Journal of Cellular Biochemistry 66:98–111 (1997)

Intracellular Distribution of Heat-Induced Stress Glycoproteins

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Abstract Cellular heat stress results in elevated heat-shock protein (HSP) synthesis and in thermotolerance development. Recently, we demonstrated that protein glycosylation is also an integral part of the stress response with the identification of two major stress glycoproteins, GP50, associated with thermotolerance, and P-SG67, the "prompt" stress glycoprotein induced immediately during acute heat stress. In the present study, we characterized the subcellular location and redistribution of these proteins during the cellular injury and recovery phase. In unheated and heated CHO cells, both stress glycoproteins were present in each subcellular fraction isolated by differential centrifugation. However, the subcellular redistribution in the course of cellular recovery after heat stress was specific for each stress glycoprotein. GP50 was present in all subcellular fractions before heat stress, but showed relatively little redistribution after heat stress. By 24 h of recovery following stress, GP50 showed partial depletion from lysosomes and microsomes, and was mainly present in the mitochondria. Glycosylated P-SG67 was redistributed in a more complex fashion. It was seen predominantly in the lysosomes and microsomes immediately following heat-stress, but after 6 h of recovery following heat stress, it largely disappeared from the microsomes and was present mainly in the cytosol. By 24 h of recovery following heat stress, it was found predominantly in the nucleus-rich fraction and mitochondria. The localization of GP50 and P-SG67 by subcellular fractionation is consistent with immunolocalization studies and contrasts with the translocation of HSP70 after heat stress from cytosol to nuclei and nucleoli. These results reflect a characteristic distribution for each stress glycoprotein; their presence in virtually all subcellular fractions suggests multifunctional roles for the various stress glycoproteins in the cellular heat stress response. J. Cell. Biochem. 66:98–111, 1997.

Key words: hyperthermia; thermotolerance; protein glycosylation; subcellular distribution

Cellular injury caused from heat or other environmental insults is generally associated with increased expression of heat shock proteins (HSPs) [Gething and Sambrook, 1992; Hendrick and Hartl, 1993]. Heat stress can also induce thermotolerance development [Henle, 1987; Hendrick and Hartl, 1993; Henle et al., 1990a], possibly as a result of elevated HSPs [Weber, 1992]. Previous studies from our laboratory have shown that heat stress increased not only HSP expression, but also enhanced the glycosylation of specific proteins [Henle et al., 1988a,b, 1990b, 1993, 1994; Henle and Nagle, 1991].

Chinese hamster ovary (CHO) cells exhibit two types of heat stress-induced glycosylation responses: a "classical" or "late" glycosylation response [Henle et al., 1988b, 1994] and "prompt" glycosylation [Henle et al., 1993]. The "late" glycosylation response occurs minutes or hours following acute heat stress and is primarily associated with the specific glycosylation of GP50 in parallel with expression of thermotolerance and the accumulation of HSPs [Henle and Nagle, 1991]. "Prompt" glycosylation occurs immediately during acute heat stress and is specific for the glycosylation of "prompt" stress glycoproteins (P-SGs), P-SG67 and P-SG64

Contract grant sponsor: National Cancer Institutes, contract grant number CA-33405; contract grant sponsor: Office of Naval Research, contract grant number ONR4114502.

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Received 5 June 1996; accepted 11 March 1997

[Henle et al., 1993]. GP50 was recently identified as the J6 gene product [Henle et al., 1994], whereas, P-SG67 and P-SG64 were both identified as calreticulin [Jethmalani et al., 1994; Jethmalani and Henle, 1994]. The phenomena of stress-induced protein glycosylation are not limited to CHO cells, but have also been documented in other cell lines [Henle et al., 1995].

The functional role of stress glycoproteins remains unknown. Defining the subcellular location and redistribution of these glycoproteins both before and after stress may provide initial information regarding their potential physiological function(s). Therefore, the present study was designed to characterize the subcellular distribution of both "late" and "prompt" stress glycoproteins in CHO cells. These studies include both differential centrifugation and immunolocalization as complimentary techniques. The results indicate that glycosylated GP50 and the P-SGs, P-SG67 (calreticulin) are generally present in all subcellular fractions, consistent with multifunctional roles for these proteins.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Materials

CHO cells were grown asynchronously in McCoy's 5A medium, supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) [Henle et al., 1988a,b, 1993, 1994; Henle and Nagle, 1991]. Cells were subcultured twice weekly to maintain cultures in an exponential growth pattern. Cells were grown in T-75 plastic culture flasks; for heat treatments, flasks were horizontally submersed in well-circulated, precision-controlled ($< \pm 0.05$°C) water baths [Henle et al., 1988b]. D-[2-^{3}H]mannose (23 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA); common chemicals were from Sigma (St. Louis, MO).

Heating and Labeling of Proteins

Cells were labeled with 50 µCi/ml of D-[2-^{3}H]mannose in Earle's Balanced Salt Solution, supplemented with 50 µM D-glucose, 2 mM L-glutamine, and 50 mM MOPS, pH 7.4, as previously described [Henle and Nagle, 1991]. Control cells were labeled for 1 h at 37°C; thermotolerant cells were labeled during a 1 h period, beginning at 5 h after a heat treatment at 45°C for 10 min [Henle et al., 1988a,b, Henle and Nagle, 1991]. For "prompt" heat stress-induced glycosylation, cells were heat-shocked at 45°C for 30 min during which they were simultaneously labeled with D-[2-^{3}H]mannose [Henle et al., 1993]. Following labeling, the medium was removed and culture flasks were rinsed twice with fresh medium without label. From this point onwards, the redistribution of D-[2-^{3}H]mannose label was studied by further incubation of cells at 37°C; thermotolerant cells were incubated for 0, 1, 3, 6, and 18 h following labeling, whereas cells labeled under "prompt" heat stress conditions were incubated for another 0, 2, 6, and 24 h. After the respective incubation period, cells were processed as stated below.

Subcellular Fractionation

Cell fractionation was carried out by differential centrifugation as described [Onoda and Djakiew, 1993; Hamel et al., 1993]. At the end of the various incubation periods, the cells were washed, scraped, and resuspended in 1 ml of buffer containing 0.25 M sucrose, 20 mM Tris, 0.1 mM PMSF, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, pH 7.4. The cell suspensions were homogenized on ice at 4°C with 100 strokes each, using a Dounce glass homogenizer and the resultant crude homogenate was subjected to sequential centrifugations. Each successive supernatant was used for the next centrifugation. The first centrifugation was at 112*g* for 10 min at 4°C to yield nuclei and unbroken cells as a pellet. The nuclear membrane pellet was obtained by centrifugation at 600*g* for 10 min at 4°C, whereas the mitochondrial pellet, lysosomal pellet, microsomal pellet, and the cytosol were obtained after centrifugation at 800*g* for 8 min, 20,000*g* for 8 min, and 100,000*g* for 60 min at 4°C, respectively. Protein concentration was measured by the Coomassie Blue dye method (Bio-Rad protein assay kit, Bio-Rad, Richmond, CA), using bovine serum albumin as a standard. All fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described below. The activity of cytochrome C oxidase was measured as described earlier [Cooperstein and Lazarow, 1951].

