crystal-to-detector distance was used. Intensities for 15,417 unique reflections representing 94% of possible data to 1.8 Å resolution were obtained from 55,263 independent measurements taken on 21 images (R_{sym} = 0.061). The initial *R* factor was 0.40. After crystallographic refinement by simulated annealing [A. Brunger, J. Kuriyan, M. Karplus, *Science* **235**, 458 (1987)], the *R* factor had dropped to 0.26, and electron density maps calculated at this stage revealed the bound conformation of cyclic urea **3**. Geometric refinement parameters for the inhibitor were derived from its single-crystal structure (the crystal structure of **3** alone was determined by J. Calabrese). The final *R* factor was 0.195 for 12,695 reflections to 1.8 Å resolution for which $F \geq 2$ s (*F*). Coordinates will be deposited in the Brookhaven Data Bank [F. Bernstein *et al.*, *J. Mol. Biol.* **112**, 535 (1977)], and additional details will be published elsewhere.

33. Y. Iwakura *et al.*, *AIDS* **6**, 1069 (1992). M-H T. Lai *et al.*, *J. Acq. Immune Defic. Syndr.* **6**, 24 (1993).
34. G. Jonak *et al.*, unpublished observations.
35. T. J. Dueweke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4713 (1993); M. Tisdale, S. D. Kemp, N. R. Parry, B. A. Larder, *ibid.*, p. 5633.
36. M. J. Otto *et al.*, paper presented at the 2nd International Workshop on HIV Drug Resistance, Noordwijk, The Netherlands, 3 to 5 June 1993.
37. All compounds reported here have satisfactory spectroscopic and elemental analysis.
38. K_i values were determined with recombinant single-chain dimeric HIV protease and a fluorescent substrate [described by Y. S. E. Cheng *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9660 (1990)]. The use of single-chain dimeric protease allows enzyme concentrations as low as 0.0625 nM to be used. Reaction products were separated by high-performance liquid chromatography with a Pharmacia Mono Q anion-exchange column, and the product was quantified by fluorescence. The ability of test compounds to block cleavage of the HIV-1 gag polyprotein was assessed with [^{35}S]methionine-labeled in vitro translation product corresponding to *gag* p17 plus the first 78 amino acids of *gag* p24 and recombinant HIV PR as described [S. Erickson-Viitanen *et al.*, *AIDS Res. Hum. Retroviruses* **5**, 577 (1989)]. Cyclic ureas such as compound **1** and **4** were found to be competitive inhibitors of HIV PR.

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Regulation of MHC Class I Transport by the Molecular Chaperone, Calnexin (p88, IP90)

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Assembled class I histocompatibility molecules, consisting of heavy chain, β_2-microglobulin, and peptide ligand, are transported rapidly to the cell surface. In contrast, the intracellular transport of free heavy chains or peptide-deficient heavy chain–β_2-microglobulin heterodimers is impaired. A 90-kilodalton membrane-bound chaperone of the endoplasmic reticulum (ER), termed calnexin, associates quantitatively with newly synthesized class I heavy chains, but the functions of calnexin in this interaction are unknown. Class I subunits were expressed alone or in combination with calnexin in *Drosophila melanogaster* cells. Calnexin retarded the intracellular transport of both peptide-deficient heavy chain–β_2-microglobulin heterodimers and free heavy chains. Calnexin also impeded the rapid intracellular degradation of free heavy chains. The ability of calnexin to protect and retain class I assembly intermediates is likely to contribute to the efficient intracellular formation of class I–peptide complexes.

