC	IPR000242	Tyrosine specific protein phosphatase
	IPR000572	Eukaryotic molybdopterin oxidoreductase
	IPR000608	Ubiquitin-conjugating enzymes
	IPR000751	M phase inducer phosphatase
	IPR000788	Ribonucleotide reductase large subunit (Ribred2)
	IPR001763	Rhodanese-like
FeB	IPR000358	Ribonucleotide reductase large subunit (Ribred1)
H	IPR000345	Cytochrome *c* heme-binding site
	IPR002016	Haem peroxidase
MeB	IPR000815	Hg_reductase
	IPR001748	G10
	IPR004843	M-ppestrase
	IPR007113	Cupin_sup
SS	IPR000063	Thioredoxin type domain
	IPR000103	Pyridine nucleotide-disulfide oxidoreductase, class-II (Pyr-A)
	IPR001310	Histidine triad (HIT) protein
	IPR001327	FAD-dependent pyridine nucleotide-disulphide oxidoreductase
	IPR004123	Pre-mRNA splicing protein (TrxRel)
ZnB	IPR000197	TAZ finger
	IPR000433	Zn-finger, ZZ type
	IPR000571	Zn-finger, C-x8-C-x5-C-x3-H type
	IPR000822	Zn-finger, C2H2 type
	IPR000834	Zn carboxypeptidase A metalloprotease (M14)
	IPR000994	Metallopeptidase family M24
	IPR001138	Fungal transcriptional regulatory protein, N-terminal
	IPR001510	Zn-finger, NAD^+ ADP-ribosyltransferase
	IPR001607	Zn-finger in ubiquitin thiolesterase
	IPR001628	Zn-finger, C4-type steroid receptor
	IPR001841	Zn-finger, RING
	IPR001876	Zn-finger, Ran-binding
	IPR001878	Zn-finger, CCHC type
	IPR001965	Zn-finger-like, PHD finger
	IPR002219	Protein kinase C, phorbol ester/diacylglycerol binding
	IPR002857	Zn-finger, CXXC type
	IPR002867	Zn-finger, cysteine-rich C6HC
	IPR002893	Zn-finger, MYND type
	IPR003126	Zn-finger (putative), N-recognin
	IPR003613	Zn-finger, modified RING

AC, active/binding site cysteine; FeB, iron binding; H, heme; MeB, metal binding; SS, disulfide; ZnB, zinc binding; IPR, InterPro.

indicates the overall reduction of the intracellular redox environment of cells progressing through the cell cycle.

Identification of protein redox motifs. The final IPR redox motif list included 536 entries that were distributed into functional categories as follows: 3.2% (17 motifs) active/functional-site cysteines (AC); 2.1% (11 motifs) electron transport; 8.2% (44 motifs) heme (H); 12.5% (67 motifs) iron binding (FeB); 23.3% (125 motifs) metal binding (MeB, exclusive of zinc and iron); 23.9% (128 motifs) disulfide (SS); and 26.9% (144 motifs) zinc binding (ZnB). A total of 38 motifs representing 6 categories (AC, FeB, H, MeB, SS, and ZnB) were found in the candidate redox-regulated proteins (Table 1). The entire motif list is in the Data Supplement (available through the *Physiological Genomics* web site).[1]

Cell cycle protein lists. An initial list of 1,634 cell cycle-related proteins was assembled from combined entries from the Swiss-Prot and SpTrEMBL protein databases. Of these, 1,508 proteins had at least one IPR accession number and were subsequently cross-referenced with the IPR redox motif list to identify putative redox-regulated proteins. The remaining 126 cell cycle proteins lacked an IPR accession number and were thus excluded from the analysis. The complete cell cycle protein list is available in the Data Supplement (mentioned above).

Candidate redox-regulated proteins and functional categories. By cross-referencing the cell cycle protein and redox motif lists, 92 candidate redox-regulated cell cycle proteins were identified. Major functional categories for candidate proteins include transcription (e.g., transcription factors or coactivators), nucleotide metabolism (e.g., methyltransferases and ribonucleotide reductases), (de)ubiquitinylation (ubiquitin pathway), (de)phosphorylation (kinases/phosphatases), and other (e.g., protein inhibitors, assembly proteins, and spindle proteins, and others). The candidate protein list was distributed by functional category as follows: 15.2% transcription (14 proteins), 15.2% nucleotide metabolism (14 proteins), 17.4% ubiquitinylation (16 proteins), 27.2% kinase/phosphatase (25 proteins), and 25.0% other (23 proteins).

