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Calreticulin Associates With Stress Proteins: Implications for Chaperone Function During Heat Stress

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Abstract Acute heat stress leads to the glycosylation of a ''prompt'' stress glycoprotein, P-SG67/64, identified as calreticulin. In the present study, we used immunoprecipitation to investigate the interactions of P-SG/calreticulin with other proteins during cellular recovery from heat stress. In heat-stressed CHO and M21 cells, both glycosylated and unglycosylated P-SGs interact with HSP90, GRP94, GRP78, and the other prompt stress glycoprotein, P-SG50, in an ATP-independent manner. Specificity of HSP-P-SG interactions was determined by chemical cross-linking with the homo-bifunctional agent DSP (3,3′-dithiobis[succinimidyl propionate]). Characterization of the cross-linked complexes involving calreticulin and heat shock proteins (HSPs) showed an average mass of 400–600 kDa by gel filtration chromatography. Overall, the consistent association of glycosylated and unglycosylated calreticulin with P-SG50 and unglycosylated HSPs suggests that P-SG/calreticulin is an active member of the cast of glycone/aglycone chaperones that cooperate to achieve cellular recovery from acute heat stress. J. Cell. Biochem. 69:30–43, 1998.

Key words: hyperthermia; calreticulin; chaperone complexes; prompt glycosylation

Environmental stresses, including heat stress, cause cellular injury and are commonly associated with increased expression of heat shock proteins (HSPs) [Gething and Sambrook, 1992; Hendrick and Hartl, 1993]. Heat stress also induces thermotolerance development [Henle, 1987; Henle et al., 1990; Hendrick and Hartl, 1993], linked to elevated levels of HSPs [Weber, 1992]. In addition to elevated HSP synthesis, thermotolerant cells exhibit a parallel increase in glycosylation of specific proteins [Henle et al., 1988, 1993, 1995, 1997].

Two types of heat-induced protein glycosylation phenomena are associated with the cellular stress response: (1) the "classical" or "late" response that is primarily associated with glycosylation of GP50 and GP62 and parallels the expression of thermotolerance and accumulation of HSPs [Henle et al., 1988, 1994, 1995, 1997], and (2) the "prompt" glycosylation response that occurs immediately during the heat shock and is associated with the selective glycosylation of "prompt" stress glycoproteins (P-SGs), P-SG67 and P-SG64 [Jethmalani and Henle, 1994; Henle et al., 1993, 1995, 1997]. GP50 is a glycosylated variant of the J6 gene product [Henle et al., 1994], whereas GP62 is a partial homologue of HSP70 [Jethmalani and Henle, 1997a,b]. Both P-SG67 and P-SG64 have been identified as calreticulin [Jethmalani et al., 1994; Jethmalani and Henle, 1994; Henle et al., 1997].

The functional role of P-SG67/64 during heat stress remains unknown. Recently, subcellular localization of P-SG67 by differential centrifugation and imunolocalization showed that P-SG67 was present in all subcellular fractions in CHO cells, both before and after stress [Jethmalani et al., 1997], possibly reflecting a multifunctional role for calreticulin in normal and heat-injured cells. A chaperone function for calreticulin has been supported by numerous reports [Dedhar, 1994; Nigam et al., 1994; Coppolino et al., 1995; Nauseef et al., 1995; Wada et al., 1995; Hebert et al., 1995; Meldolesi et al., 1996; Wiuff and Houen, 1996; Van Leeuwen and Kearse, 1996; Krause and Michalak, 1997], although the function of the glycosylated

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calreticulin in the form of a P-SG remains unclear. The present study was designed to determine the interactions of P-SG/calreticulin with other proteins, particularly HSPs; these observations may support a chaperone function for calreticulin during recovery from acute heat stress. Our results indicate that P-SG/calreticulin specifically associates with several members of the HSP family and with the other stress glycoprotein, P-SG50. This finding supports the concept that P-SG/calreticulin functions in a protein chaperone complex along with HSPs and stress glycoproteins during cellular recovery from acute heat stress.

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). Rat-1 and M21 cells were grown in DMEM medium with 10% FBS (Hyclone, Logan, UT), as reported elsewhere [Henle et al., 1997]. Cells were subcultured twice weekly to maintain cultures in exponential growth. D-[2-^{3}H]mannose (specific activity, 23 Ci/mmol) and ^{35}S-Trans label (cat. no. 51006, 70% L-methionine, [^{35}S] and ~15% L-cysteine, [^{35}S]; specific activity, 1,000 Ci/mmol) were purchased from ICN Radiochemicals (Irvine CA); [α-^{32}P]dCTP was obtained from NEN Research Products (Boston, MA). For immunoprecipitation and Western blotting, polyclonal rabbit anti-calreticulin antibody (Affinity BioReagents, Inc., Neshanic Station, NJ), monoclonal rat anti-HSP90 and anti-GRP94 antibodies (StressGen Biotechnologies Corp., Victoria, BC, Canada), and polyclonal rabbit anti-GP50/J6 antibody (gift from Dr. S.Y. Wang, SUNY, Albany, NY) were used. Secondary goat anti-rabbit and anti-rat antibodies linked to alkaline phosphatase were obtained from BioRad Laboratories (Hercules, CA) and Sigma (St. Louis, MO), respectively. Protein A-sepharose, protein G-sepharose, and Sepharose CL-6B were obtained from Sigma. Protein concentration was measured as described elsewhere [Henle et al., 1997; Jethmalani and Henle 1997a].

Heat Treatments and Labeling of Proteins

For heat treatments, cells were horizontally submersed in a precision-controlled ($<\pm 0.05°$), well-circulated water bath, as described elsewhere [Henle et al., 1993, 1997]. Cells were labeled with either 40 µCi/ml of ^{35}S-Trans label or 50 µCi/ml of D-[2-^{3}H]mannose, as previously described [Jethmalani and Henle, 1998]. Control cells were labeled for 30 min at 37°C; for "prompt" heat stress-induced glycosylation, cells were heat shocked for 30 min at 45°C, during which time they were simultaneously labeled [Henle et al., 1993]. In pulse-chase experiments ("cold chase"), cells were incubated at 37°C in label-free fresh medium for an additional 0, 2, 6, or 24 h.

Immunoprecipitation Studies

Cellular proteins were solubilized in buffer A, containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, and 1 µg/ml pepstatin; immunoprecipitation was performed as previously described [Jethmalani and Henle, 1998]. Briefly, solubilized proteins were preabsorbed with protein A-agarose or protein G-sepharose beads, supernatants were incubated overnight at 4°C with the respective antibodies: anti-GP50, anti-calreticulin (5 µl each) or anti-HSP90, anti-GRP94 (10 µl each) antibodies [Jethmalani and Henle, 1998]. On the next day, protein A-agarose/protein G-sepharose beads were added, and samples were incubated with gentle rotation for another 2 h at 4°C [Jethmalani and Henle, 1998]. Thereafter, beads were washed, and bound proteins were eluted under reducing conditions (60 µl of Laemmli's buffer) with heating for 10 min at 110°C [Jethmalani and Henle, 1998]. Residual beads were pelleted by centrifugation, and clear supernatants were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Although overnight incubations may increase nonspecific coprecipitations that could cause an increase in background, we used these conditions for optimal immunoprecipitation of glycosylated calreticulin (D-[2-^{3}H]mannose-labeled P-SGs). This same protocol was followed in all other experiments so that valid comparisons could be made.

Chemical Cross-Linking With DSP and ATP-Dependence of the Protein Interactions

Cells were labeled with ^{35}S-Trans label and lysed in buffer B, which contained 0.5% Nonidet P-40, 0.2% digitonin, and 0.23 mM PMSF in phosphate buffered saline (PBS, pH 8.0; lysis buffer) in the presence or absence of the cross-linker dithiobis (succinimidyl propionate; DSP; Pierce, Rockford, IL), as described elsewhere [Jethmalani and Henle, 1998]. The cross-linking reactions were terminated by the addi-

tion of 50 mM glycine [Jethmalani and Henle, 1998]. Thereafter, supernatant fractions were precleared, and immunoprecipitation was performed, as described above. In a separate experimental series, we characterized the effects of permanently unfolded proteins on interactions between P-SG/calreticulin and other stress proteins via immunoprecipitation of P-SG/calreticulin in the presence of reduced carboxymethylated α-lactalbumin (RCMLA; Sigma; 10 μM, 30 min). The ATP dependence of protein interactions was examined in another experimental series. In this series, bound proteins were eluted from beads after immunoprecipitation with buffer A, which contained 2.5 mM ATP, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ for 30 min at 4°C [Jethmalani and Henle, 1998]. In parallel experiments, 5 U of apyrase (Sigma) were added to solubilized protein lysates to deplete endogenous ATP [Jethmalani and Henle, 1998]. Beads were pelleted and washed twice with buffer A, and bound proteins were eluted with Laemmli's buffer, as described above.