Class I molecules of the major histocompatibility complex (MHC) present peptides derived from endogenously synthesized proteins to cytotoxic T cells. Assembly of the class I heavy chain with β_2-microglobulin (β_2m) occurs rapidly within the ER (*1*, *2*). Peptides arising from protein degradation within the cytosol are delivered to assembling class I molecules by transporter proteins (TAP1 and TAP2) localized to membranes of the ER and *cis*-Golgi (*3*, *4*). For most class I molecules, transport to the cell surface is rapid, although allotype-specific variation is observed in the rate of transport from the ER to the Golgi apparatus (Table 1, mouse lymphoma). In murine RMA-S cells, which have a mutation in the TAP2 protein (*5*), K^b and D^b heavy chains associate with β_2m (*6*, *7*) but are largely deficient in peptide ligand (*8*–*11*) and are transported from the ER to Golgi very slowly (Table 1, RMA-S). Peptide-deficient class I molecules can also be produced by expression of murine heavy chains and β_2m in *Drosophila* Schneider cells because these cells lack auxiliary proteins required for supplying peptides to assembling class I molecules (*12*). The peptide-deficient class I molecules synthesized by *Drosophila* cells are transported much more rapidly than their counterparts in RMA-S cells. Peptide-deficient D^b, K^b, and L^d heterodimers in *Drosophila* cells acquired resistance to digestion by endoglycosidase H (reflective of transport through the Golgi) with half-times of 25, 60, and 80 min, respectively, as compared to >180 and 100 min for D^b and K^b heterodimers in RMA-S (Table 1). These findings suggested that a mechanism exists in mammalian cells for retaining incompletely assembled class I molecules, a mechanism that is nonexistent or impaired in *Drosophila*.

Calnexin is a membrane-bound chaperone of the ER that interacts transiently with a diverse array of wild-type proteins at early stages in their synthesis and in a prolonged fashion with misfolded or incompletely assembled proteins (*1*, *6*, *13*–*18*). As yet, there have been no direct demonstrations of the functions that calnexin may serve in these interactions. Because calnexin binds quantitatively to newly synthesized class I heavy chains in mouse cells (*1*), we assessed its ability to influence the transport of incompletely assembled class I molecules in *Drosophila* cells. Stably transfected *Drosophila* cell lines were prepared that expressed murine K^b, D^b, or L^d heavy chains with or without β_2m in the absence or presence of calnexin (*19*). A protein immunoblot of lysates from these transfectants revealed that cells transfected with the calnexin complementary DNA (cDNA) expressed calnexin in amounts comparable to those observed in murine tumor cells, whereas cells lacking this cDNA exhibited only trace amounts of two endogenous proteins of ~90 kD (Fig. 1). Thus, *Drosophila* cells either do not possess substantial amounts of a calnexin homolog or the similarity in structure is not sufficient to permit cross-reactivity with antibodies to calnexin.

Expression of calnexin slowed ER to Golgi transport of peptide-deficient D^b-β_2m heterodimers from a half-time ($t_{1/2}$) of 25 to 125 min (Fig. 2A). In cells lacking calnexin, the intensity of the heavy chain band increased after prolonged periods of time. This was due to slow assembly of the D^b heavy chain with β_2m, an event that was required for immunoreactivity (*20*). In cells expressing calnexin, assembly occurred much more rapidly, suggesting that calnexin may promote assembly of the D^b heavy chain with β_2m in addition to slowing the transport of the heterodimer. To confirm that a complex was indeed formed between peptide-deficient D^b-β_2m heterodimers and calnexin, we performed a comparable pulse-chase experiment, except that cells were lysed in the presence of the homobifunctional cross-linker DSP. In cells lacking calnexin, the only cross-linked species recovered had an apparent molecular size of ~60 kD (Fig. 2B). However, in cells expressing calnexin, other cross-linked species were observed as a doublet of ~170 kD

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(Fig. 2B). The 60-kD species represents heavy chain cross-linked to β_2m, and the 170-kD species is the heavy chain–calnexin complex (*1*). (The doublet represents complexes with and without an additional cross-link to β_2m.) The cross-linked D^b-calnexin complex disappeared slowly over time. Comparison of the rate of complex dissociation with the rate of ER to Golgi transport revealed that the two processes occurred with comparable kinetics (Fig. 2C).

