We previously described a novel molecular chaperone (designated p88) that participates in the assembly of murine class I histocompatibility molecules (Degen, E., and Williams, D. B. (1991) J. Cell Biol. 112, 1099-1115). Our findings suggest that p88 may either promote proper assembly of class I molecules or retain them, probably within the endoplasmic reticulum (ER), until assembly of the ternary complex of heavy chain, β2m-microglobulin, and peptide ligand is complete. In this report, we compare p88 to calnexin, a calcium-binding 90-kDa phosphoprotein of the ER membrane (Wada, I., Rindress, D., Cameron, P. H., Ou, W.-J., Doherty, J.-J., II, Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) J. Biol. Chem. 266, 19599-19610). We show that p88 and calnexin share antigenic epitopes defined by a polyclonal anti-calnexin antiserum. Furthermore, both proteins were immunoprecipitated in association with an intracellularly retained variant of the class I H-2Kb molecule. Since p88 and calnexin were also indistinguishable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, were resistant to digestion with endoglycosidase H, and exhibited virtually identical patterns of peptide fragments following digestion with either V8 protease or trypsin, we conclude that p88 and calnexin represent the same protein. The identification of the p88 chaperone as a phosphorylated, calcium-binding protein of the ER membrane suggests possible means whereby its interaction with class I molecules may be regulated.

Class I molecules of the major histocompatibility complex are cell surface glycoproteins composed of a highly polymorphic transmembrane heavy chain associated with a polypeptide of limited variability termed β2m-microglobulin (β2m). 1

Peptide fragments arising from the degradation of endogenously synthesized self or foreign proteins bind to a site in the extracellular domain of the newly synthesized heavy chain and are transported to the cell surface. The class I-peptide complexes are surveyed by cytotoxic T-cells and, if the complex is recognized as foreign, the antigen presenting cell is destroyed (reviewed in Ref. 1).

Following synthesis, assembly of the class I heavy chain and β2m occurs rapidly in the endoplasmic reticulum (ER) with a t1/2 of ~2-5 min (2-4). Subsequent transport to the cell surface occurs at distinct rates (t1/2 = 15 to >300 min) depending on the particular heavy chain with export from the ER apparently being the rate-limiting step (5-7). Whether peptide binding precedes or follows the association of β2m with heavy chain is unknown. Also, the subcellular site where peptide ligand binds to newly synthesized class I molecules is not clearly established. Evidence obtained from studies with drugs that disrupt normal exocytic transport (8, 9) or with cells that are defective in delivering peptide ligands to class I molecules (10-12), implicate the ER to cis-Golgi portion of the exocytic pathway. Assembly of the ternary complex of heavy chain, β2m, and peptide is clearly important both in the acquisition of a stable, mature conformation of the class I molecule as well as in rendering it competent for rapid and efficient intracellular transport. Cell lines deficient in the synthesis of β2m accumulate conformationally aberrant free heavy chains that in most cases are not transported (4, 13-15). In cells deficient in generating or delivering peptide ligands to class I molecules, the heavy chain-β2m heterodimers form but are unstable at 37 °C and are only slowly transported (16-19).

We previously reported the identification of an 88-kDa chaperone protein (p88) that participates in the assembly of murine class I molecules (2). In normal cells, p88 binds rapidly and quantitatively to newly synthesized heavy chains and remains associated during and after heavy chain assembly with β2m. Subsequently, p88 dissociates at a rate closely resembling the distinctive rate of exchange of the particular class I molecule is transported from the ER to the Golgi apparatus. Recently, we examined the relationship between p88 interaction and assembly of class I molecules in more detail. In cells lacking β2m, p88 remains permanently associated with free heavy chains throughout their lifetime in the ER. In cells that are deficient in delivering peptide ligands to class I molecules, p88 exhibits prolonged association with heavy chain-β2m heterodimers, slowly dissociating in parallel with the slow transport of the peptide-free class I molecule. 2 These findings led us to speculate that p88 functions as a retention molecule that interacts with incompletely assembled forms of

The abbreviations used are: β2m, β2m-microglobulin; endo H, endoglycosidase H; ER, endoplasmic reticulum; FCyS, fetal calf serum; SSRRα and SSRRβ, α and β subunits of the signal sequence receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

1 Degen, E., Cohen-Doyle, M. F., and Williams, D. B. (1992) J. Exp. Med., in press.

2 Degen, E., Howard, D. M., and Williams, D. B. (1992) J. Biol. Chem. 267, 11,099-1115.

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** Recipient of a University of Toronto Open Fellowship.

