Post-translational Arginylation of Calreticulin

A NEW ISOSPECIES OF CALRETICULIN COMPONENT OF STRESS GRANULES*

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Post-translational arginylation consists of the covalent union of an arginine residue to a Glu, Asp, or Cys amino acid at the N-terminal position of proteins. This reaction is catalyzed by the enzyme arginyl-tRNA protein transferase. Using mass spectrometry, we have recently demonstrated in vitro the post-translational incorporation of arginine into the calcium-binding protein calreticulin (CRT). To further study arginylated CRT we raised an antibody against the peptide (RDPAIYFK) that contains an arginine followed by the first 7 N-terminal amino acids of mature rat CRT. This antibody specifically recognizes CRT obtained from rat soluble fraction that was arginylated in vitro and also recognizes endogenous arginylated CRT from NIH 3T3 cells in culture, indicating that CRT arginylation takes place in living cells. Using this antibody we found that arginylation of CRT is Ca2⁺-regulated. In vitro and in NIH 3T3 cells in culture, the level of arginylated CRT increased with the addition of a Ca2⁺ chelator to the medium, whereas a decreased arginine incorporation into CRT was found in the presence of Ca2⁺. The arginylated CRT was observed in the cytosol, in contrast to the non-arginylated CRT that is in the endoplasmic reticulum. Under stress conditions, arginylated CRT was found associated to stress granules. These results suggest that CRT arginylation occurs in the cytosolic pool of mature CRT (defined by an Asp acid N-terminal) that is probably retrotranslocated from the endoplasmic reticulum.

The complex mechanism of post-translational modification of proteins is important for the regulation of cell functions. One of these post-translational modifications remaining poorly understood is the incorporation of arginine into the N terminus of proteins (1–3). Protein arginylation is catalyzed by the arginyl-tRNA protein transferase (ATE 1), which transfers arginine to a variety of peptides and proteins, all containing acidic amino acids or cysteine as N-terminal residue (4–6). This enzyme is likely to be cytosolic because it lacks motifs characteristic of either endoplasmic reticulum (ER)3 targeting or membrane-spanning helical segments (7). In addition, arginyl-tRNA protein transferase fused to green fluorescent protein has been observed both in the cytosol and nucleus of NIH 3T3 cells (8).

We recently identified by mass spectrometry the in vitro post-translational incorporation of arginine into calreticulin (CRT) (9). Calreticulin is a Ca2⁺-binding protein that is highly concentrated in the lumen of the ER. This protein functions as a chaperone involved in the control of newly synthesized proteins and as a modulator of Ca2⁺ signaling and Ca2⁺ homeostasis (10–13). The primary translation product of rat CRT contains a 17-amino acid N-terminal signal peptide that leads the nascent protein to the ER. As the protein translocates to the lumen of the ER this peptide is co-translationally cleaved, giving rise to the mature protein that contains an aspartic acid exposed at the N terminus (14). The C terminus end of CRT contains a KDEL ER retrieval sequence (15–16). Nevertheless, numerous functions of this protein such as nuclear export receptor activity, anti-thrombotic activity, modulation of cell adhesion, and its integrin-dependent Ca2⁺ signaling (17–20) have led to the proposal that CRT has functions in the cytoplasm, the nucleus, and on the cell surface. The presence of CRT in the cytosol, as well as the origin of this cytosolic CRT, is still a matter of controversy. It has been reported that cytosolic CRT could be produced either by retrotranslocation from the lumen to cytoplasmic compartments or by an inefficient CRT translocation to the ER (21–22).

In the study describing CRT as an in vitro acceptor of arginine (9) it has had not been determined whether arginine was incorporated on native CRT or on mature CRT (which contains an acidic amino acid as acceptor residue; Ref. 14). Our hypothesis is that arginine is incorporated in mature CRT (9) by the arginyl transferase that specifically adds arginine to acidic N-terminal amino acids (4). Because of the cytosolic distribution of this enzyme, it was unclear whether arginylation of CRT was an ER luminal or a cytosolic event. In the present study we demonstrate that arginylation of CRT occurs on a cytosolic pool of mature CRT and that this modification is regulated by Ca2⁺.