Gel Filtration Chromatography

Protein complexes formed within intact cells among P-SG, P-SG50, and HSPs were characterized further by gel filtration chromatography [Jethmalani and Henle, 1998]. Both CHO and M21 cells were labeled with 40 μCi/ml of ^{35}S-Trans label and were washed five times. Proteins were solubilized with 1% Nonidet P-40 in buffer C (50 mM Tris, pH 7.4, 100 mM NaCl, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 1 h at 4°C. Samples were then diluted with buffer C to reduce Nonidet P-40 concentration to 0.1% and centrifuged at 13,000*g* for 15 min. The resulting supernatant (1 ml) was loaded onto a Sepharose CL-6B column (30 × 1 cm), and proteins were eluted with buffer C containing 0.1% Nonidet P-40, as described elsewhere [Jethmalani and Henle, 1998]. Each fraction collected was assayed for radioactivity and analyzed by SDS-PAGE. Fraction 10 demonstrated the simultaneous presence of P-SG/calreticulin, P-SG50, and various HSPs, suggesting the isolation of the intact chaperone complex in this fraction.

Electrophoresis, Electroblotting, and Western Blotting

SDS-PAGE and electroblotting of proteins to PVDF membranes was performed, as described elsewhere [Henle et al., 1997; Jethmalani and Henle, 1997a]. Membranes were exposed to Kodak X-Omat films and subjected to Western blotting [Henle et al., 1997; Jethmalani and Henle, 1997a]. The Amplified alkaline phosphatase detection kit (Bio-Rad, Richmond, CA) was used to visualize the antigen–antibody complex [Jethmalani and Henle, 1997a].

Indirect Immunofluorescence Staining

The intracellular distribution of P-SG64 and HSP70 was determined by using specific antibodies, as described elsewhere [Jethmalani et al., 1997]. The subcellular distribution of HSP70 is well characterized in the literature both before and after heat stress and was included as a positive control [Lindquist and Craig, 1988; Welch et al., 1991; Welch, 1992; Hattori et al., 1993]. M21 cells were grown on glass coverslips (22 × 22 mm) in 35-mm culture dishes. Cells were subjected to "prompt" heat stress (45°C, 30 min) but without radiolabeling [Jethmalani et al., 1997]. After rinsing with cold PBS, cells were permeabilized, fixed in cold methanol, and incubated with 10% normal goat serum in PBS for 1 h at room temperature to inhibit nonspecific antibody binding [Jethmalani et al., 1997]. For immunofluorescence staining, cells were incubated for 1 h at 37°C with the first antibody mixture, consisting of rabbit anti-calreticulin polyclonal antibody and anti-HSP70 antibody in 10% goat serum. Cells were washed extensively and incubated further for 1 h at 37°C with a second antibody mixture of fluorosceine isothiocyanate (FITC)-conjugated and rhodamine-conjugated antibodies [Jethmalani et al., 1997]. Isotypic controls were used for each primary antibody. Cells were washed extensively, and coverslips were mounted with anti-fade medium (Oncor, Gaithesberg, MD). Fluorescence was analyzed by a confocal argon-ion laser scanning microscope (LSM 300, Zeiss) with excitation wavelengths of 488 nm and 520 nm. Photomicrographs were taken with a conventional fluorescence microscope (Olympus Model BH-2, Tokyo, Japan) equipped with an automatic exposure device.

RESULTS AND DISCUSSION

Interactions of P-SGs With Classical HSPs

Figure 1 shows the association of ^{35}S-labeled and D-[2-^{3}H]mannose-labeled (glycosylated) P-SG/calreticulin with other labeled proteins,

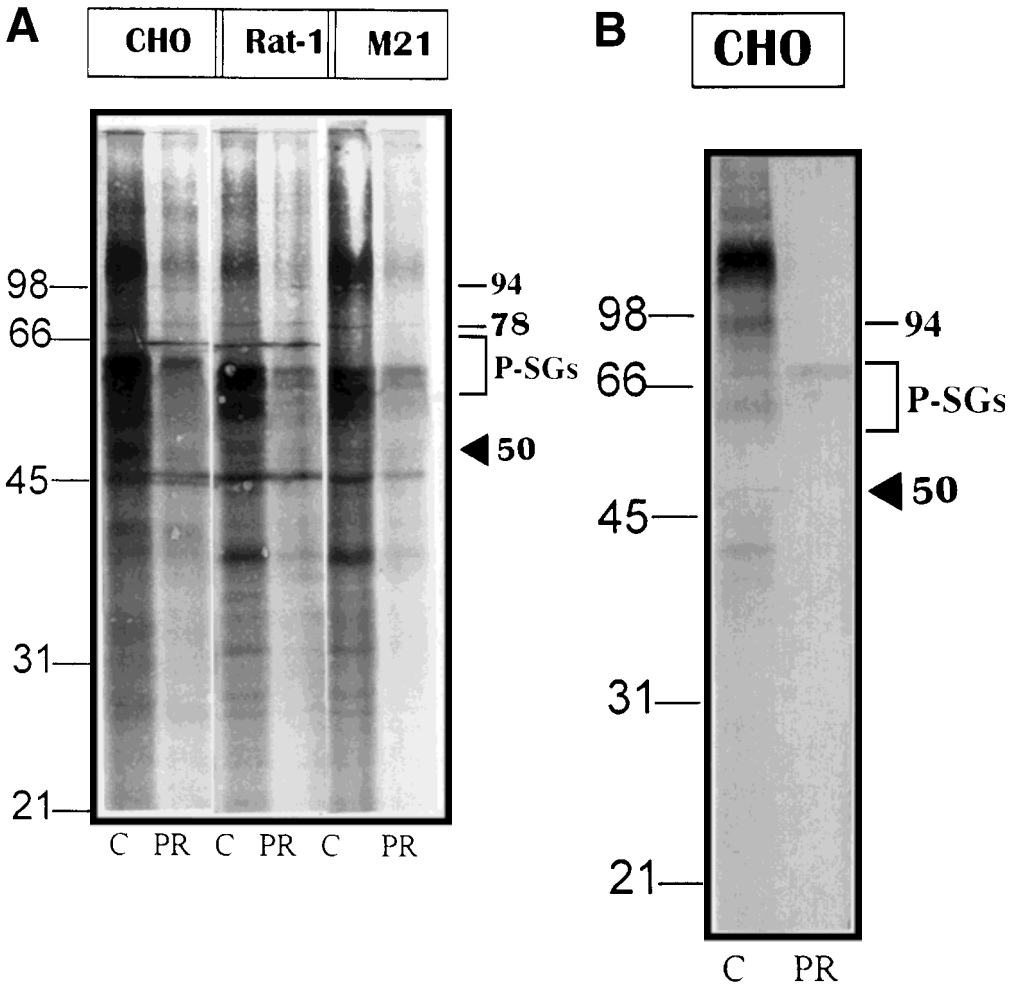

Fig. 1. Associations of newly synthesized proteins with P-SG/calreticulin. Cells were heat stressed at 45°C, labeled with either [^{35}S]-Trans label or D-[2-^{3}H]mannose, and proteins were solubilized (see Materials and Methods). Immunoprecipitation was performed with anti-calreticulin antibody. Bound proteins were eluted, separated by SDS-PAGE, and electroblotted to PVDF membranes. The autoradiograms indicate the standard molecular weight markers on the left side. On the right side, the positions of major coprecipitating proteins are indicated by their molecular mass in kilodaltons. P-SGs collectively refer to non-glycosylated calreticulin and the glycosylated P-SG67/P-SG64. **A:** ^{35}S-labeled proteins eluted under reducing conditions. **B:** Interactions of glycosylated P-SG/calreticulin with other glycoproteins. D-[2-^{3}H]mannose-labeled cells were used in experiments shown in B. C, control unstressed cells; PR, "prompt" heat-stressed cells.

including members of the HSP families. The autoradiogram of ^{35}S-labeled proteins in Figure 1A represents parallel experiments with three cell lines, CHO, Rat-1, and M21, and yielded qualitatively similar results. Immunoprecipitation with anti-calreticulin antibody, followed by elution of bound proteins under reducing conditions (Fig. 1A), showed that calreticulin specifically associates with a number of newly synthesized proteins with molecular masses of 94, 78, and 50 kDa that were further identified by Western blotting as GRP94, GRP78, and GP50, respectively (see Fig. 2B). Figure 1A also shows that the molecular weight of the P-SGs ranged from 58 to 67 kDa, which is consistent with the presence of variably glycosylated forms of calreticulin [Jethmalani and Henle, 1994]. Interactions between calreticulin and stress proteins were characterized further in subsequent coprecipitation and cross-linking experiments (Figs. 2, 3).