Electrophoresis, Fluorography, and Western Blotting

SDS-PAGE was performed as described previously [Henle and Nagle, 1991; Henle et al., 1993]. Gel lanes were loaded for equal radioactivity. After electrophoresis, gels were stained

and soaked in En^{3}Hance (NEN Products, Boston, MA), dried, and exposed to X-Omat film (Eastman Kodak Company, Rochester, NY) at −70°C up to 60 days. Films were developed, and fluorograms were analyzed by densitometry (Computing densitometer, Model 300A, Molecular Dynamics, Sunnyvale, CA) when film exposures fell within the linear range of O.D. greater than 0.25 AU [Laskey and Mills, 1975] and less than 3.0 AU (manufacturer's calibration data). For Western blotting, proteins were electroblotted onto PVDF membranes, as described [Jethmalani, et al., 1994]. Both GP50 and calreticulin antibodies were obtained as discussed below and used for immunoblotting at 1:500 dilution. Blots were developed first using the Immunelite II Chemiluminescent Protein detection kit (Bio-Rad, Hercules, CA) and fluorograms were obtained; following this, blots were developed with the Amplified Alkaline Phosphatase Immuno-Blot assay kit (Bio-Rad, Hercules, CA). The fluorograms were analyzed by densitometry as described above.

Indirect Immunofluorescence Staining

The intracellular distribution of GP50, P-SGs, and HSP70 was determined using specific antibodies. CHO cells were grown on glass coverslips (22 × 22 mm) in 35-mm culture dishes. Cells were subjected to heat stress, as described above, but without radiolabeling. After specific heat stress treatments, cells were rinsed with cold PBS, permeabilized, and fixed in cold 100% methanol (−20°C) for 10 min. Cells were then rinsed with PBS and incubated with 10% normal goat serum in PBS (Sigma Immunochemicals) for 1 h at room temperature to inhibit nonspecific antibody binding. For immunofluorescence staining, cells were incubated with the polyclonal anti-GP50/J6 antibody (1/66 dilution, S.-Y. Wang), the polyclonal anti-calreticulin antibody (1/100 dilution, Affinity BioReagents Inc., Neshanic Station, NJ), or the monoclonal anti-HSP70 antibody (1/100 dilution, Stressgen Biotechnologies, Corp., Victoria, B.C., Canada, cat. no. SPA 810) as the first step. The second antibody was an FITC-conjugated goat anti-rabbit IgG (1/100 dilution, Jackson Immunochemicals, Raritan, NJ) or rhodamine-conjugated goat anti-mouse antibody (1/100 dilution, Jackson Immunochemicals). For double immunofluorescence staining, cells were first incubated at 37°C for 1 h with the first antibody consisting of a mixture of either rabbit anti-GP50/J6 polyclonal antibody and mouse anti-HSP70 monoclonal antibody, or a mixture of rabbit anti-calreticulin polyclonal antibody and mouse anti-HSP70 monoclonal antibody in 10% goat serum. Isotypic controls were used for each primary antibody. Cells were then washed extensively with cold PBS and then incubated at 37°C for 1 h with the second antibody that was a mixture of FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse antibody. Cells were now extensively washed and coverslips were mounted with anti-fade medium (Oncor, Gaithersburg, MD). Fluorescence was analyzed by a confocal Argon ion laser scanning microscope (LSM 300, Zeiss, Thornwood, NY) with excitation wavelengths of 488 and 520 nm. Photomicrographs were taken with a conventional fluorescence microscope (Olympus, Japan) equipped with an automatic exposure device.

RESULTS

Subcellular Redistribution of Glycosylated GP50 (Induced by Thermotolerant Heat Stress)

Figure 1A shows fluorograms that define the redistribution of glycosylated GP50 in subcellular fractions, including the nucleus-rich fraction (N + UN), nuclear membrane (NM), mitochondria (MIT), lysosomes (LYS), microsomes (MIC), and cytosol (CYT). Parallel fractions were isolated from unstressed controls and thermotolerant cells (heat-stress: 10 min at 45°C, plus 5 h, 37°C recovery). GP50 was present in all subcellular compartments in both unstressed control and heat-stressed thermotolerant cells. The subcellular fractions were assessed for activity of the mitochondrial marker enzyme, cytochrome C oxidase (Table I). The mitochondrial fraction from control cells contains 80% of total cellular cytochrome C oxidase activity, whereas that from the thermotolerant cells showed 66–76% of activity, excluding the unbroken cells/nucleus-rich fraction (Table I). Thus, a modest overlap does exist between subcellular fractions and must be considered when low levels of stress glycoproteins appear in specific subcellular fractions where their presence may be due to minor contaminations from other subcellular fractions.

Figure 1B shows subcellular redistribution of glycosylated GP50 in control and thermotolerant cells when D-[2-^{3}H]mannose labeling was followed by 1–18 h of "cold" chase. The data shown are a representative set of experiments;