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**EXPERIMENTAL PROCEDURES**

*Chemical Reagents—Chemical reagents, protease inhibitors, and prestained molecular weight standard mixture were purchased from Sigma.*

**Preparation of Rat Brain Soluble Extract—**Soluble brain extract from rat was prepared as described (23). Briefly, brains were homogenized in 10 mM Tris-HCl buffer, pH 7.8, containing 0.25 M sucrose, 6 mM 2-mercaptoethanol, 1 mM EGTA, and a mixture of protease inhibitors (100 μM leupeptin, 100 μM bestatin, 200 μM N-p-tosyl-L-arginine methyl ester, 2 μg/ml apro tinin, and 100 μM aminotatin). The homogenate was centrifuged at 100,000 × g for 1 h, and the supernatant was filtered through a Bio-Gel P6 column (Bio-Rad) equilibrated in 10 mM Tris-HCl buffer, pH 7.8, containing protease inhibitors. All procedures were performed at 4 °C.

**Incorporation of [14C]Arginine into Proteins of Soluble Brain Extract—**Proteins of soluble extracts were arginylated as described by Hallak et al. (23). The incubation system consisted of 7 mg/ml of protein from soluble brain extract, 10 μM [14C]arginine (3 μCi/ml) (PerkinElmer Life Sciences), 1 mM ATP, 100 mM KCl, 5 mM MgCl2, Tris-HCl buffer, pH 7.8, and protease inhibitors. Standard [14C] incorporation assay contained also 1 mM EGTA. To determine the effect of Ca2⁺ in the [14C]arginine incorporation into CRT, CaCl2 was varied from 0 to 2 mM and EGTA from 0 to 1 mM. Where indicated, 60 pg/ml of bovine CRT (Sigma) was added to the soluble brain extract. After incubation at 37 °C for 45 min, arginine incorporation was stopped by the addition of Laemmli sample buffer (24).

**Cell Cultures—**NIH 3T3 cells were plated in 100-mm Petri dishes coated with poly-L-lysine and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). For intracellular Ca2⁺ depletion, NIH 3T3 cells were washed twice with Dulbecco’s modified Eagle’s medium and incubated for 15 min with Dulbecco’s modified Eagle’s medium supplemented with 3 mM EGTA and 2 μM thapsigargin (TG) (dissolved in Me2SO 0.65% w/v) or 10 μM BAPTA AM and 2 mM TG. The concentration of Me2SO in the media was 0.02% (v/v) also in control cells.

**Preparation of Cell Lysate—**NIH 3T3 cells were grown to 90% confluence, washed twice with phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4, and disrupted in lysis buffer (radioimmune precipitation buffer) containing 1% w/v Nonidet P-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 10 mM Na2PO4, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, pH 7.2. After 10 min at 0 °C the cells were scraped. Total protein concentration was determined using the BCA protein assay kit (Pierce).

**Immunoprecipitation—**The stress granule component TIA-1 was immunoprecipitated using an anti-TIA-1 pAb C20 (Santa Cruz Biotechnology) using standard protocols. In brief, 3 μl of anti-TIA-1 polyclonal Ab were incubated for 1 h at 4 °C with 25 μl of protein G-Sepharose (Amersham Biosciences) in 1 ml of radioimmune precipitation buffer. The suspension was briefly centrifuged and the pellet washed twice with radioimmune precipitation buffer. Then, cell lysate (300–500 μg of protein) was added to the suspension, and the mixture was rotated overnight at 4 °C. The suspension was briefly cen-

fuged and the supernatant discarded. The protein G-Sepharose-C20 antibody-TIA-1 complex was washed five times with 500 μl of radioimmune precipitation buffer prior to the addition of Laemmli sample buffer.

