Brief Definitive Report

Calnexin Retains Unassembled Major Histocompatibility Complex Class I Free Heavy Chains in the Endoplasmic Reticulum

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Summary

The assembly of major histocompatibility complex (MHC) class I molecules involves the association of heavy (H) chain with β_2-microglobulin (β_2m) and peptide. Unassembled class I H chains do not exit the endoplasmic reticulum (ER) and this is exemplified by the β_2m-deficient human melanoma FO-1 where free class I H chains are unable to complete assembly. In pulse chase experiments involving FO-1 cells, unassembled free class I H chains were shown to be stably associated with calnexin (IP90/p88), a 90-kD integral membrane molecular chaperone of the ER. To establish a role for calnexin in mediating this retention, we transfected FO-1 cells with a cytoplasmic tail deletion mutant of calnexin. Since the cytoplasmic tail contains the ER retention motif, these mutant calnexin molecules leave the ER and progress to the cell surface. In these stable transfectants of FO-1, free class I H chains also exited the ER and trafficked to the cell surface with calnexin, thus establishing a role for calnexin in the quality control of MHC class I assembly through mediating the ER retention of incompletely assembled class I H chains.

In the endoplasmic reticulum, MHC class I H chains associate with β_2 microglobulin (β_2m) and peptide to form the complete class I complex (1). Such fully assembled complexes exit the endoplasmic reticulum (ER) and rapidly traffic to the cell surface. Studies of abnormal cell lines such as the β_2m-deficient lymphoma, Daudi (2, 3) and the peptide transporter associated with antigen processing (TAP)–mutant RMA-S cells (4, 5) have indicated that in the absence of one of the components of the MHC class I complex (either β_2m or peptide), free H chains are predominantly retained in the ER and are not expressed on the cell surface. This ER retention of unassembled class I molecules serves as an example of quality control since it assures that only fully assembled complexes can reach the cell surface. Whereas the mechanisms of the quality control machinery operative in the ER are not fully understood, it has been hypothesized that molecular chaperones play a major role in the stabilization and retention of unassembled class I molecules in the ER until the assembly of peptide and β_2m with the H chain is completed. Recently, an ER resident integral membrane molecular chaperone termed calnexin (IP90, p88) was identified (6–8). Calnexin associates transiently with a multitude of proteins shortly after synthesis (9). Similarly, in normal class I assembly, calnexin associates transiently with free class I H chains soon after their synthesis. Calnexin then dissociates from completely assembled class I complexes that progress to the cell surface (6). In contrast, in cell lines like Daudi and RMA-S, which are deficient in one component of the MHC class I complex, there is prolonged association of incompletely assembled class I H chains with calnexin (10). Thus, previous data suggest that calnexin might function to retain free H chains in the ER until assembly with β_2m is complete and/or promote the assembly of the ternary class I complex.

The melanoma cell line FO-1 is an example of a cell line where there is incomplete assembly of MHC class I resulting in the absence of cell surface class I expression. In FO-1 cells, a lesion in the β_2m gene results in an absence of β_2m gene transcription and complete lack of β_2m protein synthesis (11). Thus, although there is abundant class I H chain protein produced, it cannot assemble with β_2m. Therefore, the unassembled H chains are stably retained in the ER and do not progress to the cell surface. In this study, we assess the role of calnexin in the ER retention of free class I H chains in FO-1 cells. We show that calnexin associates stably with the unassembled free H chain and determines its intracellular location, thus accounting for the retention of free class I H chains in the ER.

Materials and Methods

Cells and Antibodies. The FO-1 melanoma cell line was obtained from Dr. Soldano Ferrone (New York Medical College, Valhalla, NY) (11). The SK-MEL-28 melanoma cell line was obtained from

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Dr. Lloyd Old (Memorial Sloan-Kettering Cancer Center, New York) (12). The cell lines were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1% nonessential amino acids, and antibiotics. The mAbs used were as follows: negative control Ab P3 (13), anti-human β_2m Ab BBM1 (14), and W6/32 (15), recognizing HLA-A, B, and C locus products complexed to β_2m, were obtained from the American Type Culture Collection (Rockville, MD). mAb HC10, specific for MHC class I free H chain was obtained from Hidde Ploegh (Massachusetts Institute of Technology, Cambridge, MA) (16). mAb AF8 specific for human calnexin was generated in our laboratory (8).