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class I molecules, retaining them (and perhaps stabilizing them) intracellularly until formation of the ternary complex of heavy chain, $\beta_m$, and peptide is complete.

In this report, we compare p88 to a 90-kDa phosphoprotein that was previously identified as a membrane protein of the ER with four regions of sequence identity to calreticulin, the major calcium binding protein within ER lumina (20). This integral membrane phosphoprotein, which was termed calnexin, binds calcium and is part of a complex with three other proteins including the $\alpha$ and $\beta$ subunits of the signal sequence receptor (SSR), and an additional 25-kDa glycoprotein of unknown function. Our findings indicate that p88 and calnexin are identical.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Purified Proteins**—The R1E-K$^+$-3 cell line was prepared by transfecting $\beta_m$-deficient R1E ribosome thymoma cells with a K$^+$ heavy chain gene lacking exon 4 (which encodes the $\alpha$ extracellular domain; Ref. 19). This cell line was a gift of Dr. Richard Flavel, Yale University. Cells were grown at 37°C in a 5% CO$_2$/air atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Anti-8 rabbit antisera was raised against the peptide encoded by exon 8 of the K$^+$ gene and is capable of recognizing the cytoplasmic tail of the K$^+$ (or other K$^+$) heavy chain whether or not $\beta_m$ is associated (21). This antisera was provided by Dr. Brian Barber, University of Toronto. Rabbit anti-serum directed against intact ER of canine pancreas (anti-ER) was obtained from Dr. Daniel Louvard, Institut Pasteur, Paris. For anti-calnexin, a portion of the canine calnexin cDNA encoding amino acids 8–286 was fused to the C terminus of the glutathione S-transferase gene using the PGE2-2T plasmid (Pharmacia LKB Biotec Inc.). The fusion protein was purified using glutathione-agarose (Sigma), and the calnexin fragment released by thrombin digestion was used to raise antisera in rabbits. Calnexin and pgp35 (35 kDa) were purified as described previously with the final step involving excision of the polypeptides corresponding to calnexin (90 kDa) and pgp35 (35 kDa) from an SDS-PAGE gel followed by electrophoretic elution (20).

**Metabolic Radio labeling and Immunoprecipitation**—R1E-K$^+$-3 cells (7.5 × 10$^6$) were washed and then preincubated at 37°C for 30 min in methionine-free RPMI 1640 containing 9 mM Hepes (Met-free 1640). They were subsequently resuspended in 5 ml of labeling medium (4 parts Met-free 1640, 1 part RPMI 1640, 2% FCS, and 6% dialyzed FCS) containing 0.5 mCi of [35S]Met (Amersham Corp., Oak Brook, IL) and were incubated for 5 h at 37°C. At this time an additional 0.125 mCi of [35S]Met in 5 ml of Met-free 1640 was added and the incubation continued for an additional 30 min. Subsequent steps were developed to preserve the complex between class I molecules and p88 and were performed at 4°C. Radiolabeled cells were washed twice with PBS, pH 8, and lysed in 1 ml of PBS, pH 8, containing 0.8% CHAPS, 10 mM iodoacetamide, 0.23 mM phenylmethylsulfonyl fluoride, and 1% aprotinin (Sigma) for 5 min. The lysate was centrifuged at 100,000 × g for 15 min. Immune complexes were harvested by shaking for 1 h with 90 μl of a 33% suspension of Affi-Gel-protein A (Bio-Rad) in 0.6% CHAPS, PBS, pH 8. Agarose beads were washed 8 times with CHAPS/PBS before the antigen was eluted by heating at 70°C in 90 μl of 10 mM Tris, 1% SDS, pH 7.3. All samples were precipitated with 10 μl of acetonitrile overnight at –20°C. Precipitates were subjected to digestion with endoglycosidase H (endo H) and analysis by SDS-PAGE (10% gel) and fluorography as described previously (2).