**Electrophoresis, Autoradiography, and Immunodetection of Proteins—**Proteins from soluble brain extract, cell lysate, and immunoprecipitated materials were separated using SDS-PAGE (24) and were electrophoretically transferred onto nitrocellulose membrane (25). As primary antibodies, we used mouse anti-CRT mAb (1:2500) (BD Biosciences) that specifically recognizes the C-terminal end of CRT, goat anti-TIA-1 (C20) pAb (1:200) (Santa Cruz Biotechnology), and a rabbit anti-arginylated-CRT (R-CRT) pAb (dilution 1:60) specific for arginylated calreticulin (this work). The polyclonal antibody R-CRT was custom designed by Eurogentec (Belgium) and directed against the peptide RDPAlYFK coupled to keyhole limpet hemocyanin. This peptide corresponds to the N-terminal sequence of the mature form of calreticulin (devoid of the signal peptide amino acids 1–17), with an arginine residue (bolded) added at the N-terminal end. The rabbit IgGs with affinity for the peptide RDPAlYFK were affinity-purified on a column linked with the peptide RDPAlYFK and then eluted with 0.5 M SCNK. In a second affinity chromatography, the peptide KDPAlYFK was used to retain IgGs nonspecific of the arginylated peptide. As secondary antibodies we used biotiny- lated goat anti-rabbit and anti-mouse IgG (H+L) and horse anti-goat IgG (H+L) (1:15000) (Vector Labs). The nitrocellulose membrane was probed with horseradish peroxidase-labeled streptavidin (1:60000) (Amersham Biosciences) that was visualized by the ECL Western blotting detection system (Amersham Biosciences). After immunostaining, the [14C]-arginy lated proteins were visualized by autoradiography.

To determine the relative sensitivity of the R-CRT antibody with respect to the CRT antibody, known amounts of purified rat CRT were [14C]-arginylated in vitro (see above), and the samples were transferred to a nitrocellulose membrane and subjected to autoradiography and to Western blot analysis with anti-R-CRT and anti-CRT antibodies. To quantify the degree of arginylation, the band corresponding to CRT was excised from the membrane and the radioactivity was measured in a scintillation counter. The corresponding densitometric analysis of CRT from each antibody was performed using the image analysis system function of the METAMORPH 3.0 software. From the analysis of these data, it was found that the R-CRT antibody is 170 times more sensitive than the CRT antibody.

**Immunofluorescence—**For immunofluorescence, NIH 3T3 cells were grown on glass coverslips to 60% confluence. Cell cultures were washed twice with PBS and fixed with 3% (w/v) formaldehyde in PBS containing 4% w/v sucrose for 30 min at room temperature. After washing with PBS, cells were permeabilized with 0.2% v/v Triton X-100 in PBS for 10 min and then treated with 5% w/v bovine serum albumin for 2 h before the addition of antibodies. Cells were incubated for 4 h at 4 °C with primary antibodies and for 2 h at room temperature with secondary antibodies. All antibodies were diluted in PBS containing 5% w/v bovine serum albumin. As primary antibodies, we used rabbit R-CRT pAb (1:60) to detect arginylated CRT, mouse anti-CRT mAb (1:2500) and rabbit anti-CRT (PA3–900).
pAb (1:100) (Affinity Bioreagents) to detect CRT, and goat anti-TIA-1(C20) pAb (1:100) to detect TIA-1. As secondary antibodies we used Alexa Fluor 543-conjugated goat anti-rabbit IgG (1:1000) (Molecular Probes), Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000) (Molecular Probes), and Alexa Fluor 488-conjugated donkey anti-goat IgG (1:1000) (Molecular Probes). Control for the cross-talk between the fluorophores was negligible. However, for double staining, TIA-1 and R-CRT (or CRT) labeling were performed sequentially, starting with TIA-1 labeling.

