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Regulation of Protein Compartmentalization Expands the Diversity of Protein Function

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Summary

Proteins destined for the secretory pathway are translocated into the endoplasmic reticulum (ER) by signal sequences that vary widely in their functional properties. We have investigated whether differences in signal sequence function have been exploited for cellular benefit. A cytosolic form of the ER chaperone calreticulin was found to arise by an aborted translocation mechanism dependent on its signal sequence and factors in the ER lumen and membrane. A signal sequence that functions independently of these accessory translocation factors selectively eliminated cytosolic calreticulin. In vivo replacement of endogenous calreticulin with a constitutively translocated form influenced glucocorticoid receptor-mediated gene activation without compromising chaperone activity in the ER. Thus, in addition to its well-established ER lumenal functions, calreticulin has an independent role in the cytosol that depends critically on its inefficient compartmentalization. We propose that regulation of protein translocation represents a potentially general mechanism for generating diversity of protein function.

Introduction

Approximately half of all eukaryotic proteins have destinations other than their site of synthesis in the cytosol. During or shortly after their synthesis, localization signals in these proteins are recognized by dedicated machinery to facilitate their trafficking and cellular compartmentalization. Although the basic principles and essential machinery for segregation of proteins to the various cellular organelles have been established, both the quantitative and regulatory aspects have been largely neglected. Proteins are often implicitly presumed to be segregated to their putative destination with essentially 100% fidelity and efficiency. However, this is unlikely to be the case given that any multistep cell biological process has inherent limitations to its efficiency. The actual range of efficiencies in vivo for protein segregation to various intracellular organelles is generally poorly quantified. Furthermore, the fate of the nonsegregated fraction and the consequences of its presence for cell physiology are not considered in most studies of protein localization and function.

The mechanistic basis of protein translocation into the ER has been defined by the study of a few particularly efficient, and experimentally simple, model substrates (reviewed by Rapoport et al., 1996; Johnson and van Waes, 1999). In the mammalian system, a signal sequence in the substrate to be translocated across the ER is recognized as it emerges from the ribosome by the signal recognition particle (SRP) (Walter and Johnson, 1994). This complex of SRP bound to the ribosome-nascent chain (RNC) is targeted to the membrane via a GTP-dependent interaction with the SRP receptor. The RNC is then transferred to a translocon whose central channel is formed by the Sec61 complex. Productive insertion of the nascent chain into this channel requires signal sequence recognition by the Sec61 complex (Jungnickel and Rapoport, 1995). Once the chain has engaged the translocation channel to gain access to the ER lumen, it is transported concurrently with its synthesis by a ribosome that remains tightly bound to the Sec61 complex.

As these basic steps and machinery of translocation have become better understood, some attention has recently turned toward exploring the differences rather than commonalities among substrates. One long observed and striking difference is the tremendous diversity in the signal sequences that mediate translocation (von Heijne, 1985). Although they all share certain features such as hydrophobicity that allow them to be uniformly recognized by SRP, the signal sequence of each substrate is unique. This diversity was generally thought to represent a lack of selective pressure to maintain any exact sequence motif necessary for the basic signal sequence functions. However, it is becoming more apparent that sequence variations among signals have several functional consequences: different efficiencies of interactions with the translocon (Rutkowski et al., 2001, 2003; Kim et al., 2002), different requirements for accessory translocon components such as TRAM (Voigt et al., 1996) or the TRAP complex (Fons et al., 2003), and the choice of translocation pathway taken by the substrate (Ng et al., 1996; Wittke et al., 2002).

In the few cases in which they have been examined, such differences in signal function can lead to effects on translocation or secretion efficiencies (Kim et al., 2002; Rutkowski et al., 2003; Rane et al., 2004; Holden et al., 2005), glycosylation (Rutkowski et al., 2003), or, in the specialized case of the prion protein, membrane integration and topology (Rutkowski et al., 2001; Kim et al., 2002; Kim and Hegde, 2002; Ott and Lingappa, 2004). Remarkably, the signal-translocon interaction is sufficiently variable among substrates that it can be exploited to selectively inhibit the translocation of some, but not other, proteins by small molecules (Garrison et al., 2005). Thus, productive translocon engagement by signal sequences is emerging as a key step in translocation that involves multiple components, is widely variable among substrates, and has consequences for subsequent events in protein localization and matura-

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tion. These observations raise the possibility that functional maturation of a protein could be influenced by regulating the mode or efficiency of signal-translocon interactions. Indeed, a recent analysis quantifying the efficiency of protein segregation into the ER revealed widely varying amounts of a nonsegregated population depending on the choice of signal sequence (Levine et al., 2005). This result provided proof-of-concept that a signal-containing protein can reside and potentially function in the cytosol as a consequence of inefficient translocation into the ER. Whether differential translocation is indeed exploited by the cell to generate multiple functional populations of some proteins remains largely unexplored.

Many studies have incidentally observed unexpected cellular locations for signal sequence-containing proteins ostensibly intended for the secretory pathway (summarized in Levine et al., 2005). One such example is calreticulin (Crt), an abundant ER lumenal chaperone with well-established roles in protein quality control and calcium homeostasis (Ellgaard and Helenius, 2003; Michalak et al., 2002). In addition to these functions, Crt has been implicated by several independent studies in other cellular activities that occur in locations outside the ER (Coppolino and Dedhar, 1998; Michalak et al., 1999). These functions include binding to the cytosolic tails of integrins to modulate their function (Coppolino et al., 1997), translational regulation of certain mRNAs (Iakova et al., 2004), modulation of gene expression by steroid hormone receptors (Dedhar et al., 1994; Burns et al., 1994), and nuclear export (Holaska et al., 2001). Whether Crt mediates these effects directly by its proposed presence in the cytosol, or indirectly through its well-established functions in the ER lumen, remains controversial (Michalak et al., 1996). An indirect consequence of ER lumenal Crt is exceedingly difficult to exclude given the central roles of Crt in protein maturation and calcium homeostasis, both of which can have a wide array of downstream consequences.

Based on our work on signal sequence function, we hypothesized that at least a small fraction of Crt would fail to be segregated into the ER lumen. Given the high abundance of Crt in the ER, small amounts of non-ER Crt (e.g., even less than 5% of the total) that would easily elude reliable detection techniques could nonetheless be biologically significant. If this nontranslocated population of Crt were to escape degradation and fold appropriately, it could contribute to distinct functions in the cytosol, in addition to its ER lumenal roles. We have investigated this hypothesis and identified the pathway by which a functional Crt is generated in the cytosol. These findings are synthesized into a more broadly applicable framework for considering protein segregation to the ER as a precisely controlled and potentially regulated process that imparts greater diversity to the localization and function of proteins.

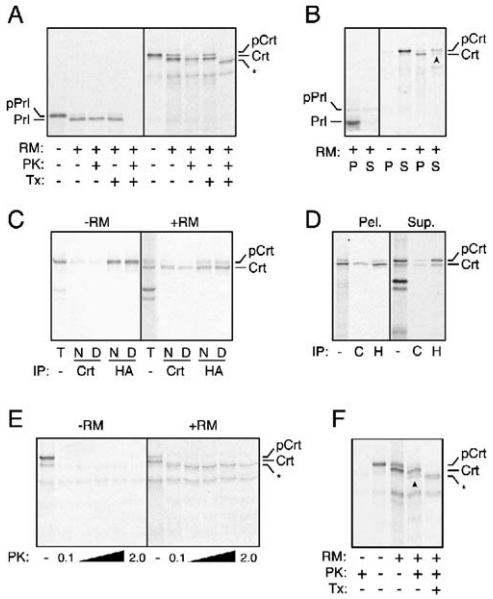

Figure 1. Reconstitution of Selective Dual Localization of Crt In Vitro

(A) Prolactin and Crt were synthesized in vitro with reticulocyte lysate (RL) lacking or containing ER-derived rough microsomes (RM) as indicated below the autoradiograph. After translation, samples were divided into equal aliquots for incubation on ice without or with proteinase K (PK) and/or Triton X-100 (Tx) as indicated. Following termination of the protease digestion, samples were analyzed by SDS-PAGE and autoradiography. The positions of the precursors (pPrl and pCrt) and mature forms (Prl and Crt) of the products are indicated. In addition, a protease-resistant core of Crt generated by digestion of folded product (see below) is indicated by an asterisk (*). Note that although subtle, the percentage of Crt that is processed and subsequently protected from PK digestion was consistently observed to be lower than that seen with Prl.

(B) Prl and Crt, translated without or with RM, were separated by sedimentation into a membrane pellet (P) and soluble supernatant (S). Equal aliquots of each fraction were analyzed. Note that in the Crt sample with RM, a small amount of Crt at the position of the mature form is found in the supernatant (arrowhead).

(C) Crt was translated without or with RM. An aliquot of the total products was reserved for direct analysis (T), while the remainder was divided into four equal aliquots for immunoprecipitation with antibodies against either Crt or HA under both native (N) and denaturing (D) conditions. Note that mature Crt is immunoprecipitated comparably well with both antibodies. By contrast, the immature form of Crt is selectively precipitated by the HA antibody.

(D) The pellet and supernatant from a Crt translocation reaction fractionated as in (B) were either analyzed directly (T) or immunoprecipitated with Crt (C) or HA (H) antibodies under native conditions.