The effects of coexpression of calnexin on the intracellular transport of peptide-deficient K^b-β_2m and L^d-β_2m molecules were also examined (Fig. 2D). Calnexin slowed the intracellular transport of these class I molecules as well (Table 1). Furthermore, DSP cross-linking revealed a complex with calnexin in each case that dissociated at a rate similar to that observed for ER to Golgi transport (*20*). Calnexin retarded the intracellular transport of peptide-deficient class I molecules to the extent that the rates of transport were similar to those in mouse RMA-S cells (Table 1). These experiments were done at 24°C, a temperature at which the *Drosophila* cells were viable and peptide-deficient class I molecules were stable. To ensure that the results were relevant to those obtained in mammalian cells at higher temperatures, we repeated the experiments at 30°C. This was the maximum temperature possible, because at higher temperatures class I molecules lacking peptide ligands are unstable and the heavy chains dissociate from β_2m at the cell surface (9). Slight increases in transport rates were noted in all cell lines, but the proportional decrease in transport rate owing to the presence of calnexin was maintained at 30°C (*20*). The rates of transport for the three peptide-deficient class I molecules in the absence of exogenous calnexin were distinctly different (Table 1). These molecules may have intrinsic differences that influence their transport, such as a propensity to form reversible aggregates or their degree of interaction with endogenous components of the *Drosophila* cells (although our cross-linking experiments failed to detect any discrete complexes). In this context it is noteworthy that the rates of transport fall into the same rank order as the thermal stabilities for these heterodimers (*12*) ($D^b\beta_2m > K^b\beta_2m > L^d\beta_2m$).

We also examined the effect of calnexin on the intracellular transport of class I heavy chains expressed without β_2m. In murine or human cells that lack β_2m, different free heavy chain allotypes are either not transported or are transported at barely detectable rates (*21, 22*). However, in *Drosophila* cells expressing free D^b heavy chains in the absence of calnexin, a portion (22% after 40 min of chase) of D^b heavy chains was transported and acquired resistance to endo H digestion (Fig. 3A). In

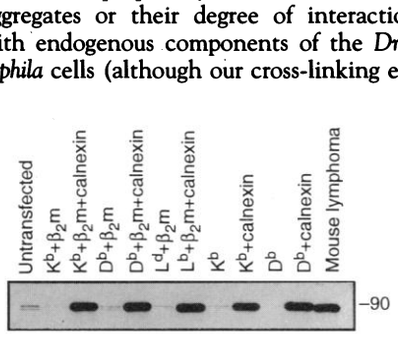

Fig. 1. Expression of calnexin in transfected *Drosophila* cells and in mouse MDAY-D2 cells. Lysates of cells transfected with the indicated cDNAs were subjected to protein immunoblot analysis with antibodies to calnexin (*19*). The molecular size marker is indicated on the right (in kilodaltons).

Table 1. Intracellular transport rates of peptide-containing and peptide-deficient class I molecules in various cells. All values were determined by assessment of the rate at which Asn-linked oligosaccharides on newly synthesized class I molecules were converted to a form that was resistant to cleavage by endo H. Enzymes that process oligosaccharides to the endo H–resistant form are localized to different Golgi subcompartments in different cell types (*30*). Data for class I–peptide complexes in mouse lymphoma cells were obtained with MDAY-D2, RMA, and EL-4 cells (*1, 6*). Rates for peptide-deficient molecules in mouse RMA-S cells were determined at both 26°C and 37°C (*6, 20*). Transport rates were similar at both temperatures, although at 37°C the mature molecules were unstable. The data for peptide-deficient molecules in *Drosophila* cells are summarized from the experiments described in Fig. 2.

Class I molecule	Rate of ER to Golgi transport ($t_{1/2}$ in minutes)			
	Mouse lymphoma	Mouse RMA-S	*Drosophila* (− calnexin)	*Drosophila* (+ calnexin)
D^b-β_2m	55	>180	25	125
K^b-β_2m	20	100	60	120
L^d-β_2m	55	–	80	175