**Cleveland Mapping**—With V9 Protease—R1E-K$^+$-3 cells (2.0 × 10$^7$) were radiolabeled for 3 h with 2 mCi of [35S]Met in 20 ml of labeling medium and subsequently pulsed with 0.5 mCi of [35S]Met for an additional 30 min. Cells were divided into 4 equal aliquots and each was lysed in 750 μl of lysis buffer. Two aliquots were treated with 1 μl of anti-8 for isolation of p88 and two with 2.5 μl of anti-ER antisera for isolation of calnexin. Immune complexes were recovered as above except that the Affi-Gel-protein A was eluted with 80 μl of SDS-PAGE sample buffer containing 40 mM dithiothreitol. Samples were subjected to SDS-PAGE (7.5% gel) and the gel was dried on Bio Gel Wrap (Bio Design, Inc.) and protein bands were visualized by autoradiography. The ~90-kDa protein bands were excised from the gel, rehydrated in 0.5 ml of 0.1% SDS, boiled for 5 min, and shaken overnight at room temperature. This first eluate was removed and the procedure repeated two more times to maximize peptide recovery. Eluates from the duplicate samples were pooled and analyzed by Cleveland mapping. An additional sample of p88, obtained by cross-linking p88 to the K$^+$-3 molecule, was prepared as described previously (2) and also subjected to Cleveland mapping.

For Cleveland mapping (22), all samples were dried under vacuum and dissolved in 90 μl of 0.1% SDS, 10 mM iodoacetamide, 6.8% glycerol and 1 mM EDTA. The pH of each sample was adjusted to 6.8–7.0 prior to boiling and loading on a 17.5% SDS-PAGE gel. Each sample was overlaid with 15 μl of a 50 μg/ml solution of S. aureus V8 endoproteinase in Tris-glycine-EDTA buffer. Electrophoresis was carried out at 110 V until the samples were concentrated at the stacking/separating gel interface. Protease digestion was allowed to continue for 45 min with the current turned off before SDS-PAGE was run in the usual manner. The gels were fixed in 10% trichloroacetic acid and then analyzed by fluorography using 1 M salicylate.

**Tryptic Peptide Mapping**—R1E-K$^+$-3 cells were radiolabeled as described above, except that the K$^+$ was preincubated with [35S]Met (Tran3'S-label, ICN) and the wash and labeling media lacked methionine and cysteine. The immunosolubilization of p88 and calnexin and their subsequent recovery from SDS-PAGE gels were also performed as detailed in the preceding section. The purified, radiolabeled proteins were reduced, alkylated, and digested with trypsin using the method of Swiedler et al. (23). Digests were fractionated on a Beckman UltraspHERE C$_{18}$ reverse phase column (4.6 × 250 mm) in conjunction with a Beckman System Gold HPLC system. The column was equilibrated in 0.1% trifluoroacetic acid and was run at a flow rate of 1 ml/min. Peptides were eluted using a 90-min linear gradient of 0–37% acetonitrile in 0.1% trifluoroacetic acid followed by 15 min at 50% acetonitrile in 0.1% trifluoroacetic acid. Half-minute fractions were collected and radioactivity was quantitated by liquid scintillation counting.

**Western Blotting**—R1E-K$^+$-3 p88 Complexes—R1E-K$^+$-3 cells (2 × 10$^7$) were washed twice with PBS, pH 8, and lysed in 5 ml of CHAPS lysis buffer. Following centrifugation, the lysate was preclarified and divided into three equal parts. Two samples were incubated with 7.5 μl of anti-8 and the third with 7.5 μl of rabbit preimmune serum. Immune complexes were recovered with Affi-Gel-protein A and subjected to SDS-PAGE (7.5% gel). Subsequent transfer of proteins to nitrocellulose and blocking of non-specific sites with 5% non-fat dry milk in TBS was carried out as described by Towbin et al. (24). The nitrocellulose sheet was cut such that one portion contained the preimmune control and an anti-8 precipitate and the other portion contained only an anti-8 precipitate. The former portion was incubated with a 1:25 dilution of anti-calnexin and the latter with a 1:25 dilution of anti-p88 serum for 2 h at 22°C. The strips were rinsed and then each was incubated with 125I-protein A (Amersham Corp., 3.0 × 10$^6$ cpm/ml) for 1 h at 22°C. Dried strips were exposed to x-ray film using an intensifying screen.

**RESULTS**

The murine $\beta_m$-deficient cells used in this study (R1E-K$^+$-3) express a variant of the class I H-2K$^b$ heavy chain lacking the $\alpha_2$ extracellular domain. K$^+$-3 heavy chains are conformationally aberrant, and the bulk of these molecules accumulate intracellularly, stably associated with p88 throughout their lifetime (15).

