Retrotranslocation of the Chaperone Calreticulin from the Endoplasmic Reticulum Lumen to the Cytosol

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Received 28 June 2005/Returned for modification 12 July 2005/Accepted 20 July 2005

Polypeptide folding and quality control in the endoplasmic reticulum (ER) are mediated by protein chaperones, including calreticulin (CRT). ER localization of CRT is specified by two types of targeting signals, an N-terminal hydrophobic signal sequence that directs insertion into the ER and a C-terminal KDEL sequence that is responsible for retention in the ER. CRT has been implicated in a number of cytoplasmic and nuclear processes, suggesting that there may be a pathway for generating cytosolic CRT. Here we show that CRT is fully inserted into the ER, undergoes processing by signal peptidase, and subsequently undergoes retrotranslocation to the cytoplasm. A transcription-based reporter assay revealed an important role for the C-terminal Ca^{2+} binding domain in CRT retrotranslocation. Neither ubiquitylation nor proteasome activity was necessary for retrotranslocation, which indicates that the pathway is different from that used by unfolded proteins targeted for destruction. Forced expression of cytosolic CRT is sufficient to rescue a cell adhesion defect observed in mouse embryo fibroblasts from *crt*^{-/-} mice. The ability of CRT to retrotranslocate from the ER lumen to the cytosol explains how CRT can change compartments and modulate cell adhesion, transcription, and translation.

Calreticulin (CRT) is a calcium-binding chaperone that is highly concentrated in the lumen of the endoplasmic reticulum (ER). Cotranslational insertion of CRT into the ER lumen involves the same machinery used for the biosynthesis of luminal, secreted, and membrane proteins. Recognition of the N-terminal signal peptide by signal recognition particle stalls translation and facilitates mRNA-ribosome complex targeting to the translocon, the protein-conducting channel in the ER membrane (24). Upon emergence on the luminal side of the translocon, the N-terminal signal peptide is cleaved by signal peptidase. Within the ER lumen, CRT participates in protein folding and calcium homeostasis (16, 25, 35).

Over the past decade, CRT has also been implicated in a variety of processes that occur outside of the ER lumen. Cell surface CRT has been shown to function in both antigen presentation and complement activation (18, 19). More perplexing are the activities of CRT reported to occur in the cytoplasm. For example, CRT binds to the sequence KXGFFKR found in the cytoplasmic tail of α -integrins (40), an observation that prompted investigation into the role of CRT in cell adhesion. While there is general agreement that CRT expression levels are correlated with enhanced cell adhesion, it has remained an issue of debate whether the underlying mechanism involves physical interactions between CRT and integrins in the cytoplasm or whether it involves the influence of CRT from within the ER lumen (10, 34).

Because the CRT binding sequence in α -integrins is also

found in the DNA binding domain of nuclear receptors, several laboratories have analyzed the interplay between CRT and the receptors for glucocorticoids, androgen, retinoic acid, and vitamin D. CRT antagonizes nuclear receptor transcription activity in cells and blocks nuclear receptor binding to cognate DNA response elements in gel shift assays, findings that suggest nuclear CRT may limit nuclear receptor interactions with promoter elements (6, 14, 45, 50).

Independent evidence that CRT has nuclear functions came from our biochemical purification of cytosolic factors that stimulate nuclear protein export in a permeabilized-cell model. HeLa cell-derived and recombinant CRT can stimulate nuclear export of glucocorticoid receptor in vitro, and glucocorticoid receptor export is defective in *crt*^{-/-} cells grown in culture (2, 21). Extending the theme of CRT functions outside of the ER are UV cross-linking experiments showing that CRT binds directly to structured elements in certain cellular and viral RNAs (1, 20, 23, 47, 51). In cell-based assays, CRT negatively regulates the translational efficiency of transcripts encoding the cyclin-dependent kinase inhibitor p21 and the myeloid transcription factor C/EBP (20, 23, 47).

The aforementioned examples are difficult to reconcile with a CRT distribution that is restricted to the ER lumen. Cell surface localization of CRT would require escape from KDEL-based retention and recycling mechanisms in the secretory pathway (3). Even more problematic are the CRT functions that occur in the cytoplasm and nucleus, which are predicted to depend either on CRT protein synthesis outside of the ER or on a mechanism that facilitates CRT relocation from the ER lumen to the cytosol. In the course of experiments addressing CRT biosynthesis, we found that CRT is fully inserted into the ER and that it subsequently undergoes retrotranslocation to the cytosol. CRT is the first example of a eukaryotic protein that undergoes retrotranslocation in order to function outside the ER lumen.