Metabolic Labeling, Immunoprecipitations, and Gel Electrophoresis. FO-1 cells (2×10^6 cells/ml) were labeled for 4 h with 0.2 mCi of [^{35}S]methionine (EXPRE^{35}S^{35}S; DuPont, New England Nuclear, Boston, MA) in methionine-free, cysteine-free RPMI 1640/5% dialyzed FCS medium. At the end of the labeling period, cells were washed three times in cold PBS and lysed by solubilization in 0.3% CHAPS lysis buffer (140 mM NaCl, 50 mM Tris, pH. 7.6, 7.5 mM iodacetamide, 1 mM PMSF). For pulse chase experiments, 5×10^6 cells were labeled for 5 min with 0.3 mCi of [^{35}S]methionine in methionine-free, cysteine-free labeling medium. After the short pulse, cells were washed three times in cold PBS, and incubated in complete RPMI medium at 37°C. At each chase time point, aliquots were washed and lysed as described above. Labeled cells were immunoprecipitated as described (17) and analyzed by SDS-PAGE under reducing conditions.

For endoglycosidase H digestions, immunoprecipitates were boiled in 40 μl of 1% SDS/0.14 M 2-ME. After cooling, the mixture was diluted in 360 μl of 0.15 M acetate buffer, pH 5.5, containing 1 mM PMSF. 5 μl of Endo H (1 U/ml; Genzyme Corp., Cambridge, MA) was incubated with half the above solution for 16 h at 37°C, while the other half was mock incubated. After digestion, 10 μg BSA was added as carrier and samples were recovered by TCA precipitation. Protein pellets were taken up in sample buffer and analyzed by SDS-PAGE.

Immunofluorescence Labeling. Immunofluorescence localization was done as described previously (18). Briefly, FO-1 cells grown on glass coverslips were fixed in 3.7% paraformaldehyde in PBS for 10 min, permeabilized in cold methanol, and incubated with either the HC10 or AF8 mAbs, followed by FITC-conjugated goat anti-mouse Abs.

DNA Transfection and Selection of Wild-type and Mutant Calnexin Expressing FO-1 Cells. FO-1 cells were cotransfected with 20 μg of either full-length or cytoplasmic tail-deleted (cyt-deleted) calnexin in Ap^{r}M8 (18) plus 1 μg of the G418 resistance encoding vector pSRαneo (19) by calcium phosphate precipitation. Transfected cells were selected for G418 resistance by their ability to grow in DMEM supplemented with 10% FCS in the presence of 1.5 mg/ml G418 (Geneticin; Sigma Chemical Co., St. Louis, MO). Isolated G418 resistant colonies were cloned using cloning cylinders and grown in separate wells. To identify those that expressed the truncated cyt-deleted calnexin, a panel of clones was metabolically labeled with [^{35}S]methionine, lysed in TBS/2% Triton X-100 lysis buffer, and calnexin was immunoprecipitated using anti-calnexin mAb, AF8.

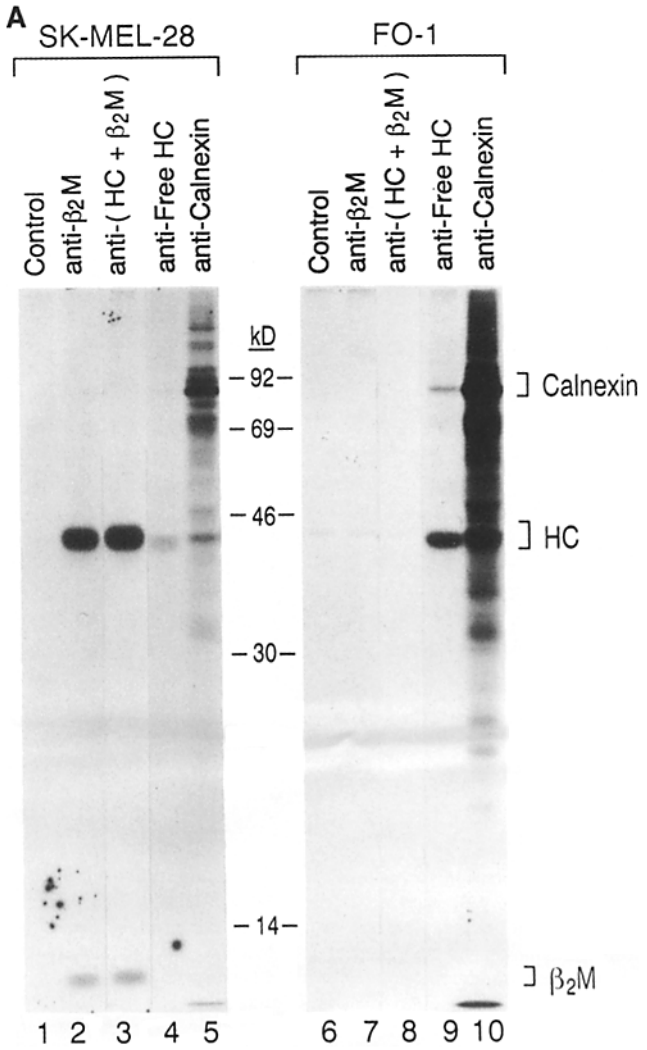
Flow Cytometry. Cells were stained with primary mAbs P3, HC10, AF8, and W6/32, followed by goat anti-mouse Ig conjugated to PE (Tago Inc., Burlingame, CA) for 1 h on ice in staining buffer (PBS containing 5% fetal bovine serum and 0.1% sodium azide). After washing in staining buffer, cells were analyzed on a FACSORT® flow cytometer (Becton Dickinson & Co., Mountain View, CA). Dead cells were excluded by staining with propidium iodide.

