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

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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.
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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.
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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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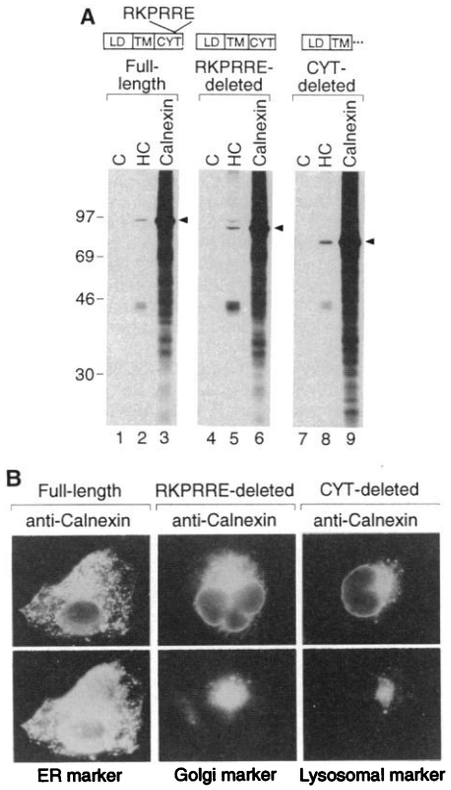

Fig. 1. Deletion of the calnexin ER retention motif allows its exit from the ER. (**A**) Immunochemical analysis of the expressed calnexin proteins in COS-7 cells transfected with full-length calnexin, RKPRRE-deleted calnexin, or CYT-deleted calnexin (*24*). Transiently transfected COS-7 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 5 hours (*25*). Lysates were immunoprecipitated with control monoclonal antibody TCRδ1 (C), monoclonal antibody to MHC class I free heavy chain, HC10 (HC), and monoclonal antibody to human calnexin, AF8 (calnexin). Immunoprecipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) (10.5% gel) under nonreducing conditions. Solid arrowheads denote position of the recombinant calnexin protein. (**B**) Immunofluorescence localization of full-length and truncated calnexin in COS-7 cells (*23*). Cells were transfected with full-length calnexin (left), RKPRRE-deleted calnexin (middle), or CYT-deleted calnexin (right) and stained with anti-calnexin (top row). The bottom row shows the same cell as that above stained with anti-gp96 (left), Golgi marker lens culinaris lectin (middle), or anti–LAMP-1 (right).

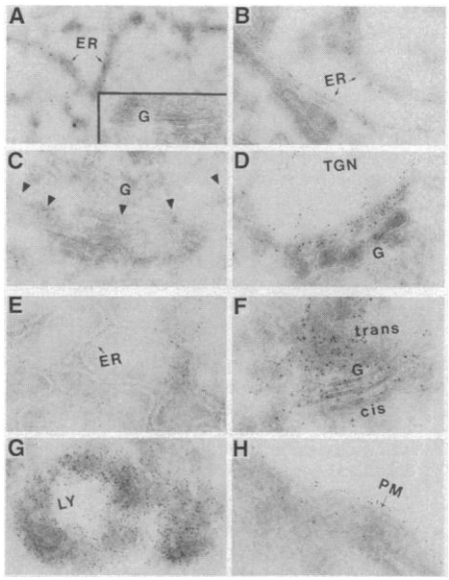

Fig. 2. Immunoelectron microscopic localization of full-length and truncated calnexin in transfected COS-7 cells. Immunolabeling was done on ultrathin cryosections with affinity-purified monoclonal antibody to calnexin (AF8) as primary antibody and a gold conjugate of goat antibody to mouse IgG as secondary antibody (Amersham, Arlington Heights, Illionis). (**A**) Full-length calnexin is localized in the ER of COS cells. Immunogold particles were present in the cisternae of the rough-surfaced ER (arrows) but absent from the Golgi (see inset of the same COS cell) (magnification, ×47,500). (**B** to **D**) Localization of RKPRRE-deleted calnexin to ER and Golgi. (B) Localization to ER (arrows; ×72,500); (C) Golgi localization, with stronger labeling at the trans face of the Golgi stacks (arrows; ×72,500); and (D) localization to the trans golgi network (TGN) vacuoles (×108,750). (**E** to **H**) Localization of CYT-deleted calnexin in secondary lysosomes and plasma membrane. Localization in the ER [(E); ×95,000], Golgi [(F); ×95,000], multivesicular type secondary lysosomes (LY) [(G); ×95,000], and on the plasma membrane (PM) [(H); ×108,750]. Immunoelectron microscopic methods were as described in (*26*).