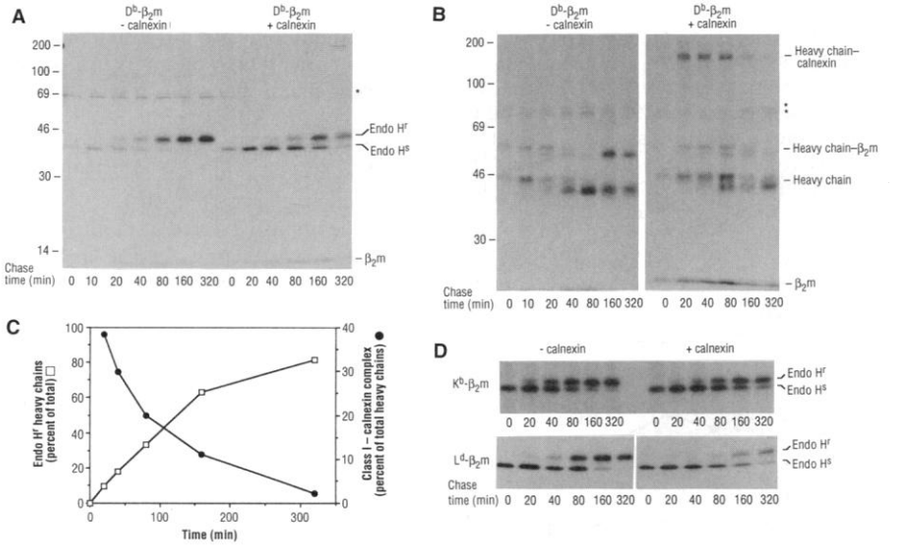

Fig. 2. Intracellular transport of peptide-deficient class I heterodimers in the absence and presence of calnexin. *Drosophila* cells expressing various peptide-deficient class I molecules in the absence or presence of calnexin were incubated with [^{35}S]Met for 10 min and then in the presence of excess unlabeled Met for the times indicated (*29*). (**A**) Immunoisolated D^b-β_2m molecules were digested with endo H and analyzed by reducing SDS-PAGE. The mobilities of the endo H–resistant (endo H^r) and endo H–sensitive (endo H^s) heavy chains are indicated. Molecular sizes are indicated on the left (in kilodaltons). (**B**) D^b-β_2m molecules immunoisolated from DSP-treated cell lysates and analyzed by nonreducing SDS-PAGE. The mobilities of the various cross-linked species are shown. In *Drosophila* cells Golgi processing leads to a mature heavy chain that is smaller than the immature heavy chain. These mobility differences are reversed after removal of immature oligosaccharides by endo H treatment (A). (**C**) Comparison of the rate of dissociation of the class I–calnexin complex with the rate of ER to Golgi transport. The amounts of D^b heavy chain that were endo H–resistant [panel (A), right] and that could be cross-linked to calnexin [panel (B), right] were quantitated by densitometry and expressed as a percentage of total heavy chains recovered at each chase time. (**D**) K^b-β_2m and L^d-β_2m molecules synthesized in the absence or presence of calnexin were digested with endo H and analyzed by reducing SDS-PAGE. In (A) and (B), asterisks denote contaminating bands that were present in control samples treated with an unrelated antibody.

contrast, when the experiment was repeated in the presence of calnexin, the transport of free D^b heavy chains was largely prevented. Only after a prolonged period of time was any endo H–resistant species detected (Fig. 3A). Cross-linking of cell lysates revealed that in cells expressing calnexin, a heavy chain–calnexin complex could be detected at all times (Fig. 3B). Unlike the situation with D^b-β_2m heterodimers (Fig. 2B), the amount of cross-linked D^b-calnexin complex remained relatively constant, indicating that little dissociation had occurred. This is consistent with the very low amount of D^b heavy chain transport observed in these cells.