Results and Discussion

Class I Chains Do Not Assemble into Mature Class I Molecules in the β_2m-deficient Melanoma FO-1. The human malignant melanoma SK-MEL-28 is an example of normal MHC class I assembly involving the association of H chain, β_2m, and peptide, leading to high levels of cell surface expression of class I molecules (12). SK-MEL-28 cells were metabolically labeled with [^{35}S]methionine for 4 h, lysed, and immunoprecipitated with anti-class I complex mAbs (Fig. 1 *A*). As expected, Abs against β_2m (mAb BBM-1) (Fig. 1 *A*, lane *2*) and assembled class I (mAb W6/32) (lane *3*) coimmunoprecipitated the class I H chain (~43 kD) complexed with β_2m (12 kD). Ab specific for the free class I H chain (mAb HC10) coimmunoprecipitated low levels of free H chain (~43 kD). In addition, immunoprecipitates obtained using calnexin-specific mAb, AF8 (Fig. 1 *A*, lane *5*), immunoprecipitated calnexin, a 90-kD ER resident protein, and confirmed the coimmunoprecipitation of a number of proteins associated with it as previously described (9).

In contrast, in the β_2m-deficient FO-1 cell line, although there is efficient H chain synthesis, no further assembly of these class I molecules into β_2m-associated mature class I molecules occurred. 4-h metabolic labeling of FO-1 cells followed by cell lysis and immunoprecipitation analysis revealed the presence of free class I H chains (Fig. 1 *A*, lane *9*) but the absence of β_2m (lane 7) and completed class I complexes (lanes 7 and *8*). Moreover, calnexin was readily coimmunoprecipitated with free H chains (Fig. 1 *A*, lane *9*) in these β_2m negative cells, suggesting a possible role for calnexin, itself an ER resident protein, in the ER retention or retrieval of unassembled free H chains. Free H chains do not exit the ER and progress to the medial Golgi apparatus as shown by the sensitivity of free H chains to the enzyme Endo H in metabolically labeled FO-1 cells even after a 2-h chase in unlabeled medium (Fig. 1 *B*). Furthermore, immunofluorescence staining of FO-1 cells using Ab against free H chain (*HC10*) or Ab against calnexin (*AF8*) reveals a characteristic reticular ER staining pattern consistent with the localization of free H chains in the ER (Fig. 1 *C*).

Free Class I H Chains in FO-1 Appear Retained in Association with Calnexin. The coimmunoprecipitation of free H chain in FO-1 with calnexin suggested that calnexin may be functioning to retain the unassembled H chain in the ER. To examine this possibility further, pulse chase analysis was performed to examine the fate of these free class I H chains over time. After a short 5-min pulse labeling of FO-1 cells with [^{35}S]methionine, in the anti-calnexin immunoprecipitates, there were numerous proteins associated with calnexin, including class I H chain (Fig. 2, lane *4*). It is striking that after a 1-h chase in media lacking ^{35}S label, the majority of the transiently associated proteins dissociated from calnexin, whereas class I H chains were stably associated with calnexin in the FO-1 cell line over this time period (Fig. 2, lane *8*). We further confirmed the association of calnexin with class I H chains by performing two-dimensional gel electrophoresis (nonequilibrium pH gel electrophoresis-SDS/PAGE) on these coimmunoprecipitating proteins. Mixtures of anti-