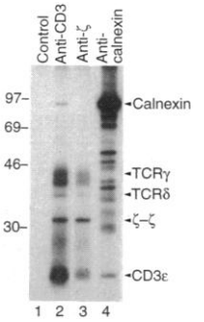

Fig. 3. Association of calnexin with T cell receptor chains. Proteins in lysates of intracellularly ^{125}I-labeled MOLT 13 cells were immunoprecipitated with control monoclonal antibody P3 (lane 1), monoclonal antibody to CD3ε, UCHTI (lane 2), monoclonal antibody to TCRζ, TIA-2 (lane 3), or monoclonal antibody to calnexin, AF8 (lane 4). Immunoprecipitates were resolved by SDS-PAGE (10.5% gel) under nonreducing conditions (*27*).

domains of integral membrane proteins. To determine whether calnexin mediates the retention of incompletely assembled TCR subunits in the ER, we engineered mutants lacking the COOH-terminal charged residues RKPRRE (E, Glu; K, Lys; P, Pro; R, Arg), which contain a −3 arginine and a −5 lysine (underlined) similar to the consensus motif implicated in the ER retention of other transmembrane ER resident proteins (*10*). Truncation mutants lacking the COOH-terminal six amino acids (RK-PRRE-deleted) or the entire cytoplasmic tail (CYT-deleted) (Fig. 1) were expressed in COS cells. After 48 hours, the transfectants were incubated with [^{35}S]methionine and [^{35}S]cysteine, and proteins were immunoprecipitated with antibody to human calnexin that does not react with endogenous monkey calnexin. The full-length calnexin (90 kD), RKPRRE-deleted calnexin (86 kD), and CYT-deleted calnexin (75 kD) are shown in Fig. 1. Both truncated forms of calnexin coimmunoprecipitated with many associated proteins as is typical of the wild-type calnexin (Fig. 1A) (*6*). The association of each of these recombinant calnexin proteins with COS cell β_2-microglobulin–free MHC class I heavy chains was also apparent (Fig. 1A).

The intracellular localization of the full-length and truncated calnexin forms was assessed by immunofluorescence microscopy (IM) and immunogold electron microscopy (EM) after staining with monoclonal antibody specific for human calnexin (Figs. 1B and 2). Wild-type calnexin localizes to the nuclear envelope and rough ER (*3*). The transfected full-length calnexin was present in the ER (Fig. 1B), and this was confirmed by EM (Fig. 2A). There was no detectable wild-type calnexin in the Golgi (Fig. 2A, inset), other cellular organelles, or the plasma membrane (*11*). In contrast, the RKPRRE-deleted calnexin exited the ER, and in addition to limited nuclear envelope and ER expression, staining also was detected in the Golgi as confirmed by the localization of lens culinaris lectin as a Golgi marker (Fig. 1B) (*12*). EM analysis also confirmed both ER and Golgi localization of RKPRRE-deleted calnexin (Fig. 2, B to D). CYT-deleted calnexin also exited the ER and was visualized not only in the Golgi, but also in vesicular compartments (punctate staining) that colocalized with the lysosome-associated membrane protein marker LAMP-1 (*13*) (Fig. 1). EM analysis confirmed the presence of the CYT-deleted calnexin not only in the Golgi, but also in secondary lysosomes and on the plasma membrane (Fig. 2, E to H).

Having engineered mutants of calnexin that no longer localized exclusively to the ER, we then tested whether calnexin functions in the retention of unassembled chains of the multisubunit TCR. The TCR complex consists of a TCR (αβ or γδ) in association with CD3 (γ, δ, and ε) and the ζ-ζ or ζ-η dimer (*1, 14*). These subunits assemble in the ER, and partial complexes that lack any TCR or CD3 chain are retained in the ER and are eventually degraded there or in lysosomes. Thus, incomplete complexes are not expressed on the cell surface, and only completed complexes as signified by the addition of the ζ chain progress to the cell surface (*15–18*). Intracellular iodination of permeabilized cells of