(E) Crt was translated without or with RM, and it subsequently digested for 1 hr with increasing concentrations of PK from 0.1 to 2.0 mg/ml. While the precursor (generated in the absence of RM; left panel) is digested fully even at the lowest PK concentration, mature Crt is resistant to digestion at the highest PK concentration.

(F) Crt translated in the absence or presence of RM was digested with PK as in (A). Note that the PK-resistant fragment generated by mature Crt can be observed in the sample with intact RM (arrowhead), suggesting that at least some mature Crt is present in the cytosol.

Results

Reconstitution of Cytosolic Crt Generation In Vitro

The synthesis and ER segregation of an HA-tagged rat calreticulin (hereafter referred to simply as Crt) was examined in a mammalian in vitro translation and translocation system (Figure 1A). Expression of Crt in reticulocyte lysate (RL) resulted in the synthesis of a ~55 kDa product corresponding to a full-length Crt precursor.

The majority of this band was shifted to a slightly smaller, signal sequence-cleaved product (corresponding to mature Crt) when translation was carried out in the presence of ER-derived rough microsomes (RM). The mature Crt product (but not the Crt precursor) was protected from protease digestion, indicating its localization in the lumen of RM (Figure 1A).

These results were qualitatively similar to the translocation properties of prolactin (Prl), a well-studied secretory protein (Figure 1A). However, over the course of several similar experiments, we consistently noticed subtle quantitative differences. First, the efficiency of translocation, as assessed by the percentage of total synthesized material that was subsequently protected from protease digestion, was always less for Crt than for Prl (~65% versus ~90%). This was observed in two distinct ways. First, the percentage of signal sequence cleavage, a marker for access of the N-terminal domain of the substrate to the lumenal space of the ER, was lower for Crt than for Prl. And, second, of the signal-cleaved material, the percentage that was protected from protease was always slightly less for Crt than for Prl (~80% versus ~95%).

Sedimenation analysis of the translocation reactions (Figure 1B) confirmed that both of these observations can be explained by slightly reduced translocation efficiency of Crt relative to Prl. Hence, the cytosolic fraction contained essentially all of the Crt precursor and a small, but discrete, proportion of the processed Crt product (arrowhead, Figure 1B). The majority of the processed Crt was in the RM fraction, consistent with its translocation into the lumen and protection from PK digestion. By contrast, nearly all of the synthesized Prl was converted to the signal-cleaved form of Prl and was found in the sedimented fraction; again, this is consistent with its quantitative protection from PK digestion. Together, these results demonstrate that the translocation efficiency of Crt is lower than for Prl in the in vitro system, resulting in the generation of a small, but detectable, population of cytosolic Crt under conditions in which very little Prl remains in the cytosol. Importantly, at least some of this cytosolically located Crt is of a size that comigrates with signal sequence-cleaved, mature Crt that is in the ER lumen.

We used an immunological approach to determine if the lower-molecular weight cytosolic population of Crt is in fact full-length processed Crt (i.e., signal cleaved). To do this, we took advantage of a fortuitous observation that our anti-Crt antibody preferentially recognized the N terminus of processed, but not precursor, Crt (Figure 1C). Hence, in contrast to an antibody against the C-terminal HA tag, the anti-Crt antibody immunoprecipitates the signal-cleaved form of Crt with higher efficiency than the precursor. When this antibody was used for immunoprecipitations of the cytosolic population of Crt, the lower band was preferentially immunoprecipitated with higher efficiency than the upper band (Figure 1D), confirming that it represented Crt that had been processed. Furthermore, both bands in the cytosolic fraction were immunoprecipitated equally well with the anti-HA antibody, confirming that both bands contain an intact C terminus. This argues that the lower band in the cytosolic fraction represents full-length processed Crt that is generated when the Crt is synthesized in the presence of RM. This lower band was not seen if the Crt was synthesized without RM (e.g., Figure 1C), or if RM was added to the reaction after Crt had been synthesized (data not shown). Thus, it appears to be generated as a product of the Crt translocation reaction, and it is selective to Crt since a similar population was not observed for Prl. This form of Crt will hereafter be referred to as cyCrt, a cytosolically localized Crt that is processed, and therefore distinguishable from precursor Crt (pCrt), which simply failed to be translocated at all into the ER.

To assess the folding status of in vitro-generated cyCrt, we used resistance to limited digestion with proteinase K (PK) as a probe of folded domains. ER lumenal Crt that is properly folded acquires a conformation that generates a core folded domain of ~45 kDa that resists further digestion by PK (Figure 1E; indicated by an asterisk). The precursor Crt does not attain this same conformation, as evidenced by its complete digestion under identical conditions (Figure 1E, left panel). If the products of a Crt translocation reaction include any cytosolic Crt that acquires a similar fold to the ER lumenal Crt, digestion with PK (in the absence of detergent) should convert it to the characteristic 45 kDa fragment. Indeed, careful examination of the PK digestion products reveals this 45 kDa band in amounts that correspond to the levels of properly processed Crt observed in the supernatant by the sedimentation assay (Figure 1F, arrowhead). As expected, digestion after permeabilization of the membrane with detergent generates the 45 kDa product from the ER lumenal Crt. From these data, we conclude that cyCrt is a full-length, signal-cleaved protein that appears to have a similar folded conformation to ER lumenal Crt, but one that is different than pCrt. Thus, the events leading to the selective generation of a cytosolic form of Crt can be reconstituted in vitro.

Signal Sequence-Dependent Control of Cytosolic Crt

Generation of a cytosolic form of an otherwise translocated protein was observed for Crt, but not for Prl. To investigate the basis for this selectivity, we analyzed chimeric constructs between Crt and Prl. The domains of both proteins that direct their translocation into the ER are their respective signal sequences (Figure 2A). These not only differ widely in their sequence characteristics, but also potentially in their functional properties and efficiencies. To determine whether such differences could account for the generation of cyCrt, we analyzed Prl-Crt, a construct in which the native Crt signal sequence has been replaced with that from Prl. Direct comparison of Prl-Crt with Crt in translocation assays (Figures 2B and 2C) revealed a significantly reduced amount of cyCrt generated from Prl-Crt. In particular, very little Prl-Crt was found in the cytosolic fraction after sedimentation analysis (Figure 2B), and protease digestion of this sample did not generate the 45 kDa core domain characteristic of cyCrt (Figure 2C).

Conversely, Crt-Prl, in which the signal of Prl is replaced with the Crt signal sequence, was observed to be translocated slightly less efficiently than pPrl when analyzed by sedimentation assays (Figure 2D). These

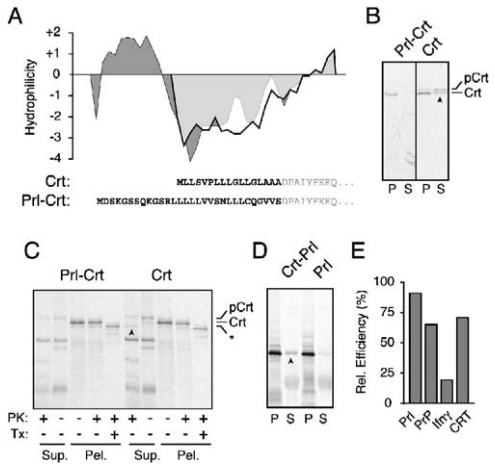

Figure 2. The Dual Localization of Crt Is Influenced by the Signal Sequence

(A) The signal sequences of Prl and Crt are shown (bold type) below their superimposed hydropathy analyses.

(B) Prl-Crt and Crt were translated in the presence of RM and fractionated by sedimentation into a cytosolic supernatant (S) and membrane pellet (P). The arrowhead indicates the increased amount of Crt in the supernatant relative to the corresponding Prl-Crt sample.

(C) Prl-Crt and Crt were translated in the presence of RM and separated into cytosolic and membrane fractions (Sup. and Pel., respectively) that were each analyzed by PK digestion. The arrowhead indicates the protease-resistant core generated from mature Crt that is found in the supernatant of the Crt sample, but not the corresponding Prl-Crt sample.

(D) Crt-Prl and Prl were translated in the presence of RM and fractionated by sedimentation into a cytosolic supernatant (S) and membrane pellet (P). The arrowhead indicates the increased amount of Crt-Prl in the supernatant relative to the corresponding Prl sample.

(E) Bar graph of the relative efficiencies of translocon gating activity by the indicated signal sequences using a previously characterized reporter assay (Kim et al., 2002; see Figure S1).

results suggest that the signal sequence plays a key role in the generation of cyCrt. Analysis of the efficiency of the Crt signal sequence function by using previously characterized assays (Kim et al., 2002) confirmed and extended this conclusion. In this experiment, we observed that the targeting function of the Crt signal is comparable to the targeting seen with other signals, including the Prl signal (data not shown). By contrast, the posttargeting function of initiating translocation through the translocon was slightly reduced for the Crt signal relative to the Prl signal (Figure 2E and Figure S1; see the Supplemental Data available with this article online). In fact, the efficiency was almost equal to that observed for the signal sequence from the prion protein (PrP), which, in recent studies, has been shown to also generate a cytosolic form (termed cyPrP) both in vitro and in vivo (Rane et al., 2004). Thus, we conclude that Crt, like PrP, has a signal sequence that displays a slight, but detectable, inefficiency in its posttargeting functions of translocon gating and/or initiation of translocation. This inefficiency appears to be the basis of cyCrt generation since it can be selectively eliminated by the use of the more efficient signal sequence from Prl.