The ^{35}S-labeled 50-kDa protein, operationally labeled as P-SG50, also showed increased glycosylation during "prompt" heat stress [Jethmalani et al., 1997] and is probably identical to GP50. GP50 is the most abundant stress glycoprotein in CHO cells but not in rat lines Rat-1 and M21 [Henle et al., 1997]. The equivalence

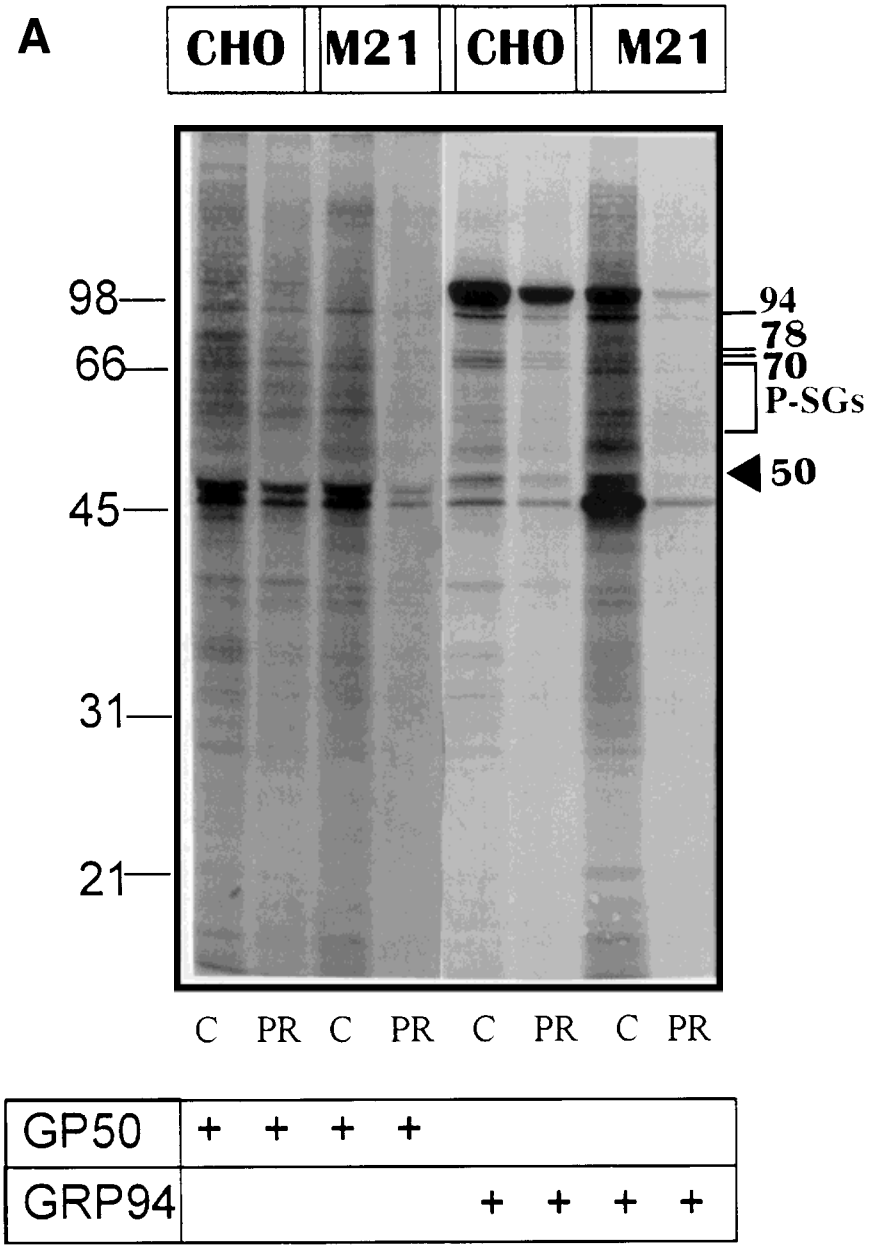

Fig. 2. Coprecipitation of P-SG/calreticulin with specific HSPs. Cells were labeled with [^{35}S]-Trans label as in Figure 1, and proteins were analyzed by SDS-PAGE. Proteins were electroblotted to a PVDF membrane that was exposed to film. The table at the bottom of the figure indicates the specific antibodies (either GP50 or GRP94) used for immunoprecipitation of the different samples. **A:** The autoradiogram indicating the SDS-PAGE pattern of the electroblotted ^{35}S-labeled proteins. **B:** Western blotting identifying specific coprecipitating proteins. In these experiments, the respective portions of the membrane were cut, and Western blotting was performed using GRP94 (top), calreticulin (middle), and GP50 (bottom) antibodies. Molecular weight markers are shown on the left side; the molecular masses of HSPs and P-SG50 are shown on the right side together with the location of the P-SGs (nonglycosylated calreticulin and the glycosylated P-SG67/P-SG64). C, control unstressed cells at 37°C; PR, "prompt" heat-stressed cells.

of P-SG50 and GP50 is supported by immunoprecipitation experiments with anti-GP50 antibody that show consistent coprecipitation of the various forms of P-SG/calreticulin (Fig. 2). The ~110-kDa protein that coprecipitates with GRP94 (Fig. 2A, especially lanes 5–8) may be HSP110 [Lee-Yoon et al., 1995], although this remains to be established. The identity of another protein with a molecular mass of ~120 kDa remains unknown.