Motif frequency and distribution by cell cycle phase. The frequency and distribution of redox motifs by cell cycle phase within the candidate list of proteins are shown in Table 2. The most prominent motif categories in the candidate proteins were AC, MeB, and ZnB, whereas only a small number of proteins contained FeB, H, or SS motifs. AC-harboring proteins were primarily functional during G_2/M phase, with only a few functional during G_1 and S phases. The only FeB-containing protein (ribonucleotide reductase small subunit; 12) functions in S phase. H-containing proteins (Speedy/Spy1 and YPL006W) participate exclusively in G_2/M. Similar to AC-

[1]The Supplementary Material for this article is available online via **http://physiolgenomics.physiology.org/cgi/content/full/00058.2004/DC1**.

Table 2. *Redox motif frequency and distribution per cell cycle phase*

Motif Types	Frequency of Motif in Candidate Proteins	Cell Cycle Phase				
		G_1	S	G_2/M	All	Unknown
AC	27.2% (25/92)	16.0% (4)	12.0% (3)	64.0% (16)		8.0% (2)
FeB	1.1% (1/92)		100.0% (1)			
H	2.2% (2/92)			100% (2)		
MeB	18.4% (17/92)	11.7% (2)		76.5 (13)	5.9% (1)	5.9% (1)
SS	6.5% (6/92)	50.0% (3)		16.7% (1)	16.7% (1)	16.7% (1)
ZnB	44.6% (41/92)	26.8% (11)	12.2% (5)	34.2% (14)	7.3% (3)	19.5% (8)

Percentage of proteins with redox motif type per cell cycle phase is shown, and protein numbers are in parentheses. See legend to Table 1 for definitions of abbreviations.

containing proteins, MeB-containing proteins were functional primarily during G_2/M phase. In contrast to AC- and MeB-containing proteins, SS- and ZnB-containing proteins were distributed equally across all cell cycle phases.

Candidate protein characteristics by cell cycle phase. G_1 phase proteins represented 20.7% (19 proteins) of the redox-regulated proteins identified (Table 3). The distribution of motif types among G_1 proteins (i.e., percentage of AC, MeB, etc. . . per G_1 phase) was similar to that of the entire candidate protein population. The distribution of proteins by function was 31.6% transcription (6 proteins), 5.3% nucleotide metabolism (1 protein), 10.5% ubiquitinylation (2 proteins), 26.3% kinase/phosphatase (5 proteins), and 26.3% (5 proteins) other (dehydrogenase, cell adhesion, p53 degradation inhibitor, Cdc28 inhibitor, and unreported). S phase proteins represented only 10.9% (10 proteins) of the total candidate redox-regulated cell cycle proteins (Table 3). Motif category representation within the S phase proteins was similar to the entire candidate protein population. The distribution by function of S phase proteins is 70% nucleotide metabolism (7 proteins), 10% transcription (1 protein), and 20% ubiquitinylation (2 proteins).

Redox motif-harboring proteins functional during G_2/M phase represented the largest group, making up 50.0% (46 proteins) of the total list of candidates (Table 4). A greater number of AC- and MeB-containing proteins and fewer ZnB-containing proteins function during G_2/M phase compared with the total candidate protein population. The distribution of G_2/M phase proteins by function was 4.3% (2 proteins) transcription, 10.9% (5 proteins) nucleotide metabolism, 17.4% (8 proteins) ubiquitinylation, 41.3% (19 proteins) kinase/phosphatase, and 26.1% (12 proteins) other.

Five proteins (5.4% of total proteins) were reported to function in all cell cycle phases (Table 5). These proteins harbored primarily ZnB motifs (3 of the 5 proteins). The proteins were categorized functionally as 40% (2 proteins) transcription, 20% (1 protein) ubiquitinylation, and 40% (2 proteins) other. The cell cycle phase of activity for the remaining 13.0% (12 proteins) of the candidate cell cycle proteins remains unassigned (Table 5). This group was predominated by ZnB-containing proteins. The distribution of proteins by functional category was 25.0% (3 proteins) transcription, 8.5% (1 protein) nucleotide metabolism, 25.0% (3 proteins) ubiquitinylation, 8.5% (1 protein) kinase/phosphatase, and 33.0% (4 proteins) other.