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MATERIALS AND METHODS

Subcellular fractionation. Digitonin was used to selectively permeabilize the plasma membrane of cells and release cytosolic proteins. Adherent cells were trypsinized, collected by centrifugation at $1,500 \times g$, washed in transport buffer (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA), and diluted to 5×10^6 cells/ml in transport buffer supplemented with protease inhibitors (1 μ g/ml each of aprotinin, leupeptin, and pepstatin) and 2 mM dithiothreitol. Cells were permeabilized in 0.005% digitonin for 5 min on ice. Permeabilization was stopped by a twofold dilution with ice-cold transport buffer. Following centrifugation, the supernatant and pellet were harvested. Cell equivalents of intact cells, digitonin-permeabilized cells, and the digitonin-released fraction were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using primary antibodies to CRT, Grp94, protein disulfide isomerase, and Erp72 (all from StressGen, Vancouver, British Columbia, Canada) or to green fluorescent protein (Molecular Probes, Eugene, OR). The yellow fluorescent protein (YFP) construct containing the CRT signal sequence and ER retention signal was purchased (Clontech, Palo Alto, CA), and used to generate YFP lacking the signal sequence (*YFP).

Protease digestion. To determine whether cytosolic and ER-derived CRT have different conformations, we performed protease digestion experiments. Digitonin-released and ER fractions were isolated from Cos7 cells as described above. Extracts were dialyzed into 50 mM Tris, pH 8.0, and fractionated on a MonoQ column using a linear NaCl gradient from 100 mM to 1 M. CRT-containing fractions from each MonoQ run were digested with trypsin (1:100 trypsin-CRT) for 30 min at 30°C in the presence or absence of 20 mM Ca^{2+} . Reactions were analyzed by immunoblotting using anti-CRT primary antibody and by silver staining.

Transcription assay. NIH 3T3 cells were plated in 35-mm wells at a density of 2.5×10^5 cells/ml and grown for 24 h. Transfections were performed using Cytofectene (Bio-Rad, Hercules, CA) transfection reagent with 60 ng *Renilla* luciferase, 540 ng Gal4-luc, and 600 ng of ERX plasmid as indicated. Transfections were performed in triplicate, measured using the Dual Luciferase System (Promega, Madison, WI) 24 h posttransfection, and plotted as the mean $\pm$ standard deviation. ERX contained the 17-amino-acid N-terminal signal sequence from mouse *CRT1* (MLLSVPLLLGLLGLAAA), the Gal4 DNA binding domain (amino acids 2 to 94 in Gal4), and the p53 activation domain (amino acids 1 to 37 in p53). ERX also contained a hemagglutinin (HA) epitope which allowed comparison of expression levels by immunoblotting with anti-HA 16B12 (Babco, Berkeley, CA). The levels of ERX expression for all constructs (which range in size from 25 kDa to 90 kDa) were analyzed on a single blot. Only the relevant molecular weight region is shown. The ERX and CRT forms that lack the N-terminal signal sequence are designated *ERX and *CRT, respectively. The minimal signal peptidase cleavage site inserted between CRT and YFP contained 10 amino acids from the CRT signal sequence (LLGLLGLAAA).

Immunoprecipitation. Cos7 cells were grown overnight in 100-mm plates, and transfected with plasmids encoding CRT-HA and T7-ubiquitin; 24 h posttransfection, cells were treated with 40 μ M MG132 or vehicle (dimethyl sulfoxide) overnight. Following two washes in ice-cold phosphate-buffered saline (PBS), cells were scraped into PBS supplemented with protease inhibitors and dithiothreitol and collected by centrifugation. The resulting pellet was snap-frozen in liquid nitrogen. Cell pellets were then resuspended in lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% NP-40) supplemented with protease inhibitors and dithiothreitol and incubated for 20 minutes on ice. Immunoprecipitations were performed overnight at 4°C using the anti-HA 16B12. Bound and unbound fractions were analyzed by SDS-PAGE and immunoblotting using HA (Babco) and T7 (Novagen, Madison, WI) antibodies.

Cell-binding assays. We coated 96-well plates with 10 μ g/ml collagen IV dissolved in 0.05 N HCl (Sigma, St. Louis, MO) for 1 hour at room temperature. After coating, wells were rinsed three times with PBS. The *crt*^{-/-} cells were transfected with plasmids encoding CRT or *CRT (lacking the N-terminal signal sequence), or empty vector; 24 h posttransfection, *crt*^{+/+} and *crt*^{-/-} mouse embryo fibroblasts (MEFs) were seeded in 96-well plates and incubated for 90 min at 37°C. Cells were washed three times in warm PBS, and bound cells were fixed in 4% paraformaldehyde for 20 minutes. After washing six times in PBS, cells were stained with crystal violet (Sigma) for 3 h at 4°C. Crystal violet was extracted from cells with 0.2% Triton X-100, and the amount of dye was measured by absorbance at A_{570} .

Boyden chamber cell migration assays. BioCoat inserts (8- μ m pore size) (Becton Dickinson, Bedford, MA) were used in 24-well dishes to measure cell migration. The underside of each insert was treated with 10% fetal bovine serum prior to the assay. *crt*^{+/+} and *crt*^{-/-} MEFs were washed, resuspended in serum-free medium (10^5 cells/ml), and applied (5×10^4 cells) to the top chamber. The

bottom chambers were filled with 750 μ l medium containing 10% serum to stimulate migration. Following incubation at 37°C for 48 h to allow migration, the chambers were rinsed with PBS and fixed with 4% paraformaldehyde for 20 min. Cells that underwent migration were detected by 4',6'-diamidino-2-phenylindole (DAPI) staining and counted using a 20X objective on an upright Nikon fluorescence microscope (Melville, NY; model number E800) equipped with a Hamamatsu C-4742-95 charge-coupled device camera. Image acquisition was performed using Openlab v. 3 software. *crt*^{-/-} cells did not display obvious spreading defects when viewed by phase microscopy.