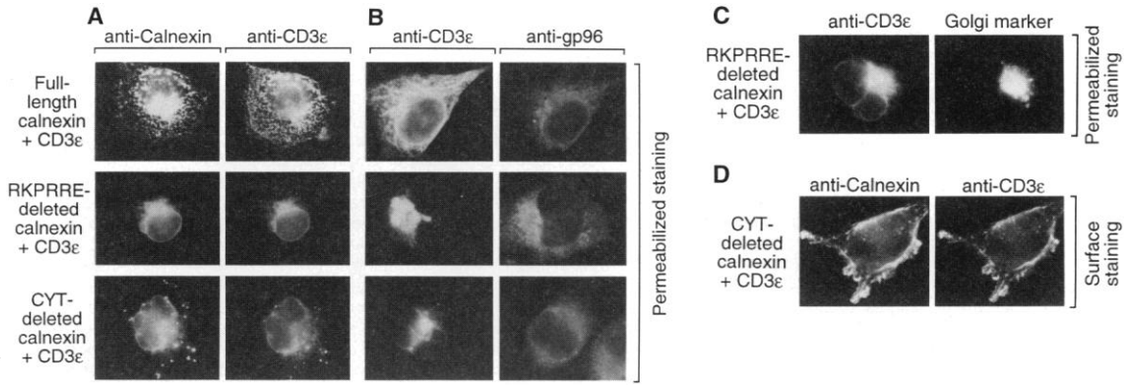

Fig. 4. Colocalization of truncated calnexin lacking the ER retention motif with associated CD3ε. (**A**) Double immunofluorescence staining of COS-7 cells coexpressing the different forms of calnexin shown and CD3ε (*28*). Two days after transfection, cells were fixed and permeabilized (Fig. 2) and stained. In each paired set, the same cell was stained with anti-calnexin (AF8) and anti-CD3ε (SP34) and visualized with rhodamine-conjugated and FITC-conjugated goat antibodies to mouse IgG (Tago, Burlingame, California), respectively. (**B**) Double immunofluorescence staining of transfected CD3ε and endogenous gp96 in COS-7 cells coexpressing full-length or truncated calnexin and CD3ε. The cotransfected cells (A) were stained with anti-CD3ε (SP34) and with rabbit antiserum to endogenous gp96, an ER resident marker protein. Shown in each pair is the same cell costained to detect the shift in localization of CD3ε compared to the ER localization of gp96. (**C**) Double immunofluorescence of the same cell cotransfected with RKPRRE-deleted calnexin and CD3ε and stained with anti-CD3ε (SP34) and Golgi marker lens culinaris lectin. (**D**) Double immunofluorescence analysis of cell surface expression of CD3ε and calnexin in COS-7 cells coexpressing CYT-deleted calnexin and CD3ε.

the TCRγδ T cell tumor line MOLT 13 confirmed the association of calnexin with intracellular components of the TCR, but not with completely oligomerized complexes (Fig. 3) (6). For example, immunoprecipitates prepared with antibody to CD3ε recognized both partial and completed complexes of CD3ε (20 kD) with other TCR components such as ζ-ζ (32 kD), TCRδ (40 kD), and TCRγ (44 kD) and revealed the coimmunoprecipitation of calnexin (90 kD) with components of the CD3 complex. However, a monoclonal antibody to TCRζ immunoprecipitated completed TCR complexes that are competent to exit the ER, and these complexes were not associated with calnexin.

In the absence of other subunits of the T cell receptor, CD3ε is stably retained in the ER in nonlymphoid COS cells (*19*). We coexpressed the CD3ε chain of the TCR complex along with full-length and truncated forms of calnexin in COS cells. Immunofluorescence microscopy revealed colocalization of calnexin with CD3ε in COS cells expressing either the full-length or truncated calnexin. Although CD3ε coexpressed with the full-length calnexin was retained in the ER (Fig. 4A), CD3ε was exported from the ER to the Golgi when coexpressed with RKPRRE-deleted calnexin (Fig. 4A). The Golgi localization of CD3ε was confirmed with the Golgi marker lens culinaris lectin (Fig. 4C). Similarly, when expressed with the CYT-deleted calnexin, CD3ε was carried out of the ER and was found in the Golgi, in lysosomes, and on the cell surface (Fig. 4, A and D). Cell surface localization of CYT-deleted calnexin and CD3ε was also confirmed by flow cytometry (*11*). In each set of transient transfections, approximately 10 to 30% of the COS cells expressed the introduced calnexin and CD3ε as determined by single staining with the relevant antibodies (*11*). Furthermore, controls to exclude cross-reaction of the antibody reagents were done to establish specificity of the staining in the double labeling experiments. For each cotransfection of CD3ε with the different forms of calnexin, control immunofluorescence double staining was done with antibodies specific for CD3ε and the ER resident protein gp96/GRP94 (*20, 21*) (Fig. 4B). Although changes in cell compartment for CD3ε that paralleled calnexin were documented in each instance, no changes were noted in the ER localization of endogenous gp96 in the same transfected cell. These control experiments emphasize both the specificity of the antibody staining and the selectivity of calnexin's effect on CD3ε localization. Localization of CD3ε was compared with that of Golgi marker lens culinaris lectin and the lysosomal marker LAMP-1 (Fig. 4C) (*11*). We confirmed these studies using TCRδ, another subunit of the TCR-CD3 complex, which yielded identical results (*11*).