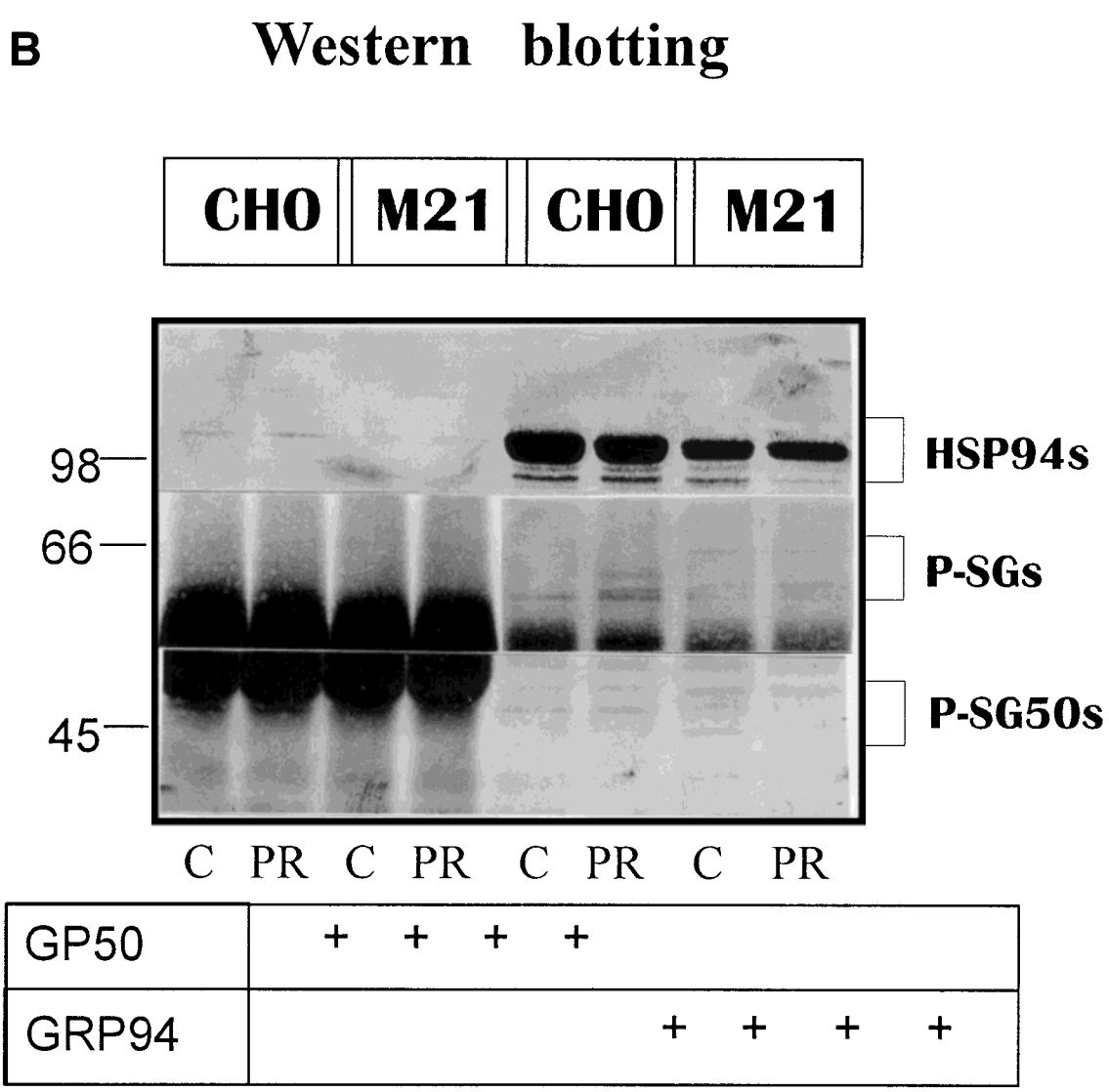

Figure 2. (Continued.)

Figure 1B shows interactions of D-[2-^{3}H]mannose-labeled (glycosylated) P-SG67/64/calreticulin with other glycoproteins, including GRP94, GRP78, and P-SG50 in CHO cells. The presence of interacting glycoproteins with P-SG/calreticulin was more noticeable in control cells (Fig. 1B, lane C) than in "prompt" heat-stressed cells. A major reason for this observation is the profound reduction in protein synthesis, accompanied by selective glycosylation of P-SG67 that occurs during "prompt" heat stress [Henle et al., 1993]. The identity of the coprecipitating proteins with molecular masses of ~110 and 120 kDa in control CHO cells has not been established. The results, however, support a significant interaction of variably glycosylated P-SGs with other glycoproteins.

Coprecipitation of P-SGs With Specific HSPs

The coprecipitation of GRP94, GRP78, and P-SG50 with the "prompt" stress glycoprotein calreticulin shown in Figure 1 may be specific or nonspecific. If specific, immunoprecipitation with antibodies toward specific stress proteins should also coprecipitate calreticulin. Figure 2A shows results of immunoprecipitation experiments with antibodies to GP50/P-SG50 (lanes 1–4) and GRP94 (lanes 5–8) obtained in parallel with CHO and M21 cells. Both antibodies accomplished the coprecipitation of calreticulin among other proteins, suggesting that the calreticulin–HSP interactions are specific. It is interesting to note that immunoprecipitation with anti-GRP94 antibodies also showed coprecipita-

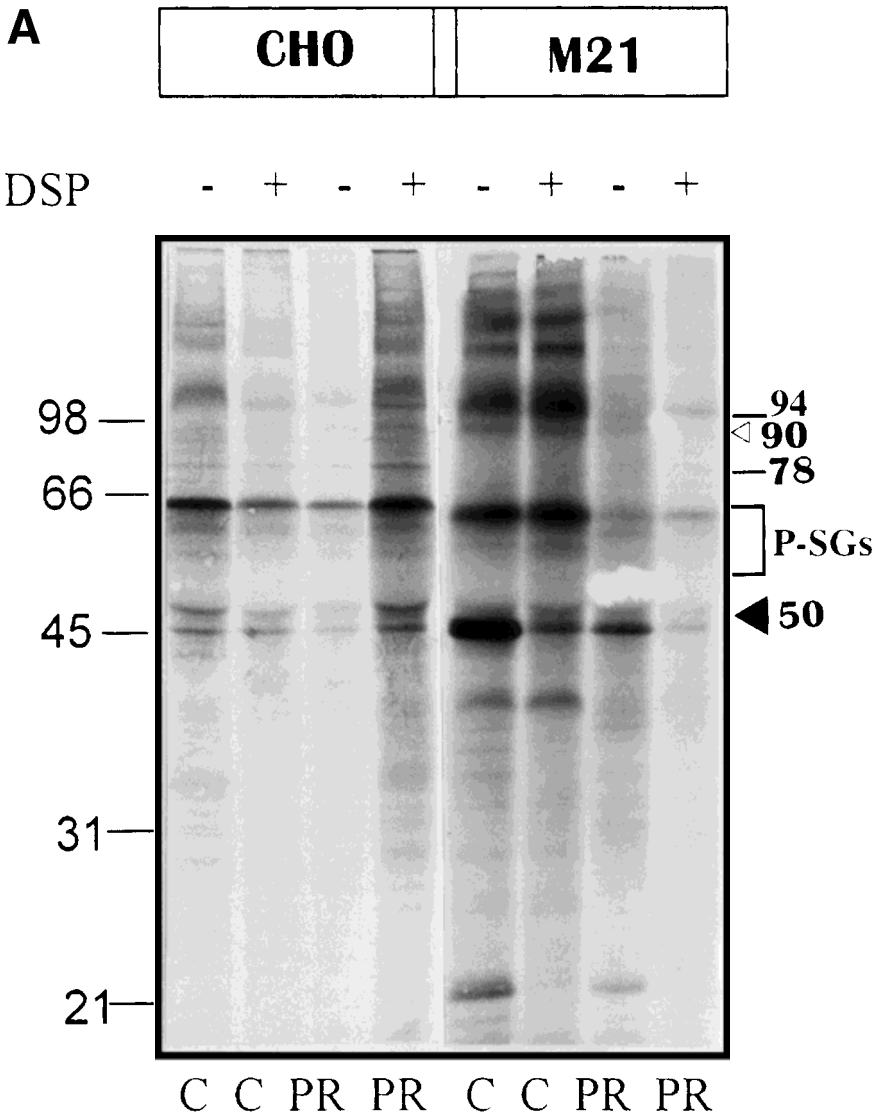

Fig. 3. Specificity and ATP-dependence of the protein interactions. **A:** Cells were metabolically labeled with [^{35}S]-Trans label and lysed in the presence (+) or absence (-) of the cross-linker DSP (see Materials and Methods). Proteins were immunoprecipitated with anti-calreticulin antibodies and analyzed by SDS-PAGE. **B:** Effect of ATP on the interaction of P-SG/calreticulin with other proteins. Immunoprecipitation was performed by using the anti-calreticulin antibody in the presence of either ATP that was added after immunoprecipitation to elute bound proteins or apyrase (5 U) that was added to deplete endogenous ATP. +Ab, immunoprecipitation using anti-calreticulin antibody; Apy, addition of apyrase; ATP, elution of bound proteins with ATP after immunoprecipitation.

tion of a 70-kDa protein, HSP70, especially in control M21 cells (Fig. 2A, lane 5). Interactions among HSP70, GRP94, and GP50 in thermotolerant cells also have been reported [Jethmalani and Henle, 1998]. Specific coprecipitating stress proteins in these experiments were further identified by Western blotting (Fig. 2B) as HSP94, P-SGs, and P-SG50, an observation that is consistent with that of previous reports [Henle et al., 1994; Jethmalani and Henle, 1994; Jethmalani and Henle, 1998]. The amount of GRP94 that coprecipitated with GP50 antibodies (Fig. 2A,B) was lower in M21 cells than in CHO cells, probably reflecting lower cellular concentrations of GP50 in M21 versus CHO cells [Henle et al., 1997]. The presence of multiple bands of P-SGs in the immunoprecipitate obtained with GRP94 antibodies (Fig. 2B, lanes 5–8) is consistent with variably glycosylated forms of calreticulin that associate with GP50 and GRP94. These results, therefore, further support the observations shown in Figure 1.