Additional investigation of the literature revealed 22 proteins that have been shown previously to be redox-regulated or are closely related to proteins demonstrated to be redox-regulated (Table 6). These proteins are involved primarily in nucleotide metabolism, ubiquitinylation, and (de)phosphorylation. The proteins active in G_1 phase make up 22.72% (5) of the total, whereas 68.18% (15 proteins) are active in G_2/M phase, 4.55% (1 protein) are in all phases, and 4.55% (1 protein) are unassigned. Finally, 70% of these proteins contain an AC motif, although proteins containing FeB, MeB, SS, and

Table 3. *Candidate redox-regulated cell cycle proteins: G_1 and S phases*

Motif	Common Name	Species	Accession No.	Function	Details
G_1 phase					
AC	Cdc25A	H, M, R	P30304	Phosphatase	M phase inducer phosphatase 1/A
AC	Cdc25A	X	P30308	Phosphatase	Tyrosine phosphatase Cdc25A
AC	MPK1	H, M, R	P28562	Phosphatase	Dephosphorylates MAPK ERK2; oxidation induced
AC	Cdc34	Sp	P14682	Ubiquitin ligase	Mediates G_1-S phase transition
MeB	DLDH	Sp	O00087	Dehydrogenase	Required for G_1-S phase transition
MeB	PPP6	H, R	O00743	Phosphatase	Possibly involved in the G1-S phase transition
SS	IL1RL1	H, M	P14719	Cell adhesion	Possibly involved in T cell regulation
SS	Hit family protein 1	S	Q04344	Nucleotide hydrolase	Interacts with Cdk7 and Kin28
ZnB	Cdc68	S, Kl	P32558	Tnx regulator	Contributes to cyclin expression for passage of Start
ZnB	CdkI FAR1	S	P21268	Cdk inhibitor	Inhibits Cdc28
ZnB	MAT1	H, Mg, M, X	P51948	Kinase assembly	Stabilizes cyclin H-Cdk7
ZnB	PMH1	Sp	O94684	MAT1 homolog	Mat1 homolog
ZnB	Mdm4	H	O35681	Mdm2 inhibitor	Inhibits Mdm2 degradation
ZnB	p300	H	Q09472	Tnx adaptor	Adapter for regulatory element activity
ZnB	NURR1	H	P43354	Tnx factor	Nuclear receptor/general Tnx coactivator
ZnB	GFI1	H, M, R	Q99684	Tnx factor	Possible Tnx factor that regulates S phase genes
ZnB	TAFII-230	D	P51123	Tnx regulator	Essential for G_1 phase progression
ZnB	ZFP RME1	S	P32338	Tnx regulator	Involved meiosis control
ZnB	Mdm2	Cf, E, H, M	Q00987	Ubiquitin ligase	Inhibits p53/p73-mediated cell cycle arrest/apoptosis
S phase					
AC	RR1 M1 chain	H, M, Sp, S	P23921	RR subunit	Provides DNA precursors
AC	RR1 large chain 1	S	P21524	RR subunit	Constitutive RR1
AC	RR1 large chain 2	S	P21672	RR subunit	Inducible version of RR1
FeB	RR1 small chain	Sp	P36603	RR subunit	Combines with RR1 and RR3
SS	RR	An	Q9HEW8	RR	Ribonucleotide reductase
ZnB	P38-2G4	H	Q9UQ80	DNA repair	Possibly involved in DNA repair
ZnB	DNMT1	H	P26358	Methyltransferase	Methylates CPGs; regulates Tnx by binding HDAC2
ZnB	Rep2	Sp	Q09824	Tnx activator	Tnx activator interacts with Res2 and Cdc10
ZnB	BARD-1	H	Q99728	Ubiquitin ligase	Implicated in BRCA1-mediated tumor suppression
ZnB	BRCA1	H, M	P38398	Ubiquitin ligase	Tnx regulator of p21-DNA damage response

Species: *Aspergillus nidulans* (An), *Canis familiaris* (Cf), *Drosophila melanogaster* (D), *Equus caballus* (E), *Homo sapiens* (H), *Kluyveromyces lactis* (Kl), *Marthasterias glacialis* (Mg), *Mus musculus* (M), *Rattus norvegicus* (R), *Saccharomyces cerevisiae* (S), *Schizosaccharomyces pombe* (Sp), *Xenopus laevis* (X). Swiss-Prot accession numbers represent one example of the corresponding protein. Tnx, transcription; Cdk, cyclin-dependent kinase; RR, ribonucleotide reductase. See legend to Table 1 for further definitions of abbreviations.