Biochemical studies of both TCR and MHC class I assembly indicate that there is early transient interaction of calnexin with unassembled chains; however, in variants deficient in a given subunit, there is prolonged association with partner chains that are retained in the ER (*6, 8*). The data described here explain those observations by demonstrating that calnexin retains assembling TCR components in the ER. Moreover, as shown in Fig. 1A, a large number of proteins associate with calnexin shortly after their synthesis (*6*), suggesting also that calnexin may play a similar role in the ER retention of these proteins. Additional mechanisms of ER retention for assembling subunits may exist to ensure retention of individual subunits during their assembly either before or after their interaction with calnexin. For example, CD3ε also appears to be retained by a motif intrinsic to its cytoplasmic tail (*22*). Presumably, this CD3ε motif is masked by the association of CD3ε with calnexin or with other components of the TCR-CD3 complex.

Calnexin affects critical quality control by preventing inappropriate expression of partial complexes of multimeric proteins on the cell surface. The retention of individual chains or partial complexes by calnexin might also be viewed as a molecular chaperone function in the assembly of multisubunit protein complexes because retention would facilitate the opportunity for the oligomerization of individual chains.

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23. For immunofluorescence staining, cells were washed in phosphate-buffered saline (PBS), fixed in paraformaldehyde (3.7%) in PBS, permeabilized in cold methanol, and blocked in PBS containing goat serum (5%) and Triton X-100 (0.3%) (blocking buffer). Cells were then incubated with anti-calnexin in blocking buffer, washed in PBS, followed by incubation with fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse immunoglobulin G (IgG). Control double staining was done with lens culinaris lectin (E-Y Labs.) and anti–LAMP-1 [J. W. Chen *et al.*, *J. Cell Biol.* **101**, 85 (1985)]. Positive cells were examined on a Nikon Opti-phot 2 fluorescence microscope and photographed with Kodak T3200 film.
24. A 2-kb Spe I–Spe I fragment containing the coding region of calnexin was cloned into the Xba I site of expression vector Ap^{r}M8 (*6*) to generate the full-length calnexin construct. The RKPRRE-deleted calnexin cDNA was constructed by polymerase chain reaction (PCR) with the following oligonucleotide primers: 5′ primer, GGGGAATTCATGGAAGGGAAGTGG; 3′ primer, GGGAATTCTTAGTTTCTTGGTGATCTG. The CYT-deleted calnexin cDNA was also generated by PCR using the above 5′ primer with the following 3′ primer: GGAATTCTTATTCTTTCCAGAACAGCAG. Note that in the CYT-deleted calnexin, three anchor residues belonging to the cytoplasmic tail are present. After PCR amplification of the truncated calnexin cDNAs, the reaction products were digested with Eco RI and ligated into the Eco RI site of Ap^{r}M8 strain. DH5α-competent *Escherichia coli* were then transformed with the ligation products, and clones with correct inserts were used for transfections.
25. COS-7 cells were transfected by the DEAE-dextran method as described (*6*). Two days after transfection, cells were metabolically labeled for 5 hours with [^{35}S]methionine and [^{35}S]cysteine, lysed in 0.3% CHAPS lysis buffer, and immunoprecipitated as described (*6*).
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27. MOLT 13 cells were permeabilized with 8 μM digitonin, iodinated with lactoperoxidase, and solubilized in 1% digitonin in tris-buffered saline (TBS), and proteins were immunoprecipitated as described [F. Hochstenbach *et al.*, *J. Exp. Med.* **168**, 761 (1988)].
28. CD3ε cDNA [D. Gold *et al.*, *Nature* **321**, 431 (1986)] was ligated into the Eco RI site of the expression vector Ap^{r}M8. COS-7 cells were cotransfected with calnexin and CD3ε and permeabilized cells were stained as described (*23*). For double immunofluorescence analysis, cells were stained first with anti-CD3ε (SP34) and FITC-conjugated goat antibodies to mouse IgG. After blocking of the cells with isotype-matched myeloma MOPC 21 (Sigma), cells were stained either with AF8 or anti-gp96 (*20*) and then with the relevant rhodamine-conjugated secondary antibodies.
29. We thank D. Friend, L. B. Chen, and B. Brunkhorst for advice on immunofluorescence staining; H. Slayter for electron microscopy; and P. Srivastava, C. Terhorst, P. Anderson, and H. Ploegh for antibodies and cDNA clones. We also thank V. David, M. Sugita, C. Parker, and S. Porcelli for critically reading the manuscript. S.R. is a postdoctoral fellow of the Irvington Institute for Medical Research, and M.B.B. is a scholar of the Leukemia Society of America. Supported by grants from the National Institutes of Health.