Fluorescence microscopy. Cells were plated on glass coverslips overnight prior to transfection, and processed for immunofluorescence microscopy 24 h posttransfection. Coverslips were rinsed in PBS, fixed in PBS/3.7% formaldehyde for 20 min at room temperature, rinsed in PBS, and permeabilized in PBS/0.2% Triton X-100 for 5 minutes. Coverslips were blocked with 2% serum and 2% serum albumin (in PBS) for 20 min at room temperature prior to staining with antibodies to CRT (StressGen) or T7 (Novagen). Cells were visualized using a Nikon microscope (40x oil immersion lens) and image acquisition was performed using Openlab 3 software.

Cell culture. Stable cell lines expressing CRT, *CRT, YFP (containing the CRT N-terminal signal sequence), and *YFP were generated using Flp-In CHO cells (Invitrogen, Carlsbad, CA). Protein fusions were cloned into the BamHI and XhoI sites of the pFRT/TO vector and cotransfected with the pOG44 plasmid that encodes Flp recombinase (Invitrogen). Transfectants were selected in 0.5 mg/ml hygromycin B. The *crt*^{+/+} and *crt*^{-/-} MEFs (32) were maintained in medium supplemented with 10% fetal calf serum. Retrotranslocated CRT was 5 to 15% of total cellular CRT, depending on the cell line.

RESULTS

We set out to determine how the results from a variety of systems that invoke CRT function in the cytoplasm and nucleus can be reconciled with CRT biosynthesis as an ER protein. The best biochemical evidence for a cytosolic pool of CRT is based on classical subcellular fractionation, which revealed that cells contain both microsomal and cytosolic pools of CRT (21). The cytosolic pool can be released by treating cells with digitonin, a cardiac glycoside that selectively permeabilizes the cholesterol-rich plasma membrane but leaves organelles, including the ER and nuclear envelope, intact. Using this approach, we assessed the relative levels of cytosolic CRT in different cell types (Fig. 1A).

The percentage of CRT that was released by digitonin varied with cell type and was highest in Cos7 cells, where it represented ~14% of the total CRT. As expected, the ER luminal chaperones Grp94 and PDI were not released from cells upon digitonin treatment, indicating that the presence of CRT in the cytosolic fraction is not due to permeabilization of the ER membrane (Fig. 1B). To further establish that the ER membrane was not compromised by digitonin treatment, we used immunofluorescence microscopy to test whether an antibody could bind to an ER-targeted yellow fluorescent protein. The anti-YFP antibody failed to detect YFP in the lumen of the ER in cells treated with digitonin unless the sample was permeabilized with Triton X-100 (0.2%) (Fig. 1C). Because soluble proteins are released under the same conditions that leave the ER intact, digitonin permeabilization provides a facile assay to distinguish between proteins that are cytosolic versus proteins that are found in membrane compartments including the ER.

Cytosolic CRT is derived from the ER pool. We next set out to determine the origin of the cytosolic pool of CRT. Isoform diversity was eliminated as the basis for generating cytosolic CRT because both *CRT* genes in mammals encode protein isoforms that appear to contain an N-terminal signal sequence (*CRT1*, MLLSVPLLLGLLGLAAA, and *CRT2*, MVSARALLWAICR

FIG. 1. Cytosolic fraction of CRT can be released from cells by treatment with digitonin. (A) The cells used throughout our studies were treated with 0.005% digitonin for 5 min. Antibody binding to CRT was quantitated using the Odyssey gel system, and the percent of cellular CRT released was calculated by dividing the amount of CRT in the released fraction by the amount of CRT present in intact cells. (B) Cell permeabilization assay to determine cytosolic and ER compartmentalization of endogenous proteins. Intact cells, digitonin-permeabilized cells (containing the ER fraction), and the released fractions were analyzed by immunoblotting. Permeabilization of the plasma membrane releases the cytosolic pool of CRT, which is not the case for chaperones restricted to the ER lumen (Grp94 and protein disulfide isomerase [PDI]). (C) The integrity of the ER membrane is preserved at low concentrations of digitonin. Cos7 cells were transfected with ER-targeted YFP. Cells were treated with digitonin (to permeabilize the plasma membrane) or Triton X-100 (to permeabilize all membranes) and probed with an antibody that detects YFP. At low concentrations of digitonin, the antibody cannot access YFP expressed within the ER.

