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PROMPT GLYCOSYLATION OF CALRETICULIN IS INDEPENDENT OF Ca^{2+} HOMEOSTASIS

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Summary - Selective glycosylation of "prompt" stress glycoproteins (P-SG), mainly P-SG67 and P-SG64 (M_r of 64,000, pI=5.1), occurs immediately during acute heat-stress. In the present study, P-SG64 was purified by sequential gel filtration, anion-exchange, affinity chromatography, and two-dimensional isoelectric focusing/ SDS-PAGE. Purified P-SG64 was further characterized by microsequencing of a peptide fragment, PT-61, which showed a 100% sequence homology with calreticulin, suggesting that P-SG64 is identical to calreticulin. PT-61 also showed 55%, 58% and 63% sequence homologies with calnexin, HIV-1 gp120 and HIV-2 envelope polyprotein, respectively. $^{45}Ca^{2+}$ overlay studies confirmed Ca^{2+}-binding of P-SG64. P-SG67 was also recently identified as calreticulin (8), which suggests that CHO cells either have two isoforms of calreticulin or express variable states of calreticulin glycosylation during acute heat stress. The role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) during heat-induced "prompt" glycosylation was also examined and indicated an 8-fold increase in $[Ca^{2+}]_i$. Chelation of this increased cytoplasmic Ca^{2+} by BAPTA reduced glycosylation of P-SG67/P-SG64/calreticulin only by ~20%. This observation suggests that altered $[Ca^{2+}]_i$ homeostasis is not directly linked to calreticulin glycosylation, instead, heat-induced calreticulin glycosylation is a Ca^{2+}-independent effect.

Heat stress results in preferential synthesis and accumulation of both "heat shock proteins" (HSPs) (1, 2) and enhanced protein glycosylation (3, 4). Increased levels of cellular HSPs have been proposed as the major mechanism of thermotolerance, partially based on their known functions in polypeptide folding, transport and assembly (5). However, enhanced glycosylation of the major heat-induced 50-kDa glycoprotein, GP50, in CHO cells, was also related to the expression of cellular thermotolerance (3). GP50 is closely related to the retinoic acid-inducible J6 gene product (6) which belongs to the multifunctional serpin family (7). Similarly, identification of a major prompt stress glycoprotein (P-SG) (8), as the multifunctional protein calreticulin (9) suggests that there are proteins other than "classical" HSPs that play equally important, and possibly multifunctional roles in the cellular response to heat-stress.

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Abbreviations: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetoxymethyl ester; CHO, Chinese hamster ovary cells; ER, endoplasmic reticulum; HPLC, high pressure liquid chromatography; HSP, heat shock protein; PAGE, polyacrylamide gel electrophoresis; P-SG, prompt stress glycoproteins; PVDF, polyvinylidene difluoride.

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The identification of the most prominent P-SGs is a first step towards determination of the functional significance of prompt glycosylation. Following the recent identification of P-SG67 in CHO cells, as calreticulin (8), we concentrated on the characterization of the second most prominent P-SG, P-SG64. The data presented in this report show that P-SG64 is also identical to calreticulin, based on amino acid sequencing of a major peptide fragment. Furthermore, we determined the relationship between intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the enhanced glycosylation of both P-SG67 and P-SG64, since calreticulin contains known Ca^{2+}-binding domains (10).

MATERIALS AND METHODS

Cell Culture, Heating and Materials - CHO cells were grown in T-75 plastic culture flasks in McCoy's 5A medium, supplemented with 10% fetal bovine serum(FBS), and heated as previously described (3, 4). D-[2-^{3}H]mannose (sp act, 23 Ci/mmol) and $^{45}CaCl_2$ (sp act, 8.5 mCi/mg Ca^{2+}) were purchased from ICN Radiochemicals (Irvine, CA). Fluorescent probes, Fura-2/AM and BAPTA/AM, were obtained from Molecular Probes (Eugene, OR). Both fura-2 and BAPTA were solubilized in dimethyl sulfoxide (DMSO) as 1mM stock solutions, stored at -20°C and protected from light until use. Stock solutions were freshly diluted with respective buffers to the final concentrations stated below. DMSO in the culture medium was always maintained below 0.05%. Films obtained after fluorography were analyzed *via* laser densitometry (300A Molecular Dynamics).