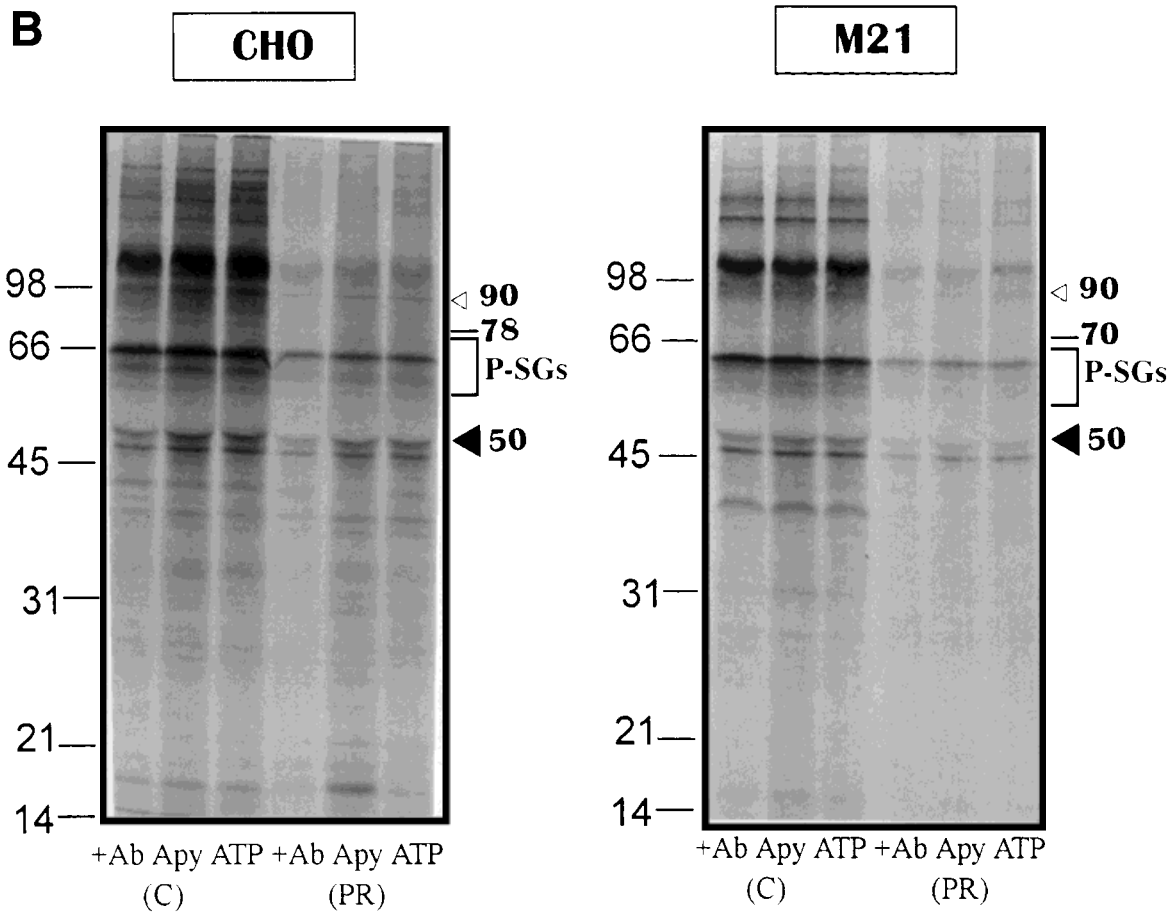

Figure 3. (Continued.)

Chemical Cross-Linking With DSP

Low-affinity proteins can be difficult to detect by immunoprecipitation techniques. However, such low-affinity binding can be easily studied when proteins are effectively trapped through the use of the homobifunctional cross-linker, DSP, which can be cleaved under reducing conditions. In our experiments using both CHO and M21 cells, immunoprecipitation with the anti-calreticulin antibody after protein cross-linking with DSP consistently resulted in the coprecipitation of proteins with molecular masses of 94, 90, 78, and 50 kDa (Fig. 3A, lanes 2, 4, 6, and 8). These proteins were identified as GRP94, GRP78, HSP90, and P-SG50 based on the Western blotting shown in Figure 2B. Although similar proteins coprecipitated in the presence and absence of DSP, the binding of specific proteins (molecular masses of 94, 90, 78, and 50 kDa) to P-SG/calreticulin in the presence of DSP was clearly evident against a lower background. This result implies that binding of calreticulin to these proteins is highly specific and that low-affinity binding perhaps plays a minor role in the formation of these intermolecular associations.

ATP Dependence of Protein Interactions

Protein interactions with stress proteins are often ATP dependent; this is true, for example, for the interaction of GP50 and HSPs in thermotolerant cells [Jethmalani and Henle, 1998]. To determine whether protein interactions involving P-SGs were similarly ATP dependent, we designed a series of experiments in which exogenous ATP was provided after immunoprecipitation for the potential release of proteins from immune complexes (Fig. 3B). Conversely, apyrase was added to replicate samples to block endogenous ATP use. The immunoprecipitation profiles were unaffected by the addition of either apyrase or exogenous ATP (Fig. 3B). This result indicates that interactions between P-SG67/64 and associated stress proteins are

ATP independent. This result is also in agreement with those of previous reports of calreticulin-protein interactions [Otteken and Moss, 1996] but differ from the ATP-dependent interactions that exist between GP50 and HSPs in thermotolerant cells [Jethmalani and Henle, 1998].

Kinetics of Associations Between P-SGs and HSPs

The half-life of P-SG67/64/calreticulin is in excess of 24 h [Jethmalani et al., 1997]. The time dependence of P-SG interactions with other stress proteins was studied in kinetic experiments in which immunoprecipitation of calreticulin was carried out after a "cold" chase of 0, 2, 6, and 24 h after metabolic labeling (Fig. 4). In spite of a slow turnover of certain constituent proteins, as shown by a gradual reduction in their labeling intensity (see 6-h and 24-h lanes for M21 cells and 24-h lane for CHO cells), our results, overall, showed no significant alterations in the immunoprecipitation profiles, which indicates that interactions between P-SGs and other stress proteins are stable, persisting up to 24 h.

In other experiments, we also examined how addition of a permanently unfolded protein, RCMLA, affected the interaction of P-SG67/64 with other stress proteins. Immunoprecipitation with calreticulin antibodies in the presence of RCMLA slightly increased (~20%) coprecipitation of the 50-, 94-, and 110-kDa proteins with P-SGs in heat-stressed CHO and M21 cells but did not change the qualitative pattern of associated proteins (data not shown). This observation shows that the presence of denatured/unfolded proteins has some effect on ternary or quarternary complex formation of P-SG67/64 with stress proteins. These results remain to be confirmed by using purified proteins within an in vitro system.