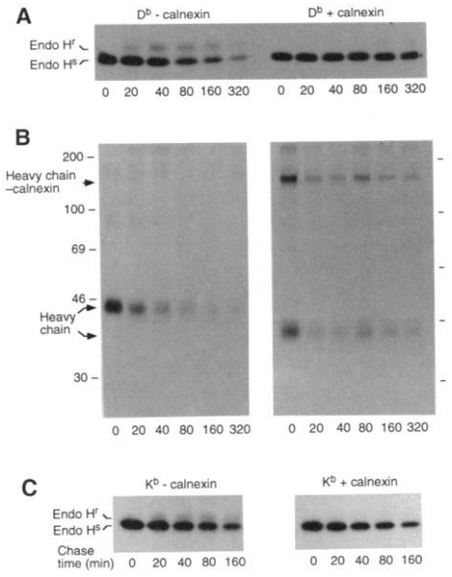

Fig. 3. Intracellular transport of class I heavy chains in the absence and presence of calnexin. *Drosophila* cells expressing free class I heavy chains in the absence or presence of calnexin were incubated with [^{35}S]Met for 10 min and then in the presence of excess unlabeled Met for the times indicated (*29*). (**A**) Immunoisolated D^b heavy chains digested with endo H. (**B**) D^b heavy chains immunoisolated from DSP-treated cell lysates. The mobilities of the heavy chain and cross-linked heavy chain–calnexin complex are indicated. Molecular sizes are indicated on the left and right (in kilodaltons). (**C**) Immunoisolated K^b heavy chains digested with endo H.

Similar results were obtained when free K^b heavy chains were examined (Fig. 3C). The extent to which these molecules acquired endo H resistance in the absence of calnexin (15% at 40 to 80 min of chase) was less than that for free D^b heavy chains. However, when calnexin was coexpressed, the transport of free K^b heavy chains was barely detectable. As was observed for peptide-deficient heterodimers, increasing the temperature to 30°C or 37°C increased transport rates slightly but did not affect the degree to which transport was inhibited by calnexin (*20*). Clearly, calnexin functions to retain free class I heavy chains and peptide-deficient heavy chain–β_2m heterodimers.

Free heavy chains expressed in the absence of exogenous calnexin were not transported efficiently through the *Drosophila* secretory pathway. This may reflect a propensity for free heavy chains to form aggregates or to acquire some other transport-incompetent conformation. Alternatively, the slow transport may result from interaction with endogenous *Drosophila* proteins. For example, free heavy chains may interact with the ER chaperone BiP in *Drosophila* cells as they do in mammalian systems (*6*).

Calnexin also exerted a stabilizing effect on free heavy chains. In the absence of calnexin, free D^b heavy chains were degraded with half-times of 125 min (24°C), 40 min (30°C), and 26 min (37°C) (Fig. 4). With coexpression of calnexin, degradation was markedly slowed such that only 15 to 20% of the heavy chain was degraded over 160 min at 24°C or at 30°C. At 37°C, the $t_{1/2}$ of degradation in the presence of calnexin (120 min) was extended nearly five times over that in the absence of calnexin. Calnexin also impeded the degradation of free K^b heavy chains, although the effect was less pronounced. The lifetime of free K^b heavy chains was extended by about two times at 24°C and by about three times at 37°C. Thus, the association of calnexin with free class I heavy chains protects them from intracellular degradation.

In murine cells a close correlation exists between the characteristic ER to Golgi transport rates observed for different class I allotypes and the durations of their interaction with calnexin (*1*). Furthermore, impaired intracellular transport of free heavy chains and peptide-deficient heavy chain–β_2m heterodimers is accompanied by a corresponding prolonged association with calnexin (*6*). These correlations can now be explained by the demonstration that calnexin retains class I assembly intermediates intracellularly. During the course of normal class I biogenesis, such retention may provide additional time to ensure the efficient assembly of the complete complex of heavy chain, β_2m, and peptide ligand. In this manner, incompletely assembled molecules would be largely prevented from reaching the cell surface where they could potentially bind exogenous antigenic peptides and lead to the destruction of otherwise normal cells by cytotoxic T lymphocytes. The ability of calnexin to impede the intracellular destruction of free heavy chains may also contribute to an increased efficiency of class I assembly. This property may be particularly important in many nonlymphoid tissues where the amount of class I expressed is reduced and the steady-state concentration of class I subunits during intracellular assembly is presumably low (*23*).