LIVA) (36). The signal sequence of the isoform encoded by the *CRT1* gene directs efficient ER insertion when fused to a YFP reporter. After insertion into the ER lumen, YFP was not released from cells treated with digitonin (Fig. 2A). This result suggests that the cytosolic pool of CRT does not arise from translation on free ribosomes as a consequence of poor recognition of the CRT signal sequence by signal recognition particle or inefficient engagement with the translocon (41). In addition, CRT synthesized from a single copy transgene in CHO cells results in both ER and cytosolic pools of CRT, demonstrating that a transcript encoding the full-length, signal sequence-containing gene gives rise to both forms of the protein (Fig. 2A). Finally, it is unlikely that cytosolic CRT is generated by internal initiation because translation from methionines downstream of the authentic start site would generate a polypeptide at least 10 kDa smaller than the size of CRT.

To formally establish that cytosolic CRT is derived from the

pool in the ER lumen, an epitope cleavage assay was designed to register whether CRT has been processed in the ER. The assay is based on specific recognition of the signal peptide cleavage site by signal peptidase, a multisubunit enzyme that is restricted to the lumen. The T7 epitope (11 amino acids) was fused to the N terminus of two forms of CRT, one that contained the signal sequence (T7-CRT) and one that lacked the signal sequence (T7-*CRT) (Fig. 2B). Immunofluorescence microscopy of cell lines stably expressing these proteins revealed T7 reactivity only in the form that was targeted to the cytoplasm (T7-*CRT) (Fig. 2C and 2D, lower panels), confirming that cytoplasmic factors do not alter T7 immunoreactivity. Cell fractionation and immunoblotting established that the T7 epitope was absent from both the ER and cytosolic forms of CRT that are first targeted to the ER (Fig. 2D, middle panels). Because epitope removal from CRT in this assay is dependent on ER targeting and processing by signal peptidase,

FIG. 2. CRT signal sequence directs efficient insertion into the ER. (A) CHO cells stably expressing YFP engineered with or without the N-terminal CRT signal sequence (YFP, targeted to ER; *YFP, targeted to cytoplasm) were used in the cell permeabilization assay. Except where indicated (*), the constructs used in this study contain an N-terminal signal sequence. CHO cells stably expressing mouse CRT either lacking or containing its signal sequence were assayed in the same manner, and detected using a CRT antibody that recognizes mouse but not hamster CRT. Note that the concentration of digitonin used was sufficient to permeabilize 90 to 95% of the cells (determined by trypan blue staining). The release efficiency of a protein will be influenced by protein-protein interactions and nuclear localization; thus, the fraction of CRT identified as cytosolic is probably an underestimate. (B) Signal sequence cleavage assay to establish that cytosolic CRT is processed through the ER. Diagram of constructs used to show N-terminal T7 epitope is removed by signal peptidase cleavage (blue arrow). (C) CHO cells stably expressing T7 epitope-tagged mouse CRT were assayed for signal sequence cleavage by immunofluorescence microscopy. T7 immunoreactivity is lost when CRT is synthesized as an ER protein but retained when CRT is synthesized as a cytosolic protein. (D) Cell permeabilization assay to show the T7 epitope was absent from cytosolic CRT that was synthesized as an ER protein (bkgd, background band).

the data show that at least the N terminus of CRT is inserted into the ER lumen prior to the appearance of CRT in the cytoplasm.

While the above data clearly indicate that CRT translation is initiated at the ER, it was important to address whether or not translation is completed at the ER. Biosynthesis of cytosolic CRT could, in principle, be the consequence of premature

ribosome release from the ER membrane, after which translation is completed in the cytoplasm. Alternatively, CRT might be synthesized as an ER protein; following cotranslational insertion into the ER lumen, CRT could undergo retrotranslocation into the cytoplasm. To date, the only classes of proteins known to undergo retrotranslocation are misfolded proteins, major histocompatibility complex (MHC) class I heavy

FIG. 3. CRT is fully inserted into the ER lumen prior to retrotranslocation. (A) Diagram of constructs used to show ER insertion. A minimal signal peptidase cleavage site from the CRT signal sequence (black box) was inserted between CRT and YFP. (B) Immunoblotting (anti-YFP) of the CRT-YFP fusions expressed in Cos7 cells. CRT-YFP targeted to the ER via the endogenous CRT signal sequence is cleaved at the minimal signal peptidase cleavage site, yielding the ~25-kDa YFP. In the cell permeabilization assay, the cleaved YFP product is not released by digitonin. In contrast, the CRT-YFP fusion that is not targeted to the ER is not cleaved at the minimal signal peptidase cleavage site, but is released by digitonin in the cytosolic fraction.

chain, and certain bacterial toxins including the cholera toxin A subunit (28, 44, 48, 49). After retrotranslocation, misfolded proteins and MHC are degraded, whereas bacterial toxins re-fold and mediate ADP-ribosylation of protein substrates in the cytosol.

To determine whether CRT undergoes partial or complete insertion into the ER prior to its appearance in the cytoplasm, we engineered a CRT fusion protein that would register exposure of the CRT C terminus to the ER lumen. A minimal signal peptidase cleavage site was inserted in-frame between the C terminus of CRT and the N terminus of YFP (Fig. 3A). If CRT is fully translated at the ER, the CRT-YFP fusion should be cleaved into ~50-kDa (CRT) and ~25-kDa (YFP) polypeptides. If, however, cytoplasmic CRT is generated by premature ribosome release during CRT translation, we should observe a ~75-kDa CRT-YFP product by immunoblotting because the minimal peptidase cleavage site will not be exposed to signal peptidase within the ER lumen.