Labeling of Proteins - Prompt glycosylation was monitored by labeling CHO cells with D-[2-^{3}H]mannose, as described (4, 8). Label was added ~1 min prior to submersing flasks in the hyperthermic water bath. All heat treatments were for 30 min at 45°C; corresponding controls were submersed for 30 min at 37°C (4). Following heating, cells were rinsed; scraped in the cold (4), resuspended in 20 mM Tris, pH 7.2, and sonicated for 30 sec. Proteins were solubilized and samples were processed further as described earlier (8). Protein concentration was measured by the Coomassie blue dye method (Bio-Rad protein) with bovine serum albumin as a standard.

Purification of P-SG64 - P-SG64 was purified by sequential gel filtration, ion-exchange, and affinity chromatography, as reported for P-SG67 (8). Appropriate peak fractions were pooled and final samples were lyophilized after the affinity chromatographic step.

Electrophoresis, Electroblotting and Amino Acid Sequence Analyses - Lyophilized sample was solubilized and subjected to two-dimensional electrophoresis, as described earlier (8, 11). Proteins were electroblotted using a Trans-Blot system (Bio-Rad); stained with Ponceau S, and P-SG64 was excised as described (8). Excised membrane fragments were rinsed with 1 ml of d/H_2O, and stored wet at -70°C till further analysis. A total of 16 PVDF membrane fragments containing electroblotted P-SG64 were sent to Harvard Microchem (Cambridge, MA) for sequence analysis. Amino acid composition was determined; lysozyme C digestion was performed and the peptide fragments obtained were separated by HPLC. Three peptide fragments were characterized by matrix-assisted laser desorption mass spectrometry (MALD-MS) (12); one peptide fragment was selected for sequence analysis. Sequence homology with other known proteins was determined *via* the BLAST network service at the National Center for Biotechnology Information.

Calcium studies - Ca^{2+}-binding of P-SG64 was determined by $^{45}Ca^{2+}$ overlay after transfer to the membrane as described earlier (8). $[Ca^{2+}]_i$ was measured using fura-2 as described (13). Briefly, CHO cells were trypsinized and cells (5×10^6 cells) were loaded with 2 μM fura-2/AM at 37°C for 40 min in 2 ml of RPMI 1640 medium with FBS. After incubation, cells were washed twice with 5 ml of the following buffer: 140 mM NaCl, 5 mM KCl, 10 mM Hepes, 10 mM glucose, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4. Cells were finally resuspended in 2 ml of the same buffer and transferred to a quartz cuvette. The cuvette was immediately placed into a thermostatically controlled cuvette holder at 37°C (Perkin Elmer LS 50 spectrofluorometer), with continuous stirring of the cell suspension. Fluorescence measurements were monitored for 5 min at 37°C with excitation at 340 nm and emission at 510 nm. Cells were then heated by increasing the temperature of the water bath attached to the circulator controlling the cuvette temperature to 45°C, which was constantly maintained for further 40-50 min during which the fluorescence of the cells was monitored at 5 min intervals. At the end of the experiment, Triton X-100 and EGTA were used to calibrate the assay and the concentration of $[Ca^{2+}]_i$ was calculated as described (13).

Prompt Glycosylation and Ca^{2+}-Buffering Using BAPTA - The effect of Ca^{2+}-buffering on prompt glycosylation was studied using the Ca^{2+} chelator BAPTA (14). CHO cells in T-75 flasks were loaded at 37°C with BAPTA (5 μM) for 60 min (15) after which they were heated for 30 min at 45°C. During the same time, cells were labeled with D-[2-^{3}H]mannose. Preliminary experiments using two different BAPTA concentrations (5 μM and 25 μM) showed that irrespective of the μM concentration, there was a similar increase in intracellular BAPTA levels as measured by its absorbance at 254 nm. Hence, 5 μM BAPTA concentrations were chosen for subsequent studies. The same BAPTA concentration was earlier shown to significantly reduce intracellular Ca^{2+} that was associated with reduced HSP-26 synthesis (15). Total exposure to extracellular BAPTA in all our

experiments was 1.5 h. Following labeling, cells were scraped and rinsed; proteins were solubilized, and SDS-PAGE was performed as described previously (11). The relative change in protein glycosylation, caused by BAPTA was determined by densitometric scanning of the film obtained after fluorography.