**Image Acquisition**—Confocal images were captured with an inverted Zeiss LSM 5 Pascal laser confocal microscope (Carl Zeiss, Jenna, Germany), using a 63/1.4 or 100/1.4 Plan-Apochromat objective. Pinholes were set for a nominal axial resolution $<0.6 \mu m$. Excitation on the LSM 5 Pascal laser confocal microscope was with a 25-milliwatt argon laser emitting at 488 nm or with a 1-milliwatt helium/neon laser emitting at 543 nm. Emissions were collected using a 505 to 530-nm band pass filter for Alexa 488 or a 560 to 615-nm band pass filter for Alexa 543 emission.

**RESULTS**

**Immunodetection of $^{14}$C-Arginylated Calreticulin by Anti-R-CRT Antibody**—We raised an antibody against a peptide containing the first 7 N-terminal amino acids of mature rat CRT with an additional arginine residue at the N terminus of the peptide ($R_{DPAIYFK}$). This antibody (anti-R-CRT) and one that specifically recognizes CRT (anti-CRT) were used to analyze the *in vivo* arginylation of CRT.

To demonstrate that anti-R-CRT antibody specifically recognizes R-CRT, the soluble fraction of rat brain homogenates was *in vitro* arginylated with $[^{14}$C]arginine for different periods of times. Autoradiography of the $^{14}$C-arginylated proteins shows increasing $[^{14}$C]arginine incorporation during time into several proteins, including CRT at 57 kDa (Fig. 1A). Total CRT remains constant during the time course as determined by Western blot of the extracts with an antibody (anti-CRT) that recognizes the C-terminal region of CRT (Fig. 1B). In contrast, the anti-R-CRT immunostaining progressively increases, at least up to 140 min of incubation (Fig. 1C), with concomitant increase in the $[^{14}$C]arginine incorporated by this protein (Fig. 1A). This result indicates that the anti-R-CRT antibody directed against the putative N terminus of the arginylated mature CRT is specific for the arginylated form of CRT, because it was the only band detected after the *in vitro* arginylation.

**Effect of Ca$^{2+}$ on the CRT Arginylation**—Calreticulin is a Ca$^{2+}$-binding protein whose tertiary structure is modified by this cation (26–27). Therefore, we examined whether the argi-
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Arginylation of CRT is affected by the concentration of Ca$^{2+}$. A soluble fraction from rat brain was $^{14}$C-arginylated in vitro in the presence of different Ca$^{2+}$ concentrations. We found that the increase of Ca$^{2+}$ concentration specifically inhibits the $^{14}$C-arginine incorporation into CRT (Fig. 2A). The $^{14}$C-arginine incorporation was also assayed as a function of the Ca$^{2+}$ chelator EGTA. Increasing EGTA concentration increases the amount of $^{14}$C-arginine incorporated into CRT (Fig. 2B), further indicating that Ca$^{2+}$ has an inhibitory effect on the arginylation of CRT. When EGTA was omitted in the incubation system, anti-R-CRT immunostaining became negligible, whereas no variation was observed for total CRT (Fig. 2C, compare lane 2 with lane 1). To improve sensitivity in the $^{14}$C-arginine incorporation assay, we added purified mature bovine CRT to the incubation system and arginylation assays were performed in the presence of different amounts of Ca$^{2+}$ or EGTA. Mature bovine CRT was chosen as substrate of arginylation because it has a potential arginine acceptor (glutamic acid) at the N terminus and N-terminal sequence similar to that in rat CRT. We observed that bovine CRT arginylation is also modulated by Ca$^{2+}$ levels (Fig. 2, A' and B'), Ca$^{2+}$ modulation of the $^{14}$C-arginine incorporation seems to be specific for CRT because the $^{14}$C arginylation of the other protein substrates was not affected by the addition of calcium or of EGTA (Fig. 2, A and B).