Cos7 cells were transfected with a YFP fusion that is directed to the ER by the CRT N-terminal signal sequence (CRT-YFP) or a YFP fusion that lacks an ER-targeting signal sequence (*CRT-YFP) and is therefore translated in the cytoplasm. Following transfection, cells were treated with digitonin to release cytosolic proteins. The fractions were then analyzed by immunoblotting with an anti-YFP antibody. We observed robust cleavage of CRT-YFP and retention of YFP

FIG. 4. Cytosolic CRT is protease resistant. Cells were treated with digitonin to release cytosolic proteins. The remaining permeabilized cells were put through two freeze-thaw cycles to permeabilize organelles. ER and cytosolic fractions were enriched for CRT by filtration over a MonoQ anion-exchange column. Peak fractions of each run were digested with trypsin (1:100 trypsin-protein) for 30 min at 30°C. Trypsin digests were then probed for CRT. As expected, CRT in the ER fraction was degraded by trypsin in the absence of Ca²⁺, whereas it was protease resistant in the presence of high Ca²⁺. Cytosolic CRT, however, was protease resistant in the presence and absence of Ca²⁺, suggesting that cytosolic CRT has a different structure. (B) The enriched cytosolic fractions do not contain trypsin inhibitors. Silver staining of the trypsin digests indicates that trypsin degrades cytosolic proteins in the presence and absence of Ca²⁺.

in the permeabilized cell fraction, indicating that the CRT signal sequence directed highly efficient ER targeting and that the cleavage site between CRT and YFP was exposed to the ER lumen (Fig. 3B, lanes 1 to 3). Translating the fusion protein directly in the cytoplasm (*CRT-YFP) to preclude exposure of the minimal peptidase cleavage site to signal peptidase generated an uncleaved ~75-kDa protein that was released from cells treated with digitonin (Fig. 3B, lanes 4 to 6). Proteolytic cleavage in this assay demonstrates luminal exposure of the minimal cleavage site, and shows that CRT undergoes complete insertion into the ER lumen. These data, together with our epitope cleavage results (Fig. 2), suggest that CRT within the lumen of the ER is the precursor to cytosolic CRT.

Conformation of cytosolic CRT. The conformation of CRT can be influenced by a variety of factors, including Ca²⁺, Zn²⁺, and ATP binding, and the presence of a disulfide bond (11). The conformation of CRT, in turn, is thought to help specify substrate recognition by determining whether it binds directly to protein or to carbohydrate moieties in the ER (37, 42). The ER lumen contains a high Ca²⁺ concentration and is an oxidizing environment whereas the cytoplasm contains low Ca²⁺ levels and is reducing. We therefore hypothesized that ER- and cytosol-derived CRT would display conformational differences that could be detected by limited proteolysis.

We prepared CRT-enriched fractions from the ER and from the cytosol and used limited proteolysis to probe CRT conformation. As reported by other laboratories (11), ER-derived CRT was rapidly degraded by trypsin in the absence of Ca²⁺,

FIG. 5. Transcription-based assay to measure retrotranslocation of CRT. (A) Design of the assay and constructs used for transfection. ERX is a fusion of the DNA-binding domain (DBD) from Gal4p and the activation domain (AD) from p53 and contains the N-terminal CRT signal sequence. Because ERX is retained in the ER, it generates only a background level of luciferase activity. Protein disulfide isomerase (PDI) is an ER luminal protein similar in size and isoelectric point to CRT. (C) CRT promotes retrotranslocation of ERX, measured by the level of luciferase activity (CRT-ERX). (D) CRT domains involved in retrotranslocation. Deletion of the N domain of CRT resulted in a high level of retrotranslocation, whereas deletion of the C domain resulted in no retrotranslocation. (E) The C domain is sufficient for retrotranslocation when fused to ERX or protein disulfide isomerase. Luciferase assays were normalized to cotransfected *Renilla* luciferase, performed in triplicate, and are plotted with the standard deviation. The levels of ERX reporters were analyzed by immunoblotting on a single gel, but only the region of the gel containing the ERX fusion is shown.

but was rendered protease resistant when preincubated with Ca^{2+} (Fig. 4A, lanes 4 to 6). This is thought to occur because Ca^{2+} drives the formation of a more compact conformation of CRT (26). Strikingly, cytosol-derived CRT was resistant to trypsin digestion even in the absence of Ca^{2+} , suggesting that cytosolic CRT has adopted a different structure than ER CRT (Fig. 4A, lanes 1 to 3). Analysis of the digests by silver staining showed that trypsin degraded the bulk of cytosolic and ER proteins in the samples regardless of Ca^{2+} addition (Fig. 4B).