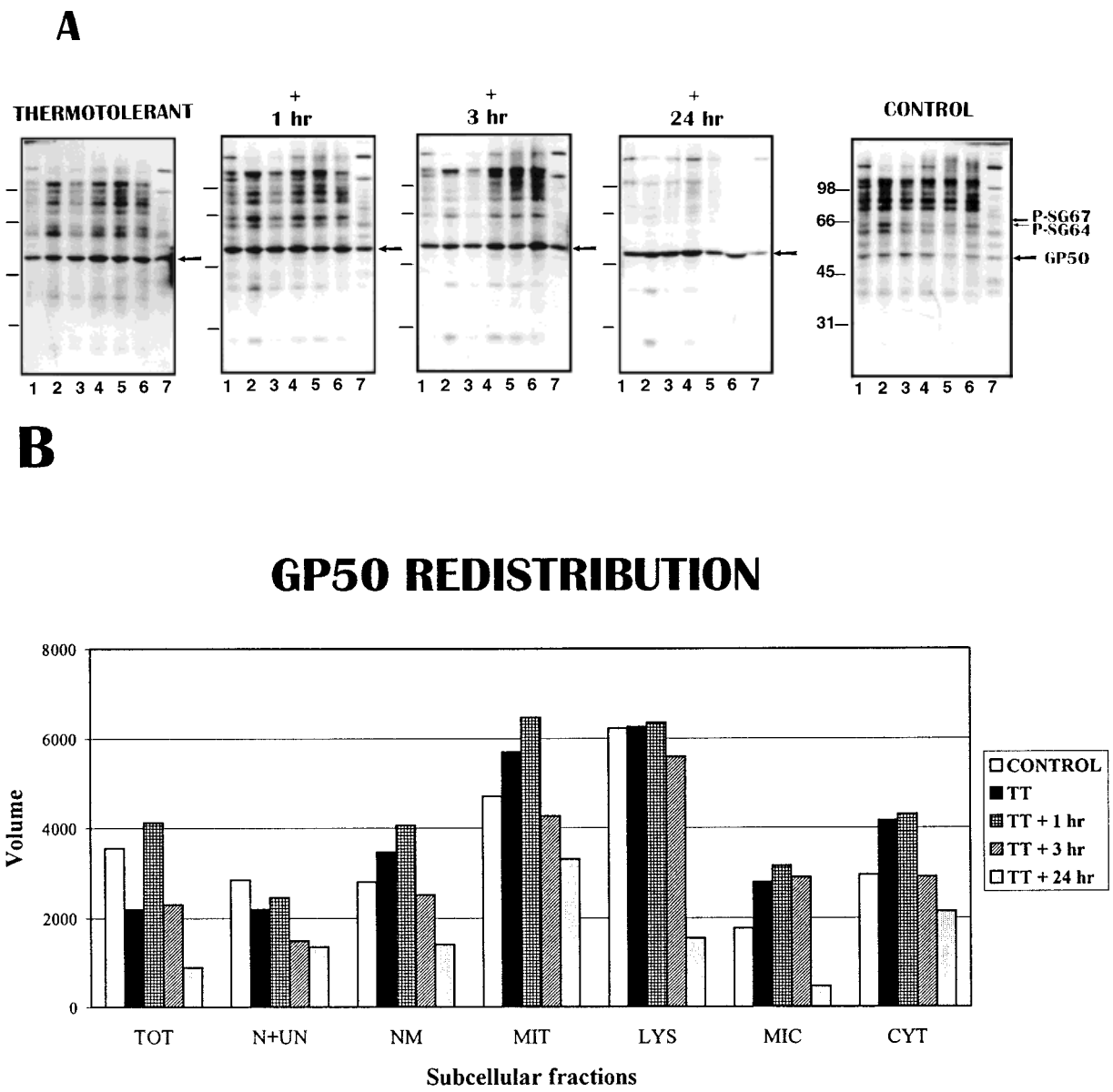

Fig. 1. Subcellular distribution of GP50. CHO cells were labeled with D-[2-^{3}H]mannose (50 µCi/ml) for 1 h at 37°C after recovery of cells (for 5 h) following heat stress at 45°C for 10 min (thermotolerant conditions); control cells were labeled with D-[2-^{3}H]mannose (50 µCi/ml) for 1 h at 37°C. In some experiments, following labeling, cells were incubated further in label-free culture medium for 0, 1, 3, and 24 h. Cells were resuspended in 1 ml of buffer containing 0.25 M sucrose, 20 mM Tris, 0.1 mM PMSF, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, pH 7.4, and fractionation was carried out by differential centrifugation following which the various fractions were subjected to SDS-PAGE (see Materials and Methods for details). **A:** Fluorogram indicating changes in GP50 glycosylation in various subcellular fractions in both control and thermotolerant cells. Gel lanes were loaded for equal radioactivity (~15,000 dpm). **Lane 1,** total cellular fraction; **lane 2,** nuclei and unbroken cells; **lane 3,** nuclear membrane fraction; **lane 4,** mitochondrial fraction; **lane 5,** lysosomal fraction; **lane 6,** microsomal fraction; **lane 7,** cytosolic fraction. Molecular weight markers are shown on the left side. Arrows indicate positions of GP50, P-SG67, and P-SG64. **B:** Bar graph indicating the relative changes in GP50 glycosylation in various subcellular fractions (after 0, 1, 3, and 24 h of postlabeling recovery) as determined by densitometric scanning of the fluorogram (A). The Y-values shown here represent ratios of densitometric scan volumes per 50 µg of protein for each data point. The data presented here are representative of results from one set of experiments.

TABLE I. Distribution of Marker Enzyme Cytochrome C Oxidase in Various Subcellular Fractions*

	Cytochrome C oxidase (enzyme activity/mg)[a]				
	C (2)	TT (2)	TT + 1 (2)	TT + 3 (1)	TT + 24 (1)
N + UN	22.7 ± 5.0	26.9 ± 5.7	24.3 ± 2.9	28.2	34.6
NM	9.7 ± 0.6	11.9 ± 1.1	13 ± 5.1	5.6	10.6
MIT	145 ± 12.3	95.1 ± 35.1	67.4 ± 19.9	35.8	88.0
LYS	5.5 ± 1.5	5.1 ± 0	6 ± 0	N.D.	7.2
MIC	17.6 ± 0.1	11.5 ± 0.04	11.2 ± 1.6	9.2	10.0
CYT	3.2 ± 0.1	2.2 ± 0.8	0.8 ± 0.1	1.04	2.4

*Each value represents mean ± S.E. Number of samples is indicated in parentheses. N.D., not determined.
[a]Enzyme activity represents Δlog[Ferricytochrome C]per minute.

reproducibility was independently verified. Under non-stressed conditions, D-[2-^{3}H]mannose-labeled (glycosylated) GP50 levels were highest in the mitochondrial and lysosomal fraction (Fig. 1B). In thermotolerant cells, glycosylated GP50 was elevated in all subcellular fractions except the nucleus-rich fraction; highest concentrations remained evident in mitochondria and lysosomes (Fig. 1B). This pattern persisted when labeling was followed by a 1 h "cold" chase. After 3 h of "cold" chase, glycosylated GP50 levels were reduced by one third in most subcellular fractions with smaller reductions in lysosomes and microsomes. By 18 h of "cold" chase, equivalent to 24 h after heat-stress, glycosylated GP50 was still present at approximately 50% of peak values in nuclei, mitochondria, and cytosol, with highest levels remaining in mitochondria (Fig. 1B).

Redistribution profiles of glycosylated GP50 differ for each individual subcellular fraction isolated at various time points between 1–18 h of "cold" chase. For example, glycosylated GP50 levels peaked at 1 h of "cold" chase in the nucleus-rich, nuclear membrane, mitochondria, and cytosol; thereafter GP50 levels gradually decreased between 3 and 18 h of cold chase. In contrast, glycosylated GP50 levels in lysosomes and microsomes also peaked at 1 h of "cold" chase, but declined sharply by the end of 18 h of "cold" chase. The most rapid clearing of GP50 between 3 and 18 h cold chase period occurred in the microsomal fraction, which contains ER, the major site of protein glycosylation.

The redistribution of GP50 was also studied by Western blot analysis using anti-GP50 antibodies (Fig. 2A), which recognize both the aglycone and glycosylated forms of GP50. GP50 was detected in all subcellular fractions, with higher concentrations in the mitochondria of control unstressed and heat-stressed cells (Fig. 2B), consistent with the above-mentioned results (Fig. 1A, B).

The subcellular localization and redistribution of GP50 within intact cells was studied relative to that of HSP70 by co-localization via double immunofluorescence staining. These measurements detect the distribution of the total amount of nascent and glycosylated GP50. In unstressed cells, GP50 was concentrated in the cytoplasm and in the ER network, which extends across the cytoplasm and is marked by intense green fluorescence. GP50 was also localized in the nucleus with generally lower levels of green fluorescence; however, localized regions within nuclei, possibly nucleoli, displayed fluoresence that was comparable to that in cytoplasm (Fig. 3A). Similarly, HSP70 was also cytoplasmic with less intense red fluorescence in a fraction of the nuclei (Fig. 3A). After heat stress at 45°C and with thermotolerance development at 37°C, GP50 appeared localized mainly at perinuclear sites with intense green fluorescence, in cytoplasm and ER networks, with extensions marked by less intense green fluorescence reaching towards the cell edge (Fig. 3B). The cell nucleus appeared with reduced intensity of green fluorescence, but contained localized regions resembling nucleoli with concentrated fluoresence. Localization of HSP70 to the nucleus and to nucleolar sites of heat-stressed cells was also visible by intense red fluorescence (Fig. 3B) and is similar to that in earlier observations [Hattori et al., 1993; Milarski et al., 1989]. Overall, the intensity of HSP70 staining was less than that of GP50 both in the unstressed and in heat-stressed cells. Also, GP50 and HSP70 were localized in the nuclei of the *same* cells after thermotolerant induction, consistent with a potential co-localization of GP50 and HSP70.