**Arginylation of CRT in Cultured Cells**—In addition to the soluble fraction of rat brain, the incorporation of $^{14}$C-arginine in CRT was also found in the soluble fraction from COS, Chinese hamster ovary, and NIH 3T3 cells. It was deemed of importance to examine whether CRT arginylation occurs in vivo. This was addressed in mouse NIH 3T3 fibroblasts in culture. These cells showed one of the highest $^{14}$C-arginine incorporation rates in CRT; in addition, the N-terminal sequence used to generate the anti-R-CRT antibody is common to both mouse and rat CRT.

The existence of an arginylated isoform of CRT in NIH 3T3 cells was evident in both immunocytochemistry and immunoblot assays. Immunocytochemical assay of NIH 3T3 cells with the anti-R-CRT antibody reveals a scarce, cytosolic-like immunostaining of R-CRT (Fig. 3A, b) with occasional cluster distribution. It is worth noting that the immunostaining for R-CRT lacks the typical ER appearance characteristic of CRT (Fig. 3A, a) and that both stainings do not overlap (Fig. 3A, c). These results indicate that R-CRT is present in cells and that it shows an intracellular localization different from that of luminal CRT. Moreover, when total proteins from NIH 3T3 cells are subjected to immunoblot and probed with the R-CRT antibody, a 57-kDa band is recognized by this antibody (Fig. 3B, lane 1), indicating the presence of endogenous R-CRT. Arginylated CRT and CRT from NIH 3T3 cells show the same electrophoretic mobility, as determined by immunostaining with an antibody that recognizes the C-terminal region of CRT (Fig. 3B, lane 3). Taking into account the different sensitivities of the used antibodies (see “Experimental Procedures”), we determined by scanning of the Western blots and subsequent densitometric quantification of R-CRT and CRT signals that in whole cells R-CRT represents 0.05% of total CRT. Considering that cytosolic CRT in these cells represents 2% of the total CRT (21), the arginylated isoform accounts for 2.5% of the cytosolic CRT.

To address whether CRT arginylation in cells is also regulated by Ca$^{2+}$, NIH 3T3 cells were treated with 2 μM TG, a sarcoplasmic endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor capable of depleting luminal Ca$^{2+}$ (28), plus 3 mM EGTA or with 2 μM TG plus 10 μM BAPTA-AM, a membrane-permeable Ca$^{2+}$ chelator. The use of the Ca$^{2+}$ chelators EGTA or BAPTA-AM with the inhibitor TG leads to a decrease in the levels of cytosolic Ca$^{2+}$ (29–30). We found a significant increase in the level of R-CRT after TG/EGTA treatment both by immunocytochemistry with the anti-R-CRT antibody (Fig. 3A, compare e with b) and by Western blot (Fig. 3B, compare lane 2 with lane 1). After scanning of the Western blots, a densitometric quantification of R-CRT levels reveals 100% increase of arginylated calreticulin in TG/EGTA-treated cells with respect to control cells (Fig. 3C, a). The level of total CRT, detected with the antibody that recognizes the C-terminal region of CRT (anti-CRT) (Fig. 3C, b), was also increased in TG/EGTA-treated cells, although this increment is lower than that for R-CRT. In accordance with these results, it has been reported that TG treatment promotes an increase in CRT gene expression (31). Immunocytochemistry analysis reveals that TG/BAPTA-AM and TG/EGTA treatments induce a prominent clustering of R-CRT (Fig. 3A, e and h), whereas for total CRT only a small condensation in the perinuclear zone is seen (Fig. 3A, d and g). As observed in control cells, treated cells do not show colocalization of R-CRT and CRT (Fig. 3A, f, d, and i). Our results indicate that CRT arginylation occurs in living cells and that it is modulated by Ca$^{2+}$ levels. The reason why we do not observe colocalization between R-CRT and CRT is considered below.