Previously, we used a chemical cross-linking approach to demonstrate interaction between class I heavy chains and p88 (2). We now show that by solubilizing cells in CHAPS detergent and using gentle immunoprecipitation conditions, the complex can be isolated by co-immunoprecipitation with anti-heavy chain antisera. As shown in Fig. 1 (anti-8 lanes), two major bands were observed corresponding to p88 and the 35-kDa K$^+$-3 heavy chain. As expected, the K$^+$-3 heavy chain which bears immature N-linked oligosaccharides was sensi-
antigen competition experiment was performed. The anti-ER antiserum was preincubated either with purified calnexin (20) or with an unrelated phosphoglycoprotein (pgp35) and then tested for its ability to immunoprecipitate proteins from an R1E-K\textsuperscript{b}-3 cell lysate. As shown in Fig. 1, preadsorption of the antiserum with calnexin completely blocked the isolation of the 88-kDa protein. Furthermore, co-precipitation of the 35-kDa presumptive K\textsuperscript{b}-3 heavy chain was also blocked. In contrast, preadsorption with pgp35 had no effect on the isolation of either the 88- or 35-kDa proteins. This evidence demonstrates that the 88-kDa protein isolated with the anti-ER antiserum is indeed calnexin. Furthermore, because the isolation of calnexin and the presumptive K\textsuperscript{b}-3 heavy chain were both blocked in this experiment, these findings suggest that calnexin may be equivalent to p88. It is noteworthy that substantially less K\textsuperscript{b}-3 heavy chains were precipitated in these experiments with the anti-ER antiserum than with the anti-8 antiserum. This is due to the fact that antibodies against calnexin in the anti-ER antiserum are of relatively low titre resulting in the isolation of a fraction of the total calnexin pool. This pool appears to be in substantial excess over K\textsuperscript{b}-3 heavy chains and hence only a small portion of the heavy chains could be recovered. In other experiments, using several-fold larger amounts of antiserum, a corresponding increase in the intensities of the calnexin and K\textsuperscript{b}-3 bands was observed (data not shown).

To assess the relationship between p88 and calnexin more directly, we used Cleveland mapping to compare p88 precipitated with the anti-heavy chain antiserum to calnexin isolated with the anti-ER antiserum. Since our previous studies characterizing p88 were based on chemical cross-linking, we prepared an additional authentic p88 sample by first cross-linking it to K\textsuperscript{b}-3, isolating the complex, breaking the cross-link, and recovering p88. All three 88-kDa samples were subjected to digestion with V8-protease and analysis by SDS-PAGE (Fig. 2A). Comparison of the three digestion patterns revealed that they were identical. Three major digestion products with molecular masses of 41, 33, and 22 kDa were observed; six minor fragments of 49, 44, 36, 31, 29, and 26 kDa were also detected. As a second independent test, we compared tryptic peptides derived from immunoprecipitated p88 and calnexin by reverse phase HPLC (Fig. 2B). The peptide maps were virtually identical although, upon close inspection, some minor variations were apparent. A peak-by-peak comparison revealed that most of these variations were quantitative in nature; a particular fragment was clearly present in both samples but simply differed in amount. In our experience, this is typical of results obtained with replicate digests of identical samples. In only a single case was a qualitative difference observed: in the region of fractions 27–30. The reason for this difference is unknown but given the identical pattern of fragments in the rest of the map, it is most likely a technical artifact possibly resulting from incomplete digestion. The results obtained from the two peptide mapping approaches strongly suggest that p88 and calnexin are identical.

Additional support for this suggestion was obtained by probing immunoprecipitated K\textsuperscript{b}-3-p88 complexes in a Western blot using an independently derived anti-calnexin antiserum. Unlabeled R1E-K\textsuperscript{b}-3 lysates were incubated either with preimmune serum or with anti-heavy chain serum, and then immune complexes were subjected to SDS-PAGE and transferred to nitrocellulose. When probed with the anti-calnexin antiserum (Fig. 3A), only a single band was detected that was specific to the anti-heavy chain immunoprecipitate.
Fig. 2. Comparison of p88 and calnexin by peptide mapping. Panel A, Cleveland mapping using V8 protease. [35S]Met-labeled samples of p88 and calnexin were prepared and subsequently digested with V8 protease as detailed under "Experimental Procedures." Lane 1, p88 isolated by chemically cross-linking the K'3 heavy chain to p88. Lane 2, p88 isolated by co-immunoprecipitation with anti-8 heavy chain antiserum. Lane 3, calnexin isolated by immunoprecipitation with anti-ER antiserum. The mobilities of the nine distinct peptide fragments present in the digests of each sample are indicated. Panel B, tryptic peptide mapping by reverse phase HPLC. Samples of p88 and calnexin, radiolabeled with [35S]Met and [35S]Cys, were digested with trypsin and analyzed using a C18 reverse phase HPLC column as described under "Experimental Procedures."

