Enhanced Catalysis of Ribonuclease B Folding by the Interaction of Calnexin or Calreticulin with ERp57*

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The endoplasmic reticulum is the site of folding, disulfide bond formation, and N-glycosylation of secretory proteins. Correctly folded proteins are exported from the endoplasmic reticulum, whereas incorrectly folded proteins are retained by a quality control system. The type I membrane-protein calnexin and its soluble homologue calreticulin are constituents of this system that recognize monoglucosylated N-linked glycans that are present on unfolded glycoproteins. Although several components of the quality control apparatus are well characterized, it is not known whether and how they interact with enzymes that catalyze protein folding. The endoplasmic reticulum protein ERp57 is homologous to protein-disulfide isomerase and can be cross-linked to the same monoglucosylated glycoproteins that bind to calnexin and calreticulin. The present study demonstrates that the disulfide isomerase activity of ERp57 on the refolding of monoglucosylated ribonuclease B is much greater when this glycoprotein is associated with calnexin or calreticulin. This result is in contrast to protein-disulfide isomerase, whose activity on monoglucosylated ribonuclease B is decreased in the presence of these lectins. No direct binding of monoglucosylated ribonuclease B or monoglucosylated glycans to ERp57 could be detected, but we show that ERp57 interacts directly with calnexin.

The quality control system of the endoplasmic reticulum (ER)1 ensures that only folded proteins proceed further along the secretory pathway. Some of the abundant ER proteins are components of molecular chaperone systems that bind to unfolded proteins, retaining them in the ER. The folding enzymes are also abundant in the ER and comprise several disulfide isomerase and prolyl peptidyl isomerases (1, 2). How chaperones and folding enzymes interact to facilitate protein folding in the ER is not known.

Calnexin and calreticulin participate in a molecular chaperone system that integrates the processes of N-glycosylation and quality control (3, 4). They both are lectins that bind N-glycans of the form GlcNAc2Man9Glc1 that result from the removal of the two outer glucoses from GlcNAc2Man9Glc3 oligosaccharides by the sequential action of glucosidases I and II. Removal of the last glucose by glucosidase II prevents binding by calnexin and calreticulin. Then, if the glycoprotein is unfolded, a glucose residue is added back to the high mannose core by the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT), which recognizes unfolded proteins (5). Consequently, during folding glycoproteins undergo cycles of binding and release from calnexin and calreticulin, which are driven by the addition and removal of a glucose residue (4). As a result of the specificity of UGGT, only unfolded glycoproteins bind to calnexin and calreticulin in vivo (6, 7), even though these lectins do not recognize the conformation of their protein substrates (8, 9).

Monoglucosylated glycoproteins, in addition to binding to calnexin and calreticulin, can also be cross-linked to the ER protein ERp57 (also known as ER60, ERP60, ERp57, GRP58, P58, HIP-70, or Q-2; Ref. 10; Ref. 11 and references therein). ERp57 is homologous to protein-disulfide isomerase (PDI) and has been shown to exhibit thiol-disulfide oxidoreductase activity in vitro (12).

We demonstrate here, using an in vitro system that reconstitutes the binding of monoglucosylated RNase B (G1-RNase B) to the lumenal domain of calnexin, that ERp57 exhibits a remarkably increased disulfide isomerase activity on a calnexin-bound substrate.

MATERIALS AND METHODS

Proteins—Human ERp57 was produced in Escherichia coli (BL21(DE3), pLysS) with a pET9 vector (13). The construct encoded the protein without a signal sequence (residue 25–505) (14) but with an additional alanine residue at the N terminus. The soluble cell extract was loaded onto a heparin-Sepharose column equilibrated with 10 mM Tris-HCl (pH 7.4). Proteins were eluted stepwise with 200 and 600 mM NaCl in the same buffer. Rabbit antiserum was generated against the purified protein. Human PDI, calnexin ATMC, and calreticulin were prepared as described previously (6, 15, 16). The cDNA of UGGT from Drosophila melanogaster was expressed in Sf9 insect cells with a baculovirus vector. The signal sequence was replaced with that of mellitin (17), and the ER retention sequence HGEL was changed for six histidines.2 The protein was purified from the culture medium on a Ni-NTA agarose protein-disulfide isomerase; RNase B, ribonuclease B; G1, monoglucosylated; UGGT, UDP-glucose:glycoprotein glucosyltransferase; ATMC, lumenal domain of canine calnexin; CB, Coomassie Blue; AR, autoradiography; PAGE, polyacrylamide gel electrophoresis; Endo H, endo-β-N-acetylglucosaminidase H.