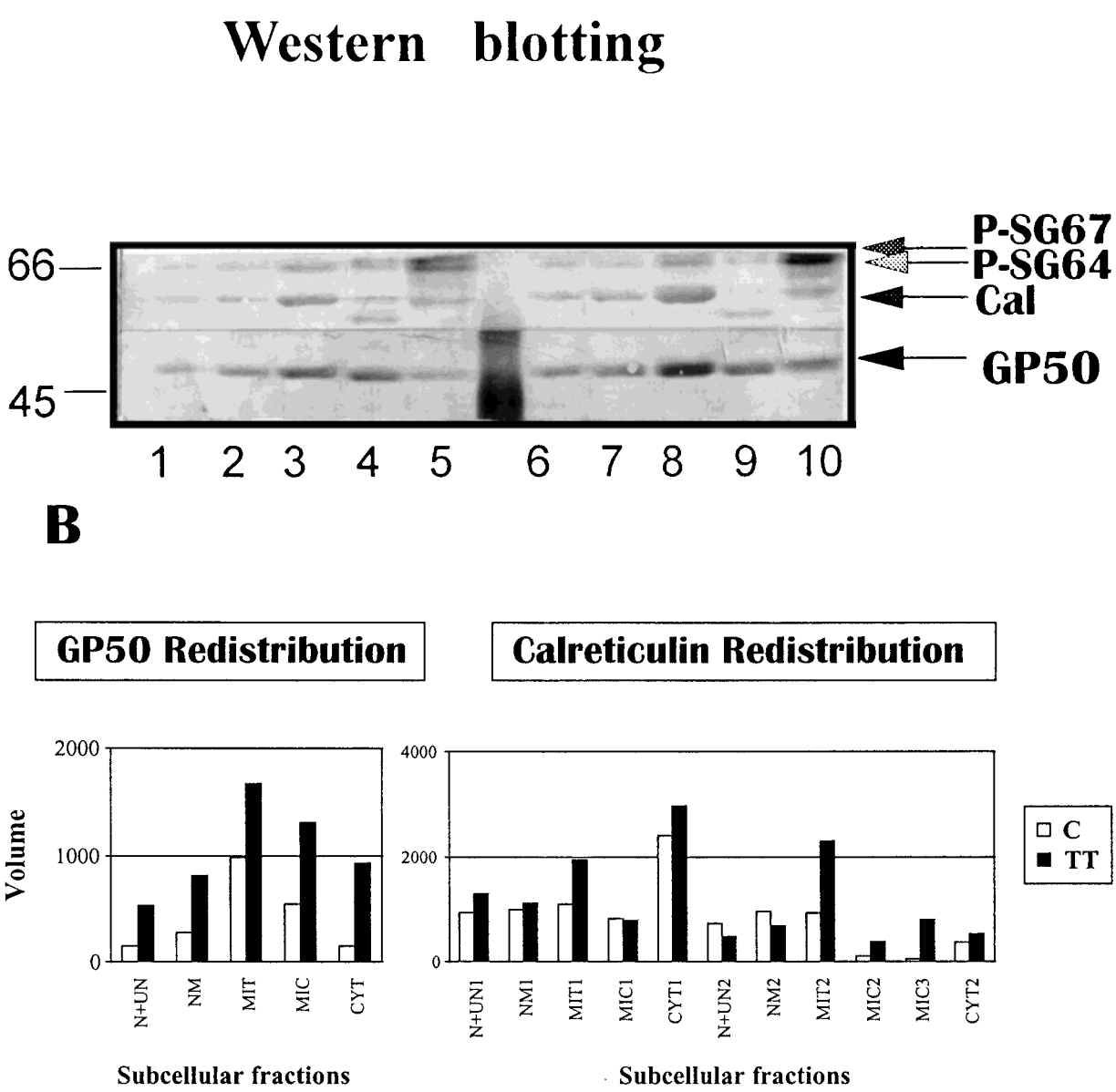

Fig. 2. Western blot analysis of GP50 and calreticulin in subcellular fractions from thermotolerant cells. Cells were heat-stressed and the subcellular fractions were obtained as described in Figure 1. Fractions were analyzed by SDS-PAGE with gel lanes loaded for equal protein (80 µg). Proteins were electroblotted to a single PVDF membrane, which was cut into various pieces based on the molecular weight range, and developed with appropriate antibodies. **A:** Western blots showing positive reactions with both anti-GP50 and anti-calreticulin antibodies. The bands were first developed by chemiluminescence detection kit; final visualization was by development with the amplified alkaline phosphatase detection kit (see Materials and Methods). **Lanes 1** and **6,** nuclei and unbroken cells from control and thermotolerant samples; **lanes 2** and **7,** nuclear membrane fractions from control and thermotolerant cells; **lanes 3** and **8,** mitochondrial fractions from control and thermotolerant cells; **lanes 4** and **9,** microsomal fractions from control and thermotolerant cells; **lanes 5** and **10,** cytosolic fractions from control and thermotolerant cells, respectively. Prestained molecular weight markers are seen between lanes 5 and 6. **B:** Bar graph indicating the change in total (aglycone and various glycosylated molecules) amounts of GP50 and P-SG67/64/calreticulin in various subcellular fractions, determined by densitometric scanning of the Western blots shown in A. 1, glycosylated P-SG67/64 calreticulin protein; 2, protein band with lower molecular weight than the glycosylated protein; 3, ~55,000 kDa band in the microsomal fraction. C, control cells; TT, thermotolerant cells.