RESULTS

Both P-SG67 and P-SG64 rapidly incorporated D-[2-^{3}H]mannose during acute 45°C-heat stress and were characterized by a pI=5.1 (8, 11). Table I shows the comparison of amino acid composition of both P-SG64 and P-SG67 with calreticulin from rabbit skeletal muscle. Subtle differences in several amino acid residues between P-SG64 and P-SG67 contrast with more significant differences between the P-SGs and calreticulin from rabbit skeletal muscle. The latter differences appear especially in threonine, proline, glycine, methionine, and phenylalanine residues (Table I). However, the most notable difference between the P-SGs and rabbit skeletal muscle calreticulin was in the aspartic and glutamic acid residues that were relatively higher in both P-SG64 and P-SG67 (Table I), and probably related to the lower pI values of these P-SGs.

Fig. 1 shows the peptide map of fragments obtained after *in situ* lysozyme C digestion of electroblotted P-SG64 and their separation by HPLC. The digestion resulted in 66 distinct peptide fragments; of these, three major fragments (PT-49, PT-54 and PT-61) were characterized by MALD-MS. PT-61 was further selected for sequence analysis which yielded a 27-amino acid sequence Residues at positions 13 and 25 were not identified with certainty. The high confidence portion of

TABLE I

Amino acid composition of P-SG64 and P-SG67 from CHO cells and calreticulin from rabbit skeletal muscle

Amino acid	P-SG64		P-SG67[a]		Calreticulin[b]	
	res/mol[c]	*(%)*	*res/mol*[c]	*(%)*	*res/mol*[c]	*(%)*
Asp and Asn	109.3	17.1	117.7	17.7	75.0	14.0
Thr	31.0	4.9	29.6	4.5	18.0	3.4
Ser	30.0	4.7	39.9	6.1	33.0	6.2
Glu and Gln	100.5	15.7	121.0	18.3	91.0	17.1
Pro	41.6	6.5	41.9	6.4	34.0	6.4
Gly	61.0	9.5	62.2	9.4	45.0	8.5
Ala	36.4	5.7	32.4	4.9	28.0	5.3
Val	31.2	4.9	27.7	4.2	25.0	4.7
Met	0.0	0.0	3.7	0.6	5.0	0.9
Ile	27.2	4.3	26.0	3.9	20.0	3.8
Leu	37.2	5.8	33.7	5.1	26.0	4.9
Tyr	18.8	2.9	18.1	2.8	14.0	2.6
Phe	29.4	4.6	27.5	4.2	40.0	7.5
Lys	57.2	8.9	52.7	8.0	51.0	9.6
His	13.0	2.0	10.9	1.7	10.0	1.9
Arg	17.0	2.7	15.9	2.4	13.0	2.5
Cys	N.D.	N.D.	N.D.	N.D.	4.0	0.8
Trp	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Nleu	0.0	0.0	N.D.	N.D.	N.D.	N.D.

P-SG64 was purified, electroblotted onto PVDF membrane and its amino acid content analyzed as discussed under "Materials and Methods". N.D., not determined.

[a]Reference 8.

[b]Reference 9.

[c]Residues/polypeptide chain obtained assuming a monomeric M_r~ 64,000 derived from SDS gels.

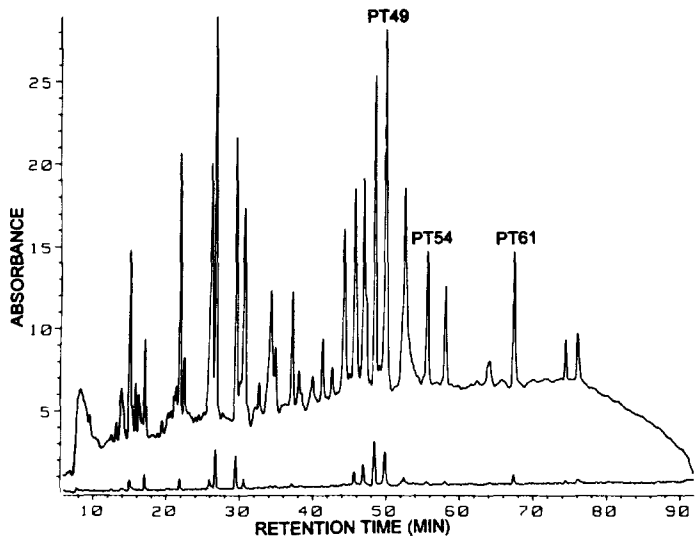

FIG. 1. **HPLC map of peptides released after *in situ* lysozyme C digestion of P-SG64.** PT49, PT54 and PT61 represent peptide fragments that were characterized by MALD-MS. PT61 is the peptide for which sequence analysis was carried out.