The interaction of calnexin with incompletely assembled class I molecules is reminiscent of the interaction of BiP during assembly of immunoglobulin (Ig) molecules. BiP binds to Ig heavy chains, retaining them intracellularly, until displaced by light chains. In the absence of BiP binding, Ig molecules are secreted more rapidly and in various stages of assembly (*24*). Calnexin also resembles BiP in that it interacts with incompletely assembled forms of a diverse array of proteins including subunits of the T cell receptor and the membrane Ig receptor (*14, 15, 17*). Calnexin may function to facilitate assembly of these complexes as well by retaining or protecting assembly intermediates. Calnexin's functions are not restricted to assembly of multisubunit complexes. Calnexin also interacts with folding

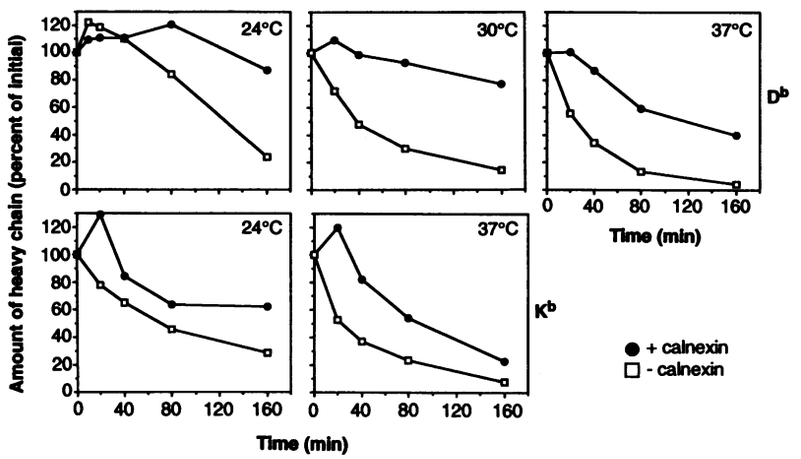

Fig. 4. Effect of calnexin on the intracellular degradation of free class I heavy chains. Experiments were done as described (Fig. 2) except that the cells were incubated with excess unlabeled Met at 24°, 30°, and 37°C (*29*). Fluorograms similar to those depicted in Fig. 3, A and C, were analyzed by densitometry, and the amount of heavy chain recovered at each time point was expressed as a percentage of the amount present at the 0 min time point.

intermediates of monomeric secretory glycoproteins, suggesting an additional role in early folding events (*18*).

