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Intracellular glutathione pools are heterogeneously concentrated

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Abstract
Glutathione is present in millimolar concentrations in the cell, but its relative distribution among cellular compartments remains elusive. We have chosen the endoplasmic reticulum (ER) as an example organelle to study compartment-specific glutathione levels. Using a glutaredoxin sensor (sCGrx1pD), which rapidly and specifically equilibrates with the reduced glutathione (GSH)-glutathione disulfide (GSSG) redox couple with known equilibrium constant, we showed that the [GSH]/[GSSG] ratio in the ER of intact HeLa cells is less than 7:1. Taking into consideration the previously determined value for [GSH][GSSG] in the ER of 83 mM, this translates into a total glutathione concentration in the ER of greater than 15 mM. Since the integrated, intracellular [GSSG] was measured as ~7 mM, we conclude the existence of a [GSSG] gradient across the ER membrane. A possible homeostatic mechanism by which cytosol-derived glutathione is trapped in the ER is discussed. We propose a high [GSSG] as a distinguishing feature of the ER environment compared to the extracellular space.

Introduction
Many intracellular reduction–oxidation (redox) processes are directly or indirectly linked to the redox-active, tripeptide-like compound glutathione [1]. Glutathione is composed of the three amino acids glutamate, cysteine, and glycine and exists as a mixture of its reduced form, GSH, and its disulfide-linked dimeric form, GSSG. The biosynthesis of glutathione takes place in the cytosol, from where it is transported to other cellular compartments [2]. As intracellular glutathione concentrations are in the millimolar range, its compartment-specific distribution is considered a major determinant of intracellular redox environments [3]. The reductive (or oxidative) power of glutathione is defined by its electrochemical half cell reduction potential (E_CSH), calculated from its standard reduction potential (E° = −240 mV) and the concentrations of GSH and GSSG using the Nernst equation (Eq. (1)) where R = 8.315 J K−1 mol−1 is the gas constant, T = 298 K the temperature, and F = 96,485 C mol−1 the Faraday constant.

E_CSH = E°C SH° − RT 2F ln [GSH]2 [GSSG] (1)

It is widely accepted that E_CSH in the endoplasmic reticulum (ER) is more oxidizing (i.e. higher) than in other non-secretory organelles such as mitochondria, nucleus, or the cytosol [4]. This makes sense, because the ER is the site where disulfide bonds are being synthesized and transferred onto nascent secretory and membrane proteins [5]. Relatively oxidizing ER redox conditions were originally reported based on the measurement of the [GSH]/[GSSG] ratio in all compartments of the secretory pathway (including the ER) in hybridoma cells as 1:3–3.5:1 [6]. This was achieved by analyzing the thiol-disulfide state of a small glycopeptide, which directly, but probably not specifically reacted with GSH–GSSG. To derive E_CSH from these ratios, the authors went on to estimate the cellular concentration of total glutathione [GSHtot] (= [GSH] + [2GSSG]) as 8 mM and assumed [GSSGtot] in the secretory pathway to either equal this concentration or to be eight times lower, which returned E_CSH values of −170 to −185 mV or −133 to −165 mV, respectively [6]. Subsequent studies using isolated ER-membrane vesicles ("microsomes") reported [GSH][GSSG] ratios of 3:1–6:1 [7,8]. However, as GSH can leak through the ER...
microsomal membrane [9], these ratios and the derived $E_{GSH}$ values based on microsomal [GStot] may not reflect the situation in the ER of living cells.

Recently, $E_{GSH}$ in the ER of intact HeLa cells was directly measured as $-208 \pm 4$ mV using a glutathione-specific redox-sensitive variant of green fluorescent protein [10]. On the basis of this finding, it was concluded that either [GStot] in the ER ([GStot]ER) exceeds cellular [GStot] ([GStot]cell) or that the [GSH]:[GSSG] ratio in the ER of live cells is $\geq 11:1$. Here, we found that a single-cysteine glutaredoxin targeted to the ER is at least 92% oxidized in situ at steady state. As sCGrx1p rapidly attains equilibrium with [GSH]:[GSSG] through autocatalyzed glutathionylation [11], this suggests that in the ER, [GSH]:[GSSG] is less than 7:1 and, consequently, [GStot]ER significantly greater than [GStot]cell.