Table 4. *Candidate redox-regulated cell cycle proteins: G_2/M phase*

Motif	Common name	Species	Accession No.	Function	Details
G_2/M phase					
AC	Cdc25B	X, H, M, R	P30305	Phosphatase	M phase inducer phosphatase 1/b
AC	Cdc25C	X, H, Ma, M, P	P30307	Phosphatase	M phase inducer phosphatase 2
AC	Cdc25	Pcfsp	O94186	Phosphatase	Mitotic inducer phosphatase Cdc25
AC	PTP1	Sp	P27574	PTP	Dephosphorylates Sty1/spc1 and Wis1/Spc2/Sty2
AC	PTP2	Sp	P32586	PTP	Dephosphorylates Sty1/spc1 and Wis1/Spc2/Sty3
AC	String	D	P20483	PTP	M phase inducer phosphatase
AC	PTP-1B	H	P18031	PTP	Nontransmembrane tyrosine phosphatase
AC	PTP3	Sp	P32587	PTP	Dephosphorylates p34cdc2; counteracts Wcc1
AC	DIM1	Sp	P87215	Spindle function	Essential for entry into mitosis
AC	UBC3	H, S	P40984	Ubiquitin ligase	Required for destruction of mitotic cyclins
AC	UBC9	S	P50623	Ubiquitin ligase	Involved in degradation of cyclin B2 and B5
AC	UBCB	Ss, X	Q95044	Ubiquitin ligase	Required for destruction of mitotic cyclins
AC	UBCC	H	O00762	Ubiquitin ligase	Required for destruction of mitotic cyclins
AC	UBCD	Sp	O00103	Ubiquitin ligase	Required for progression to anaphase
AC	UBC7	Sp	O00102	Ubiquitin ligase	Essential for onset of anaphase in mitosis
AC	UBCX	X	P56616	Ubiquitin ligase	Required for destruction of mitotic cyclins
H	Speedy/Spy1	X	Q9YGL1	Activates MAPK	A novel cell cycle regulator of G_2-M transition
H	YPL006W	S	Q12200	Tnx activator	Required for G_2/M progression independent of RAD9
MeB	cwf14	Sp	O74772	Spliceosome subunit	Associates with Cdc5 as part of spliceosome
MeB	CENP-C	H	Q03188	Kinetochore protein	Required for kinetochore assembly; binds DAXX
MeB	Cdc1	S	P40986	DNA repair	May help repair dsDNA breaks via recombination
MeB	PPA1	Sp	P23635	Phosphatase	PP2A-1 catalytic subunit
MeB	PPA2	Sp	P23636	Phosphatase	PP2A-2 catalytic subunit
MeB	BIMG	En	P20654	Phosphatase	Scr/Thr protein phosphatase PP1
MeB	DIS2	Sp, S	P13681	Phosphatase	Scr/Thr protein phosphatase PP1-1
MeB	PP1-87B	D, Sp, S	P12982	Phosphatase	Scr/Thr protein phosphatase α-2 isoform
MeB	PP1-2	Sp, S	P23880	Phosphatase	Required for mitotic exit
MeB	PPE1	Sp	P36614	Phosphatase	Phosphatase csp 1
MeB	PPP5	H, M, R	P53041	Phosphatase	Protein phosphatase T
MeB	PPH-4.1	Ce	Q966Q4	Phosphatase	Centrosomal protein needed for spindle formation
MeB	PPH-4.2	Ce	Q966Q3	Phosphatase	Centrosomal protein needed for spindle formation
SS	DIB1 like protein	Ec	Q8SSE0	Spindle function	D1b1-like protein required for mitosis entry
ZnB	CG18042	D	Q9U1J4	?	Putative APC11
ZnB	FSYA	D	P25028	Nuclear envelope protein	Required for embryonic mitosis
ZnB	Dma1	Sp	Q10322	Checkpoint component	Component of spindle assembly checkpoint
ZnB	UBPG	H	Q9Y5T5	Deubiquitinase	Associated with mitotic chromosomes
ZnB	PKC-L1	S	P24583	Kinase	Required for cell growth and G_2-M transition
ZnB	CHFR	H	Q9NRT4	Mitotic checkpoint	Defines pre-metaphase checkpoint
ZnB	Nup153	H, R	P49791	Nuclear pore protein	Possible regulator of nucleopore complex in mitosis
ZnB	AKA8	H, R	O43823	PKA type II anchor	Anchor that mediates subcellular PKA compartment
ZnB	Zfp GRT1	Sp	Q9C469	Slp1 facilitator	May facilitate anaphase progression in mitosis
ZnB	DIB1	S	Q06819	Spindle function	Plays fundamental role in mitosis entry
ZnB	cwf2	Sp	P87126	Spliceosome subunit	Associates with Cdc5 as part of spliceosome
ZnB	cwf8	Sp	O14011	Spliceosome subunit	Associates with Cdc5 as part of spliceosome
ZnB	cwf24	Sp	Q9P6R8	Spliceosome subunit	Associates with Cdc5 as part of spliceosome
ZnB	SW15	S	P08153	Tnx factor	Regulates mother-specific Ho endonuclease Tnx