REFERENCES AND NOTES

1. E. Degen and D. B. Williams, *J. Cell Biol.* **112**, 1099 (1991).
2. M. S. Krangel, H. T. Orr, J. L. Strominger, *Cell* **18**, 979 (1979).
3. J. C. Shepherd *et al.*, *ibid.* **74**, 577 (1993); J. J. Neefjes, F. Momburg, G. J. Hämmerling, *Science* **261**, 769 (1993).
4. M. J. Kleijmeer *et al.*, *Nature* **357**, 342 (1992).
5. Y. Yang *et al.*, *J. Biol. Chem.* **267**, 11669 (1992).
6. E. Degen, M. F. Cohen-Doyle, D. B. Williams, *J. Exp. Med.* **175**, 1653 (1992).
7. V. Ortiz-Navarrete and G. J. Hämmerling, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3594 (1991); E.-M. Click, K. S. Anderson, M. J. Androlewicz, M. L. Wei, P. Cresswell, *Cold Spring Harbor Symp. Quant. Biol.* **57**, 571 (1992).
8. A. Townsend *et al.*, *Nature* **340**, 443 (1989).
9. H.-G. Ljunggren *et al.*, *ibid.* **346**, 476 (1990).
10. A. Townsend *et al.*, *Cell* **62**, 285 (1990).
11. S. J. Powis *et al.*, *Nature* **354**, 528 (1991).
12. M. R. Jackson, E. S. Song, Y. Yang, P. A. Peterson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12117 (1992).
13. I. Wada *et al.*, *J. Biol. Chem.* **266**, 19599 (1991).
14. F. Hochstenbach, V. David, S. Watkins, M. B. Brenner, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4734 (1992).
15. K. Galvin *et al.*, *ibid.*, p. 8452.
16. N. Ahluwalia, J. J. M. Bergeron, I. Wada, E. Degen, D. B. Williams, *J. Biol. Chem.* **267**, 10914 (1992).
17. V. David, F. Hochstenbach, S. Rajagopalan, M. B. Brenner, *ibid.* **268**, 9585 (1993).
18. W.-J. Ou, P. H. Cameron, D. Y. Thomas, J. J. M. Bergeron, *Nature* **364**, 771 (1993).
19. The cDNAs encoding murine class I heavy chains, murine β_2m, and canine calnexin (*13*) were cloned downstream of the metallothionein promoter in plasmid pRMHa3 and transfected along with the selection plasmid phshsneo into Schneider cells as described (*12*). After 4 weeks in selection medium, G418-resistant cells were treated for 24 to 48 hours with 1 mM $CuSO_4$ and tested for expression of class I molecules and calnexin by flow cytometry and enzyme-linked immunosorbent assays. Cells were maintained at room temperature in Schneider medium (Gibco/BRL) supplemented with fetal bovine serum (10%), antibiotics, and G418 (500 μg/ml). For protein immunoblotting, cells were treated for 24 hours with 1 mM $CuSO_4$ and lysate equivalent to 1 × 10^5 cells (*Drosophila* and MDAY-D2), subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with antibodies to calnexin, and bound antibodies were visualized by enhanced chemiluminescence (Amersham). Antibodies to calnexin were raised in rabbits with a conjugate consisting of keyhole limpet hemocyanin coupled to the NH_2-terminus of a peptide corresponding to the COOH-terminal 14 amino acids of canine and human calnexin (sequence, ILNRSPRNRKPRRE; E, Glu; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; R, Arg; S, Ser). Antibodies to the peptide were affinity purified with a peptide–bovine serum albumin conjugate immobilized on Affi-Gel 10.
20. M. F. Cohen-Doyle, M. R. Jackson, A. Vassilakos, D. B. Williams, unpublished data.
21. D. B. Williams, B. H. Barber, R. A. Flavell, H. Allen, *J. Immunol.* **142**, 2796 (1989).
22. K. Sege, L. Rask, P. A. Peterson, *Biochemistry* **20**, 4523 (1981).
23. A. S. Daar, S. V. Fuggle, J. W. Fabre, A. Ting, P. J. Morris, *Transplantation* **38**, 287 (1984).
24. D. G. Bole, L. M. Hendershot, J. F. Kearney, *J. Cell Biol.* **102**, 1558 (1986); L. M. Hendershot, *ibid.* **111**, 829 (1990); ———, D. Bole, G. Köhler, J. F. Kearney, *ibid.* **104**, 761 (1987).
25. L. Margolese *et al.*, *J. Biol. Chem.* **268**, 17959 (1993).
26. H. Allen, J. Fraser, D. Flyer, S. Calvin, R. Flavell, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7447 (1986).
27. W.-R. Lie *et al.*, *J. Exp. Med.* **173**, 449 (1991).
28. M. H. Smith, J. R. M. Parker, R. S. Hodges, B. H. Barber, *Mol. Immunol.* **23**, 1077 (1986).
29. Cells were treated with $CuSO_4$ (1 mM) for 24 hours and then were incubated for 10 min at 24°C in Met-free Graces medium (5 × 10^7 cells/ml) containing 0.5 mCi/ml [^{35}S]Met (>800 Ci/mmol, Amersham). Nine volumes of Schneider medium containing Met (1 mM) and fetal bovine serum (10%) were then added, and the cells were incubated at 24°C. Portions of cells (4.5 × 10^6) were removed at various times and were lysed in 1 ml of phosphate-buffered saline containing 1% NP-40, 10 mM iodoacetamide, 1% aprotinin, and 0.25 mM phenylmethylsulfonyl fluoride. Cross-linking with the thiol-cleavable cross-linker DSP (Pierce) was done as described, except that the reaction was scaled up to 1 ml (*25*). Lysates (both cross-linked and uncross-linked) were incubated on ice overnight with 10 to 15 μg of monoclonal antibody (mAb) to class I. For cells expressing D^b-β_2m, K^b-β_2m, or L^d-β_2m heterodimers, mAb B22-249.R1, 20-8-4s, or 30-5-7s were used, respectively. B22-249.R1 and 20-8-4s react with heavy chains only when associated with β_2m (*21*, *26*), whereas 30-5-7s reacts with either free or β_2m-associated L^d heavy chains (*27*). Free D^b and K^b heavy chains were recovered with mAb 28-14-8s (*26*) and with antiserum to the COOH-terminal peptide encoded by exon 8 of the K^b gene (*28*), respectively. Immune complexes were recovered with protein A–agarose, and the agarose beads were washed four times with 10 mM tris (pH 7.4) containing 0.5% NP-40, 0.15 M NaCl, and 1 mM EDTA. For cross-linked samples, immune complexes were analyzed by nonreducing SDS-PAGE (9% gel). Uncross-linked samples were heated in 0.1 ml of 0.1 M sodium citrate (pH 6) containing 0.1% SDS at 65°C for 5 min before treatment with endo H (2 mU) overnight at 30°C. Samples were analyzed by SDS-PAGE under reducing conditions (10% gel). Gels were fixed, treated with 1 M sodium salicylate, dried, and subjected to fluorography.
30. A. Velasco *et al.*, *J. Cell Biol.* **122**, 39 (1993).