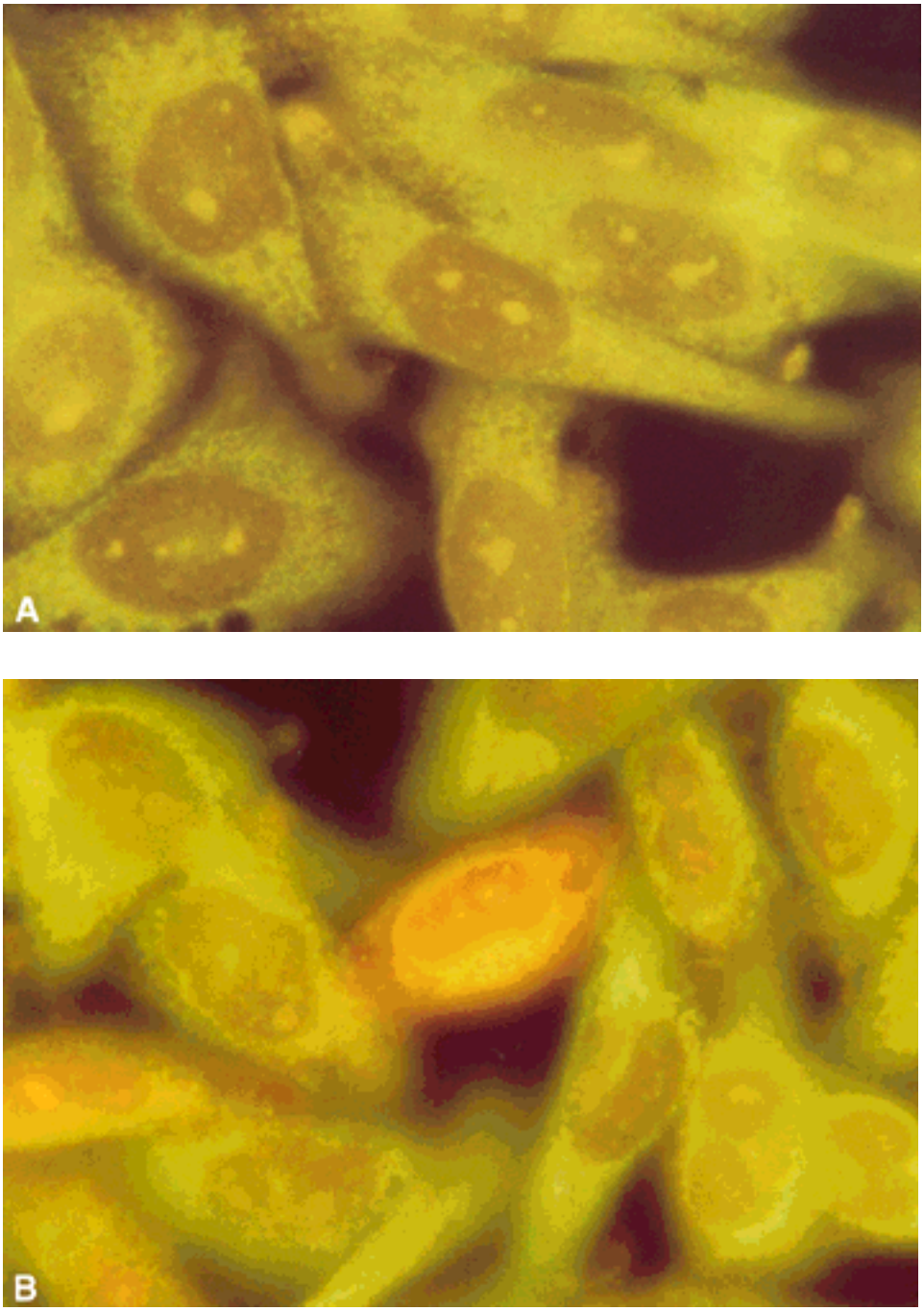

Fig. 3. Immunofluorescence analysis showing double labeling of GP50 and HSP70 in CHO cells after thermotolerant heat stress. Control unstressed cells (**A**) and thermotolerant cells heated at 45°C for 10 min with recovery for 4 h (**B**) were fixed and processed for double immunofluorescence staining with a mixture of polyclonal rabbit GP50 and monoclonal mouse HSP70 antibodies, which were visualized using a mixture of FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse antibodies (see Materials and Methods for details). Exposure times for A and B were 75 and 45 s, respectively.

Subcellular Redistribution of Glycosylated "Prompt" Stress Glycoproteins (P-SGs)

Glycosylated P-SGs were present in all subcellular fractions isolated immediately after heating (Fig. 4A). The lysosomes and microsomes were the primary locations of glycosylated P-SGs immediately after "prompt" heat stress (Fig. 4B). With a 2 h "cold" chase after heat stress, P-SGs redistributed to all other subcellular compartments, with a significant increase