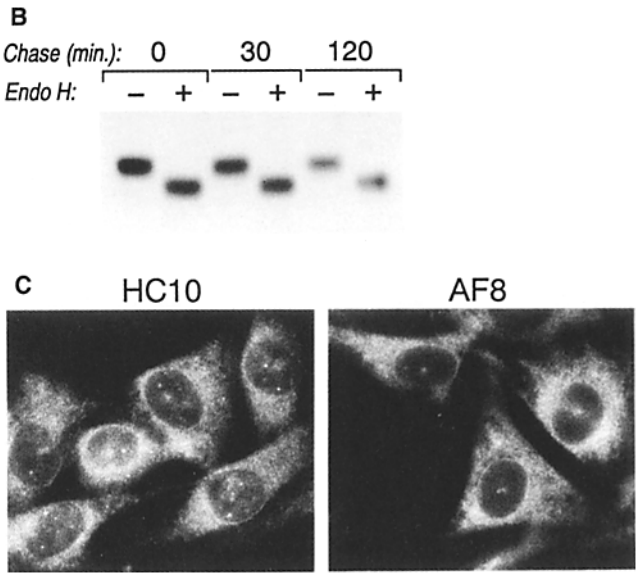


Figure 1. (*A*) Free class I H chains do not assemble into mature class I molecules in the β_2m-deficient melanoma FO-1. SK-MEL-28 cells (normal β_2m expression) (lanes *1–5*) and FO-1 cells (β_2m deficient) (lanes *6–10*) were radiolabeled with [^{35}S]methionine for 4 h. Cells were lysed in 0.3% CHAPS lysis buffer and equal aliquots of the lysates were immunoprecipitated with control mAb P3 (lanes *1* and *6*); anti-β_2m mAb BBM-1 (lanes *2* and *7*); anti-class I mAb W6/32, which recognizes H chains complexed to β_2m (lanes *3* and *8*); anti-free H chain mAb HC10 (lanes *4* and *9*); and anti-calnexin mAb AF8 (lanes *5* and *10*). The samples were analyzed on 11% SDS-PAGE under reducing conditions. (*Right*) Positions of calnexin, H chain (*HC*), and β_2m. (*B*) Free class I H chains do not exit the ER in the β_2m-deficient melanoma FO-1. FO-1 cells were pulsed with [^{35}S]methionine for 15 min and either lysed immediately (chase *0*) or chased in unlabeled medium for 30 and 120 min. At each chase time point, free class I H chains were immunoprecipitated from cell lysates. Immunoprecipitates were divided into two aliquots, incubated in the presence or absence of endo H and analyzed by 10.5% SDS-PAGE. (*C*) Free class I H chains show a reticular staining pattern characteristic of the ER. Immunofluorescence staining of FO-1 cells was done using mAb HC10 to detect class I free H chains and mAb AF8 to detect ER resident marker calnexin. This was followed by FITC-conjugated goat anti-mouse Ig as secondary Ab.

calnexin and anti-free H chain immunoprecipitates displayed indistinguishable mobilities (data not shown). These data further correlate the ER retention of free H chain with calnexin.

Cell Surface Expression of Class I Free H Chain in FO-1 Cells Stably Transfected with a Non-ER Retained Form of Calnexin. Calnexin has a COOH-terminal amino acid motif (RKPRRE) that was shown to be responsible for its retention in the ER (18). Our previous work established that deletion of this motif by truncation of the cytoplasmic tail of calnexin (cyt-deleted calnexin) resulted in the exit of this form of calnexin from the ER. In those studies, truncated calnexin protein localized in the Golgi, in secondary lysosomes and on the cell surface in transiently transfected COS cells (18).

To assess the role of calnexin in the ER retention of free class I H chains in FO-1 cells, FO-1 cells were stably transfected with cyt-deleted calnexin. Positive clones were screened by metabolic labeling followed by immunoprecipitation with anti-calnexin mAb (Fig. 3). Both truncated (◀) and endogenous full-length calnexin (◀) were visualized in the transfected FO-1 (*CT3-2*) clone (Fig. 3, lane *4*) in contrast to the wild-type FO-1 cells which lacked the truncated calnexin species (lane *2*). Note that the levels of the truncated calnexin were lower than those of the endogenous calnexin, implying that the transfected protein would have to compete with an excess of endogenous calnexin in these stable transfectants.