**Materials and methods**

**Cloning of sCGrx1pER**

The coding sequence of sCGrx1p was amplified by PCR from pOB3 [12] to include a C-terminal KDEL extension and ligated via KpnI/HindIII in frame into a plasmid harboring the ER signal sequence of Erp44 and an HA epitope upstream of a KpnI site (kindly provided by Roberto Sitia, Milan, Italy) [13].

**Cell culture and transfection**

HeLa cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.5 g/l glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg streptomycin at 37 °C in 5% CO₂ and transfected with Turbofect (Fermentas) according to the manufacturer’s protocols.

**Metabolic labeling and immunoprecipitation**

Transfected HeLa cells were washed with phosphate-buffered saline (PBS) and labeled overnight in complete DMEM cultivation medium containing 50 μCi/ml EasyTag EXPRESS 35S protein labeling mix (PerkinElmer Life Sciences). Chase was in DMEM containing 50 μCi/ml [35S]methionine. For immunoprecipitation, the cells were washed with cold PBS, lysed for 1 h on ice in lysis buffer [100 mM Na phosphate, 1% Triton X-100, pH 8, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF), and the lysate centrifuged at 17,000 g for 1 h at 4 °C]. The supernatant was added to Protein A-Sepharose beads (Life Technologies) carrying prebound anti-HA antibodies (clone 12CA5, kindly provided by Hans-Peter Hauri). After overnight incubation at 4 °C on a rotary shaker, the beads were washed four times with lysis buffer and once with PBS.

**TMM(PEG)₁₂ modification protocol**

To block the sulfhydryl groups of free cysteines in situ, cell monolayers were washed with ice-cold PBS containing 20 mM N-ethylmaleimide (NEM; Sigma), and incubated in the same buffer on ice for 20 min. After immunoprecipitation, as described above, proteins were released from the beads by incubation in 40 μl 80 mM Tris/HCl, pH 7.0, 2% SDS for 5 min in a heat block at 97 °C, followed by vortexing for 5 s. Then, 40 μl of supernatant was transferred to a tube containing 2 μl of 200 mM Tris[2-carboxyethyl]phosphine (TCEP; Sigma; $\sim 10$ mM final concentration) and incubated for 10 min at room temperature to reduce the active-site cysteine, which was then alkylated for 1 h at room temperature in 15 mM maleimide-activated polyethylene glycol (TMM(PEG)₁₂; Thermo Scientific; 5.5 μl of 125 mM stock added and carefully mixed). Excess TMM(PEG)₁₂ was removed by protein precipitation by using methanol/chloroform as previously described [14]. The extent of TMM(PEG)₁₂ modification was determined by 16% SDS-PAGE and imaging of the dried gel on a Typhoon phosphorimager (GE Healthcare).

**Determination of [GStot]cell**

A total of 1.8 × 10⁶ HeLa cells were seeded into a 10 cm dish and incubated at 37° for 48 h. To measure the cell number and diameter, cells were harvested by trypsinization and the suspension analyzed in a Luna™ automated cell counter (Logos Biosystems). Cell viability, as determined by Trypan blue exclusion, was routinely > 90%. Total cell volume was calculated by multiplying the single cell volume (by standard sphere formula) and cell count per ml of cell suspension. 1 ml of cell suspension was gently pelleted, the supernatant discarded and the cell pellet resuspended in 1 ml of 1% sulfoalicylic acid followed by incubation on ice for 15 min. Precipitated proteins were pelleted by centrifugation and the supernatant subjected to a 5.5 ‑dithiobis(2-nitrobenzoic acid)–glutathione reductase assay to measure [GStot] [15]. This concentration and the total cell volume were used to calculate [GStot]cell.

**Densitometry**

Western blots were densitometrically quantified using the ImageJ software (available at http://rsweb.nih.gov/ij/). For the quantification of Phosphorimager scans, the ImageQuant 5.2 software (GE Healthcare) was used.

**Additional methods**

Indirect immunofluorescence staining, 4-ametamido-4′-maleimidostilbene-2,2′-disulphonic acid (AMIS; Life Technologies) modification, Western blot, and the XBP1 splicing assay were performed as before [10].