this sequence (25 amino acids) was examined for homology with other known proteins and showed a 100% sequence homology with calreticulin from various species (Fig. 2). Partial homology was found with the related calcium binding protein calnexin (55%) and with other proteins that included the envelope polyprotein (63%) from human immunodeficiency virus (HIV), type 2, and glycoprotein 120 (63%) from HIV, type 1 (Fig. 2).

The above data indicate that P-SG64 is a form of calreticulin with the following amino acid substitutions in comparison to *Xenopus laevis* calreticulin: isoleucine at position 10 in PT-61 replaced methionine at position 304; alanine at position 15 replaced lysine at position 309; and phenylalanine at position 20 replaced tyrosine at position 314 (Fig. 2). Although, the amino acid residues at position 13 and 25 in PT-61 were not integrated; the amino acids corresponding to these residues in all other calreticulins from various species were always aspartic acid and tryptophan, respectively.

Recently, we reported $^{45}Ca^{2+}$ binding to P-SG64 (8) following its separation by 2-D electrophoresis and electroblotting. Results obtained indicated significant binding of $^{45}Ca^{2+}$ to P-SG64, thus, supporting the equivalency of P-SG64 to the Ca^{2+}-binding protein, calreticulin. In the present study, we examined the relationship between heat-induced glycosylation of calreticulin and the parallel increase in intracellular calcium that was earlier seen under different conditions of heat stress (15). Results obtained show that $[Ca^{2+}]_i$ at 37°C was about 30 nM in CHO cells; this gradually increased to about 225 nM after 40 min at 45°C with a doubling time of ~20 min (Fig. 3), until it reached a saturation limit near 240 nM by 50-60 min at 45°C. When 5 μM BAPTA was used to prevent this heat-induced increase in $[Ca^{2+}]_i$, prompt glycosylation was reduced only by ~20% (Fig. 4) in contrast to an insignificant change in glycosylation in control cells loaded with BAPTA; although the same BAPTA concentration was earlier shown to cause an inhibition of HSP-26 synthesis (15). This indicates little, if any, relationship between prompt glycosylation and heat induced alterations in $[Ca^{2+}]_i$ homeostasis.

PT 61	1	SGTIFDNFLITNXEAYAEEFGNETXGV	27
Calreticulin			
Bovine	327	SGTIFDNFLITNDEAYAEEFGNETWGV	353
Mouse	306	SGTIFDNFLITNDEAYAEEFGNETWGV	332
Rabbit	306	SGTIFDNFLITNDEAYAEEFGNETWGV	332
Rat	55	SGTIFDNFLITNDEAYAEEFGNETWGV	81
Xenopus laevis	295	SGTIFDNFLMTNDEKYAEEYGNETWGV	321
Calreticulin Precursor (Calregulin)			
Human	323	SGTIFDNFLITNDEAYAEEFGNETWGV	349
Mouse	323	SGTIFDNFLITNDEAYAEEFGNETWGV	349
Rat	323	SGTIFDNFLITNDEAYAEEFGNETWGV	349
Rabbit	323	SGTIFDNFLITNDEAYAEEFGNETWGV	349
Calnexin			
Human	411	SDIFFDNFIICADRRIVDDWANDGWGL	437
Dog	413	SDIFFDNFIVCGDRRVVDDWANDGWGL	439
Mus Musculus	432	SDIFFDNFIISGDRRVVDDWANDGWGL	458
Rat	432	SDIFFDNFIISGDRRVVDDWANDGWGL	458
Glycoprotein 120			
HIV-1	342	NGTKWNNTLEQIVEKLREQFGNKT	365
Envelope polyprotein			
HIV-2	68	TKWENTLKQIAKKLAEQFGNET	89

FIG. 2. **High confidence amino acid sequence and similarities of the peptide fragment PT-61 indicated in Fig. 1 with different proteins.** The amino acids underlined represent residues which are significantly different between the various proteins, whereas, the other residues represent perfect matches and conserved residues. X=residues not integrated.