Novel assay for retrotranslocation. We devised a luciferase-based transcription assay to measure CRT retrotranslocation in intact cells. The CRT signal sequence was inserted N-terminal to the DNA binding domain of Gal4 and the activation domain of p53 to generate a reporter (ERX) that is targeted to the ER lumen (Fig. 5A). If a protein is retrotranslocated, appending it to ERX will result in retrotranslocation of the reporter. Once in the cytoplasm, the ERX fusion will enter the nucleus (via the Gal4 nuclear localization signal) and activate transcription of Gal4-luc. Thus, the assay measures the retrotranslocation and transcriptional activity of ERX when fused to proteins of interest. Fusing CRT that lacks a signal sequence to the Gal4 DNA binding domain and the p53 activation domain (*CRT-ERX) generated a protein that is active for transcription (78.23 ± 3.28 relative light units [RLU]) (Fig. 5B). In

contrast, expressing severalfold more ERX, which is targeted to the ER, only resulted in near background levels of transcription (5.43 ± 1.41 RLU). CRT-ERX yielded a fivefold increase over background (28.01 ± 6.30 RLU). As a control, ERX was fused to protein disulfide isomerase (PDI), an ER chaperone with a size and isoelectric point similar to those of CRT. PDI-ERX generated only 3.11 ± 1.37 RLU, indicating that PDI does not retrotranslocate in this assay. Therefore, CRT is capable of specifically mediating retrotranslocation of the ERX reporter protein from the ER to the cytoplasm.

CRT domains and retrotranslocation. The N-terminal substrate-binding domain interacts directly with protein, carbohydrates, and RNA; the central proline-rich domain contains high-affinity, low-capacity Ca^{2+} binding sites; and the acidic C-domain contains low-affinity, high-capacity Ca^{2+} binding sites (34). Analysis of CRT deletion derivatives in the retrotranslocation assay revealed a 10-fold increase in activity ($1,024.55 \pm 34.66$ RLU) upon removal of the CRT N-terminal domain, suggesting that substrate binding could be repressive to CRT retrotranslocation. Deletion of the C-terminal domain (CRTΔC-ERX) resulted in complete loss of retrotranslocation activity. Because deletion of the C domain enhances CRT binding to substrate (37), the loss of retrotranslocation observed with CRTΔC could be due to prolonged substrate in-

teractions. We addressed this possibility by testing the sufficiency of the C domain in retrotranslocation. The C domain fused to ERX gave an activity (166.98 ± 32.21 RLU) that was comparable to that of full-length CRT fused to ERX (103.35 ± 24.87 RLU). Moreover, we found that the C domain could mediate retrotranslocation when fused to protein disulfide isomerase, another ER luminal protein (72.64 ± 26.51 RLU). Thus, the C domain is both necessary and sufficient for retrotranslocation of CRT.

CRT retrotranslocation is independent of ER-associated degradation. Misfolded proteins in the ER are retrotranslocated to the cytoplasm and degraded by the proteasome in a pathway known as ER-associated degradation (48). Although alternative ER-associated degradation pathways exist, many proteins require ubiquitylation, proteasome activity, or both in order to undergo ER-associated degradation (8, 17, 31). We considered that CRT may be retrotranslocated as part of ER-associated degradation, but a pool of retrotranslocated CRT evades degradation because it is an inefficient substrate for the proteasome. Cholera toxin, for example, is believed to escape ER-associated degradation because it lacks the lysines necessary to specify ubiquitylation (39). The large number of lysines in CRT (37 of 400 total amino acids) suggested that it could be a substrate for ubiquitylation and ER-associated degradation. If CRT retrotranslocation is linked to the ER-associated degradation pathway, CRT should accumulate as ubiquitylated intermediates in the presence of the proteasome inhibitor MG132.

We treated cells with MG132 and examined the molecular weight of hemagglutinin-tagged CRT by immunoprecipitation and blotting (Fig. 6). Inhibiting the proteasome with MG132 did not induce the accumulation of high molecular weight, ubiquitylated forms of CRT (Fig. 6A, compare lanes 3 and 6). To increase the sensitivity of the assay, T7 epitope-tagged ubiquitin was cotransfected with CRT-HA. Ubiquitin- forms of CRT were not observed when CRT was enriched by immunoprecipitation and probed for T7-ubiquitin (Fig. 6A, compare lanes 9 and 12 and lanes 21 and 24). Both MG132 and T7-ubiquitin were efficacious in the assay since ubiquitin-modified polypeptides were detected in lysates from cells treated with these reagents (Fig. 6A, lane 22). Using the *in vivo* transcription assay, we also tested whether CRT could retrotranslocate in the presence of MG132. When cells were treated with proteasome inhibitor, retrotranslocation of CRT was not affected, indicating that retrotranslocation of CRT is not dependent on proteasome activity (Fig. 6B). Retrotranslocation of ERX, however, increases twofold compared to background activity in the absence of MG132, suggesting that ERX is an ER-associated degradation substrate. The fact that CRT is not ubiquitylated under these conditions indicates that it utilizes a ubiquitin- and proteasome-independent pathway for retrotranslocation.