Species: *Caenorhabditis elegans* (Ce), *Emericella nidulans* (En), *Encephalitozoon cuniculi* (Ec), *Mesocricetus auratus* (Ma), *Sus scrofa* (P), *Pneumocystis carinii f. sp. carinii* (Pcfsp), *Spisula solidissima* (Ss). PTP, protein tyrosine phosphatase; ?, undefined. See legends Tables 1–3 for further definitions of abbreviations.

ZnB motifs were represented. A majority of the proteins are reported to be functional during G_2/M phase, with G_1 phase having the second largest distribution (Table 7).

DISCUSSION

The occurrence of redox-sensitive motifs in numerous cell cycle proteins indicates that redox may play a more central role in the regulation of cell division than is currently recognized. This assertion is reinforced by the observed reduction in the intracellular redox environment that parallels cell cycle progression from G_1 to G_2/M phase. The oscillation in the intracellular redox environment in cells progressing through the cell cycle may represent a fundamental cell cycle mechanism that contributes to the regulation of cell cycle progression through redox-regulated cell cycle proteins.

Although many reports have demonstrated that proliferating cells are more reduced and nonproliferating or differentiated cells are more oxidized (20, 23, 29, 33, 35), few have focused on differences in the intracellular redox environment by cell cycle phase. Our observations of a significant increase in GSH combined with static ROS production indicate an overall reduction of the intracellular environment as cells progress from G_1 to G_2/M phase. These data are in agreement with a recent Jurkat T lymphocyte study that demonstrated, via immunocytochemistry, the greatest GSH content in G_2/M cells (51). Reduction of the intracellular environment as cells progress from G_1 to G_2/M phase may protect genomic DNA from oxidative damage upon breakdown of the nuclear envelope. The induction of the G_1 phase checkpoint protein, p53, by ROS agrees with this hypothesis (9). Moreover, in light of the recent

Table 5. *Candidate redox-regulated cell cycle proteins: All phases and Unknown*

Motif	Common Name	Species	Accession No.	Function	Details
All					
MeB	PCM-1	H	Q15154	Centrosome protein	Associates with centrosome during G_1, S, and G_2 phases
SS	Thioredoxin II	S	P22217	Thiol buffer	Absence prolongs S and shortens G_1 phase
ZnB	ATRX	M	Q61687	Tnx regulator	Modifies gene expression by affecting chromatin
ZnB	Zac1	M	O35745	Tnx regulator	Regulator of apoptosis and cell cycle arrest
ZnB	Dorfin	H, M	Q9NV58	Ubiquitin ligase	Mediates ubiquitinylation of cellular proteins
Unassigned					
AC	SUMO-1	H, Nh	P50550	Ubiquitin ligase	Catalyzes attachment of ubiquitin-like protein sumo-1
AC	NhRAD6	Nh	P78717	Ubiquitin ligase	Involved in both morphogenesis and mitosis
MeB	Bud 31	S	P25337	Bud site selection	Bud site selection protein
SS	DIM1 like protein	H	O14834	pre-mRNA splicing	Involved in cell cycle regulation
ZnB	?	Lm	Q9GRK6	?	Possible cell cycle protein
ZnB	Histone deacetylase 6	H, M	Q9UBN7	Deacetylase	Deacetylates core histones (H2A and B, H3 and H4)
ZnB	Suppressin	R	Q62998	Inhibits cell cycle entry	Suppressor of cell cycle entry
ZnB	Cdc kinase C2F3.15	Sp	O14098	Kinase	Putative cell division protein kinase
ZnB	ZFP1	Ca	P28875	Tnx activator	May be involved in Tnx activation
ZnB	EGRP-alpha	H	O75411	Tnx factor	Early growth response gene
ZnB	SC1 (TCF19)-7	H	Q9Y242	Tnx factor	DNA binding; required for late cell cycle progression
ZnB	EDD	H	O95071	Ubiquitin ligase	Regulates DNA TopoIIBP in DNA damage response

