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Research Paper

Cysteines 208 and 241 in Ero1α are required for maximal catalytic turnover

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ABSTRACT

Endoplasmic reticulum (ER) oxidoreductin 1α (Ero1α) is a disulfide producer in the ER of mammalian cells. Besides four catalytic cysteines (Cys^{94}, Cys^{99}, Cys^{394}, Cys^{397}), Ero1α harbors four regulatory cysteines (Cys^{104}, Cys^{131}, Cys^{208}, Cys^{241}). These cysteines mediate the formation of inhibitory intramolecular disulfide bonds, which adapt the activation state of the enzyme to the redox environment in the ER through feedback signaling. Accordingly, disulfide production by Ero1α is accelerated by reducing conditions, which minimize the formation of inhibitory disulfides, or by mutations of regulatory cysteines. Here we report that reductive stimulation enhances Ero1α activity more potently than the mutation of cysteines. Specifically, mutation of Cys^{208}/Cys^{241} does not mechanistically mimic reductive stimulation, as it lowers the turnover rate of Ero1α in presence of a reducing agent. The Cys^{208}/Cys^{241} pair therefore fulfills a function during catalysis that reaches beyond negative regulation. In agreement, we identify a reciprocal crosstalk between the stabilities of the Cys^{208}–Cys^{241} disulfide and the inhibitory disulfide bonds involving Cys^{104} and Cys^{131}, which also controls the recruitment of the H_2O_2 scavenger GPx8 to Ero1α. Two possible mechanisms by which thiol–disulfide exchange at the Cys^{208}/Cys^{241} pair stimulates the catalytic turnover under reducing conditions are discussed.

1. Introduction

The synthesis of extracellular proteins is initiated at the endoplasmic reticulum (ER) where translating ribosomes translocate the nascent polypeptides into the ER lumen. Many of these polypeptides subsequently acquire critical covalent linkages between cysteine residues (termed disulfide bonds) through thiol-disulfide exchange reactions. Among the different systems for disulfide bond formation in the ER, ER oxidoreductin 1 (Ero1)-catalyzed oxidation of the active-site cysteine pair in protein disulfide isomerase (PDI) constitutes the best-conserved pathway [1,2]. Ero1 in vertebrates exists in two isoforms, Ero1α and Ero1β, whereas Ero1α is ubiquitously expressed and being viewed as the major source of disulfides in humans [3].

The flavoprotein Ero1α is an oxidase that couples the reduction of molecular oxygen (O_2) to disulfide-bond formation [3]. In the reductive phase of the Ero1α catalytic cycle, the flavin adenine dinucleotide (FAD) cofactor in Ero1α is reduced to $FADH_2$ as a result of disulfide transfer from Ero1α to reduced PDI (PDI_{red}). The reaction is catalyzed by an outer active-site cysteine pair (Cys^{94}/Cys^{99}), which shuttles two electrons from PDI_{red} *via* an inner active-site cysteine pair (Cys^{394}/Cys^{397}) to FAD. This process is tightly regulated by the reversible formation of two inhibitory disulfides (Cys^{94}–Cys^{131} and Cys^{99}–Cys^{104}) [4–6]. In the oxidative phase, FAD is regenerated by the transfer of two electrons onto O_2, which leads to the formation of one molecule of hydrogen peroxide (H_2O_2) [7]. Recent evidence indicates that H_2O_2 is instantaneously reduced to H_2O by the Ero1α-associated glutathione peroxidase family enzymes GPx7 or GPx8, which also introduce the resulting second disulfide into PDI [8,9]. We showed that access of O_2 to the buried FAD molecule is negatively regulated by the Cys^{208}–Cys^{241} disulfide [10].

The thiol-disulfide statuses of all inhibitory disulfide bonds in Ero1α are governed by canonical PDI or other PDI family members [10,11]. Accordingly, regulatory cysteines fine-tune the activation state of Ero1α in a redox environment-dependent manner by blocking either the reductive (Cys^{104}, Cys^{131}) or the oxidative (Cys^{208}, Cys^{241}) phase of the catalytic cycle through the formation of feedback-regulated inhibitory disulfides in response to oxidizing conditions. Conversely, a reducing ER environment promotes