It co-migrated precisely with a metabolically labeled standard of p88. Furthermore, probing an anti-heavy chain immunoprecipitate with preimmune serum failed to detect this species (Fig. 3B). These findings independently support the suggestion that p88 and calnexin are identical.

Fig. 3. Recognition of p88 by anti-calnexin antiserum. Panel A, immune complexes obtained by treating unlabeled CHAPS lysates of K'-3 cells either with preimmune or anti-8 sera were transferred to nitrocellulose and probed in a Western blot with anti-calnexin antiserum (see "Experimental Procedures"). As a standard for the mobilities of p88 and K'-3, a small portion of an anti-8 immunoprecipitate from [35S]Met-labeled cells was included (standard lane). Panel B, a duplicate anti-8 immunoprecipitate from K'-3 cells was probed in a Western blot with preimmune antiserum. Mobilities of molecular weight standards are indicated.

In Fig. 3A, the anti-calnexin antiserum reacted strongly with an additional species of 60 kDa present in both the preimmune and anti-heavy chain immunoprecipitates. This protein has not been identified but it is of interest to note that calreticulin, which shares four regions of sequence identity with calnexin, migrates as a 60-kDa species on SDS-PAGE (26). It is possible that some calreticulin, which is an abundant ER luminal protein (27, 28), was adsorbed nonspecifically from the R1E-K'-3 cell lysates to protein A-agarose during the immunoprecipitation procedures.

DISCUSSION

In this report we have shown that p88 and calnexin share antigenic epitopes defined by a polyclonal anti-calnexin antiserum and that each protein can be independently immunoprecipitated in association with a transport-deficient variant of the class I H-2Kb molecule. Furthermore, comparison of p88 isolated by co-immunoprecipitation with anti-class I heavy chain antiserum with calnexin isolated with anti-ER antiserum, revealed that the two proteins exhibit identical mobility on SDS-PAGE, the same resistance to endo H digestion, and virtually identical patterns of peptide fragments following digestion with either V8 protease or trypsin. Based on this experimental evidence, we conclude that p88 and calnexin are the same protein.

Calnexin was previously identified and purified on the basis of its phosphorylation by an ER-associated casein kinase II-like activity (20). Sequencing of cDNA clones revealed four regions of similarity with calreticulin, a major calcium-binding protein of ER lumina. Evaluation of 45Ca binding revealed that calnexin shares with calreticulin the ability to bind
calcium. However, unlike calreticulin, calnexin is a membrane protein with a predicted type I topology. Three additional membrane proteins co-purified with calnexin from canine pancreatic ER, two of which (SSRα and SSRβ) have been implicated in an association with newly translocated secretory proteins (29-31). Immunofluorescence studies with antibodies specific for each of the four proteins of the complex revealed reticular and nuclear membrane staining similar to the typical ER pattern observed with the marker anti-ER antisera (20).

We determined previously that the anti-ER antisera of Louvard et al. (25) clearly co-precipitates calnexin with the 35-kDa SSRα glycoprotein in Triton X-100-solubilized canine microsomes (data not shown). Since the truncated class I K0-3 molecule used in the present experiments also has a molecular mass of 35 kDa, we were unable to evaluate the presence of SSRα. However, additional studies performed with a cell line expressing a full-length class I heavy chain (45 kDa) revealed that a 35-kDa glycoprotein could be immunoprecipitated in association with calnexin and heavy chain using either anti-ER or anti-class I heavy chain antisera (data not shown). Studies to determine if this glycoprotein is indeed SSRα are in progress.

Recently, the human homologue of calnexin has been identified and shown to associate with incompletely assembled subunits of not only class I molecules but of the T-cell receptor and membrane-bound immunoglobulin as well (32). Thus calnexin apparently functions as a general chaperone, although its action may be restricted only to the assembly of normally retained proteins along the exocytic pathway. These findings suggest that in addition to being influenced by folding and subunit assembly events, BiP interaction with its substrates may be regulated by variations in calcium levels within the ER. It clearly will be of considerable interest to determine whether the binding of calnexin to its substrates is similarly influenced by calcium as well as whether the related luminal ER protein, calreticulin, also serves a chaperone function.

Acknowledgments—We thank Richard Flavell for providing the RIE-K-5 cell line and Drs. Brian Barber and Daniel Louvard for their generous gifts of antisera. We also thank Pam Cameron for expert technical assistance and Dr. D. Y. Thomas for his interest and support.

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