Species: *Candida albicans* (Ca), *Leishmania major* (Lm), *Nectria haematococca* (Nh). See legends to Tables 1–4 for further definitions of abbreviations.

report by Menon et al. (31) that demonstrated the requirement for an oxidative event in early G_1 phase for cells to proceed to S phase, p53 induction by ROS presupposes a reduction of the intracellular environment prior to S phase entry. Recent evidence that the initiation of DNA replication in yeast is coordinated with the shift from the oxidative to reductive phase of the respiratory oscillation suggests that this phenomenon may be a common mechanism for eukaryotes (26).

The events driving cell cycle progression (e.g., cyclin expression, DNA and protein synthesis, and protein degradation) are mediated by networks of proteins involved in transcription (e.g., AP-1, NF-κB, and E2F), nucleotide metabolism (e.g., ribonucleotide reductases), phosphorylation (MAPK, cyclin D/cyclin-dependent kinase 4), dephosphorylation (Cdc25A, B, and C), and ubiquitinylation (E1-E3 ubiquitin ligases), among others. If one or more proteins required for a central cell cycle process, such as nucleotide metabolism, were redox sensitive, then a significant shift in the intracellular redox environment could, in this case, attenuate DNA synthesis possibly resulting in cell cycle arrest in S phase. Three-fourths (69 of 92) of the candidate proteins identified are directly involved in transcription, nucleotide metabolism, (de)phosphorylation, or (de)ubiquitinylation, which are all essential processes for cell cycle progression. The remaining 23 proteins have diverse functions such as checkpoint proteins (Dma1, CHFR), spindle proteins (Dim1, DIB1), deacetylases (HDAC6), dehydrogenases (DLDH), protein regulators (Cdk-FAR1, Mdm2, Mdm4), nucleoporins (Nup153), kinetochore proteins (CENP-C), and

Table 6. *Cell cycle-associated proteins demonstrated to be redox regulated*

Phase	Motif	Common Name	Species	Accession No.	Function	References
G_1	AC	RR1 M1 chain	H, M, Sp, S	P23921	RR subunit	46
	AC	RR1 large chain 1	S	P21524	RR subunit	46
	AC	RR1 large chain 2	S	P21672	RR subunit	46
	FeB	RR1 small chain	Sp	P36603	RR subunit	46
	ZnB	Mdm2	Cf, E, H, M	Q00987	Ubiquitin ligase	8
G_2/M	AC	Cdc25B	X, H, M, R	P30305	Phosphatase	39, 52
	AC	Cdc25C	X, H, Ma, M, P	P30307	Phosphatase	39, 52
	AC	PTP1	Sp	P27574	PTP	30
	AC	PTP2	Sp	P32586	PTP	30
	AC	PTP-1B	H	P18031	PTP	30
	AC	PTP3	Sp	P32587	PTP	30
	AC	UBC3	H, S	P40984	Ubiquitin ligase	34
	AC	UBC9	S	P50623	Ubiquitin ligase	34
	AC	UBCB	Ss, X	Q95044	Ubiquitin ligase	34
	AC	UBCC	H	O00762	Ubiquitin ligase	34
	AC	UBCD	Sp	O00103	Ubiquitin ligase	34
	AC	UBC7	Sp	O00102	Ubiquitin ligase	34
	McB	PPA1	Sp	P23635	Phosphatase	62
	McB	PPA2	Sp	P23636	Phosphatase	62
	ZnB	PKC-L1	S	P24583	Kinase	13
All	SS	Thioredoxin II	S	P22217	Thiol buffer	32
Unknown	FeB	RR	An	Q9HEW8	RR	46

See legends for Tables 1–5 for definitions of abbreviations.