Cytosolic CRT and cell adhesion. It has been shown previously that CRT can increase cell binding to extracellular matrix. The ability of CRT to enhance cell adhesion could be due to its activity within the ER lumen (34). However, CRT can be coimmunoprecipitated with integrins in response to cell attachment to extracellular matrix (9). This observation supports the alternative view that cytosolic CRT contributes to cell adhesion.

FIG. 6. Retrotranslocation of CRT is not linked to protein degradation. (A) Assay for ubiquitylation of CRT. HA epitope-tagged CRT was transfected into Cos7 cells in the absence (lanes 1 to 6) and presence (lanes 7 to 12) of T7 epitope-tagged ubiquitin (T7-Ub), in either the absence or presence of the proteasome inhibitor MG132 (40 μ M). CRT-HA was immunoprecipitated and blotted using the HA (lanes 1 to 12) and T7 (lanes 13 to 24) antibodies. Ubiquitylated forms of CRT were not detected after MG132 treatment, and CRT was not immunoprecipitated by the HA antibody, indicating that CRT is not a substrate for the proteasome. The ladder of endogenous proteins detected with the T7 antibody demonstrates MG132 inhibition of the proteasome (lane 22). (B) Proteasome activity is not required for retrotranslocation of CRT. NIH 3T3 cells were transfected with *ERX, CRT-ERX, and ERX. After overnight treatment with MG132, retrotranslocation of *ERX and CRT-ERX was not affected, whereas ERX activity increased twofold.

To address this issue, *crt*^{+/+} and *crt*^{-/-} MEFs (32) were tested in cell motility and adhesion assays. In a Boyden chamber assay, the *crt*^{-/-} MEFs had a decreased migratory capacity compared to *crt*^{+/+} MEFs (Fig. 7A). *crt*^{-/-} MEFs also showed reduced binding to collagen IV relative to *crt*^{+/+} MEFs (Fig. 7B), which is consistent with previous results showing that CRT functions in the pathway of integrin-mediated cell attachment (10). *crt*^{-/-} MEFs were transiently transfected with CRT

FIG. 7. CRT functions outside of the ER. (A) *crt*^{-/-} MEFs displayed a reduced level of motility in a transwell migration assay compared to *crt*^{+/+} MEFs. (B) CRT expression enhances cell binding to extracellular matrix. *crt*^{+/+} and *crt*^{-/-} MEFs were assayed for binding to collagen type IV. Only *crt*^{+/+} MEFs responded to collagen IV. (C) Cytoplasmic CRT promotes cell adhesion. Transiently transfected CRT targeted directly to the cytoplasm (*CRT) stimulated *crt*^{-/-} cell binding to collagen type IV. Expression levels of transiently transfected CRT (lane 1) and *CRT (lanes 2 to 4) in *crt*^{-/-} cells are shown.

that lacks the N-terminal signal sequence (denoted *CRT) to determine whether cell binding to collagen IV is modulated by cytosolic CRT. Expression of *CRT in *crt*^{-/-} MEFs stimulated cell binding to collagen IV in a dose-dependent manner (Fig. 7C). Thus, retrotranslocation may provide a link between the ER lumen and cell adhesion machinery.

DISCUSSION

CRT functions in the ER as a Ca²⁺ storage protein and chaperone that, in concert with calnexin and protein disulfide isomerase, mediates protein folding and quality control (12, 16). The possibility that CRT functions outside the ER has been viewed with skepticism because all isoforms encode an N-terminal signal sequence and because the high concentration of CRT in the ER/nuclear envelope has not allowed convincing localization of CRT in the nucleus (38). CRT function on the cell surface is, however, viewed as a significant aspect of complement activation and antigen presentation (18, 19). As the cell surface is topologically equivalent to the ER lumen, CRT can escape KDEL-dependent ER retention and move through the secretory pathway.

CRT domains and retrotranslocation. CRT is composed of three functional domains. The N and P domains of CRT are important for protein and carbohydrate interactions with substrates in the ER lumen (27). The C domain of CRT contains multiple Ca²⁺ binding sites, which, based on structural similarities to calnexin, make direct contact with conserved regions

in the N domain to form a carbohydrate recognition site (43). Using a novel assay that measures retrotranslocation in living cells, we examined the ability of these domains to promote the movement of a reporter protein out of the ER lumen. We found that the N domain is inhibitory to retrotranslocation, the P domain displays no activity in the assay, and the C domain is both necessary and sufficient for retrotranslocation. These data highlight the importance of the C domain in retrotranslocation and suggest that it may contain a signal that is recognized by machinery in the ER lumen. Whether CRT is exported via the translocon remains to be determined.