Characterization of Protein Complexes by Gel Filtration

These immunoprecipitation data support the specific interactions between P-SGs and certain stress proteins. Further characterization of specific complexes that form within intact cells was accomplished by gel filtration chromatography without prior immunoprecipitation. This chromatographic method separates acute heat-stress-induced protein complexes from noncomplexed proteins. The ^{35}S-labeled protein elution profiles for both CHO and M21 cells exhibited

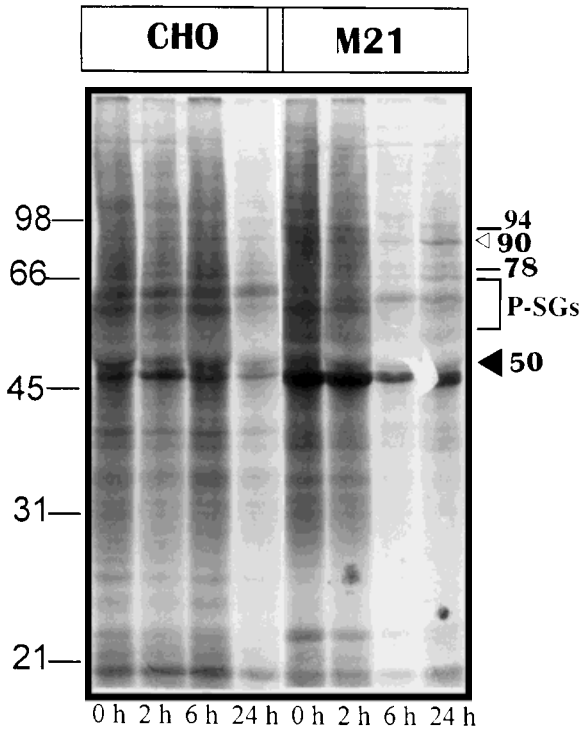

Fig. 4. Kinetics of associations between P-SG/calreticulin, P-SG50, and HSPs. Cells were labeled with [^{35}S]-Trans label, washed, and allowed to recover in fresh media without label at 37°C. Cells were harvested at the end of a "cold chase" 0–24 h after labeling. Immunoprecipitation was performed with anti-calreticulin antibody. Calreticulin is observed in variably glycosylated forms of P-SGs.

two peaks with the major peak eluting at fraction 10 (Fig. 5A). In a separate series of experiments to calibrate the column with standard proteins, we found that majority of thyroglobulin (669 kDa) and minor amounts of apoferritin (443 kDa) eluted in fraction 10. This result suggests that the ^{35}S-labeled protein complexes from intact CHO and M21 cells, which elute in fraction 10, have an apparent molecular mass of 400–600 kDa. SDS-PAGE analysis of fraction 10 further revealed the presence of the P-SG/calreticulin and the other proteins having molecular masses of 50, 78, 90 and 94 kDa (Fig. 5B) in both CHO and M21 cells. These latter proteins most likely represent the stress proteins, P-SG50, GRP78, HSP90, and GRP94 because immunoprecipitation of calreticulin from fraction 10, followed by sequential immunoprecipitation of P-SG50, GRP94, and HSP90 from the immunoprecipitate, demonstrated positive interactions between calreticulin and these particular stress proteins (data not shown). Overall, CHO cells exhibited a much higher yield of the protein complexes than did M21 cells (Fig.

5A), which is consistent with the relative amounts of protein visible by SDS-PAGE analysis (Fig. 5B). These results may reflect relative amounts of calreticulin in CHO versus M21 cells. It is noteworthy that the specific proteins found in the complexes separated by size exclusion chromatography are identical to those isolated by immunoprecipitation (Figs. 1–4). This result further supports the concept that all of these protein interactions are specific and do not represent an artifact of the immunoprecipitation technique.

Indirect Immunofluorescence Staining

Interactions of P-SG/calreticulin with other stress proteins during recovery from acute heat stress may require protein redistribution to specific subcellular organelles. Immunolocalization in intact M21 cells was used to assess the effects of acute heat stress on the redistribution of both calreticulin (green fluorescence) and HSP70 (red fluorescence) (Fig. 6). The distribution of HSP70 was used as a positive control because its subcellular localization has been extensively characterized [Lindquist and Craig, 1988; Welch et al., 1991; Welch, 1992; Hattori et al., 1993]. Unheated M21 cell populations are often quite heterogeneous in the amount of HSP70 labeling. This heterogeneity is shown in Figure 6A, where cells at the top show increased red fluorescence that obscures the distribution of green fluorescence. However, in the cells toward the bottom, intense green fluorescent staining representing variably glycosylated and aglycone forms of calreticulin is seen to localize mostly to cytoplasm, apparently concentrated in the endoplasmic reticulum (ER) network that extends toward the cellular periphery (Fig. 6A). These control cells also show punctate areas of green fluorescence in the nucleus, which is consistent with a highly localized distribution of calreticulin at this site. In these cells, HSP70 is nearly absent from the cytoplasm but is present in the nucleus with diffuse red fluorescence, suggesting relatively low concentrations of HSP70 at this site (Fig. 6A).

Acute heat stress (45°C, 30 min), followed by 1 h recovery, significantly changed the distribution patterns of both calreticulin and HSP70. The intense green fluorescence reflecting P-SG/calreticulin localization is now seen in lower concentrations in cytoplasm but mostly at perinuclear membranes that probably represent the ER network (Fig. 6B). The nucleus of these acute heat-stressed cells is characterized by a loss of the punctate green fluorescence that was visible in unheated control cells (Fig. 6A). Instead, these cells show reduced P-SG/calreticulin concentrations in the nucleus, as judged by the diffuse green fluorescence at this site (Fig. 6B). Heat stress resulted in the translocation of HSP70 mostly to the nucleus, particularly to nucleoli that exhibit intense red fluorescence. Concurrently, HSP70 is relatively less abundant in cytoplasm (Fig. 6B). Overall, there is no indication of colocalization between P-SG/calreticulin and HSP70 after heat stress (Fig. 6B), a result that is consistent with studies based on subcellular fractionation [Jethmalani et al., 1997].

In summary, the present study shows that glycosylated and unglycosylated P-SG/calreticulin associate with a discrete set of proteins that include members of the HSP family and P-SG50 (Figs. 1–5). The major components of the P-SG complex include HSP90, GRP94, GRP78, and P-SG50, which probably associate as ternary complexes (Fig. 5). These interactions are ATP independent, an observation that is consistent with that of previous reports [Otteken and Moss, 1996]. Similar associations of calreticulin with GRP78, GRP94, and the ER chaperone calnexin have been reported under nonstress conditions [Tatu and Helenius, 1997]. These interactions are specific, as supported by data from cross-linking with DSP (Fig. 3), and they appear to be stable for up to 24 h (Fig. 4). Overall, the similarity of protein interactions in both CHO and M21 cells supports a generalized function for the P-SG complexes.

Classical HSPs and stress glycoproteins localize and redistribute in a manner that is characteristic for each family member and partly reflects its physiological function [Lindquist and Craig, 1988; Weber, 1992; Hendrick and Hartl, 1993; Jethmalani et al., 1997]. For instance, both HSP70 and HSP90 are found mainly in cytoplasm under nonstress conditions and preferentially translocate to nuclei and nucleoli after heat stress, whereas GRP78 and GRP94 are mostly ER residents [Lindquist and Craig, 1988]. In contrast, stress glycoproteins such as GP50/P-SG50 and P-SG/calreticulin appear in all subcellular compartments before and after heat stress (Fig. 6) [Jethmalani et al., 1997]. Together, the data suggest that the function of P-SG/calreticulin is closely linked with that of HSP90, GRP94, GRP78, and P-SG50.