The selective generation of cyCrt during Crt translocation represents a small proportion of the total Crt, and it can be difficult to distinguish from the translocation properties of other proteins like Prl or Prl-Crt (Figure 2). While such a small amount in the cytosolic compartment in vivo could potentially be physiologically relevant (as discussed in the Introduction), the actual observations in Figures 1 and 2 are subtle and close to the limits of detectability and resolution by these assays. We therefore sought to exaggerate the pathway leading to cyCrt generation to both substantiate its presence and gain insight into the mechanisms involved. Since previous analysis of signal sequence efficiency in vivo (Levine et al., 2005) indicated that differences in cell type and culture conditions can selectively and markedly affect the function of some signals, we reasoned that manipulating the composition of ER membranes may influence cyCrt generation. We therefore examined the translocation of Crt and Prl-Crt across ER membranes fractionated to remove subpopulations of proteins (Figure 3). To monitor cyCrt levels, we followed the relative generation of the 45 kDa core domain upon digestion of the translocation reactions with PK. In this assay, fully translocated Crt is completely protected from PK by the membrane, the cytosolic precursor is completely digested, and the signal-cleaved cyCrt generates the 45 kDa band due to its folded conformation. Thus, the ratio of the fully protected 55 kDa protein to the 45 kDa core provides a quantitative measure of the percentage of ER-targeted Crt that is translocated versus converted into cyCrt. Substantially more cyCrt was observed when Crt translocation was analyzed by using RM that had been selectively depleted of lumenal proteins (LD-RM; Figure 3A). By contrast, Prl-Crt generated very little cyCrt, even in the LD-RM. Thus, although the difference in cyCrt generation is rather subtle in RM (Figure 2), depletion of lumenal proteins from the membrane markedly exaggerated the differences in translocation behavior between Crt and Prl-Crt (Figure 3A).

This difference was even more pronounced when, in addition to lumenal proteins, a subset of membrane proteins (most notably the TRAP complex) was removed from the membranes by anion exchange (Fons et al., 2003). In these membranes, the vast majority of Crt (~70%–80%) is generated as cyCrt, with the remainder being translocated completely across the membrane. By contrast, less than 10% of the Prl-Crt was generated as cyCrt in reconstituted proteoliposomes regardless of the presence or absence of the anionic membrane proteins (Figure 3B). Thus, in these reconstituted proteoliposomes, the conditions markedly favor the generation of cyCrt by Crt, but not Prl-Crt. Importantly, it is clear that even though overall translocation efficiency is lower in the reconstituted proteoliposomes, Prl-Crt still does not generate much cyCrt. This suggests that the events leading to the generation of cyCrt are not merely a consequence of low translocation efficiency mediated by the Crt signal sequence. Rather, the nature of the functional interaction between the Crt signal and translocon appears to be qualitatively different than for the Prl signal in a way that favors aborted translocation and generation of cyCrt. Taken together, these results indicate that cyCrt

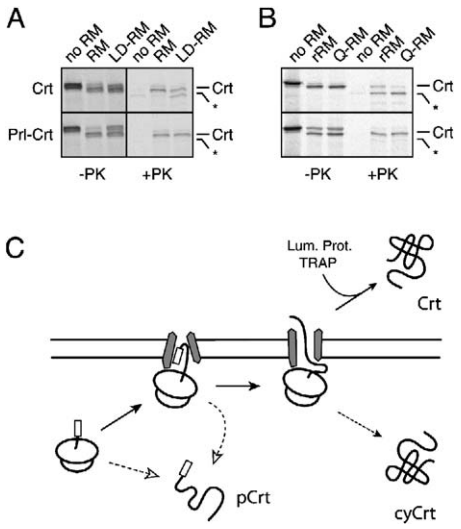

Figure 3. Cytosolic Crt Generation Can Be Modulated by *trans*-Acting Factors

(A) RM selectively depleted of lumenal contents (LD-RM; Garrison et al., 2005) were compared to mock-treated RM in their ability to support translocation of Crt and Prl-Crt. Translocation was assayed by protease protection, in which lumenal Crt is fully protected from digestion, the precursor is completely digested, and cyCrt generates the protease-resistant core indicated by the asterisk (*). Note that for Crt, but not Prl-Crt, depletion of lumenal proteins generates increased amounts of cyCrt.

(B) Translocation of Crt and Prl-Crt were analyzed with proteoliposomes prepared from either unfractionated ER membrane proteins (rRM) or after selective depletion with anion exchange (Q-RM). While this depletion has no effect on translocation of Prl-Crt, Crt generates increased amounts of cyCrt in Q-RM relative to rRM.

(C) Diagram of the pathway for generation of mature cytosolic Crt (cyCrt). Nascent Crt that fails to target or initiate translocation generates pCrt. Crt that initiates translocation sufficiently to be processed by signal peptidase generates both ER lumenal Crt and a minor population of cyCrt that is similarly folded, as judged by its identical resistance to PK digestion. The ER lumenal form is favored in the presence of lumenal proteins and the TRAP complex; in their absence, the cyCrt form is favored. These steps can be bypassed by the Prl signal, which predominantly generates the ER lumenal form regardless of ER factors.

can be generated from nascent Crt molecules that target to the ER membrane, initiate translocation sufficiently to result in signal sequence cleavage, but nonetheless fail to be translocated completely across the membrane (Figure 3C). Consistent with this interpretation, introduction of a glycosylation site close to the N terminus of Crt slightly improves its translocation into the ER lumen with a corresponding decrease in the amount left in the cytosol (Figure S2).

Detection and Manipulation of Cytosolic Crt In Vivo

Having elucidated the basic pathway and parameters of cyCrt generation in vitro, we could now turn to its analysis in vivo. We first determined whether, in cultured cells, cyCrt is generated at detectable amounts. We used both endogenously and exogenously expressed marker proteins in the cytosol and ER lumen to optimize a fractionation procedure suitable for this analysis (Figure 4A). Upon digitonin permeabilization of adherent cells (either MDCK or HeLa), a cytosolic fraction was efficiently released, while extraction of proteins in membrane bound compartments was minimal. Upon subsequent extraction with Triton X-100, the vast majority of these latter proteins were recovered, leaving behind only cytoskeletal and nuclear matrix proteins (Figure 4A). Immunoblotting demonstrated nearly quantitative recovery of HSP90 in the cytosolic fraction, while the ER lumenal chaperones BiP and PDI were in the noncytosolic fraction (Figure 4B). Identical results were obtained in transfected cells expressing cytosolic GFP or a GFP targeted to the ER lumen by fusion to hen egg lysozyme and a KDEL retention sequence (Figure 4B). Furthermore, these exogenously expressed markers behaved as expected by this fractionation procedure. While a small amount of cytosolic GFP contaminated the noncytosolic fraction, the reverse was minimal (~2%; see below).

Exogenously expressed Crt was detected at higher levels (~2- to 4-fold) than Prl-Crt in the cytosolic fraction prepared by this extraction procedure (Figure 4C). Importantly, almost all of the material in the cytosolic fraction of the Crt sample corresponded to the properly processed, mature form. By contrast, only the signal-containing precursor was observed in the cytosolic fraction of Prl-Crt-expressing cells. The noncytosolic fractions of both samples were identical, showing only mature Crt, as expected, for the ER lumenal form (data not shown). The relative amount of Crt in the cytosolic fraction was quantified by comparison to a dilution series of the noncytosolic fraction (Figure 4D). This analysis demonstrated that between ~5% and 20% of the total Crt (depending on the cell type and growth conditions) could be found in the cytosol. Importantly, only ~2% of a cotransfected ER lumenal GFP was found in the cytosolic fraction of the same samples (Figure 4D). These results indicate that, consistent with the in vitro results, a properly processed form of Crt is present in the cytosol of cultured cells. This cyCrt generated in vivo could additionally be distinguished from ER-lumenal Crt on the basis of disulfide bonding (Figure S3). While ER-lumenal Crt contained a single disulfide, cyCrt had all three of its cysteines free, as might be expected in the reducing environment of the cytosol.