**Results and discussion**

**Principle of [GSH]:[GSSG] determination in the ER**

With the aim to monitor the [GSH]:[GSSG] ratio in the ER of live cells, we fused the C30S mutant of yeast glutaredoxin 1 (sGrx1p) with N-terminal signal peptide and HA-tag and C-terminal KDEL ER retrieval motif (Fig. 1A). This mutant is known to catalyze its own glutathionylation by reacting with one molecule of GSSG according to Eq. [2] [11].

\[
\text{sGrx1pSH} + \text{GSSG} \rightleftharpoons \text{sGrx1pSSG} + \text{GSH}
\]

The equilibrium constant $K_{ox}$ of the above reaction (Eq. [3]) has been determined by two independent methods with similar outcomes [11], whereas the value of 74 ± 6 derived from MALDI-TOF mass spectrometry was considered quantitative.

\[
K_{ox} = \frac{[\text{sGrx1pSSG}[\text{GSH}]}{[\text{sGrx1pSH}][\text{GSSG}]}
\]

Autocatalyzed equilibration of the sGrx1p SH–sGrx1p SSG and the GSH–GSSG redox couples occurs rapidly in vitro [11,16]. Based on this, we assumed this reaction to be at kinetic equilibrium even in a complex mixture of redox couples as present in the lumen of the ER. Thus, using the experimentally determined [sGrx1pSH]:[sGrx1pSSG] ratio and $K_{ox}$, the [GSH]:[GSSG] ratio $R_{CS}$ can be calculated according to Eq. (4).

\[
R_{CS} = \frac{[\text{GSH}]}{[\text{GSSG}]} = \frac{K_{ox}[\text{sGrx1pSH}][\text{GSSG}]}{K_{ox}[\text{sGrx1pSSG}]}
\]
We expressed ER-targeted sCGrx1p (sCGrx1pER) in HeLa cells and confirmed its correct localization, which overlapped with that of endogenous protein disulphide isomerase (PDI), by indirect immunofluorescence microscopy using anti-HA (Fig. 1B+). Specifically, since both our antibody against the ER marker PDI and anti-HA are of mouse origin, we co-expressed the ER-targeted fluorescent protein HyPerER [17] and found co-localization with PDI (Fig. 1B) and with sCGrx1pER (Fig. 1C).

Expression of sCGrx1pER does not disturb ER homeostasis

Although PDI family members can catalyze protein deglutathionylation reactions to some extent [18], the ER in mammalian cells does not harbor bona fide glutaredoxin enzymes [19]. Accordingly, introduction of an exogenous glutaredoxin such as sCGrx1pER could disturb ER redox homeostasis. It could, for instance, catalyze formation of GSSG from glutathionylated proteins. This could result in a decrease in the [GSH]:[GSSG] ratio, which in turn would likely elicit unfolded protein response (UPR) stress signaling pathways, as has been observed before [20]. We therefore set out to test these possibilities. To this end, HeLa cells were transfected with sCGrx1pER, empty vector as negative control, or a plasmid encoding the hyperactive ER oxidase Ero1β-C100/130A [10] as positive control for ER hyperoxidation. To probe for the oxidation state of the ER in these cells, we used a previously established electrophoretic mobility shift assay based on the alkylation of cysteine residues in the ER-resident, PDI-related oxidoreductase ERp57 [14,21]. Expression of sCGrx1pER did not increase the oxidation of ERp57, whereas expression of Ero1β-C100/130A did as expected (Fig. 2A).

We next examined two established pathways of the mammalian UPR under the same experimental conditions, namely the phosphorylation of double stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) and the splicing of X-box binding protein 1 (XBP1) mRNA [22]. As shown in Fig. 2B, neither PERK phosphorylation nor XBP1 splicing was detected in cells expressing sCGrx1pER. In contrast, treatment of cells with DTT, an established trigger of the UPR, readily activated both PERK and XBP1.
of sCGrx1pER in SDS-PAGE. Autoradiography revealed that the vast majority of sCGrx1pER was predominantly glutathionylated at steady state (Fig. 3A).

We performed pulse-chase experiments using HeLa cells where sCGrx1pER was transiently expressed. Cells were metabolically labeled with 35S-methionine overnight and chased for 3 h in media containing an excess of unlabeled methionine (chase). sCGrx1pER was not significantly degraded during the chase, indicating that it is reasonably stable in the ER (Fig. 3A+B).