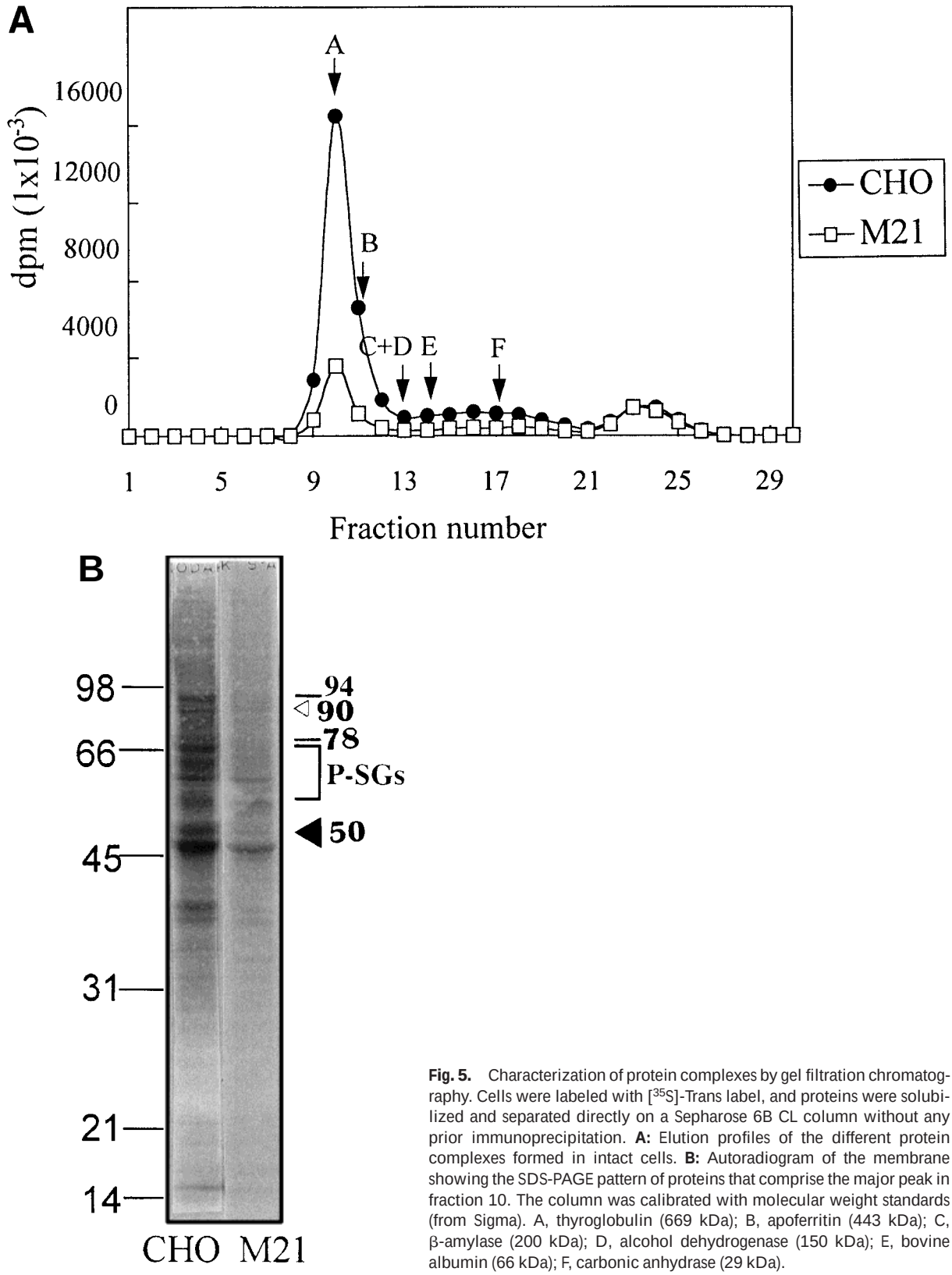

Fig. 5. Characterization of protein complexes by gel filtration chromatography. Cells were labeled with [^{35}S]-Trans label, and proteins were solubilized and separated directly on a Sepharose 6B CL column without any prior immunoprecipitation. **A:** Elution profiles of the different protein complexes formed in intact cells. **B:** Autoradiogram of the membrane showing the SDS-PAGE pattern of proteins that comprise the major peak in fraction 10. The column was calibrated with molecular weight standards (from Sigma). A, thyroglobulin (669 kDa); B, apoferritin (443 kDa); C, β-amylase (200 kDa); D, alcohol dehydrogenase (150 kDa); E, bovine albumin (66 kDa); F, carbonic anhydrase (29 kDa).

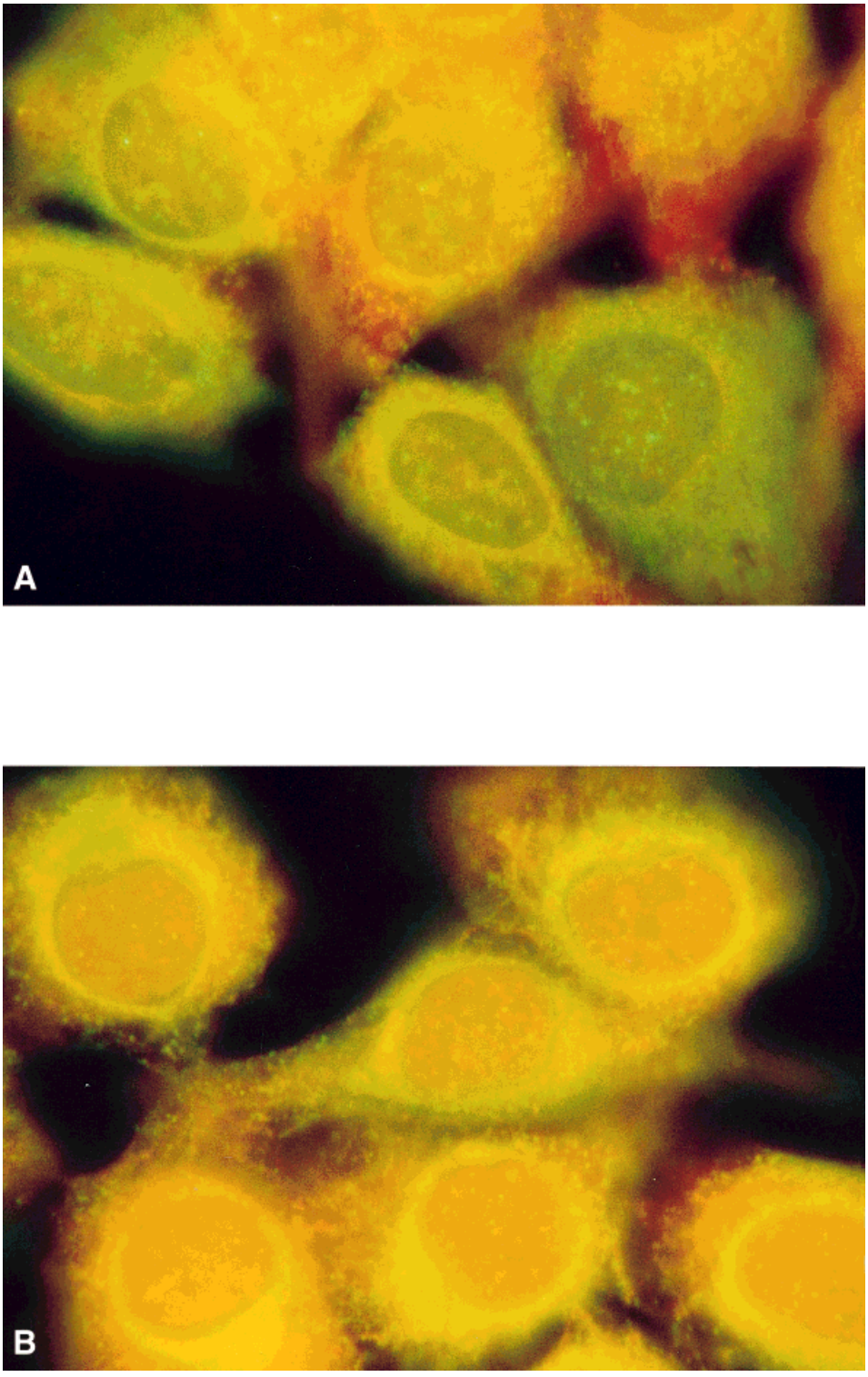

Fig. 6. Immunofluorescence labeling of calreticulin and HSP70 in M21 cells. Control unheated cells (**A**) and acute heat-stressed cells (45°C, 30 min with recovery for 1 h) (**B**) were fixed and processed for double-immunofluorescence staining. The primary antibodies used were a mixture of polyclonal rabbit anti-calreticulin and monoclonal mouse anti-HSP70 antibodies. These antibodies were detected with FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse antibodies (for details, see Materials and Methods). Exposure times for A and B were 75 s each.