2 D. C. Tessier, A. Zapun, N. J. Darby, M. Michalak, J. J. M. Bergeron, and D. Y. Thomas, unpublished data.
column (Qiagen). Affinity chromatography purified RNase B was from Sigma. Denaturation of RNase B and glucosylation with UDP-[3H]glucose were performed as described previously (9).

Refolding of RNase B—After glucosylation, refolding was initiated by dissolving freeze-dried reduced RNase B (60 μM) in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM CaCl₂, 0.5 mM oxidized glutathione (2 mM reduced glutathione, with or without ~12 μM of ATMC or calreticulin, and ~4 μM of either ERp57 or PDI at 25 °C). Aliquots were withdrawn after various times, reacted for 5 min at room temperature with 0.25 mM of 1,3 diiodoacetamide in 1.5 mM Tris-HCl (pH 8.7), and then stored on ice prior to analysis by low pH nondenaturing PAGE (18). The incorporation of [3H]glucose was detected by fluorography (Amplify, Amersham Corp.).

FIG. 1. Refolding of RNase B catalyzed by ERp57 or PDI, with or without calnexin. After monoglucosylation, refolding of reduced RNase B was initiated in a glutathione redox buffer in the presence of ERp57 (a) or PDI (b) or without catalyst (CTL, c) in the absence (−ΔTMC) or the presence (+ΔTMC) of the luminal domain of calnexin. The reaction was terminated after the indicated times, and the RNase B conformation was examined by nondenaturing PAGE. Unfolded RNase B (U) has the slowest mobility, and the native form (N) has the greatest. Most of the RNase B is detected by CB staining, but G1-RNase B is detectable only by AR. The graphs show the quantitation by densitometry of native nonmonoglucosylated RNase B (circles) and G1-RNase B (squares) with (black symbols, solid line) and without (open symbols, dashed line) ΔTMC.

RESULTS

Refolding of RNase B in the Presence of Calnexin or Calreticulin and ERp57—To demonstrate the function of ERp57, its disulfide isomerase activity on the refolding of G1-RNase B was examined. RNase B has the same sequence as RNase A but is glycosylated on residue Asn³⁴. RNase A and B both contain eight cysteine residues that form four disulfide bonds that are required for the native structure, and the proteins unfold upon reduction of these bonds. The disulfide-coupled refolding of RNase A and of RNase B have been well characterized and are similar (18, 19). Unfolded reduced RNase B is a substrate for UGGT (9), and the monoglucosylation was performed with UDP-[3H]glucose. However, the glucosylation of RNase B is rather inefficient because the preferred substrate for UGGT is the Man⁹ glycoform, which comprises only about 1.5% of the RNase B sample used in these experiments (9), thus only about 1% of the total RNase B is labeled to yield G1-RNase B. When examined by gel electrophoresis, this small fraction of G1-RNase B was only detectable by autoradiography (AR), whereas the large excess of nonmonoglucosylated RNase B was observed by Coomassie Blue (CB) staining, providing a useful internal control (9).

Refolding was initiated by dissolving the reduced RNase B in a glutathione buffer that approximates the redox conditions of the ER (21). The influence of calnexin was tested by adding a soluble recombinant form (residues 1–462) that lacks the transmembrane and cytoplasmic segments (ΔTMC) (22). The refolding reaction was terminated at various times by alkyla-

This result with ERp57 is in marked contrast to that observed with PDI. In the absence of calnexin, PDI exhibited a greater disulfide isomerase activity than ERp57 (Fig. 1, −ΔTMC, compare b and a). The monoglucosylation of RNase B (AR) did not influence the activity of PDI. The presence of calnexin did not influence the isomerization of the disulfide bonds in the non-G1-RNase B (Fig. 1b, +ΔTMC, CB). However, calnexin did inhibit the catalysis of the refolding of G1-RNase B (Fig. 1b, −ΔTMC, AR). This effect of calnexin on the activity of PDI, which we have previously reported (9), is the opposite of its effect on ERp57. Calreticulin had the same effects (data not shown). In the absence of either catalyst, the refolding of RNase B was very slow, and no effect of calnexin could be
Cooperation of Calnexin and ERp57

PDI has been studied for 35 years, and its activity seemed sufficient to account for the rapid formation of disulfide bonds in the ER. Indeed, an amount of PDI equivalent to that found in an ER extract produced similar in vitro refolding rates of bovine pancreatic trypsin inhibitor as the total extract (23). Other thiol-disulfide oxidoreductases similar in sequence to PDI were later also found in the ER, but their functions have remained elusive. Currently, five additional PDI-like proteins have been identified in mammals: ERp57, ERp72, P5 (reviewed in Ref. 1), PDIR (24), and PDIp, which is specific to the pancreas (25). That ERp57 could be cross-linked to the same substrates as calnexin and calreticulin was the first indication that the various PDI-like proteins might be dedicated to different classes of proteins (10), and we show here that this is associated with a functional difference between Erp57 and PDI.