Table 7. *Redox motif frequency and distribution per cell cycle phase of proteins reported to be redox regulated*

Motif Types	Frequency of Motif Type in Candidate Proteins	Cell Cycle Phase				
		G_1	S	G_2/M	All	Unknown
AC	68.2% (15/22)	20.0% (3)		80.0% (12)		
FeB	9.1% (2/22)	50.0% (1)				50.0% (1)
MeB	9.1 (2/22)			100.0% (2)		
SS	4.5% (1/22)				100.0% (1)	
ZnB	9.1% (2/22)	50.0% (1)		50.0% (1)		

Percentage of proteins with redox motif type per cell cycle phase is shown, and protein numbers are in parentheses. See legends for Tables 1–5 for definitions of abbreviations.

others. The abundance and diversity of function of the candidate proteins suggests that numerous cell cycle processes may be redox sensitive, implying that changes in the intracellular redox environment could affect cell cycle progression in a variety of ways.

Based on our current observations, oxidant-sensitive proteins are less likely to be oxidized (inactivated) during G_2/M (reduced) vs. G_1 phase (oxidized). Consistent with this hypothesis, 64%, 100%, and 76.5% of AC, H, and MeB proteins, respectively, whose motifs are susceptible to oxidation (27, 30, 55), function during G_2/M phase (Table 2). The broad distribution of the few SS proteins identified limits the conclusions that can be drawn from this motif category. However, the distribution of three of the SS proteins, one in G_1 phase (interleukin 1 receptor-like 1), one in S phase (ribonucleotide reductase 1), and one in all phases (thioredoxin II), may indicate that the SS proteins identified are less susceptible to oxidation than the AC, H, and MeB proteins.

In contrast to the AC, H, and MeB proteins, the oxidant-sensitive zinc-binding (ZnB) proteins were active in all phases (Table 2; 58, 61). This apparent contradiction might be explained by the spatial distribution of ZnB proteins within the cell. The majority of ZnB proteins functioning in G_1 and S phases bind DNA, necessitating their nuclear localization. Cellular compartmentalization contributes to the regulation of transcription (nucleus), protein folding (endoplasmic reticulum), and energy production (mitochondria) by providing biochemically distinct microenvironments. These organelles have dissimilar redox environments compared with the cytosol (6, 21, 40, 47). Relevant to the current study, the nucleus is more reduced than the cytosol, with GSH concentrations of up to 15 mM compared with 11 mM in the cytosol (6, 40, 50). The nuclear GSH pool is also more resistant to oxidation than the cytosolic pool in rat hepatocytes cells and cultured human melanomas treated with buthionine sulfoximine (6, 22). Moreover, the reducing potential of the nucleus is reinforced by the vicinal dithiol-containing protein thioredoxin-1 (Trx1) that shuttles reducing equivalents to nuclear transcription factors via Ref-1 (59). As well, nucleus-specific Trx1 is more reduced (by −20 mV) than its cytosolic counterpart providing additional evidence of a reducing nuclear microenvironment (57).

A reduced nucleus appears to be essential for transcriptional regulation, as cysteine residues critical for protein structure or found within DNA binding domains generally must be reduced to promote transcription in the transcription factors p53, AP-1, Myb, and Sp-1 (1, 14, 15, 41, 61). In addition to promoting the repair of oxidative damage to DNA (47), a reduced nucleus may also shield genomic DNA from oxidation. In the same way, nuclear localization may protect oxidant-sensitive proteins from oxidation, possibly explaining the equal distribution of ZnB proteins between G_1 and G_2/M phases (Table 2). In support of this hypothesis, 19 of the 22 G_1 proteins identified (including AC, H, MeB, and ZnB proteins) function within the nucleus.