Calreticulin is a multifunctional protein and appears to be present in different cellular compartments [Burns et al., 1994; Meldolesi et al., 1996]. For example, association of calreticulin with GRP78 mediates glycoprotein folding and prevents premature protein exit from the ER [Tatu and Helenius, 1997]. Calreticulin can also act as a molecular chaperone during biosynthesis of the lysosomal heme protein, myeloperoxidase [Nauseef et al., 1995]. Together with calnexin, calreticulin can form ternary complexes with the newly synthesized HIV envelope protein [Otteken and Moss, 1996]. Calreticulin also participates in the assembly of the ribonucleoprotein autoantigen, Ro/SS-A, the T-cell receptor antigen proteins [Cheng et al., 1996; Van Leeuwen and Krause, 1996]. In some cases, protein interactions are dependent on the activation state of the calreticulin-interacting protein as in the case of integrin $\alpha_2\beta_1$ [Dedhar, 1994; Coppolino et al., 1995]; in other cases, protein interactions are glycosylation dependent, e.g., in the association of calreticulin with the Glut 1 glucose transporter [Oliver et al., 1996]. The interaction of both a membrane-anchored and a soluble form of calreticulin with transferrin was dependent on glucose trimming of N-linked oligosaccharides [Wada et al., 1995]. Moreover, calreticulin demonstrates lectinlike properties when it copurifies with endomannosidase by its binding to N-linked oligosaccharide moieties or to glycosylated laminin [Spiro et al., 1996; McDonell et al., 1996]. However, the role of calreticulin in the cellular recovery from heat stress and the significance of its "prompt" glycosylation under acute heat stress remains unknown [Henle et al., 1993, 1997; Jethmalani et al., 1994]. Similarly, the functional role of both glycosylated and aglycone forms of calreticulin during cellular recovery from acute heat stress remains to be addressed in future studies.

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REFERENCES

Burns K, Atkinson EA, Bleackley RC, Michalak M (1994): Calreticulin: From Ca^{2+} binding to control of gene expression. Trends Cell Biol 4:152–154.

Cheng, S-T, Nguyen TQ, Yang Y-S, Capra JD, Sontheimer RD (1996): Calreticulin binds hYRNA and the 52-kDa polypeptide component of the Ro/SS-A ribonucleoprotein autoantigen. J Immunol 156:4484–4491.

Coppolino M, Leung-Hagesteijn C, Dedhar S, Wilkins J (1995): Inducible interaction of integrin $\alpha 2\beta 1$ with calreticulin. Dependence on the activation state of the integrin. J Biol Chem 270:23132–23138.

Dedhar S (1994): Novel functions for calreticulin: Interaction with integrins and modulation of gene expression? Trends Biochem Sci 19:269–271.

Gething M-J, Sambrook J (1992): Protein folding in the cell. Nature 355:33–45.

Hattori H, Kaneda T, Lokeshwar B, Laszlo A, Ohtsuka K (1993): A stress-inducible 40 kDa protein (hsp40): Purification by modified two-dimensional gel electrophoresis and co-localization with hsc70 (P73) in heat-shocked HeLa cells. J Cell Sci 104:629–638.

Hebert DN, Simons JF, Peterson JR, Helenius A (1995): Calnexin, calreticulin, and BiP/Kar2p in protein folding [Review]. Cold Spring Harbor Symp Quant Biol 60:405–415.

Hendrick JP, Hartl F-U (1993): Molecular chaperone functions of heat-shock proteins. Annu Rev Biochem 62:349–384.

Henle KJ (1987): Thermotolerance in cultured mammalian cells. In Henle KJ (ed): "Thermotolerance," vol. I. Boca Raton: CRC Press, pp 13–71.

Henle KJ, Norris JS, Nagle WA, Moss AJ (1988): Enhanced glycosylation of a 50 kD protein during thermotolerance development in CHO cells. Int J Radiat Biol 53:839–847.

Henle KJ, Lumpkin CK, Norris JS, Nolen GT (1990): Expression of thermotolerance following microinjection of poly(A)$^+$RNA isolated from thermotolerant CHO cells. Int J Hypertherm 6:1041–1051.

Henle KJ, Kaushal GP, Nagle WA, Nolen GT (1993): Prompt protein glycosylation during acute heat stress. Exp Cell Res 207:245–251.

Henle KJ, Wang SY, Nagle WA, Lumpkin CK (1994): Heat shock glycoprotein GP50: Product of the retinoic acid-inducible J6 gene. Exp Cell Res 210:185–191.

Henle KJ, Jethmalani SM, Nagle WA (1995): Heat stress-induced protein glycosylation in mammalian cells. Trends Glycosci Glycotechnol 7:191–204.

Henle KJ, Jethmalani SM, Li L, Li GC (1997): Protein glycosylation in a heat resistant rat fibroblast cell model overexpressing human HSP70. Biochem Biophys Res Commun 232:26–32.

Jethmalani SM, Henle KJ (1994): Prompt glycosylation of calreticulin is independent of Ca^{2+} homeostasis. Biochem Biophys Res Commun 205:780–787.

Jethmalani SM, Henle KJ (1997a): Partial homology of stress glycoproteins GP62 with HSP70. Exp Cell Res 232:8–16.

Jethmalani SM, Henle KJ (1998): Interaction of stress glycoprotein GP50 with classical heat shock proteins. Exp Cell Res (in press).

Jethmalani SM, Henle KJ (1998c): Intracellular distribution of stress glycoproteins in a heat resistant cell model expressing human HSP70. Biochem Biophys Res Commun 237:382–387.

Jethmalani SM, Henle KJ, Kaushal GP (1994): Heat shock-induced prompt glycosylation. Identification of P-SG67 as calreticulin. J Biol Chem 269:23603–23609.

Jethmalani SM, Henle KJ, Gazitt Y, Walker PD, Wang S-Y (1997): Intracellular distribution of heat-induced stress glycoproteins. J Cell Biochem 66:98–111.

Krause KH, Michalak M (1997): Calreticulin. Cell 88:439–443.

Lee-Yoon D, Easton D, Murawski M, Burd R, Subjeck JR (1995): Identification of a major subfamily of large hsp70-like proteins through the cloning of the mammalian 110-kDa heat shock protein. J Biol Chem 270:15725–15733.

Lindquist S, Craig EA (1988): The heat-shock proteins. Annu Rev Genet 22:631–677.

McDonell JM, Jones GE, White TK, Tanzer ML (1996): Calreticulin binding affinity for glycosylated laminin. J Biol Chem 271:7891–7894.

Meldolesi J, Krause KH, Michalak M (1996): Calreticulin—How many functions in how many cellular compartments. Cell Calcium 20:83–86.

Michalak M, Milner RE, Burns K, Opas M (1992): Calreticulin. Biochem J 285:681–692.

Nauseef WM, McCormick SJ, Clark RA (1995): Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. J Biol Chem 270:4741–4747.

Nigam SK, Goldberg AL, Ho S, Rohde MF, Bush, KT, Sherman MY (1994): A set of endoplasmic reticulum proteins possessing properties of molecular chaperones includes Ca^{2+}-binding proteins and members of the thioredoxin superfamily. J Biol Chem 269:1744–1749.

Oliver JD, Hresko RC, Mueckler M, High S (1996): The Glut 1 glucose transporter interacts with calnexin and calreticulin. J Biol Chem 271:13691–13696.

Otteken A, Moss B (1996): Calreticulin interacts with newly synthesized human immunodeficiency virus type 1 envelope glycoprotein, suggesting a chaperone function similar to that of calnexin. J Biol Chem 271:97–103.

Spiro RG, Zhu Q, Bhoyroo V, Soling H-D (1996): Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. J Biol Chem 271: 11588–11594.

Tatu U, Helenius A (1997): Interactions between newly synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. J Cell Biol 136:555–565.

Van Leeuwen JEM, Kearse KP (1996): The related molecular chaperones calnexin and calreticulin differentially associate with nascent T cell antigen receptor proteins within the endoplasmic reticulum. J Biol Chem 271: 25345–25349.

Wada I, Imai S, Kai M, Sakane F, Kanoh H (1995): Chaperone function of calreticulin when expressed in the endoplasmic reticulum as the membrane-anchored and soluble forms. J Biol Chem 270:20298–20304.

Weber LA (1992): Relationship of heat shock proteins and induced thermal resistance. Cell Prolif 25:101–113.

Welch WJ (1992): Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol Rev 72:1063–1081.

Welch WJ, Kang HS, Beckmann RP, Mizzen LA (1991): Response of mammalian cells to metabolic stress; changes in cell physiology and structure/function of stress proteins. Curr Topics Microbiol Immunol 167: 31–55.

Wiuff C, Houen G (1996): Cation-dependent interactions of calreticulin with denatured and native proteins. Acta Chem Scand 50:788–795.