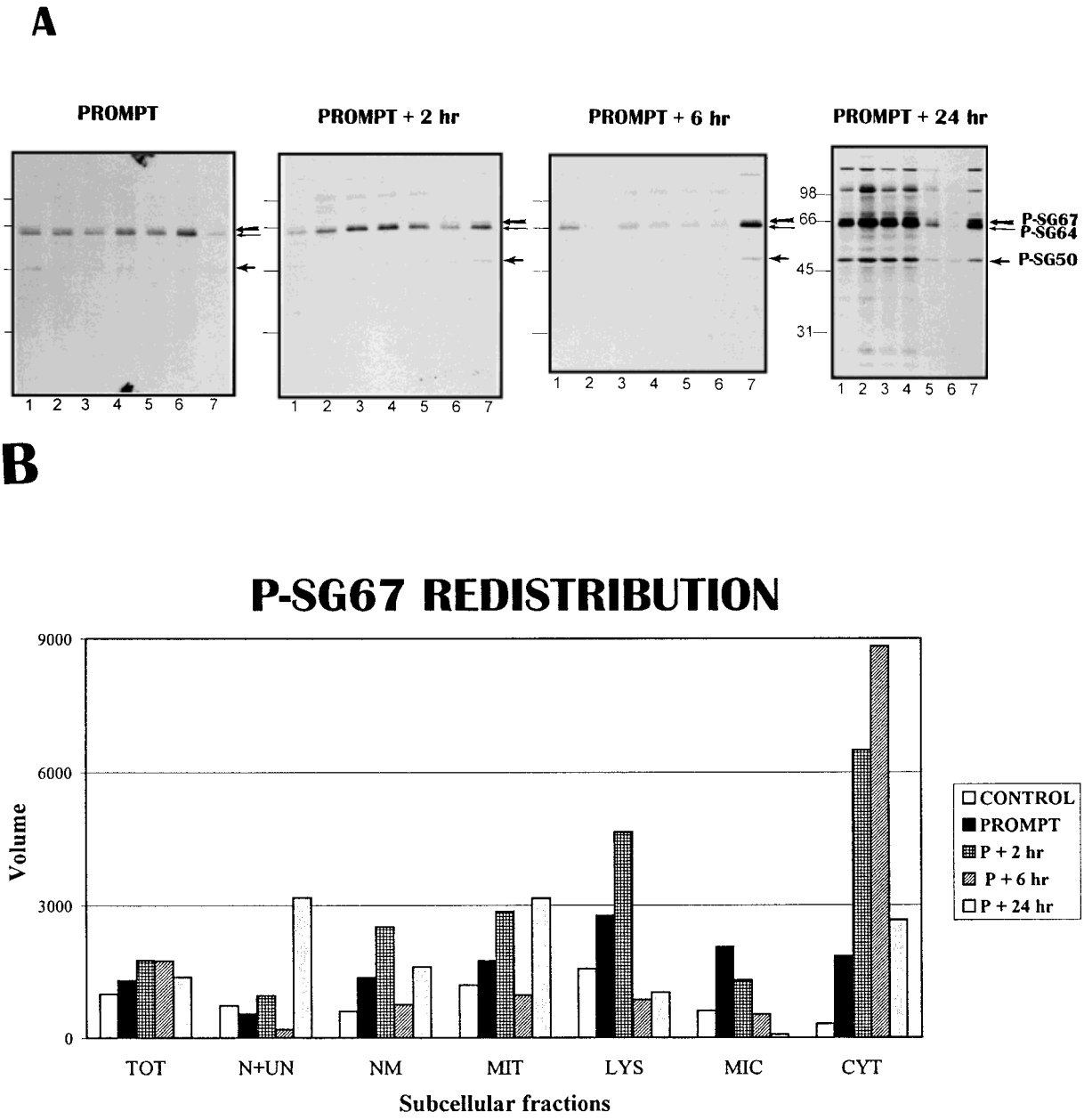

Fig. 4. Subcellular distribution of P-SG67. CHO cells were labeled with D-[2-^{3}H]mannose and allowed to recover for 0, 2, 6, and 24 h, fractionated by differential centrifugation, and subjected to SDS-PAGE as discussed under Figure 1. **A:** Fluorogram indicating changes in P-SG67 glycosylation in various subcellular fractions for both control and "prompt" heat-stressed cells. Gel lanes were loaded for equal radioactivity (~15,000 dpm). **Lane 1,** total cellular fraction; **lane 2,** nuclei and unbroken cells; **lane 3,** nuclear membrane fraction; **lane 4,** mitochondrial fraction; **lane 5,** lysosomal fraction; **lane 6,** microsomal fraction; **lane 7,** cytosolic fraction: Molecular weight markers are shown on the left side. Arrows indicate positions of P-SG50, P-SG67, and P-SG64. **B:** Bar graph indicating relative changes in P-SG67 glycosylation in various subcellular fractions as determined by densitometric scanning of the fluorogram (A). **C:** Bar graph indicating relative changes in P-SG50 glycosylation in various subcellular fractions following "prompt" heat stress (after 0, 2, 6, and 24 h of post-labeling recovery) as determined by densitometric scanning of the fluorogram (A). The results for the 24 h time point have been calculated to adjust for the increase in the amount of radiolabel added during the labeling procedure. The Y-values have been calculated as indicated in Figure 1. The data presented here are representative of results from one set of experiments.

C

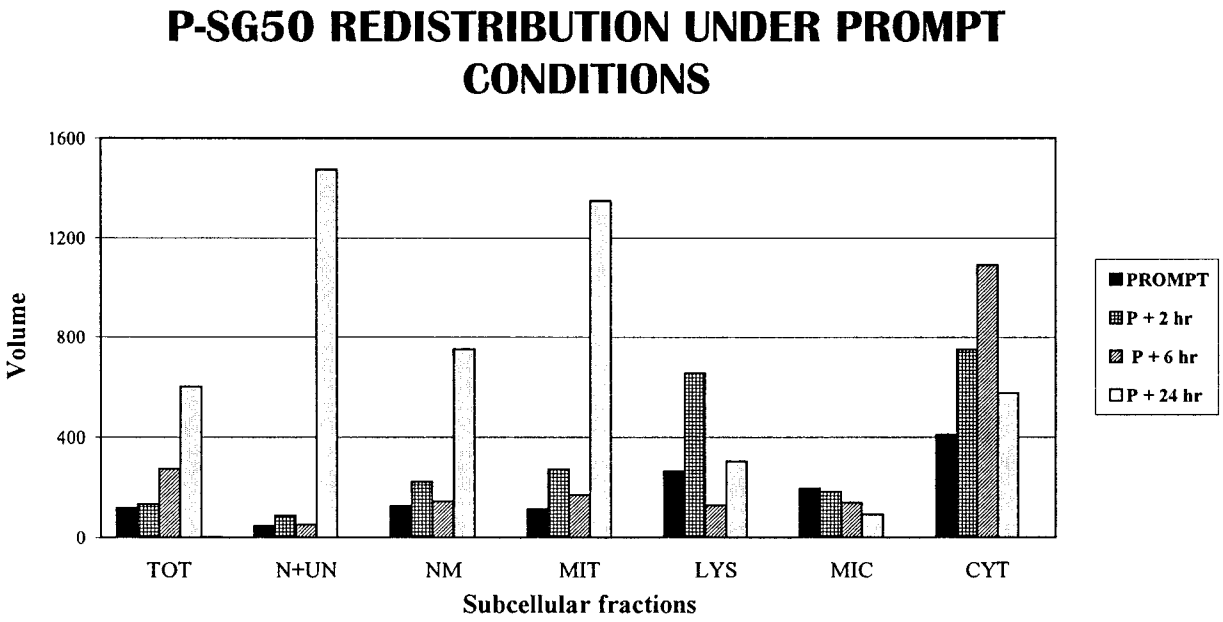

Figure 4. (Continued.)

to lysosomes and cytosol (Fig. 4B). With a 6 h "cold" chase, glycosylated P-SGs were seen predominantly in the cytosolic fraction. After a 24 h "cold" chase, glycosylated P-SGs remained high in the nucleus-rich fraction and in mitochondria (Fig. 4B), reflecting a slow turnover of glycosylated P-SGs.

Analysis of P-SG redistribution in each *individual* subcellular fraction isolated after 2–24 h of "cold" chase showed that glycosylated P-SGs were rapidly cleared from the microsomes, with little or no residue by the end of 24 h (Fig. 4B). In the cytosol, glycosylated P-SGs peaked after 6 h of "cold" chase and were rapidly cleared by 24 h. In the rest of the subcellular fractions, there were small increases by the end of 24 h of "cold" chase, consistent with a slower turnover rate.

The data from Western blot analysis (Fig. 2A) showed the presence of calreticulin in all subcellular fractions following thermotolerant heat stress (10 min at 45°C, plus 5 h, 37°C recovery), similar to the above-mentioned results obtained shortly after acute heat stress. It is interesting to note that the anti-calreticulin antibodies recognized several sets of proteins in various subcellular compartments, including mitochondria in both control and heat-stressed cells. However, in the microsomal fraction, the antibodies cross-reacted with an additional protein with a molecular weight of ~55,000 kDa. Thus, the results suggest that either calreticulin exists as several different isoforms or that calreticulin molecules undergo post-translational modifications, as reported by us earlier [Jethmalani et al., 1994; Jethmalani and Henle, 1994]. The densitometric scan of the fluorograms obtained after the chemiluminescent detection of calreticulin proteins on the blot showed changes in the redistribution of all these proteins in various subcellular compartments in both control and thermotolerant cells (Fig. 2B). All of the above results suggest that cellular calreticulin redistribution under different heat stress conditions appears to be complex.

"Prompt" labeling with D-[2-^{3}H]mannose during heat stress also showed another GP50 glycoprotein, although this protein was not labeled to the same extent as the major glycosylated P-SGs, P-SG67 and P-SG64. Positive identification of the "prompt" GP50, P-SG50 remains to be accomplished, even though its identity with the thermotolerance-associated GP50 is likely. The redistribution pattern of P-SG50 within

the various subcellular compartments differed from that of GP50, discussed above (Fig. 1B). For example, P-SG50 labeled during heat stress was localized mainly in lysosomes, microsomes, and cytosol (Fig. 4C). In contrast, glycosylated GP50 in thermotolerant cells was relatively higher in mitochondria, lysosomes, and microsomes (Fig. 1B). Furthermore, P-SG50 showed a dramatic overall increase in three fractions over the 24 h "cold" chase period—the nucleus-enriched fraction, nuclear membranes, and mitochondria—whereas glycosylated GP50 in thermotolerant cells gradually disappeared from all subcellular fractions (Fig. 1B). The unexpected rise in radiolabel of P-SG50 during the 24 h "cold" chase period could reflect translocation of P-SG50 between different organelles, or the transfer of radiolabel from carbohydrate residues of other glycoproteins with slower turnover, such as P-SGs (Fig. 4B).