We demonstrate that the catalysis of disulfide bond rearrangement by ERp57 is more efficient on G1-glycoproteins in the presence of calnexin or calreticulin. This apparent specificity does not result from a direct recognition of the glycans by ERp57. A simple model to account for the greater efficiency of ERp57 on glycoproteins that are bound to calnexin or calreticulin is depicted in Fig. 3. As both the substrate and the enzyme are brought into close proximity by their respective interaction with the lectin, their relative local concentration is increased, producing an apparent enhancement of the catalytic activity of ERp57.

In addition to the function of calnexin of retaining unfolded proteins in the ER, the affinity of calnexin for ERp57 serves to recruit this enzyme where it is needed. Disulfide bond formation and rearrangement, catalyzed by ERp57, can then occur while the glycoprotein is bound to calnexin. Therefore, the retention of unfolded proteins and the facilitation of their folding can be coordinated by calnexin and ERp57. In contrast, no cooperation between the PDI-like protein Erp72 or P5 and the chaperones BiP and GRP94 could be detected in vitro (26).

For proteins with multiple oligosaccharides such as influenza hemagglutinin, simultaneous binding to a multivalent calnexin may constrain the conformation of the protein so as to inhibit folding. Locking influenza hemagglutinin onto calnexin has been shown to inhibit the formation of disulfide bonds (27). This class of glycoprotein may require at least partial release with ERp57 or calnexin.

Fig. 2. ERp57 does not bind G1-oligosaccharides but interacts directly with calnexin. After monoglucosylation of RNase B, the oligosaccharides were released with Endo H. After incubation with (a) ERp57 or (b) ΔTMC, the mixtures were separated by gel filtration. Proteins were monitored at 280 nm (solid line), and radiolabeled G1-oligosaccharides in collected fractions were detected by liquid scintillation counting (columns). c, a small amount of ERp57 was incubated with a large excess of ΔTMC or catalase prior to separation by gel filtration. ERp57 in collected fractions was revealed by Western blotting. The peak of ERp57 detected at 280 nm is from a separate experiment with a much greater amount of protein.

reliably measured (Fig. 1c). We conclude from these results that binding of G1-RNase B to calnexin or calreticulin favors the catalysis of disulfide bond rearrangement by ERp57, whereas it hampers catalysis by PDI.

Absence of Binding of G1-Glycans to ERp57—The catalysis by ERp57 in the absence of calnexin is independent of monoglucosylation, suggesting that ERp57 does not recognize the glycan of the substrate. Nevertheless, we tested whether ERp57 directly binds G1-oligosaccharides or G1-RNase B. After monoglucosylation, glycans were released from RNase B by treatment with Endo H. The Endo H-treated protein was mixed...
from calnexin, driven by glucosidase II, to undergo folding and disulfide bond formation, which may then be catalyzed by ERp57.

Following cross-linking, ERp57 has been found in ternary complexes containing ERp57, a substrate protein and either calnexin or calreticulin (11). In view of the increased activity of ERp57 on G1-RNase B in the presence of calreticulin, it is likely that ERp57 also interacts directly with calreticulin. An interaction between PDI and calreticulin, possibly dependent on Zn$^{2+}$ ions, has also been reported (28). That PDI binds unfolded proteins is supported by the chaperone activity, which PDI exhibits (29) even on a protein without disulfide bonds (30) and which can be separated from the oxidoreductase activity by alkylating the active site cysteines (31). When substrates are bound to calnexin or calreticulin, their binding to PDI may be restricted, thus impeding the isomerase activity. Either ERp57 has a conformation that avoids this problem of steric hinderance or the role of handling the substrate is played entirely by the lectins.

The elution properties of ΔTMC in size exclusion chromatography indicate an apparent molecular mass of 280 kDa, corresponding to a pentamer or hexamer, which is in agreement with some estimates of size of the complex in vivo (6) but not others, which indicated that most calnexin may be monomeric (32). We do not yet have an explanation for these discrepancies.

The functional interaction between ERp57 and calnexin or calreticulin demonstrates that N-glycosylation does not only contribute to quality control but also to accelerating productive folding. This study also suggests that other overlooked or difficult to detect interactions may add functionality to the other members of the PDI family. The speculative ER matrix formed by a loose association of resident ER proteins (32) may be a network that creates powerful synergies between the various chaperone systems of the quality control machinery and the enzymes that accelerate folding.

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**FIG. 3.** The catalysis by ERp57 of the formation and rearrangement of disulfide bonds in a G1-glycoprotein is enhanced by the concentration effect resulting from the independent binding of glycoprotein and of ERp57 to calnexin (CNX) or calreticulin (CRT).