To confirm and directly quantify the relative levels of cyCrt generation by Crt versus Prl-Crt, we performed the analysis in a different way. For this experiment, GFP-tagged versions of Crt and Prl-Crt were used. Each was cotransfected into cells with cytosolic and ER lumenal GFP markers. After fractionation, the cytosolic and noncytosolic fractions were analyzed by blotting with GFP to simultaneously detect Crt-GFP and both markers on the same blot. Thus, Crt-GFP and Prl-Crt-GFP can be compared relative to the same marker proteins by using the same antibody. This approach avoids any potential artifacts of variability from blot to blot of detection differences between antibodies, and it permits subtle distinctions to be made. Furthermore, the amount of cyCrt was quantified by comparision of the cytosolic fraction to a titration of the noncytosolic fraction. As seen in Figures 4E–4G, ~5%–10% of the total Crt was found in the cytosolic fraction, while sub-

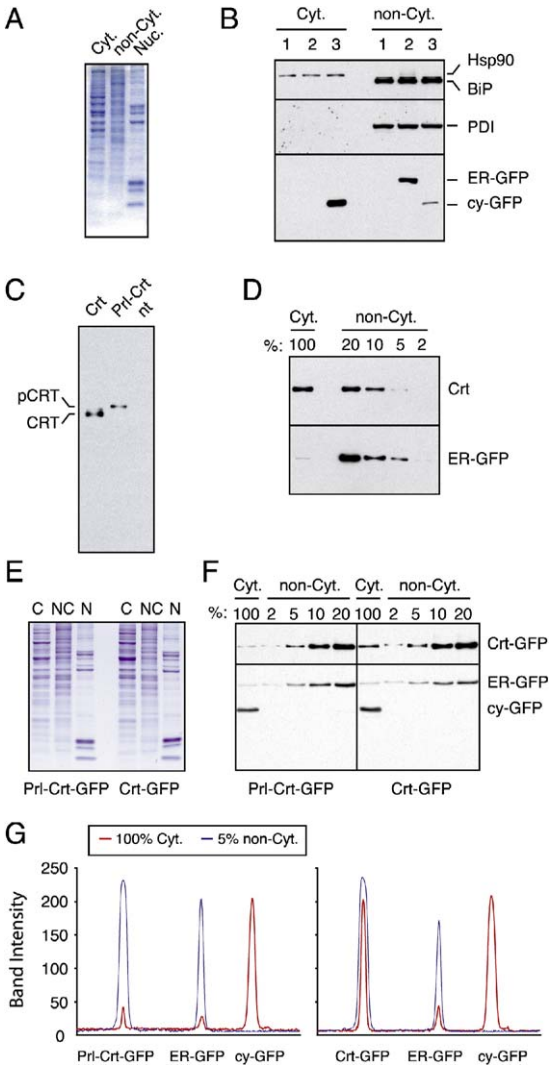

Figure 4. Detection and Modulation of Cytosolic Crt In Vivo

(A and B) HeLa cells that were either untransfected (sample 1), transfected with an ER lumenal GFP marker (sample 2), or transfected with a cytosolic GFP marker (sample 3) were separated by selective extraction into cytosolic, noncytosolic, and nuclear fractions. Equivalent aliquots of each fraction were analyzed by (A) Coomassie blue (sample 1 only) and (B) immunoblotting against several endogenous antigens and GFP markers.

(C) The cytosolic fraction of untransfected HeLa cells (nt) and cells transfected with Crt or Prl-Crt were analyzed by immunoblotting for the HA epitope tag in Crt. Note that the Crt sample has almost exclusively the processed, mature form of Crt in the cytosol. By contrast, only precursor (pCrt) is observed in the cytosol of Prl-Crt cells.

(D) HeLa cells cotransfected with Crt and ER lumenal GFP were separated into cytosolic and noncytosolic fractions. The cytosolic fraction was compared to a titration of the noncytosolic fraction to assess the relative abundances of Crt and ER-GFP.

(E and F) MDCK cells were cotransfected with cytosolic GFP, ER lumenal GFP, and either Crt-GFP or Prl-Crt-GFP. They were then separated into cytosolic (C), noncytosolic (NC), and nuclear (N) fractions that were analyzed by (E) Coomassie blue staining or (F) immunoblotting for GFP. For the immunoblot, samples were analyzed as in (D), where the cytosolic fraction was compared to a titration of the noncytosolic fraction.

(G) Densitometric comparison of the relative amounts of proteins in the cytosolic (solid line) and noncytosolic (dashed line) fractions from the analysis in (F). The "100% Cyt." and "5% non-Cyt." fractions were compared for Prl-Crt-GFP- (left panel) and Crt-GFP-expressing (right panel) cells. Both samples contained the ER lumenal and cytosolic GFP markers as internal references.

stantially less (~2%) of the Prl-Crt was observed in the cytosol. Importantly, in both samples, the cytosolic and ER lumenal GFP markers fractionated identically: cytosolic GFP was quantitatively in the cytosol, while ~2% of the ER lumenal GFP was recovered in the cytosolic fraction. Whether the ~2% of the Prl-Crt found in the cytosol is due to contamination from the noncytosolic fraction, or whether it represents genuine cyCrt, cannot be determined by these experiments. That notwithstanding, the data indicate that Crt generates a cytosolic population that is distinct from Prl-Crt or the ER lumenal GFP in several respects. Not only is it present at higher levels, but it is signal cleaved. Signal sequence processing may permit cyCrt to fold differently than it would in the presence of this extra hydrophobic domain (e.g., see Figure 1). This processing may also permit cyCrt to elude degradation by the cellular quality control pathways, thereby raising the possibility that it could have a functional role in the cytosol, as has been suggested in several previous studies.

Functional Modulation of Gene Expression by Cytosolic Crt

The ability to selectively reduce generation of cyCrt by employing the Prl signal sequence allowed us to assess its putative cytosolic functions independently of its well-established function as an ER-lumenal chaperone. One putative, but controversial, function for cytosolic Crt is its ability to modulate gene expression in response to the activation of steroid hormone receptors (Dedhar et al., 1994; Burns et al., 1994). To examine this idea, we stably expressed Crt-GFP or Prl-Crt-GFP in a previously established $Crt^{-/-}$ mouse embryo fibroblast cell line (Nakamura et al., 2000). The expression levels were evaluated by blotting, and they were shown to be the same for both Crt and Prl-Crt and were comparable to the levels of endogenous Crt (Figure 5A). Localization by fluorescence microscopy showed the expected ER pattern for both Crt and Prl-Crt (data not shown). Note that although Crt has a small proportion in the cytosol relative to Prl-Crt (e.g., Figure 4), this cannot be expected to be observed visually. When assayed for chaperone activity, both the Crt and Prl-Crt cell lines behaved identically to $Crt^{+/+}$ cells: several newly synthesized polypeptides were found associated with Crt shortly after their synthesis, but not after a subsequent chase period (Figure 5B). This is consistent with the known ability of Crt to bind a subset of proteins during their initial folding and maturation in the ER lumen (Ellgaard and Helenius, 2003). Thus, both Crt and Prl-Crt are able to identically reconstitute the ER lumenal function of Crt. In fact, since the signal sequences of Crt and Prl-Crt are cleaved upon their entry into the ER lumen, the ER of these two cell lines is essentially identical with respect to the amount and function of Crt.

To examine the role of cyCrt in the GR pathway, these cells were transfected with a plasmid encoding the lu-

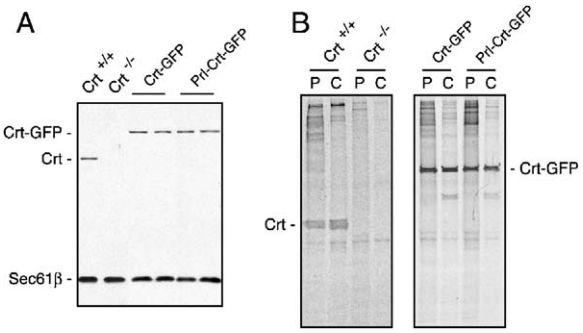

Figure 5. Construction and Characterization of Crt-GFP and Prl-Crt-GFP Cell Lines

(A) Crt$^{-/-}$ cells stably expressing either Crt-GFP or Prl-Crt-GFP were analyzed (in duplicate) by immunoblotting with anti-Crt and anti-Sec61β (an endogenous ER marker). Crt$^{+/+}$ and Crt$^{-/-}$ cells were analyzed in parallel for comparison.

(B) The cells from (A) were assessed by pulse-chase analysis for transient association of Crt with newly synthesized proteins. Cells pulse labeled for 15 min with ^{35}S-Translabel were either harvested immediately (P) or chased for 1 hr without further labeling (C) prior to native immunoprecipitation of lysates with anti-Crt. The position of the Crt and Crt-GFP products is indicated. Note that several high-molecular weight coassociated polypeptides are observed in the pulse sample that subsequently release from Crt upon chase. These are not seen in the Crt$^{-/-}$ cells.

ciferase reporter under control of glucocorticoid response elements (GREs), and the luciferase response was measured before and after stimulation with the glucocorticoid dexamethasone. If the effect of Crt on GR activity is direct, then reduction of cyCrt would be predicted to increase steroid-stimulated GR activity. By contrast, an indirect effect via ER lumenal Crt should show no difference between the Crt-GFP and Prl-Crt-GFP cells. Strikingly, the level of dexamethasone-stimulated GR activity was markedly higher in the Prl-Crt cells than in the Crt cells (Figure 6A). This response was dependent on the GRE, since a control luciferase plasmid lacking it showed no activation. Importantly, this difference in activation was not due to differences in either GR levels (Figure 6A, inset) or Crt levels (e.g., Figure 5A or Figure 6B) in these cells. Upon transfection with exogenous GR (tagged with GFP), the response to dexamethasone was significantly higher, as expected. However, the relative difference in activation between the Crt and Prl-Crt cells remained (Figure 6B) despite identical levels of GR-GFP and Crt expression in both cells (Figure 6B, inset). This differential activation of transcription was specific to the GR pathway since a heterologous transcription factor (Gal4-NFkB) mediated equally efficient activation of its corresponding reporter in both cell lines (Figure 6C). As expected, Gal4-NFkB activity was unaffected by dexamethasone, but it was dependent on a Gal4 binding site in the luciferase reporter plasmid.