Double immunostaining of intact cells using anti-calreticulin antibody (green fluorescence) and HSP70 antibody (red fluorescence) was also examined in control unheated and heat-stressed cells for subcellular localization of P-SGs. Since P-SGs are glycosylated forms of calreticulin [Jethmalani et al., 1994; Jethmalani and Henle, 1994], we used calreticulin antibodies; however, these recognize both glycosylated and aglycone forms of calreticulin. The HSP70 antibody was used for co-localization since its redistribution after heating is well characterized [Hattori et al., 1993; Welch et al., 1991; Milarski et al., 1989]. In unstressed control cells, fluoresence was generally low and P-SG/calreticulin was localized mostly to cytoplasm and the ER network extending towards the periphery of the cells, as evidenced by green fluorescent staining of these sites (Fig. 5A). In these cells, HSP70 was present in diffuse form both in the cytoplasm and nucleus, visible by non-uniform red fluorescence (Fig. 5A). Following acute heat stress at 45°C for 30 min and 1 h recovery; however, fluoresence from P-SG/calreticulin greatly intensified with localization mostly to the perinuclear membranes in the cytoplasm, including the ER network. Fluoresence from P-SG/calreticulin also appeared in the nucleus which showed less intense green fluorescence on the average, but featured punctate regions of intense green staining (Fig. 5B). Under the same conditions, HSP70 appeared with intense red fluorescence both at perinuclear sites and in the nucleus, presumably in nucleoli. Except for the perinuclear regions, HSP70 was largely absent from the cytoplasm (Fig. 5B). The most striking differences were the overall increased intensity of P-SG/calreticulin staining and the apparent lack of co-localization for P-SG/calreticulin and HSP70 in the "prompt" heating experiments (Fig. 5B).

DISCUSSION

In the present study, we determined the subcellular localization of two major types of stress glycoproteins, GP50 and the P-SGs (P-SG67, P-SG64 and P-SG50). Their localization in normal unstressed and heat-stressed cells is a first step toward defining their potential physiological function. Remarkably, both types of stress glycoproteins were present in all subcellular fractions. Moreover, each had a unique distribution profile, as determined by differential centrifugation and by immunolocalization. Furthermore, the redistribution profiles of glycosylated GP50 and P-SG50 were different even though these proteins are suspected of being identical in spite of their labeling with D-[2-^{3}H]mannose under different conditions.

The localization and redistribution of both GP50 and P-SGs to all subcellular fractions raises intriguing questions. The ER, identified here with the microsomal fraction, is the common site for protein N-glycosylation [Hirschberg and Snider, 1987]. The rapid appearance of glycosylated P-SGs and GP50 in all other subcellular fractions is unexpected and cannot be easily explained. The presence of glycosylated GP50 and P-SGs in lysosomes may reflect glycoprotein processing and turnover, steps that may be enhanced during stress [Laszlo, 1992].

GP50 is a homolog of the J6 protein [Wang, 1992] and is closely related to the ER protein gp46 [Clarke et al., 1991; Clarke and Sanwal, 1992] and to HSP47 [Hirayoshi et al., 1991]. Both HSP47 and gp46 have been implicated in collagen binding [Nakai et al., 1992]. J6 belongs to the serpin (serine protease inhibitor) protein family [Wang, 1994], but specific functions for J6 remain to be elucidated. The serpin family includes proteins with multiple, widely varied functions, ranging from protein folding and extracellular matrix remodeling to cell differentiation [Potempa et al., 1994]. GP50 and P-SG50 are probably identical molecules, based on immunoprecipitation data (unpublished data), although these stress glycoproteins become radiolabeled under different conditions and exhibit differential subcellular redistribution profiles

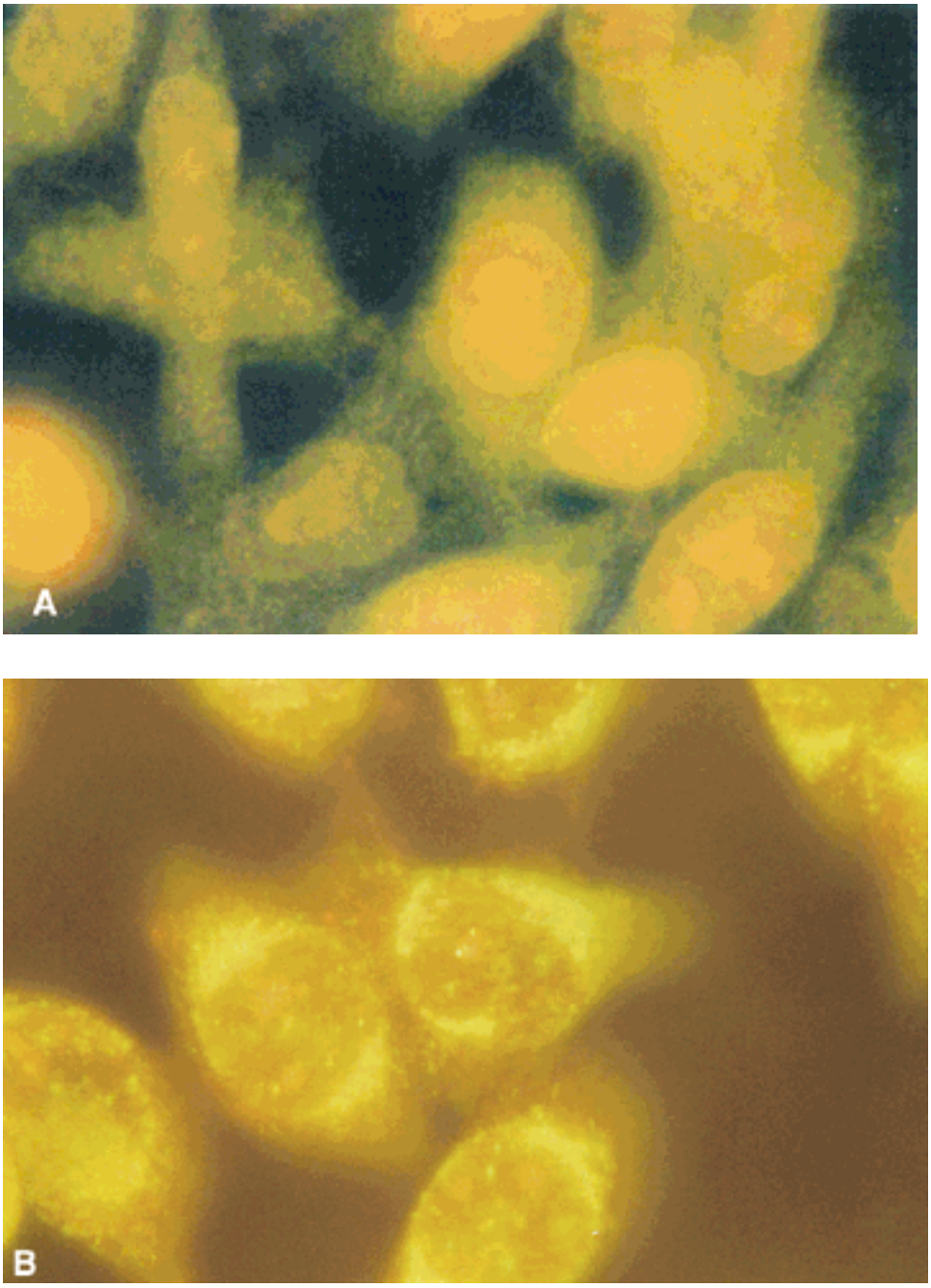

Fig. 5. Immunofluorescence analysis showing double labeling of calreticulin and HSP70 in CHO cells after "prompt" heat stress. Control unstressed cells (**A**) and acute heat-stressed cells heated at 45°C for 30 min with recovery for 1 h (**B**) were fixed and processed for double immunofluorescence staining with a mixture of polyclonal rabbit calreticulin and monoclonal mouse HSP70 antibodies, which were visualized using a mixture of FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse antibodies (see Materials and Methods for details). Exposure times for both A and B were 90 s each.