**Arginylated CRT Colocalizes with Stress Granules**—After TG treatment of NIH 3T3 cells (Fig. 3A, e and h), we found that the R-CRT is sequestered into clusters similar to the punctate cytoplasmic aggregates found in stress granules (SGs) (32). The presence of R-CRT in SGs was investigated in NIH 3T3 cells by double immunostaining with anti-R-CRT antibody and with TIA-1 antibody, a marker of SGs (32) that is specific for RNA-binding protein TIA-1. After inducing stress by treatment of cells with TG/EGTA, SG formation was detected as clusters with a robust TIA-1 staining (Fig. 4A, b). Under these experi-

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**FIGURE 3.** Immunodetection of arginylated calreticulin in NIH 3T3 in cultures. NIH 3T3 cells, treated with 2 μM TG plus 3 mM EGTA (TG/EGTA) or with 2 μM TG plus 10 μM BAPTA-AM (TG/BAPTA-AM) or with vehicle (control), were incubated for 15 min at 37 °C. A, after treatment, cells were analyzed by immunofluorescence using anti-CRT mAb (CRT) (a, d, and g) to visualize total CRT and with R-CRT pAb (b, e, and h) to visualize R-CRT. Co-localization of proteins was monitored by confocal microscopy. In all three experimental conditions, no immunocolocalization was observed in the merged images or in the zoomed regions of interest (inserts, c, f, and i). B, total protein from NIH 3T3 cells, from control cultures (lanes 1 and 3) or from cultures treated with TG/EGTA as in panel A (lanes 2 and 4), were subjected to SDS-PAGE and analyzed by Western blot with R-CRT pAb (R-CRT) (lanes 1–2) or anti-CRT mAb (CRT) (lanes 3–4). C, quantification of Western bands by densitometric scanning. The image analysis system function of the METAMORPH 3.0 software was used to calculate the total signal within the area above the threshold for each filter set. The samples were normalized to equal amounts of tubulin. Signals are expressed in arbitrary absorbance units (a.u.) as the mean ± S.D. of three experiments for R-CRT (a) and CRT (b) antibodies.
FIGURE 4. Colocalization of arginylated calreticulin and TIA-1 in NIH 3T3 cells under stress conditions. NIH 3T3 cells were cultured in the presence (A and C) or the absence (B and D) of 2 μM TG with 3 mM EGTA for 15 min. After treatment, cells were analyzed by double immunofluorescence using R-CRT pAb (a and d) and TIA-1 pAb (b and e) or anti-CRT pAb (g and j) and TIA-1 pAb (h and k). Co-localization of proteins was monitored by confocal microscopy. Yellow pseudo-color in the merged images (c, f, i, and l) and in the zoomed regions of interest (inserts) represents co-localization, resulting from R-CRT and CRT labeling in red and TIA-1 labeling in green.
indicate that (a) arginylation of CRT occurs in cultured cells, (b) arginylated CRT is localized mainly in the SGs under stress conditions.

**DISCUSSION**

Studies in a variety of tissues indicate the existence of several proteins that can be in vitro modified by post-translational arginylation (1, 23, 33); nevertheless, physiological substrates of arginylation had remained elusive until very recently. Indeed, only few proteins whose arginylation takes place in vivo had been identified: the enzyme ornithine decarboxylase (34), RGS proteins (35), an N-terminal variant of G protein χ2 subunit (36), and β actin (37). In the present study we demonstrated that CRT, recently identified as an in vitro arginine acceptor by mass spectrometry (9), also is post-translational arginylated in living cells.