DISCUSSION

In the present study, we provide evidence that P-SG64, the second major P-SG in CHO cells has 100% amino acid sequence (25/25 residues) identity to the Ca^{2+}-binding protein, calreticulin, from various species. These results, similar to those published recently (8), demonstrate that both the major P-SGs in CHO cells are calcium-binding proteins with similarity in amino acid composition and amino acid sequence to each other and to other forms of calreticulins. The difference in the molecular weight of P-SG67 and P-SG64 may reflect two isoforms of calreticulin; similar calreticulin isoforms were identified in rabbit liver where the two calreticulins differed in molecular weight by 2 kDa (16). Subtle differences in P-SG amino acid composition are indicated in Table I; however, we have no information on specific amino acid substitutions in homologous peptide fragments from P-SG64 and P-SG67. Alternatively, the differences in molecular weight between P-SG64 and P-SG67 could reflect a variable glycosylation status of calreticulin during acute heat stress. A determination of subcellular locations of both P-SG67 and P-SG64, and their redistribution between various cellular compartments during heat stress, similar to that seen for other HSPs after hyperthermia (17), may provide additional clues which may help to delineate the roles of these various calreticulin forms.

Calreticulin is a highly conserved Ca^{2+}-binding protein with strong sequence homology between species (9). The calreticulin molecule consists of 3 distinct structural domains (9, 10): an N-terminal domain that is predicted to be a globular domain; the P-domain, that is rich in proline, serine, and threonine, and includes a low-capacity, high affinity Ca^{2+}-binding site, and the C-terminal domain, that is rich in acidic residues and contains an ER retention signal, KDEL, and a high-capacity, low

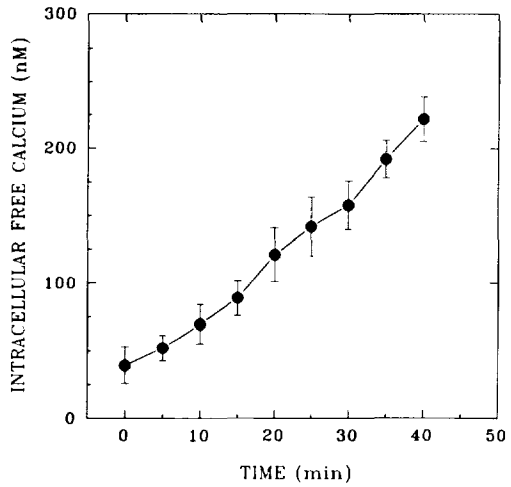

FIG. 3. **Measurement of intracellular Ca^{2+}.** CHO cells were loaded with 2 μM fura-2/AM as described (see "Materials and Methods) and fluorescence measurements were monitored initially at 37°C and then every 5 min following heat stress at 45°C. Triton X-100 (1.5% final concentration) and EGTA (20mM final concentration) were used to calibrate the assay. In the above figure, the $[Ca^{2+}]_i$ concentration at 0 min is the basal value at 37°C, whereas the $[Ca^{2+}]_i$ concentrations at 5 min and up to 40 min represent values following heating of cells to 45°C. Representative data from one of three replicate experiments are shown. Values are mean ± S.D.

affinity Ca^{2+}-binding site (10). The amino acid sequence for the peptide fragment PT-61 from P-SG64, when compared to the published sequence for rabbit skeletal muscle calreticulin (9) indicates that this fragment maps to the C-domain of the calreticulin molecule (Fig. 2). It has been suggested that the asparagine at position 327 in the rabbit calreticulin may be a potential glycosylation site (9). However, until recently, only bovine (18, 19) and rat liver calreticulins (20, 21) are reported to be glycosylated, presumably *via* glycosylation of the asparagine at position 327. Definite characterization of glycosylation patterns of calreticulin remains to be accomplished (22). The sequence analysis of PT-61 shows a potential N-glycosylation site at position 22 which corresponds to asparagine at position 327 in rabbit calreticulin (Fig. 2). We propose that this site becomes increasingly glycosylated under "prompt" heat-stress.