(Figs. 1B and 4C). Specific functions for GP50 in heat-damaged cells remain to be determined, although these may be related to those identified for other members of the serpin family.

It is noteworthy that GP50 exhibited a redistribution pattern that was different from that of HSP70. HSP70 is mostly cytoplasmic in unstressed cells and translocates to the nucleus after heat stress. In spite of these differences, however, we note overlapping distributions of HSP70 and GP50 in the nuclei of thermotolerant cells, consistent with the potential co-

localization of GP50 and HSP70 (Fig. 3). Co-localization of GP50 and HSP70 was also documented in a series of immunoprecipitation experiments using antibodies to either GP50 or HSP70. In those experiments, the two stress proteins co-precipitated with each other and also with other HSPs in heat-stressed thermotolerant cells (Jethmalani, et al., manuscript in preparation).

Another unexpected finding is the appearance of the prompt glycoprotein, P-SG, in all subcellular compartments. Since P-SGs were shown to be identical to calreticulin [Jethmalani et al., 1994; Jethmalani and Henle, 1994], we expected to find P-SGs mainly in the microsomal fraction that contains ER. Calreticulin is primarily an ER protein, with smaller amounts possibly present in the nucleus of some cells [Michalak et al., 1992]. Calreticulin has also been reported in the Golgi apparatus [Nakamura et al., 1993] and in other subcellular locations, including cell surface, perinuclear areas, and cytosolic granules [Nash et al., 1994]. The fluorograms (Fig. 4B) that show a ubiquitous subcellular distribution of glycosylated calreticulin measure a subset of total cellular calreticulin as against that revealed by immunolocalization with anti-calreticulin antibodies (Fig. 5) that detect both forms of this molecule. Thus, the earlier reports of primary ER localization for calreticulin in unheated cells do not conflict with our finding of a wider distribution of glycosylated calreticulin in heated cells and its dynamic redistribution during recovery from acute heat stress (Fig. 4). Furthermore, our finding of the presence of several sets of proteins that cross-react with the anti-calreticulin antibodies (Fig. 2A) outlines a complex pattern of calreticulin redistribution within cells.

Calreticulin is characterized by its ubiquitous tissue distribution and plays an important role in Ca^{2+} sequestration [Michalak et al., 1992]. Recently, calreticulin has also been shown to have chaperone functions with preferential binding to proteins including nascent glycoproteins [Nigam et al., 1994; Wada et al., 1995; McDonnell, et al., 1996]. The role of calreticulin during heat stress, however, is not known. It may participate in calcium homeostasis and/or protein chaperone activities, particularly in cell fractions where its concentration increases after heat shock, e.g., cytosol and the nucleus-rich fraction (Fig. 4). Interactions between P-SGs and HSP70 during acute heat stress appear unlikely, based on the immunofluorescence data in Figure 5. This inference is further supported by co-immunoprecipitation experiments using calreticulin antibodies that showed no significant association between P-SGs and HSP70 [Jethmalani et al., manuscript in preparation].

Subcellular localization and the redistribution of classical HSPs after heat stress is characteristic for each HSP family member and reflects, in part, its physiological function and/or protein targeting sequences [Lindquist and Craig, 1988; Weber, 1992; Hendrick and Hartl, 1993]. The same principles apply to stress glycoproteins; GP50 reportedly contains a C-terminal RDEL sequence, a known ER retention signal [Wang, 1992] and appeared in the microsomal fraction. Similarly, the presence of a C-terminal KDEL sequence in calreticulin molecule is a characteristic to most ER proteins [Michalak et al., 1992], even though KDEL sequence recognition is not the only mechanism for ER retention [Dorner et al., 1990]. The primary amino acid sequence of calreticulin also includes putative nuclear targeting signals [Opas et al., 1991; Michalak et al., 1992] and may explain its presence in the nucleus (Fig. 4). The presence of calreticulin in the lysosomal fraction may be explained by a recent report that shows calreticulin as a major chaperone for a lysosomal heme protein, in this case, myeloperoxidase in neutrophils [Nauseef et al., 1995].

The appearance of stress glycoproteins after heat shock in various subcellular fractions may occur by several mechanisms. For example, the attachment of cleavable amino-terminal presequences or other signal peptides can direct the import of non-mitochondrial proteins into mitochondria [Hurt and Van Loon, 1986; Skerjanc 1990]. By analogy, it may be possible that heat stress mediates the attachment of mitochondrial import sequences or nuclear retention signal to stress glycoproteins to cause their import and retention within mitochondria and nuclei. Alternatively, stress glycoproteins could exist in different isoforms within different subcellular compartments [Dedhar, 1994]. This has been shown for non-glycosylated calreticulin [Treves et al., 1992; Liu et al., 1993]. Following heat-stress, these may redistribute ubiquitously, as reported for HSP68, 70 and their various isoforms in all subcellular fractions [Napolitano et al., 1987]. Translocation of stress glycoproteins

may also be accomplished through the help of cytosolic chaperones, such as HSP70 [Skerjanc 1990; Hachiya et al., 1995]. Finally, heat stress may cause the opening of protein-conducting channels [Simon and Blobel, 1992], allowing the entry of stress glycoproteins into almost every subcellular compartment, or a heat-induced increase in membrane fluidity [Laszlo, 1992] may alter the recognition of peptide signals by receptors on membranes of different organelles with secondary translocation of stress glycoproteins.

The function of stress glycoproteins in various cell fractions may be linked to facilitated protein renaturation and protein stabilization by blocking irreversible transition states [Henle et al., 1995]. The binding of glycosylated stress proteins to misfolded, or incompletely folded proteins in the ER [Marquardt and Helenius, 1992] or to HSPs in other subcellular organelles can also modify their retention or translocation from that organelle. For example, the subcellular distribution of calreticulin was altered when calreticulin was bound to integrin [Dedhar, 1994; Coppolino et al., 1995].

In summary, this study shows that stress glycoproteins were present in all subcellular compartment. Their localization and redistribution profiles are unique and distinct for each stress glycoprotein and do not resemble that of HSP70. The ubiquitous distribution of stress glycoproteins may reflect a broadly functional role. Future studies will need to address the functional interaction of stress glycoproteins with both HSPs and other proteins in various subcellular fractions, and how such interactions affect the cellular heat resistance phenotype.

ACKNOWLEDGMENTS

The excellent technical assistance of Mr. Greg T. Nolen is greatly appreciated.

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