Calreticulin is a multifunctional Ca\(^{2+}\)-binding protein involved in a variety of cellular processes. From in vitro assays it has been shown that CRT contains a low affinity, high capacity Ca\(^{2+}\)-binding site \((K_d \sim 2 \text{ nm})\) and 22–30 mol Ca\(^{2+}\)/mol CRT localized in the C domain and a high affinity, low capacity Ca\(^{2+}\)-binding site \((K_d \sim 1 \text{ μM})\) and 1 mol Ca\(^{2+}\)/mol CRT localized in the P domain (10). Within the ER, Ca\(^{2+}\) levels regulate several functions of CRT, such as its interaction with other ER chaperones and with newly synthesized proteins (27). In the nucleocytoplasmatic compartment, Ca\(^{2+}\)-binding to CRT regulates the binding and nuclear export of steroid receptors and of proteins containing nuclear export signal. Ca\(^{2+}\)-induced CRT interaction with glucocorticoid receptor, whereas it inhibits the interaction between CRT and proteins containing nuclear export signal, like HIV-1 Rev (29). In cytosol, calreticulin binds to the cytosolic α-subunit tail of integrins and regulates integrin-mediated cell adhesion (19); this interaction is mediated by the Ca\(^{2+}\)-bound state of CRT. In summary, most of the functions mentioned for CRT seem to be related to its Ca\(^{2+}\) binding activity; thus, conditions that influence the Ca\(^{2+}\) levels are predicted to regulate the CRT activities.

In the present study we demonstrate that the incorporation of arginine in CRT is modulated by Ca\(^{2+}\) levels, being inversely proportional to the Ca\(^{2+}\) concentration. This modulation seems to be specific for this substrate, because among other soluble rat brain proteins CRT was the only one whose arginylation was inhibited by Ca\(^{2+}\) (Fig. 2, arrow). This result suggests that the enzymatic system involved in arginylation was not affected by Ca\(^{2+}\) levels and that modifications in the substrate (CRT) are modulating arginine incorporation. A possible explanation for the Ca\(^{2+}\) influence on CRT arginylation can be related to Ca\(^{2+}\) binding-induced conformational changes occurring in the tertiary structure of CRT. Upon Ca\(^{2+}\) binding, CRT adopts a more rigid and well packed tertiary structure with increased thermal stability and increased conformational rigidity (26). This conformational change also leads to the formation...
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of a protease-resistant core containing the N-terminal end of CRT (27). We propose that the high conformational rigidity of this core may also preclude the access of arginyl-tRNA protein transferase to the N-terminal end of CRT, thus preventing the covalent binding of arginine. The CRT arginylation in NIH 3T3 cells seems to be also modulated by Ca\(^{2+}\). A significant increase in the labeling of R-CRT was observed after treatment of NIH 3T3 cells with TG/BAPTA-AM or TG/EGTA. These treatments result in a decrease in both cytosolic and ER Ca\(^{2+}\) levels (29–30). The increase in R-CRT levels in treated cells is consistent with the effect of Ca\(^{2+}\) on arginylation of CRT in vitro. However, Ca\(^{2+}\) levels that inhibit CRT arginylation in vitro are in the order of 10–70 \(\mu\)M, depending on total CRT concentration (Fig. 2). Such Ca\(^{2+}\) levels are probably never reached in physiological conditions. It may be that the affinity of CRT for Ca\(^{2+}\), namely the affinity of the low affinity, high capacity Ca\(^{2+}\)-binding site, is different in cells compared with in vitro conditions.

On the other hand, another plausible mechanism by which Ca\(^{2+}\) levels alter the amount of R-CRT could be related to the stress response triggered upon ER Ca\(^{2+}\) store depletion. It has been demonstrated that such conditions (i.e. TG treatment) increase CRT gene expression (31). High amount of ER CRT during ER stress could result in an increase in the retrotranslocation of CRT to the cytosol with a subsequent increase in the arginylation levels. These mechanisms are not mutually exclusive; thus, a combination of low levels of Ca\(^{2+}\) and increased levels of CRT in both ER and cytosol could lead to the increase of R-CRT.