Partial amino acid sequence homologies were also observed between fragment PT-61 and other proteins. These included the ER membrane bound Ca^{2+}-binding protein, calnexin (Fig. 2A), and the envelope proteins, gp120 from HIV-1, and the polyprotein from HIV-2. PT-61 is homologous to a sequence in the cytosolic domain of calnexin molecule with putative chaperone functions (23). In both HIV-1 and HIV-2, PT-61 is partially homologous to the variable regions in the viral envelope proteins (24, 25) that have been implicated in immunological responses (24, 25).

In view of the Ca^{2+}-binding sites in calreticulin molecule, a role for calreticulin in cell physiology (26) relative to Ca^{2+}-binding and storage (27) is reasonable. However, the relationship between "prompt" glycosylation of calreticulin under heat stress and concurrent elevations in $[Ca^{2+}]_i$ is not known. Thus, we determined the basal levels of $[Ca^{2+}]_i$ and characterized the increase that occurs during "prompt" heat stress (Fig. 3). Both basal values of $[Ca^{2+}]_i$ at 37°C and the 8-fold increase in $[Ca^{2+}]_i$ following an acute heat stress at 45°C for 50-60 min were similar to those reported earlier (15). However, elimination of this rise in cytoplasmic Ca^{2+} using the Ca^{2+}-chelator BAPTA (14, 15) had no significant effect on the glycosylation of P-SG67/ P-SG64/calreticulin during heat stress (Fig.

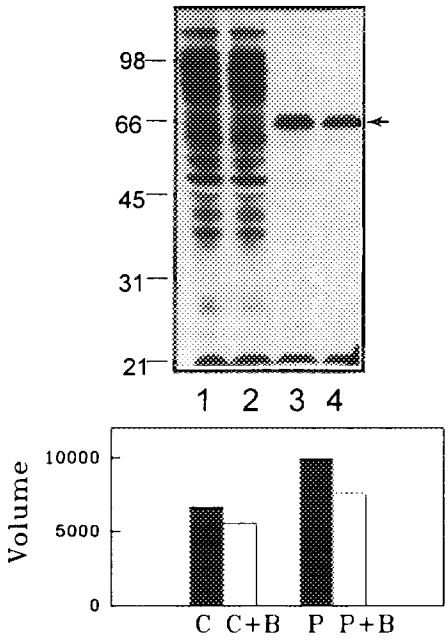

FIG. 4. **Effect of BAPTA on prompt glycosylation.** CHO cells were loaded with 5 μM BAPTA at 37°C (see "Materials and Methods"); D-[2-^{3}H]mannose (50 µCi/ml) was added and the cells were either incubated at 37°C (control conditions) or heated for 30 min at 45°C (prompt glycosylation). The relative change in protein glycosylation as determined by densitometric scanning of the fluorogram is shown in the bar graph in *lower panel* of the figure. *Lane 1 and C (bar graph)*, control CHO cells at 37°C; *lane 2 and C+B (bar graph)*, effects of BAPTA on control cells at 37°C; *lane 3 and P (bar graph)*, prompt glycosylation at 45°C; *lane 4 and P+B (bar graph)*, prompt glycosylation at 45°C in the presence of BAPTA. The amount of protein in all lanes was 200 µg. *Arrow* points to the position of major P-SGs, P-SG67 and P-SG64. In the bar graph, volume represents the volume of area corresponding to P-SGs as indicated by the arrow shown in figure above.

4). These data, therefore, suggest that "prompt" glycosylation of calreticulin and $[Ca^{2+}]_i$ homeostasis appear to be unrelated during conditions of acute heat stress.

Calreticulin is a multifunctional protein under normal physiological conditions. However, its role during acute heat stress is unknown. Interestingly, calreticulin has been implicated in modulating protein-protein interactions (28), chaperone functions (29, 30), protein synthesis (31), and in the regulation of gene transcription (32, 33). Moreover, some of these functions are also mediated by HSPs, possibly in cooperation with calreticulin to ameliorate cellular recovery. In this model, glycosylation of calreticulin may affect structural integrity, thermodynamic stability, and prevent protein aggregation (34) in ways that need to be understood in detail.

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