Because our assay relied on deletions and protein fusions, it remains formally possible that retrotranslocation mediated by the C domain is part of a pathway that senses an unfolded polypeptide. We do not favor this explanation because there was no evidence that CRT retrotranslocation is linked to a protein degradation pathway. It is also possible that retrotranslocation activity measured in certain of our assays reflects indirect effects on retention mechanisms that normally maintain chaperones in the ER lumen. For example, a carbohydrate-binding domain is formed by contact between the N and C domains in CRT (27, 43). Since deletion of the N domain reduces CRT interactions with substrates (27), the enhanced retrotranslocation observed upon deletion of the N domain could reflect relief of a luminal retention mechanism. Our data showing that the C domain mediates retrotranslocation when fused to the ER protein protein disulfide isomerase, however, establishes that retrotranslocation is not due simply to decreased interactions with substrates. We hypothesize that the C domain of CRT contains a retrotranslocation signal, in the form of either a peptide sequence or a structure that is recognized by the retrotranslocation machinery.

Early observations suggesting the presence of cytosolic CRT. CRT was initially identified in the nucleus using both subcellular fractionation and immunocytochemistry. Dedhar and coworkers demonstrated that CRT could bind to the KLGFFKR peptide, an amino acid sequence found in the cytoplasmic domain of α -integrins (14). This peptide is similar to the conserved KXFFKR motif found in steroid hormone receptors, leading to a series of studies of CRT interactions with steroid hormone receptors. Subsequent work established that CRT could interact directly with hormone receptors such as the glucocorticoid and androgen receptor and could inhibit steroid-sensitive gene transcription (6, 14). Though these findings were difficult to reconcile with the reported intracellular localization of CRT, it was suggested that, under certain physiological conditions, a nuclear pool of CRT could interact with steroid hormone receptors. This nuclear pool would be generated either by synthesis on free ribosomes due to an alternative isoform, or through export from the ER (5). In contrast to these early observations linking CRT to steroid hormone receptor activity, later reports suggested these results were due to experimental artifacts (33). Thus, despite multiple reports of cytoplasmic functions of CRT, the prevailing dogma has been that CRT acts solely from within the ER.

Diverse functions of CRT outside the ER. CRT can influence cytoplasmic pathways indirectly from within the lumen of the ER, either through Ca²⁺ buffering or modulation of Ca²⁺ channels in the ER membrane (7). A notable example is CRT regulation of calcineurin, a Ca²⁺/calmodulin-dependent pro-

FIG. 8. Targets and functions of CRT within and outside of the ER lumen. See text for discussion. GR, AR, RAR, and VDR, glucocorticoid, androgen, retinoic acid, and vitamin D receptors, respectively.

tein phosphatase that dephosphorylates and promotes nuclear import of the transcription factor NFAT (13). Nonetheless, the cytoplasmic and nuclear functions of CRT that depend on physical interactions between CRT and substrates cannot be explained by indirect effects (see Fig. 8). These include CRT binding to nuclear receptors (6, 14, 50) and viral and cellular mRNAs (1, 20, 23, 47, 51). CRT represses translation of the myeloid transcription factor C/EBP α by binding to a GC-rich stem loop in the 5' untranslated region of the transcript (23). This occurs in acute myeloid leukemia patients when the t(3, 21)(q26;q22) translocation generates a chimeric transcription factor that activates CRT expression (20). In this context, the level of cytosolic CRT resulting from retrotranslocation may be a critical determinant of growth and differentiation by modulating translation.

The cytoplasmic functions of CRT, including binding to steroid hormone receptors and integrins, are dependent upon the Ca²⁺-bound state of CRT. Within the ER, Ca²⁺ binding can induce conformational changes in CRT that influence its substrate binding affinity. In the nucleus, Ca²⁺ levels influence whether CRT exports nuclear export signal-containing substrates or hormone receptors (22). The protease resistance of cytosolic CRT shown here suggests that a calcium-independent conformational change is acquired in the cytosol, but the driving force for this conformation is unknown. Phosphorylation is a candidate mechanism for inducing conformational changes in the cytoplasm, given that CRT has been identified as a phosphoprotein by several groups (15, 29, 46) and that there is a phosphorylation site in the N terminus of CRT. Direct binding of ATP to CRT can also induce conformational changes, but it has not been established whether or not this reaction occurs *in vivo* (11).

In dissecting the biosynthetic pathway for CRT, we determined that cytosolic CRT is derived from the ER pool via a mechanism that is not based on features of the signal sequence. CRT is fully inserted into the lumen of the ER prior to its appearance in the cytoplasm, indicating that it undergoes retrotranslocation. The ability of CRT to undergo retrotranslocation and avoid ubiquitylation and degradation is a feature

shared with A/B bacterial toxins, including cholera toxin (28). However, CRT is the first example of a eukaryotic protein that undergoes retrotranslocation in order to function in a different subcellular compartment. Like chaperones that are translated directly in the cytoplasm, CRT that undergoes translocation from the ER lumen participates in diverse processes that include gene expression and cell adhesion.

ACKNOWLEDGMENTS

We thank Reid Gilmore, David Castle, Dan Burke, Dan Hebert, and especially Leonard Shank for critical comments on the manuscript. We also thank Josh Kelley for helpful discussions throughout the project. The CRT-HA construct as well as *crt*^{-/-} and *crt*^{+/+} MEFs were kindly provided by M. Michalak.

This work was supported by the NIH.

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