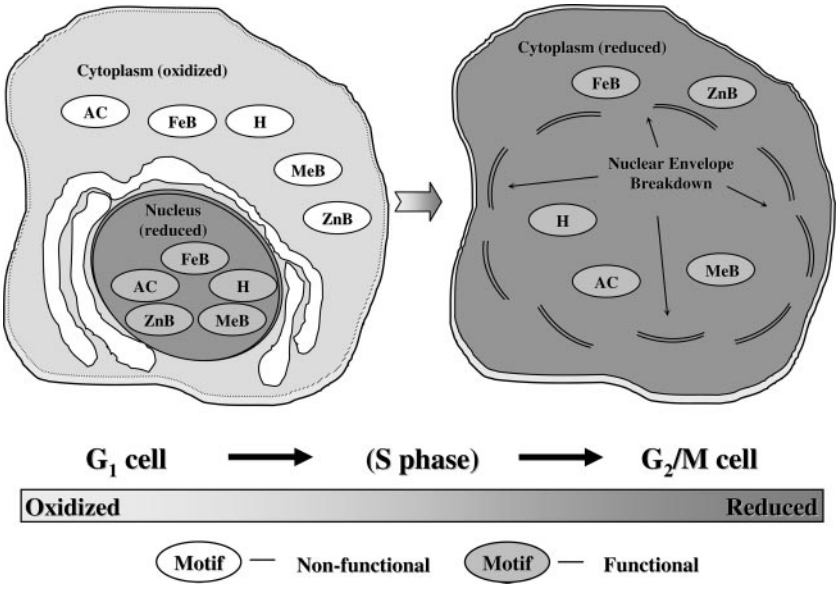

Fig. 3. Model mechanism for redox regulation of cell cycle proteins. The reduced nuclear microenvironment of G_1 cells (*left*) compared with the oxidized cytoplasm is conducive to oxidant-sensitive protein function. Prior to nuclear envelope breakdown, the intracellular redox environment of G_2/M cells (*right*) becomes more reduced (to prevent genomic DNA damage from oxidation) and in so doing, removes restrictions on oxidant-sensitive protein function. In this way, nuclear-cytoplasmic shuttling of redox-sensitive proteins might regulate the function of oxidant-sensitive proteins during G_1 phase. Once cells reach G_2/M phase, this mechanism is dismantled with the disruption of the nuclear-cytoplasmic redox gradient prior to nuclear envelope breakdown.

Another model for redox regulation of cell cycle progression highlights the potential for oxidation to initiate and terminate the cell cycle. Treatment of cells with serum, PDGF-BB, or thrombin induces second messenger ROS [via NAD(P)H oxidase], resulting in ERK2, JNK1, and p38 MAPK activation, c-Fos, c-Jun, and JunB expression, and ultimately, cell cycle initiation through AP-1 (36, 44). The subsequent reestablishment of an oxidized intracellular environment after cytokinesis could act as a cell cycle brake through the oxidation (inhibition) of cell cycle initiating proteins harboring oxidant-sensitive motifs, such as the M phase-inducing phosphatases Cdc25B and Cdc25C (39, 52). Oxidation of the catalytic cysteine residues (from their AC motifs) of Cdc25B and Cdc25C would inhibit their dephosphorylation (activation) of cyclin/cyclin-dependent kinases and arrest proliferation. Thus a dynamic intracellular redox environment may serve positive and negative regulatory and protective roles during the cell cycle. Specifically, *1*) the oxidized G_1 phase restricts oxidant-sensitive G_2/M cell cycle proteins while favoring mitogen-induced ROS second messenger signal transduction, which *2*) induces a reduction in the intracellular environment prior to S phase entry to prevent oxidative DNA damage and *3*) enables the function of oxidant-sensitive G_2/M cell cycle proteins (Fig. 3).

In summary, evidence of numerous candidate redox-regulated cell cycle proteins contributing to a diverse array of cell cycle processes demonstrates the potential for an oscillating intracellular redox environment to regulate cell cycle progression. Although the approach taken here does not demonstrate empirically how changes in the intracellular redox environment affect candidate cell cycle proteins per se, combining a novel and objective method for the prediction of redox-regulated cell cycle proteins with an in vitro characterization of the intracellular redox environment has provided valuable insight into potential redox regulatory mechanisms influencing cell division and enabled model development to guide further experimentation. Thus far, only 24% (22 of 92) of the candidate proteins identified here have been demonstrated experimentally to be redox-regulated, highlighting the need for future research in this emerging field.

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