31. We thank I. Wada and J. J. M. Bergeron for the canine calnexin gene, B. H. Barber for antiserum to peptide 8, A. Vassilakos for providing data on D^b turnover at 24°C, N. Ahluwalia for preparing antibodies to the COOH-terminus of calnexin, the Alberta Peptide Institute for peptide synthesis, F. Brodsky for insightful suggestions at the outset of this work, and E. Song and S. Pind for helpful comments. Supported by the National Cancer Institute of Canada (D.B.W.) and the National Institutes of Health (P.A.P.).

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Retention of Unassembled Components of Integral Membrane Proteins by Calnexin

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Quality control mechanisms prevent the cell surface expression of incompletely assembled multisubunit receptors such as the T cell receptor (TCR). The molecular chaperone function of calnexin (IP90, p88), a 90-kilodalton protein that resides in the endoplasmic reticulum (ER), in the retention of representative chains of the TCR-CD3 complex in the ER was tested. Truncation mutants of calnexin, when transiently expressed in COS cells, were exported from the ER and either accumulated in the Golgi or progressed to the cell surface. CD3 ε chains cotransfected with the forms of calnexin that were not retained in the ER exited the ER and colocalized with calnexin. Since engineered calnexin determined the intracellular localization of the proteins associated with it, it is concluded that calnexin interacts with incompletely assembled TCR components and retains them in the ER.

In the ER, newly synthesized components of multimeric receptor complexes must complete folding and assembly before they progress to the cell surface (*1*). This process is facilitated by transient interactions with molecular chaperones that reside in the ER lumen such as BiP, protein disulfide isomerase (PDI), and GRP94 (*2*). We identified a 90-kD human ER resident integral membrane protein, denoted calnexin (previously referred to as IP90, p88) (*3*–*5*). Calnexin associates transiently with numerous newly synthesized polypeptides (*6*) and interacts transiently with folding intermediates of secretory glycoproteins during their maturation in the ER (*7*). Calnexin also associates with partial complexes of the TCR, major histocompatibility complex (MHC) class I proteins, and B cell membrane immunoglobulin (mIg), but not with completed receptor complexes. Thus, we and others have hypothesized that calnexin may function as a molecular chaperone that retains incompletely assembled multisubunit receptors in the ER (*5*, *6*, *8*, *9*).

Calnexin is an integral membrane protein with a large ER luminal domain (LD; 461 amino acids), a transmembrane segment (TM; 22 amino acids), and a cytoplasmic tail (CYT; 89 amino acids) (*6*). This feature distinguishes calnexin from soluble chaperones such as BiP, which are located in the ER lumen and cannot interact with the transmembrane and cytosolic

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