These results indicate that the expression of Prl-Crt instead of Crt has a selective effect on GR-mediated gene expression. This effect is not due to a general transcriptional effect, and it cannot be explained by differences in either Crt or GR expression levels. Indeed, not only are Crt expression levels comparable, but the

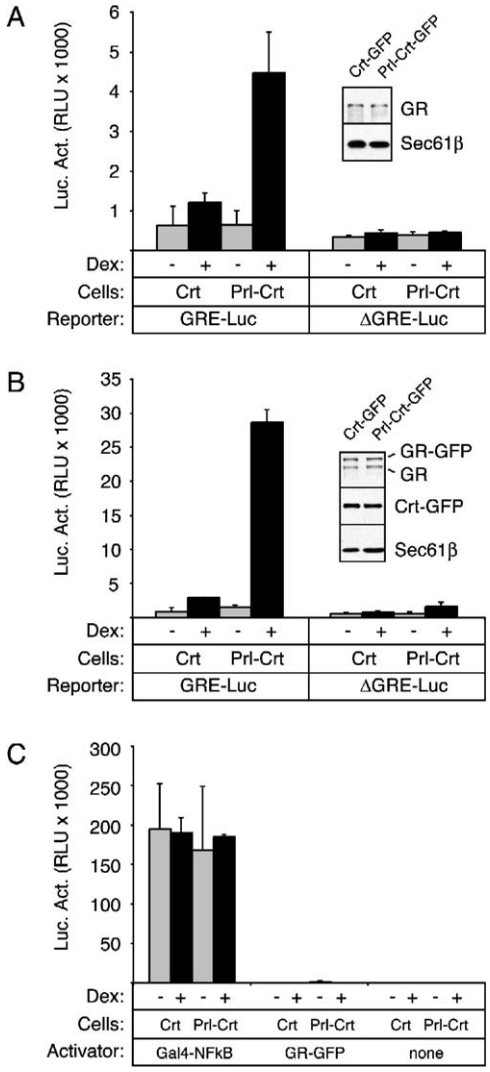

Figure 6. Functional Consequences of cyCrt on GR-Mediated Gene Expression

(A) The stable cell lines expressing Crt-GFP or Prl-Crt-GFP from Figure 5 were transfected with a luciferase reporter preceeded by glucocorticoid response elements (GRE-Luc) or a control reporter in which the GREs were deleted (ΔGRE-Luc). The cells were then left untreated (gray bars) or stimulated with 1 μM dexamethasone (Dex) for 6 hr (black bars) before analysis for luciferase activity (mean ± SD for three replicates). Crt-GFP and Prl-Crt-GFP cells displayed ~1.9-fold and ~6.9-fold stimulation, respectively, upon dexamethasone treatment. The inset shows an immunoblot for GR and Sec61β performed on the same cells as those used for the luciferase assay.

(B) An experiment performed exactly as in (A) was performed on cells additionally expressing GR-GFP (which was co-transfected at the same time as the GRE-Luc or ΔGRE-Luc plasmids). Crt-GFP and Prl-Crt-GFP cells displayed ~3.2-fold and ~19-fold stimulation, respectively, upon dexamethasone treatment. The inset shows an immunoblot for GR (and GR-GFP; top), Crt-GFP (middle), and Sec61β (bottom) performed on the same cells as those used for the luciferase assay.

(C) Crt-GFP and Prl-Crt-GFP cells were cotransfected with a luciferase reporter preceeded by the Gal4 binding element and an activator plasmid expressing either Gal4-NFkB, GR-GFP, or an empty vector. The cells were then left untreated (gray bars) or stimulated with 1 μM dexamethasone (Dex) for 6 hr (black bars) before analysis for luciferase activity (mean ± SD for three replicates).

functional activity with respect to its well-established ER chaperone role is unaffected. Thus, an effect on GR activity by an indirect consequence of compromised Crt activity in the ER lumen can be excluded. Furthermore, the cleaved Prl signal sequence (a fragment of which has been found to potentially interact with calmodulin) (Martoglio et al., 1997) did not influence GR activation independently of Crt (Figure S4). Given the very precise manner in which the Prl signal sequence decreases the generation of cyCrt both in vitro (Figure 2) and in vivo (Figure 4), we conclude that the increased translocation efficiency of Prl-Crt relative to Crt is the basis of the difference in GR-mediated gene activation.

Discussion

In this study, we have demonstrated both in vitro and in vivo that calreticulin is compartmentalized into two functional populations. The major population resides in the ER lumen, where its role as a molecular chaperone and calcium binding protein are well established. The minor population resides in the cytosol, where it is able to influence the functional activity of the glucocorticoid receptor in gene activation. The extent of partitioning between these two populations is controlled by the signal sequence of Crt. By modulating signal sequence function, the cytosolic population could be selectively reduced in vitro and in vivo. This selective modulation of cyCrt proved to be a critical advance for three important reasons.

First, establishing the existence of a very minor putatively cytosolic pool of Crt is extremely difficult without direct comparison to a matched sample that selectively lacks this population. In the absence of such a comparison, the small amount of Crt detected in the cytosolic fraction of cell lysates could easily have been ascribed to artifacts of fractionation or analysis. Hence, the ability to modulate the levels of cyCrt selectively is essential for validating its existence. Second, the demonstration that the choice of signal sequence influences cyCrt levels established that its production is a consequence of inefficient Crt translocation. Had the cyCrt arose through retrotranslocation from preexisting ER lumenal Crt, a slight increase in signal sequence efficiency would not have influenced cyCrt generation significantly. And, third, the ability to selectively reduce the levels of cyCrt allowed us to establish its direct role in GR-mediated gene expression.

Hence, it is now clear that the minor population of Crt in the cytosolic compartment is important for the previously observed effects on GR-mediated gene expression. Precisely how cyCrt influences GR function remains to be established, but it may involve a direct interaction beween the two proteins (Dedhar et al., 1994; Burns et al., 1994) to influence the nuclear export of GR (Holaska et al., 2001). The several other proposed roles for cyCrt (e.g., in integrin-mediated signaling [Coppolino et al., 1997] and translational regulation [Iakova et al., 2004]) also remain to be explored. It will be particularly interesting to elucidate the role(s) of cyCrt in the context of a whole organism. The knockout of Crt in mice displays a variety of developmental defects (Mesaeli et al., 1999; Rauch et al., 2000), the molecular basis of which remains unclear. Which of these phenotypes is due to the lack of ER lumenal versus cytosolic populations of Crt can now be examined by applying the insights from this study to selectively modulate cyCrt levels without affecting the ER lumenal population.

Our findings have illuminated another posttranscriptional mechanism for increasing the diversity of functional gene expression. By controlling the cellular compartmentalization of a protein, two distinct products with different functional roles in the cell can be generated from a single mRNA. Mechanistic analyses in vitro suggest that the decisive step in the partitioning of Crt into these two populations occurs at the translocon. After Crt nascent chains have targeted to the membrane and engaged the translocon, the majority of them complete translocation into the lumen. However, a small proportion is aborted in its forward transport and ultimately resides in the cytosol to generate cyCrt. This "slipping" back into the cytosol appears to occur at a step after the nascent Crt has had at least transient access to signal peptidase, whose enzymatic activity is located on the lumenal side of the translocon. Whether processing of cyCrt by signal peptidase is required for its cytosolic function or for it to elude degradation remains unclear. At present, we can only conclude that lack of processing results in a version of Crt that is folded differently than processed Crt, as judged by limited proteolysis. We also cannot judge whether cyCrt and ER lumenal Crt are folded similarly. Disulfide bond analysis demonstrates at least some structural differences, but it is possible that the overall fold is similar, as suggested by the same resistance to limited proteolysis. Further studies are needed to address these issues.

Transient lumenal access of nascent Crt would also explain how lumenal chaperones and the TRAP complex, which contains a large lumenal domain poised over the translocon (Menetret et al., 2005), can markedly influence the generation of cyCrt. Similarly, glycosylation at a site close to the N terminus also slightly influenced the generation of cyCrt. A plausible model would be that, at an early step after the initiation of translocation, interactions between the nascent Crt and lumenal chaperones and/or TRAP prevent it from "slipping" back into the cytosol. Such slipping into the cytosol has been observed previously for other substrates (Garcia et al., 1988; Ooi and Weiss, 1992; Hegde et al., 1998) and is consistent with the observed gap between the ribosome and translocon that would provide a route of access to the cytosol (Menetret et al., 2005).

How then does the signal sequence from Prl reduce the generation of cyCrt? We postulate that the interaction of the Prl signal with the Sec61 translocon is more stable than the corresponding interaction of the Crt signal. This increased stability would not only reduce slipping of the nascent chain prior to signal cleavage, but it would also allow the nascent chain an increased window of time during which to translocate a slightly longer stretch of polypeptide into the lumen. The initiation of folding within this longer domain on the lumenal side of the translocon would prevent its slippage (Kowarik et al., 2002), thereby increasing the overall efficiency of translocation and precluding the need for lu-

menal chaperones or the TRAP complex to bias forward transport.