We hypothesized that if calnexin mediates the retention of unassembled class I H chains in the ER, then transfection of cyt-deleted calnexin in FO-1 cells might redirect trafficking of proteins such as β_2m-free class I H chains out of the ER. To test this, the cell surface expression of β_2m-free class I H chains was compared on clones of FO-1 stably transfected with the cyt-deleted calnexin with that on control FO-1 cells transfected with full-length calnexin that is retained in the ER (9, 18). FACS® analysis revealed cell surface expression of both free class I H chains (Fig. 4 *b*) and calnexin (Fig. 4 *d*) on the cyt-deleted calnexin transfectants.

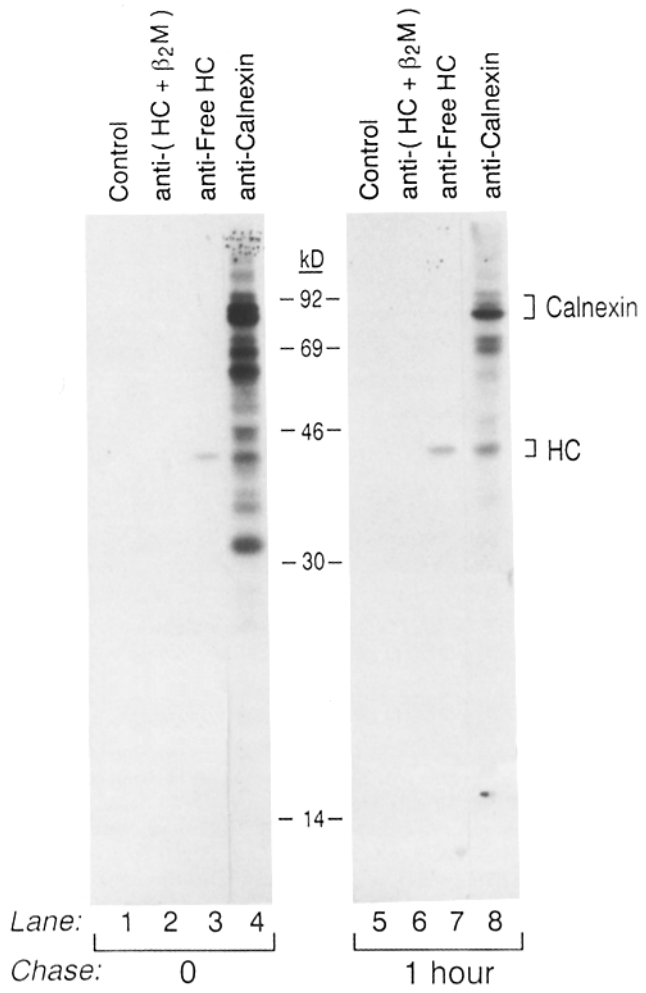
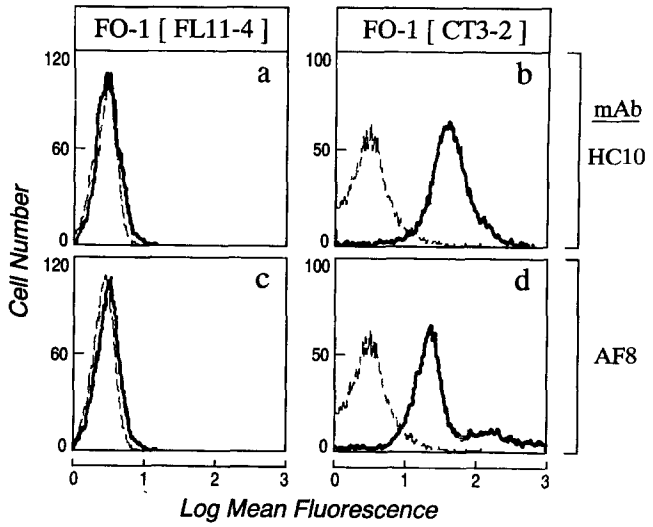


Figure 2. β_2m-free class I H chains in FO-1 are retained in association with calnexin. FO-1 cells were radiolabeled with [^{35}S]methionine for 5 min and either lysed immediately (chase *0*) or chased for 1 h (*chase 1 hour*). Cells were lysed in 0.3% CHAPS lysis buffer and equal aliquots were immunoprecipitated with control mAb P3 (lanes *1* and *5*); anti-β_2m plus H chain mAb, W6/32 (lanes *2* and *6*); anti-free class I H chain mAb, HC10 (lanes *3* and *7*); and anti-calnexin mAb, AF8 (lanes *4* and *8*). The samples were analyzed on 11% SDS-PAGE under reducing conditions. (*Right*) Positions of calnexin and H chain (*HC*).