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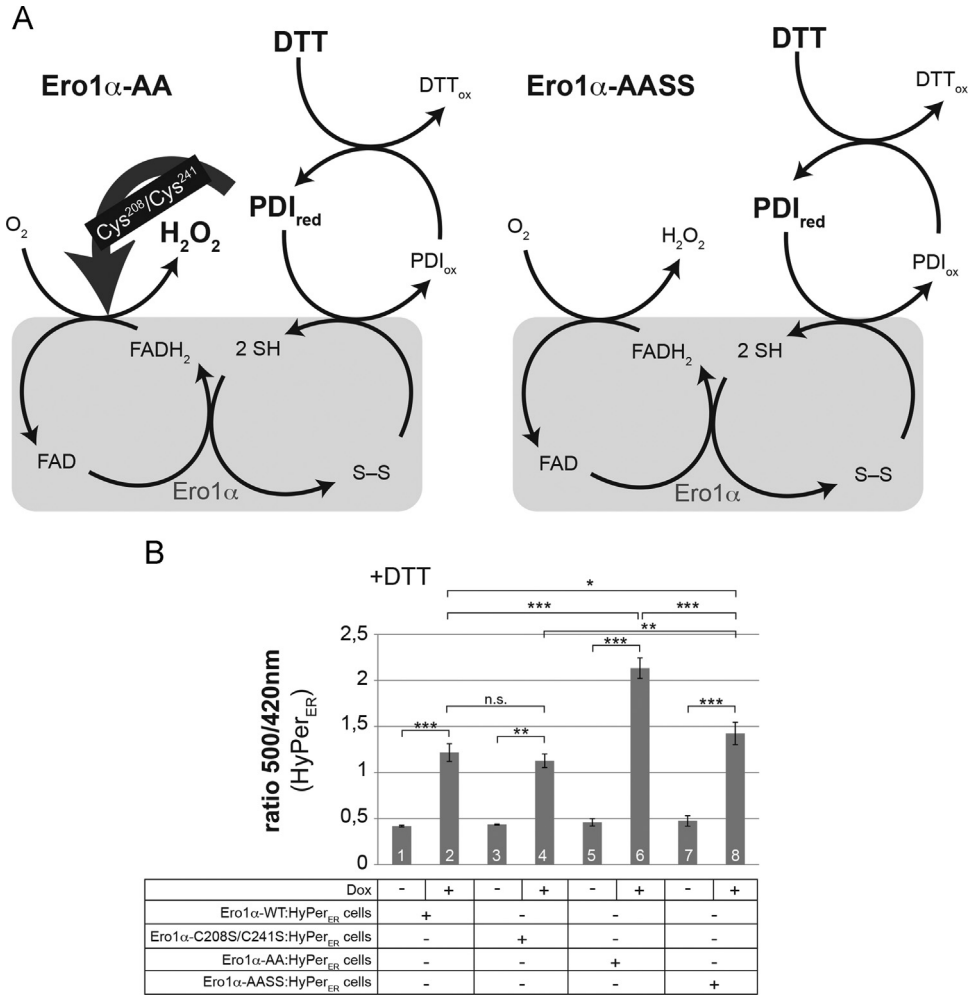

Fig. 1. (A) Schematic depicting the influence of DTT on the formation of Ero1α-derived H_2O_2 in the ER. The resulting high concentration of PDI_{red} exerts a stimulatory effect on Ero1α by catalyzing thiol-disulfide exchange at the regulatory disulfide between Cys^{208} and Cys^{241} (see main text for detailed models). (B) $HyPer_{ER}$ fluorescence excitation spectrum analyses of indicated cell lines measured 5 min after the addition of 0.5 mM DTT were performed. Plotted are the ratios of the 500 and 420 nm peak amplitudes ($n \geq 3$; mean ± SD). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Ero1α activation through PDI-catalyzed reduction of these disulfides, as could for instance be important in response to physiological hypoxia or peak concentrations of reduced glutathione (GSH) or redox-active vitamins.

Here, we report that reducing conditions, which activate Ero1α through the removal of inhibitory disulfides, more potently stimulate Ero1α activity than the mutation of all regulatory cysteines. Thus, the presence of the Cys^{208}/Cys^{241} pair is required for maximal catalytic turnover under reducing conditions. Our data indicate a new mechanism of Ero1α regulation in which thiol-disulfide exchange at Cys^{208}–Cys^{241} affects the stability of the Cys^{94}–Cys^{131} inhibitory disulfide through allosteric and/or intermolecular communication.

2. Materials and methods

2.1. Fluorescence excitation spectrum analysis

Cells stably transfected with $HyPer_{ER}$ were subjected to fluorescence excitation spectrum analysis as described before [12].

2.2. Dithiothreitol (DTT) washout assays

The cellular glutathione disulfide:total glutathione ($GSSG:GS_{tot}$) ratio after DTT washout was measured using a 5,5'-dithiobis(2-nitrobenzoic acid)/glutathione reductase recycling assay as previously described [13].

2.3. Statistics

Data sets were analyzed for statistical significance using Student's *t* test (two-tailed distribution; heteroscedastic).

2.4. Cell culture and transient transfections

The culturing of HeLa cells [14] and FlipIn TRex293 cells for doxycycline (1 μg/ml, Sigma)-inducible expression of Ero1 variants [4] has been described. The following FlipIn TRex293 cell lines have been published previously: Ero1α [4], Ero1α-AA [6], Ero1α-C208S/C241S, Ero1α-AASS [10], Ero1α-AA:$HyPer_{ER}$ [8], Ero1α-C208S/C241S:$HyPer_{ER}$ [10] and Ero1α-AASS:$HyPer_{ER}$ [10]. The Ero1α−WT:$HyPer_{ER}$ cell line was created as before [8] (with the $HyPer_{ER}$ vector kindly provided by Miklos Geiszt, Semmelweis University, Hungary).

Transient transfections of HeLa cells were carried out using Turbofect (Thermo Scientific). Transient transfections of FlipIn TRex293 cells were carried out using Metafectene Pro (Biontex).