We propose that, in contrast to the situation with Prl, a less-efficient signal sequence imposes contingencies on productive translocation by requiring that an early translocation intermediate be stabilized by additional factors (such as TRAM [Voigt et al., 1996], the TRAP complex [Fons et al., 2003], or lumenal chaperones [this study]). In the functional unavailability of some or all such factors, the fate of selected substrates (depending on the features of their signal sequences) would be changed from entering the ER to residing in the cytosol. Thus, the relative amounts of cyCrt in the cell (and hence its associated functional consequences) could be selectively and dynamically changed depending on the state of the translocation machinery of the cell. This model is consistent with the observation that the relative efficiencies of compartmentalization to the ER for different signal sequences varies in a cell type-specific manner (Levine et al., 2005). Whether such changes in compartmentalization efficiency are functionally exploited to respond to physiological or pathological changes in the cellular environment remains to be explored.

Given that highly robust and constitutive signal sequences such as that from Prl appear to be the exception and not the rule (Voigt et al., 1996; Rutkowski et al., 2001; Kim et al., 2002; Fons et al., 2003; Levine et al., 2005), translocational heterogeneity may be far more prevalent than is currently appreciated. Indeed, a wide range of proteins has been observed in unexpected cellular locales (summarized in Levine et al., 2005), the functional relevance of which is unclear. With a better mechanistic understanding of the pathways by which such alternative populations are generated, their abundances can be selectively modulated to explore their cellular roles. This general strategy, illustrated in the current study for one particular example, seems likely to reveal unanticipated or previously unexplained functions for many such proteins. The challenge for future studies will be to elucidate the parameters that regulate translocation, its mechanistic foundation, and the conditions under which this type of regulation is exploited for physiological benefit.

Experimental Procedures

Plasmid Constructions and Antibodies

HA epitope-tagged Rat Crt (RatCrt-HA) was provided by I. Wada (Wada et al., 1995) and was subcloned into the pCDNA 3.1 vector (Invitrogen) for both in vitro and cell culture expression. The GR-GFP expression plasmid was provided by G. Hager (Htun et al., 1996). The GRE-Luc reporter plasmid was provided by J. Drouin. The control ΔGRE-Luc plasmid was made by deletion of the GRE domain by using flanking Hind3 sites. Plasmids encoding Prl-Crt, PrP-Crt, Crt-GFP, Prl-Crt-GFP, Prl, cytoplasmic GFP, ER lumenal GFP, Gal4-NFkB transcription factor (pBD-NFkB), and Gal4-Luc reporter (pFR-Luc) have either been described or made by standard methods (Kim et al., 2002; Fons et al., 2003; Snapp et al., 2004; Levine et al., 2005). Complete details of the subcloning are provided in Supplemental Data. Antibodies were obtained from the following sources: rabbit anti-Crt (catalog no. PA3-900) and rabbit anti-GR (Affinity Bioreagents); mouse anti-HA (Roche; clone 12CA5); mouse anti-GFP (Clontech; clone JL-8); mouse anti-Hsp90, mouse anti-BiP, and rabbit anti-PDI (Stressgen); rabbit anti-Sec61β (Fons et al., 2003).

In Vitro Analysis

In vitro transcription, translation, protease digestions, sedimentation assays, signal sequence gating assays, and immunoprecipitations were performed as described (Kim et al., 2002; Fons et al., 2003), with minor modifications that are specified in either the figure legends or Supplemental Data.

Cell Culture Analysis

All cells (HeLa, MDCK, and the Crt cell lines) were cultured in DMEM containing 10% FBS at 37°C in a humidified environment containing 5% CO_2. Immortalized mouse embryonic fibroblasts from $Crt^{+/+}$ and $Crt^{-/-}$ embryos (clones K41 and K42, respectively) have been described (Nakamura et al., 2000). Stable clones of $Crt^{-/-}$ cells expressing Crt-GFP and Prl-Crt-GFP were isolated by selection of transfected cells with zeocin, after which they were sorted by fluorescence-activated cell sorting (FACS) (by using GFP fluorescence) to remove any nonexpressing cells. Transient transfection of plasmids utilized lipofectamine 2000 (Invitrogen) or FuGENE (Roche). Fractionation of cells into cytosolic and noncytosolic proteins by selective detergent extraction was performed as described (Levine et al., 2005). Pulse-chase analysis for the assessment of Crt chaperone function was performed on cells at ~70% confluency in 6-well dishes. After preincubation for 30 min in cysteine-methionine-free media, cells were pulse labeled with 150 μCi/ml ^{35}S TransLabel (ICN) for 15 min at 37°C. They were either harvested immediately or washed into complete media and further cultured for 1 hr at 37°C. Cells were harvested by first rinsing in PBS and subsequently lysing them in 1 ml ice-cold IP buffer containing 1× Complete Protease Inhibitor Cocktail (Roche). Following removal of insoluble materials by centrifugation (10 min at maximum speed in a microcentrifuge at 4°C), the cleared lysate was immunoprecipitated with antibodies against Crt. For luciferase assays of dexamethasone-induced GR activation, the appropriate plasmids were transfected with lipofectamine as indicated in the figure legends. After 4 hr, the media was exchanged to DMEM containing 10% charcoal-stripped FBS (GIBCO-BRL) and cultured for ~16 hr. The cells were then either treated with 1 μM dexamethasone or solvent for 6 hr prior to analysis for luciferase activity (by using materials from Promega).

Miscellaneous

SDS-PAGE was performed on 10% glycine (for optimal separation of Crt products) or 12% tricine gels (for most other applications). Hydropathy analysis was performed with MacVector software and utilized the Kyte-Doolittle scale with a window size of 7. Quantification of autoradiographs was done by phosphorimaging with the Typhoon system (Molecular Dynamics). Gel images for figures were digitized from scanned films and assembled with Adobe Photoshop and Illustrator software.

Supplemental Data

Supplemental Data including four figures are available at http://www.developmentalcell.com/cgi/content/full/9/4/545/DC1/.

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Supplemental Data

REGULATION OF PROTEIN COMPARTMENTALIZATION EXPANDS THE DIVERSITY OF PROTEIN FUNCTION

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Supplementary Methods

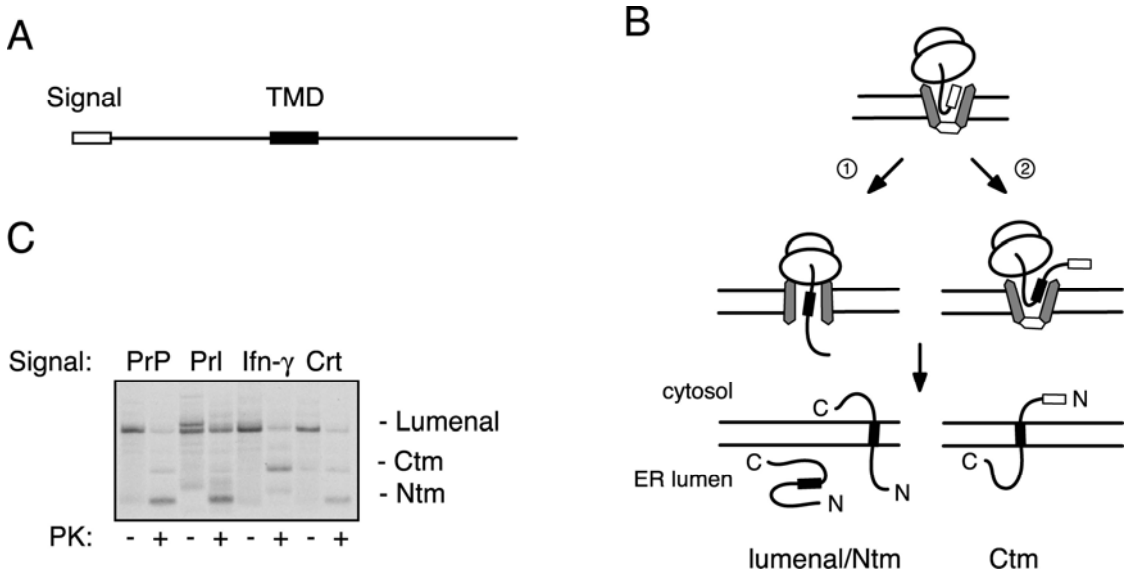
Plasmids. Rat Crt containing an HA epitope tag just preceeding the C-terminal KDEL sequence (RatCrt-HA) was provided by I. Wada and has been described (Wada et al., 1995). The complete open reading frame from RatCrt-HA was amplified by PCR and subcloned into the pCDNA 3.1 vector (Invitrogen) to make the Crt expression plasmid used for both in vitro and cell culture expression. The matched Prl-Crt and PrP-Crt plasmids were made by PCR amplification of the mature domain of RatCrt-HA (i.e., lacking the signal sequence) and subcloned into previously described cassettes (Kim et al., 2002) containing the Prl or PrP signal sequences in the pCDNA 3.1 vector. The plasmid encoding Crt-Prl was made similarly, using a comparable cassette containing the Rat Crt signal sequence and a PCR product encoding the mature domain of bovine preprolactin. No additional linker codons were introduced at the fusion points between the signal sequences and mature domains of these chimeric constructs. The in vitro expression plasmid for Prl has been used previously (Fons et al., 2003). GFP-tagged versions of Crt and Prl-Crt were made by inserting the coding region of monomeric EGFP into a unique Not1 site located just preceeding the C-terminal KDEL sequence. The cytoplasmic GFP marker was expressed from plasmid pEGFP-C1 (Clontech). The ER-lumenal GFP marker has been characterized, and is a