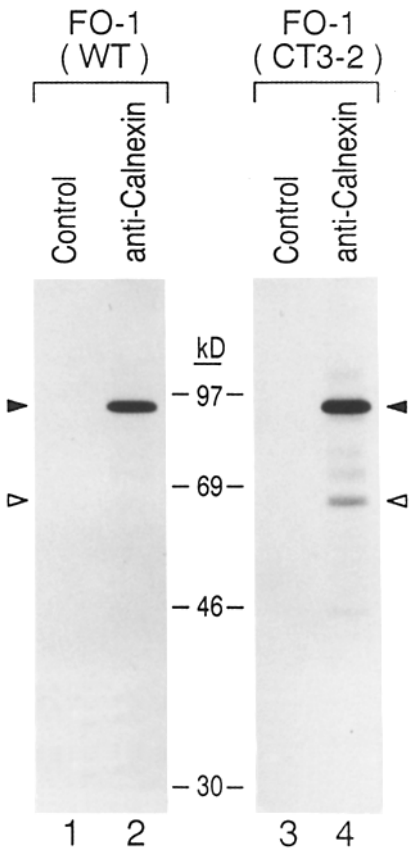


Figure 3. Expression of a non-ER retained form of calnexin in transfected FO-1 cells. Wild-type FO-1 cells (*FO-1 WT*) and a clone of FO-1–expressing cyt-deleted calnexin (*FO-1 [CT3-2]*) were radiolabeled with [^{35}S]methionine for 4 h and lysed in 2% Triton X-100. Equal aliquots of lysates were immunoprecipitated with either control mAb P3 (lanes *1* and *3*) or anti-calnexin mAb AF8 (lanes *2* and *4*). Samples were analyzed on 10.5% SDS-PAGE gels under reducing conditions. The positions of endogenous calnexin (◀) and the truncated cyt-deleted calnexin (◀) are indicated.

This was in marked contrast to the absence of cell surface expression of both proteins on control full-length calnexin transfectants (Fig. 4, *a* and *c*).

Here, the expression of a non-ER resident calnexin that trafficked to the cell surface of FO-1 cells overcame in part, the ER retention of free class I H chains and resulted in free class I H chain expression on the cell surface. These data demonstrate that calnexin determined the intracellular localization of associated class I molecules, indicating that calnexin mediates the retention of unassembled class I H chains in the ER. Thus, we reason that in normal class I biogenesis, ER resident calnexin prevents the cell surface expression of newly

Figure 4. Cell surface expression of class I β_2m-free H chains in FO-1 cells stably transfected with a non-ER retained form of calnexin. Control FO-1 cells transfected with the full-length calnexin gene (clone *FO-1 [FL 11-4]*; *a* and *c*) and FO-1 transfected with the cyt-deleted calnexin gene (clone *FO-1 [CT 3-2]*; *b* and *d*) were stained with HC10 or AF8 mAbs. This was followed by staining with goat anti-mouse Ig conjugated to PE and stained cells were analyzed by flow cytometry. Histograms with dashed lines in each overlay represent background fluorescence with negative control mAb P3.

synthesized class I molecules which are not fully assembled by retaining them in the ER.

The assembly of H chain/β_2m/peptide complexes in the ER, the process of antigen loading, is a critical step in antigen presentation. Calnexin plays a key role in the antigen presentation pathway in that it facilitates efficient assembly of the class I complex by retaining assembling components in the ER. In this regard, Jackson et al. (20) have recently shown that calnexin impedes the intracellular transport of incompletely assembled H chains by transfecting *Drosophila* cells with murine class I subunits in the absence or presence of calnexin. In addition, in the absence of a role for calnexin in the retention of incomplete class I complexes, there would be unregulated expression of incompletely assembled class I on the cell surface which could potentially bind exogenous antigenic peptides and lead to the recognition and destruction of normal cells by CTL (21, 22). Like the peptide transporters, (4, 5, 23), we propose that the molecular chaperone calnexin is an integral component of the class I antigen presentation pathway.

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