Under stress conditions, R-CRT was segregated in clusters that colocalized with the SG marker TIA-1 (Fig. 4). This result is in agreement with previous studies that showed that the disruption of normal cell homeostasis, like that occurring upon TG treatment, induces the formation of SGs (38–41). A variety of proteins are found among the components of SGs, mainly RNA-binding proteins that regulate mRNA stability (42, 32). SGs also contain some chaperones such as heat shock proteins that seem to be required to disperse aggregated TIA-1 in living cells (43). Calreticulin is a well known ER luminal chaperone and also an mRNA-binding protein (44–48), so the functional role of CRT in SGs could be related to these activities. Further studies will elucidate whether arginylation of CRT is required for its association to SGs.

Almost all known functions of CRT involve CRT-substrate interaction; nevertheless, a consensus sequence for binding to CRT does not exist. In the ER, CRT binds to recently synthesized glycoproteins as well as to non-glycosylated substrate proteins (10, 11). In cytosol, CRT binds to the DNA-binding domain of glucocorticoid receptor and to nuclear export signal-containing proteins in two completely different ways (17). It is widely accepted that the great conformational flexibility of CRT regulates specific interactions with very different kind of substrates, thus providing a large variety of functions for CRT. Thus, conformational changes of CRT promoted by the alteration in the intracellular levels of Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), and ATP would modify CRT-substrate interaction (27).

It is accepted that in the ER, CRT mainly mediates substrate recognition via its N-terminal domain (10, 17). At least for some peptides, it has been demonstrated that the incorporation of arginine in the N-terminal end could lead to a change in the peptide conformation (5); thus, we speculate that changes in CRT conformation triggered by arginylation could regulate such CRT-substrate interaction, modulating some of the functions that this protein carries out in the cytosol. In addition, further studies are required to determine whether Ca\(^{2+}\) (or Zn\(^{2+}\) or ATP) binding to R-CRT promotes the same conformational changes promoted in non-arginylated CRT.

In summary, in the present study we demonstrate that the post-translational incorporation of arginine into CRT occurs in NIH 3T3 cells and that this modification is regulated by Ca\(^{2+}\). We also demonstrate that the site of arginine incorporation in CRT arises from the cleavage of the N-terminal signal peptide. These conclusions arise from the specificity of the antibody that recognizes the mature N-terminal sequence of CRT plus arginine (Fig. 1) and furthermore from the incorporation of arginine in purified mature CRT (Fig. 2, A’ and B’). In agreement with the cytosolic localization of arginyl tRNA protein transferase (8), in vivo arginylation of CRT suggests that CRT is first co-translationally translocated to the ER for the cleavage of the signal peptide and then retrotranslocated to the cytosol for the post-translational N-terminal incorporation of arginine.

Despite the KDEL ER retrieval sequence of CRT (16, 15), numerous functions of this protein have been proposed to occur outside the ER (10, 17–19). The major biochemical evidence localizing CRT in the cytoplasm consists of the purification of this protein from HeLa cell cytosol in a search for novel nuclear export factors (17). The authors demonstrated the participation of CRT in nuclear export events of several proteins, including the glucocorticoid receptor, both in permeabilized and in vivo export assays. Recently, some mechanisms by which CRT could reach the cytosol have been proposed (21–22). Cytosolic CRT could be produced by retrotranslocation from the ER lumen to the cytoplasmic compartments (21) or, alternatively, could be inefficiently translocated to the ER (22). Both mechanisms agree in that cytosolic CRT has lost the N-terminal signal peptide in the ER before being released to the cytosol, which is a requirement for the generation of the arginylation site. Even though biochemical and functional evidence suggests the presence of CRT in the cytosol and some mechanisms by which CRT could reach this localization have been proposed, up to now cytosolic CRT had been difficult to visualize within the cell. By using the anti-R-CRT antibody we were able to distinguish for the first time by immunocytochemistry ER-lumen CRT from cytosolic CRT. Further investigations will be required to establish whether CRT arginylation is required for its cytosolic functions.

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