fusion protein containing Hen egg lysozyme, EGFP, and a C-terminal KDEL sequence (Snapp et al., 2004). This coding region was subcloned by standard methods into the pCDNA 3.1 vector for these studies. The expression plasmid for GR-GFP was provided by G. Hager (Htun et al., 1996). The GRE-Luc reporter plasmid, containing two glucocorticoid response elements preceeding the Firefly luciferase open reading frame, was provided by J. Drouin. The control ΔGRE-Luc plasmid was made by deletion of the GRE domain using flanking Hind3 sites. Plasmids encoding the Gal4-NFkB transcription factor (pBD-NFkB) and the corresponding Gal4-Luc reporter (pFR-Luc) were from Stratagene and have been characterized before (Levine et al., 2005). The Crt-CHO construct (Supplementary Fig. S2) was made by site directed mutagenesis of the RatCrt-HA plasmid using the Quickchange method (Stratagene). The Tryptophan codon at position 37 was changed to Serine, thereby introducing a consensus site for glycosylation. The control constructs for analysis of signal sequence gating activity (Supplementary Fig. S1) have been described (Kim et al., 2002). The Crt signal-containing construct for this assay was made in an identical fashion by fusing oligonucleotides encoding the signal sequence of Crt to the PrP-based reporter. CrtΔSS was made by PCR amplifying a portion of the mature domain of Crt (from codon 18 to a unique downstream EcoRV site) and using this fragment to replace the corresponding region of Crt from the start codon to the EcoRV site. An in frame start codon was introduced into the PCR product at the 5' end. This resulted in a deletion of codons 2 thru 17 from Crt to generate CrtΔSS. Prl-GFP-KDEL was prepared by introducing a PCR product coding for mGFP containing a C-terminal KDEL sequence into a previously described cassette containing the signal sequence of Prl (Kim et al., 2002). The localization of this construct to the ER was verified by fluorescence microscopy (data not shown). The construct coding for YFP (pEYFP-C1) was from Clontech.

In vitro analysis. Transcription, translation, and translocation reactions utilized rabbit reticulocyte lysate and canine pancreatic rough microsomes (RM) as previously described (Kim et al., 2002; Fons et al., 2003 and referenes therein). Translation was for 1 h at 32° C. Protease protection assays for translocation were performed on ice for 1 h with 0.5 mg/ml proteinase K (PK) as before (Kim et al., 2002; Fons et al., 2003). Reactions were terminated with excess PMSF (~5 mM) and rapidly transferring the sample to 10 volumes of boiling 1% SDS, 0.1 M Tris, pH. 8. Sedimentation of RM from translation reactions was performed at 4°C by layering the reaction (typically 20 ul) onto 100 ul of a 0.5 M sucrose cushion in physiological salt buffer (100 mM KAc, 50 mM Hepes, pH 7.4, 2 mM MgAc2) and centrifugation at 70,000 rpm for 10 min in a TL100.3 rotor (Beckman). Equivalent proportions of the supernatant and pellet fractions were either analyzed directly, or subjected to further treatments (either immunoprecipitation or protease digstion, as described in the Figure legends) prior to analysis. Immunoprecipitations under native conditions were done at 4°C on samples diluted 10 fold into IP buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 100 mM NaCl) prior to addition of antibodies. Denaturing immunoprecipitations were done on samples first heated to 100° C in the presence of 1% SDS prior to 10 fold dilution into ice cold IP buffer and addition of antibodies. Antibody incubations were for between 2-12 h at 4° C. Immune complexes were recovered with immobilized Protein A or Protein G beads, washed several times in IP buffer, and eluted with SDS-PAGE sample buffer. The assay for measuring the relative efficiencies of signal sequence-mediated gating of the translocon utilized the assay described previously (Kim et al., 2002; also described further in the legend to Supplementary Fig. S1). Proteoliposomes lacking components of the membrane

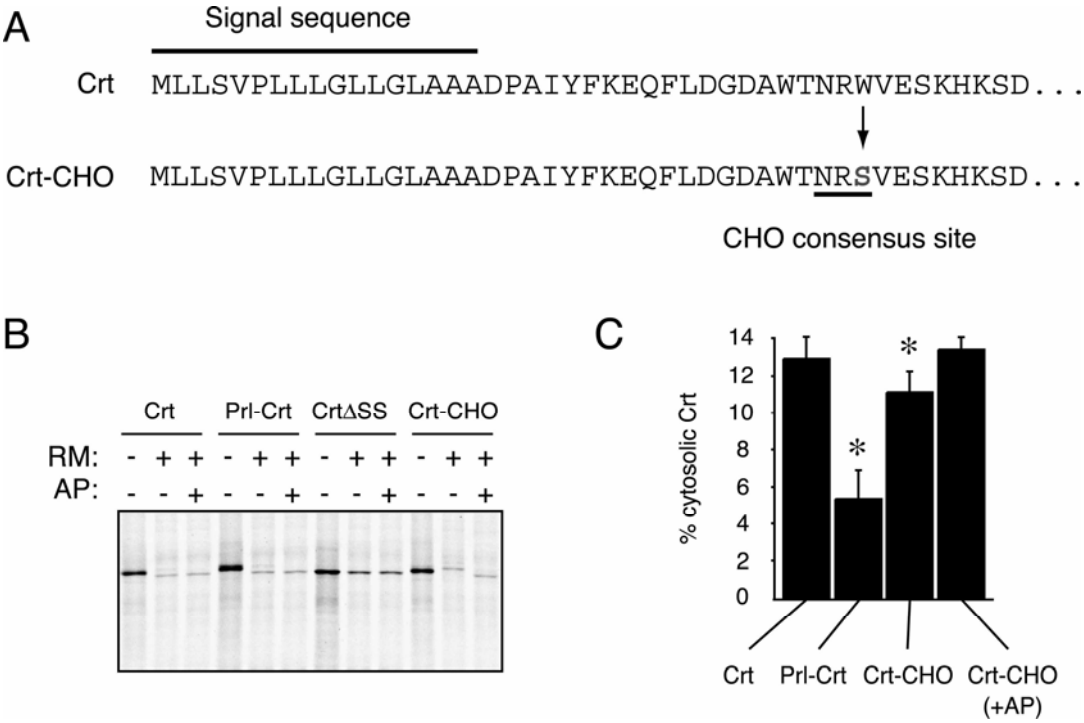
(either lumenal proteins or a subset of membrane proteins) were prepared, characterized, and used for translocation exactly as described before (Garrison et al., 2005; Fons et al., 2003).

Analysis of free cysteines by PEG-maleimide modification. PEG-maleimide was purchased from Nektar Therapeutics and dissolved at a concentration of 12 mM in 2% SDS, 50 mM Hepes, pH 7.4, just before use. Samples to be reacted with PEG-maleimide were adjusted to 2% SDS, 50 mM Hepes, pH 7.4, mixed with freshly prepared PEG-maleimide at a final concentration of 4 mM, and incubated at 37° for 30 min. The reaction was terminated by the addition of one fourth volume of 5X sample buffer containing 0.5 M DTT. Samples were subsequently analyzed directly by SDS-PAGE and immunoblotting. Hela cells were transfected 24 hours prior to analysis with the constructs indicated in Supplementary Fig. S3. For modification of total cell lysates (Fig. S3B), the cells in one well of a 6-well dish were rinsed once in PBS and lysed directly in 500 ul 2% SDS, 50 mM Hepes, pH 7.4. Of this, 100 ul was used for modification with PEG-maleimide, and after completion of the reaction, one twentieth of the sample was analyzed by immunoblotting. For analysis of fractionated cell lysates, the cytosolic and non-cytosolic fractions were prepared as described in Experimental Procedures, precipitated with TCA, and dissolved in 2% SDS, 50 mM Hepes, pH 7.4 prior to modification with PEG-maleimide and immunoblotting. This assay was adapted from previously published procedures (Guo, Z.-Y. et al., Biochemistry, 2005, 44:6537-6546).