**sCGrx1pER is not rapidly degraded**

Since the native environment of Grx1p is the yeast cytosol [23], we next tested the stability of sCGrx1p in the ER. To this end, we performed pulse-chase experiments using HeLa cells where sCGrx1pER was transiently expressed. Cells were metabolically labeled with 35S-methionine to steady state overnight followed by incubation for 0–3 h in media containing an excess of unlabeled methionine (chase). sCGrx1pER was not significantly degraded during the chase, indicating that it is reasonably stable in the ER (Fig. 3A+B).

**sCGrx1pER is predominantly glutathionylated at steady state**

Via its only cysteine in the mature protein, Cys27, sCGrx1pER reacts with GSSG leading to glutathionylation of Cys27 (Eq. (2)). To examine the extent of Cys27 glutathionylation in living cells, we employed a cysteine-specific alkylation protocol of immunoprecipitation, which was previously used to assay the thiol-disulfide state of PDI [14]. Thus, sCGrx1pER was expressed and metabolically labeled in HeLa cells, and the non-glutathionylated fraction of Cys27 (in the thiol form) was allowed to react with NEM added to the cell monolayer. Following immunoprecipitation, glutathionylation was reversed with TCEP, and the resulting reduced cysteine alkylated with TMM(PEG)12, which decreased the mobility of sCGrx1pER in SDS-PAGE. Autoradiography revealed that the vast majority of sCGrx1pER was TMM(PEG)12-modified at steady state (Fig. 4).

Conversely, sCGrx1pER that was subjected to NEM-alkylation after treatment of cells with DTT ran at the mobility of the unmodified protein (compare Figs. 3 and 4), indicating that reduced sCGrx1pER can quantitatively react with NEM in situ.

The percentage of sCGrx1pER glutathionylation (OxD) was determined by quantifying the intensity of TMM(PEG)12-modified and -non-modified bands by densitometry. To exclude incompletely folded sCGrx1pER from the analysis, we only considered sCGrx1pER 35S-signals that were obtained after a 1 h chase period (Fig. 4).

**Calculation of [GStot]ER**

Having obtained a value for [GSH]:[GSSG] ratio of 0.033 (Table 1), we conservatively concluded that [GSH]:[GSSG] in the ER of HeLa cells is less than 7:1 (Table 1). We then set out to calculate [GStot]ER. To achieve this, we used the published value of \( EC_{50(ER)} \) of -208 mV [10], which can also be
Using the ER membrane appears to maintain an ascending \([\text{GStot}]\) gradient despite the fact that glutathione synthesis takes place in the cytosol, that \([\text{GStot}]\) in the cytosol is significantly lower than \([\text{GStot}]\) in the ER. Thus, as the concentration of GSH can equilibrate between luminal and cytosolic side of the ER membrane, \([\text{GStot}]\) is significantly higher in the ER than in the cytosol.

### Table 1

| Parameter (ER, HeLa) | Value       | Reference |
|----------------------|-------------|-----------|
| \([\text{GStot}]\)   | 6.9 ± 2.6 mM| This study|
| \([\text{GStot}]\) in HeLa cells | 6.9 ± 2.6 mM | This study |

### Table 2

| Parameter (HeLa) | Value         |
|------------------|---------------|
| Average cell volume | 2.2 pl (1 pl = 10^3 \(\mu\)m\(^3\)) |

### Conclusions

Measuring compartment-specific \([\text{GS}]_{\text{tot}}\) is a challenging task [1]. In this study, we addressed the question whether or not glutathione is uniformly distributed among two subcellular compartments, cytosol and ER. For this purpose, we implemented a novel strategy to calculate \([\text{GS}]_{\text{tot}}\) in the ER by using a combination of two readouts, which report \([\text{GS}]_{\text{tot}}\) and \([\text{GS}]_{\text{tot}}\) according to Eq. (5).

\[
[\text{GS}]_{\text{tot}}(M) = \frac{0.083 + 2\times 0.083}{R_{\text{CS}}} 
\]

Using \(R_{\text{CS}} = 7\), Eq. (5) returns a value for \([\text{GS}]_{\text{tot}}\) of 15.2 mM. However, since \(R_{\text{CS}}\) is likely less than 7 (see above), the total glutathione concentration in the ER may in reality significantly exceed 15 mM (Table 1).

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