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Cell Membrane Resealing by a Vesicular Mechanism Similar to Neurotransmitter Release

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After injury to the cell membrane, rapid resealing of the membrane occurs with little loss of intracellular contents. This process has been studied by measurement of the rate of dye loss after membrane puncture in both the sea urchin embryo and 3T3 fibroblasts. Resealing of disrupted cell membranes requires external calcium that can be antagonized by magnesium. Block of multifunctional calcium/calmodulin kinase, which regulates exocytotic vesicle availability at synapses, and of kinesin, which is required for outward-directed transport of vesicles, inhibited membrane resealing. Resealing was also inhibited by botulinum neurotoxins B and A, suggesting that the two synaptosomal-associated proteins synaptobrevin and SNAP-25 also participate in resealing. This pattern of inhibition indicates that the calcium-dependent mechanisms for cell membrane resealing may involve vesicle delivery, docking, and fusion, similar to the exocytosis of neurotransmitters.

The resealing of plasma membranes is observed in experiments in which material is delivered to the cytoplasm by microinjection, chemical permeabilization, or electroporation (*1–5*). Indeed, it is not uncommon to observe rapid resealing of plasma membranes with little loss of intracellular contents. This property of cells must reflect the need to repair plasma membranes in the normal course of events experienced by cells. Transient plasma membrane disruptions commonly occur in cells that experience mechanical stress, such as gut, skin, endothelium, and muscle (*6–10*). Although erythrocytes will reseal and lipid bilayers can passively fuse together under some circumstances without Ca^{2+} (*11–15*), there is no evidence to suggest that similar passive processes are used by cells to repair their membranes. On the contrary, the fact that cells require Ca^{2+} to reseal (*3, 16–18*) suggested to us that an influx of Ca^{2+} might signal cell injury and trigger active mechanisms, similar to exocytosis, to rapidly seal membranes and ensure cell survival. We disrupted cell membranes by micropuncture with glass micropipettes and measured the resealing by observing the rate of fluorescent dye loss and monitoring the levels of free intracellular Ca^{2+} (*19*). We took advantage of the difference between unfertilized sea urchin eggs, with vesicles already docked in abundance at the plasma membrane, and the fertilized egg (embryo) in which docked vesicles had been depleted (*20*).

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In sea urchin eggs or embryos, which have a low surface to volume ratio, successful healing is rapid enough so that little or no dye loss is detected. In Swiss 3T3 cells the fraction of dye loss after micropuncture is a much larger fraction of the total and the wound to the plasma membrane involves a larger proportion of the surface. In 3T3 cells, therefore, it is easier to get more exact estimates of the time to reseal because a larger fraction of the dye is rapidly lost after micropuncture. Successful resealing required 10 to 30 s, but took longer when Ca^{2+} concentrations were low, as long as 90 to 120 s in some cases. We found threshold concentrations of Ca^{2+} in external solutions for plasma membrane resealing after micropuncture. Figure 1 illustrates examples of both successful resealing above the threshold Ca^{2+} requirement (Fig. 1A, embryo; Fig. 1C, fibroblast) and unsuccessful membrane repairs (Fig. 1B, embryo; Fig. 1D, fibroblast) below the threshold.

The threshold Ca^{2+} concentration, below which resealing did not take place, varied by cell type and cell state, and the concentration of Mg^{2+}. Unfertilized eggs in artificial seawater without Mg^{2+} healed at 300 μM Ca^{2+} and above (Fig. 2A). In artificial seawater with normal Mg^{2+} (55 mM), the threshold for healing was 1.2 to 1.3 mM Ca^{2+} (Fig. 2B). In 1 mM Mg^{2+} seawater the Ca^{2+} threshold was similar to that in 55 mM Mg^{2+} seawater, indicating a strong antagonism at low concentrations of Mg^{2+}. The state of the egg cortex determined the Ca^{2+} threshold for resealing. In fertilized eggs (embryos) or activated portions of egg surface, where the vesicles had already fused, the Ca^{2+} threshold was high-