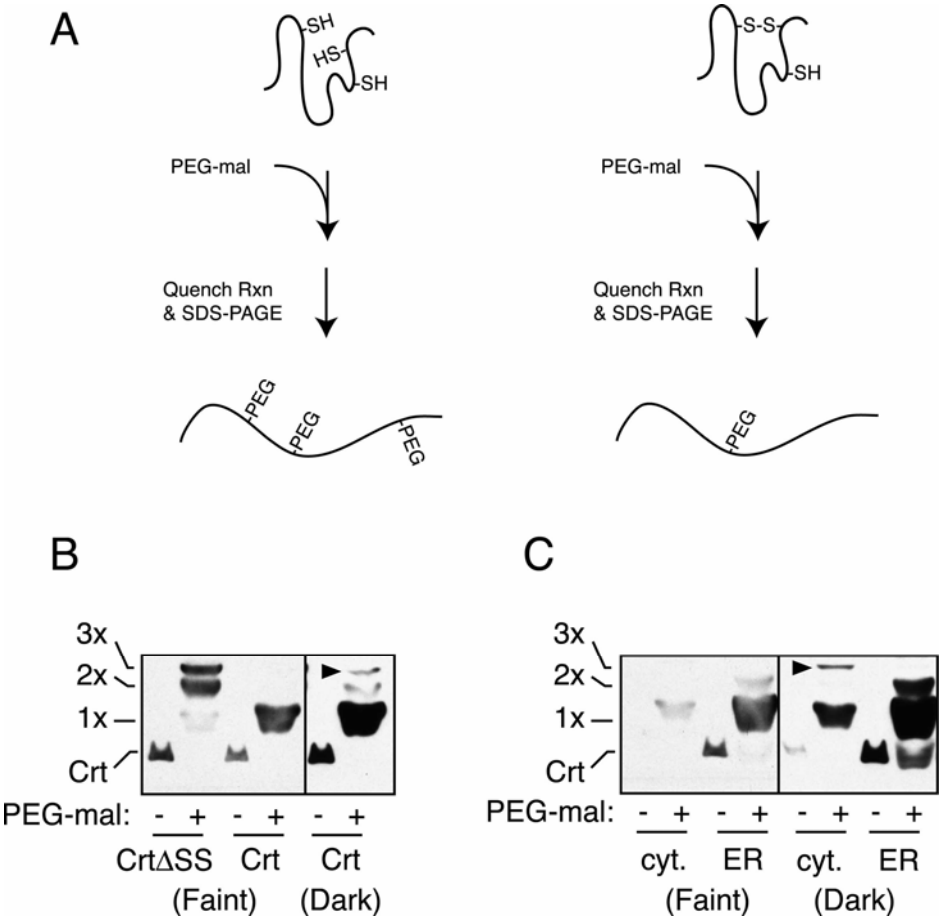
Supplementary Fig. S1 - Analysis of Crt signal sequence efficiency. (A, B) Experimental design. A signal sequence is fused to a reporter construct (based on the Prion protein) that contains within it a single potential transmembrane domain (TMD). After targeting to the translocon, the signal sequence has a small window of time before the TMD is synthesized and emerges from the ribosome. If the signal has successfully gated the translocon and initiated translocation before this occurs (option 1), the nascent chain becomes fully translocated into the lumen or made as a transmembrane protein with the N-terminus in the lumen (termed Ntm). If gating has not occured during this time (option 2), the TMD competes for the translocon and causes the protein to be made as a transmembrane protein with the C-terminus in the lumen (termed Ctm). After the translocation reaction, the relative amounts of the different forms can be determined using a protease protection assay. In this assay, fully translocated protein is completely protected from protease digestion, while the Ntm and Ctm forms are partially digested to generate characteristic sized fragments. The amount of total synthesized protein made in the lumenal/Ntm forms is a direct indicator of the gating efficiency of the signal sequence. Conversely, poorly-gating signal sequences result in increased generation of the Ctm form.

Diagram and assay adapted from Kim et al. (2002). (C) Results from the signal sequence gating assay for the Crt signal compared to previously characterized signals from the Prion protein (PrP), Prolactin (Prl), and Interferon-g (Ifn-g). The positions of the lumenal, Ntm, and Ctm forms of the protein are shown. Quantitation of these results is shown in Fig. 2E.



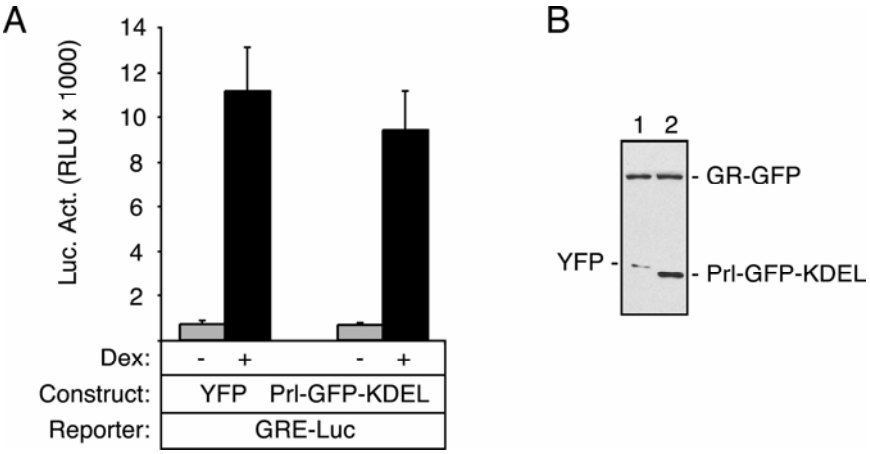
Supplementary Fig. S2 - Influence of Crt glycosylation on generation of cyCrt. (A) Crt was modified to introduce a consensus site for glycosylation 18 residues following the signal sequence to generate the Crt-CHO construct. The sequences of the wild type and mutant constructs are shown, with the mutation indicated with an arrow and consensus site underlined. (B) Crt-CHO is glycosylated. The indicated constructs were translated in reticulocyte lysate in the presence or absence of RM. Where indicated, a peptide acceptor of glycosylation (AP) was included to inhibit glycosylation. CrtDSS is a construct lacking the signal sequence and provides a marker for signal-cleaved, unglycosylated Crt. Note that Crt and Prl-Crt shift to a small molecular weight product in the presence of RM due to signal sequence cleavage. By contrast, Crt-CHO shifts to a higher molecular weight product in the presence of RM due to its glycosylation. If glycosylation is inhibited, the product of the Crt-CHO translation comigrates with the other constructs. (C) Crt, Prl-Crt, and Crt-CHO were analyzed by translocation and sedimentation assays as in Fig. 1B to evaluate the amount of cytosolic Crt that is generated. Crt-

CHO was analyzed both in the absence and presence of the acceptor peptide inhibitor of glycosylation. The results of four separate measurements for each condition were quantified by phosphorimager analysis and shown on the graph. Asterisks indicate a statistically significant difference from Crt ($p<.01$). Note that as expected, Prl-Crt generates substantially less cyCrt. Although subtle, Crt-CHO generates ~14% less cytosolic Crt than wild type. This decrease in cyCrt is a consequence of glycosylation and not the mutation per se, since inhibition of glycosylation of Crt-CHO restores the amount of cytosolic Crt to wild type levels. These results are consistent with a model of cyCrt generation in which a small proportion of nascent chains, after signal sequence cleavage, slip back through the translocon into the cytosol. Glycosylation at a site just after signal cleavage appears to reduce slippage for a proportion of such nascent chains.



Supplementary Fig. S3 - Disulfide bond analysis of cyCrt and ER Crt. (A) Experimental design. A protein containing three cysteines (such as Crt) is treated with PEG-maleimide. If no disulfide bonds are present, the protein can be modified on up to all three cysteines with PEG. The presence of a disulfide bond would result in only one cysteine being modified. These forms can be resolved by SDS-PAGE and immunoblotting to deduce the disulfide bonding status. (B) Wild type Crt or Crt deleted of its signal sequence (CrtDSS) were expressed in Hela cells, recovered under denaturing (but non-reducing) conditions, and treated with PEG-maleimide. The positions of unmodified Crt and Crt modified by one, two, or three PEG molecules are indicated to the left. Note that Crt is modified almost exclusively on a single cysteine, while CrtDSS is readily modified on up to three cysteines. Thus, the majority of Crt is in a disulfide-bonded form. Notably, longer exposures of the blot ('dark' lanes) revealed a small population of 3x-modified

Crt (arrowhead) that could potentially represent a cytosolic, non-disulfide bonded population. (C) To determine whether the 3x-modified Crt is indeed cytosolic, we separated Crt-expressing Hela cells into cytosolic and non-cytosolic (ER) fractions before treating each fraction with PEG-maleiminde. As expected, the majority of Crt is found in the ER fraction (see faint exposure of the blot at left) and this is modified primarily with only one PEG (i.e., disulfide bonded) . Note that a small proportion of this singly-modified population of Crt is also found in the cytosolic fraction, presumably due to a small degree of contamination from the ER fraction. Importantly, the minor population of Crt that can be modified with three PEGs (i.e., non-disulfide bonded) was recovered exclusively in the cytosolic fraction (arrowhead on the dark exposure of the blot). These results provide additional support for the existence of a cytosolic population of Crt that can be distinguished from ER Crt on the basis of disulfide bonding differences.



Supplementary Fig. S4 - Analysis of the Prl signal sequence on GR activity. (A) $Crt^{-/-}$ cells were co-transfected with plasmids containing the GRE-Luc reporter, GR-GFP, and either cytosolic YFP or an ER-localized GFP containing an N-terminal Prl signal sequence and C-terminal KDEL sequence. The cells were then left untreated or treated with 1 uM dexamethasone for 6 hours before analysis for luciferase activity (mean +/- SD; n=5). One replicate of cells from each transfection was also harvested in parallel for immunoblotting with anti-GFP antibodies to confirm expression of the transfected proteins (panel B). Note that expression of a protein containing the Prl signal sequence has no significant influence on the ability of GR to activate the luciferase reporter in response to dexamethasone.