Regulated motion of glycoproteins revealed by direct visualization of a single cargo in the endoplasmic reticulum

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Introduction

During interphase, the ER is a single structure consisting of interconnected hollow tubes and cisternae extending throughout the cytoplasm. Nascent proteins are transported from their sites of synthesis through the lumen to specialized microdomains, which are referred to as ER exit sites (ERESs), and their folding is mostly completed during transit. The calnexin cycle and molecular chaperones such as BiP prevent nonproductive interactions of folding intermediates by repeatedly binding to the nascent proteins (Ellgaard and Helenius, 2003). Folding enzymes such as protein disulfide isomerase edit the molecules’ structural organization. If folding cannot be completed properly, the nascent proteins tend to form aggregates as a result of interactions between exposed hydrophobic patches. Because the lumenal space of the ER is narrow, such aggregates could severely affect protein maturation. To prevent their accumulation, some terminally misfolded products are selectively retrotranslocated from the ER for proteasomal degradation (Lederkremer and Glickman, 2005).

Cells are constantly exposed to a wide variety of folding stresses, and reducing the frequency of nonproductive interactions must be critical for quality control. Because the frequency of collision is proportional to displacement of the molecule over time and because the structure of nascent proteins is unstable in general, the quality of the nascent secretory cargo should inversely correlate with the rate of diffusional motion. Thus, it is conceivable that regulation of molecular motion in the ER lumen may be an effective means to decrease molecular collisions. Although the lumen seems highly crowded, FRAP analysis of the diffusional motion of GFP indicated that motion in the ER is 20–30% of that in the cytoplasm (Dayel et al., 1999). This proportion is compatible with a random walk simulation assuming a unique reticular shape of the ER, where the lumen occupies 3–10% of the entire space (Olveczky and Verkman, 1998), and suggested that cargo proteins are freely diffusible. Membrane-bound chaperones such as calnexin should reduce the simple diffusion of some misfolded, soluble, glycosylated cargo proteins, but it remains unclear whether the ER contains general mechanisms to reduce the frequency of cargo collisions. Although it has long been thought that ER molecular chaperones associate
with each other and may form a loose complex to provide a favorable environment for nascent proteins (Tatu and Helenius, 1997; Meunier et al., 2002), reversibly misfolded cargo proteins are fully mobile, and misfolding does not seem to severely affect translational diffusion (Nehls et al., 2000; Kamada et al., 2004). Indeed, molecular chaperones such as calreticulin, a soluble homologue of calnexin, are highly mobile (Snapp et al., 2006).

In this study, we explore the possibility that protein diffusion in the ER could be regulated, thus reducing the rate of collision. Using FRAP, we searched for an extreme but physiological condition that would disturb simple diffusion without affecting folding. Interestingly, we found that the mobilities of several cargo proteins were selectively abolished immediately after exposure to hypertonicity, and N-glycosylation was required for the immobilization. To understand whether this restriction occurs under growth conditions, we used fluorescence correlation spectroscopy (FCS) to directly analyze the movements of a single molecule in and out of a focal plane (Rigler and Elson, 2001). With FCS, the motion of the molecules can be measured at extremely high time resolution by averaging the time correlation of the fluctuation. However, this is limited to estimates of the mobile populations. To elucidate the reaction, we next analyzed the displacement and lifetime of the individual molecule illuminated by evanescent light. In general, observations using total internal reflection fluorescence microscopy (TIRFM) are performed on cell surface molecules because evanescent illumination exponentially decays along the z axis and is practically limited to molecules within ~200 nm of the reflection surface (Toomre and Manstein, 2001). Because part of the ER is attached to the cell surface and the depth of evanescent illumination extends into the milieu, where light is scattered (Steyer and Almers, 1999), we reasoned that direct visualization of a single cargo protein might be possible. By analyzing the dynamics of single molecules, we show that the motion of cargo proteins in the ER lumen is regulated by the actin cytoskeleton through N-glycosylation.

Results

High osmolarity abolishes the diffusion of cargo proteins in the ER

We first addressed the question of whether the simple diffusion of cargo proteins in the ER could be abolished without inducing the formation of aggregates. We focused on osmotic stress, which is one of the most common physiological stresses. For example, renal medullary cells are constantly exposed to extreme conditions of hypertonicity consisting of 1,500 mOsm of urea and ~1,000 mOsm of osmolytes such as sorbitol (Garcia-Perez and Burg, 1991). Osmotic disturbance causes a variety of responses in cells. Exposure to hypertonicity immediately activates the Rho family GTPases, triggering downstream pathways, including an increase in filamentous actin content as well as alterations in ion transport (Pedersen et al., 2001; Di Ciano-Oliveira et al., 2006). In addition, membrane traffic such as endocytosis (Heuser and Anderson, 1989) or ER to Golgi transport (Lee and Linstedt, 1999) is severely disturbed. Interestingly, it has been reported that Hsc/hsp70 are induced in the collecting ducts when exposed to 760–1,270 mOsm (Santos et al., 2003), suggesting that alterations in osmolarity may have severe effects on protein maturation.

When we studied the effects of osmolarity changes on cargo motion, we found that several cargo proteins were immobilized under hyperosmolarity. In normal growth medium, misfolded vesicular stomatitis virus glycoprotein (VSVG)–YFP showed rapid, near-complete recovery after photobleaching (Fig. 1 A). However, the recovery was almost completely abolished after placing the cells at 930 mOsm. The photobleached spots remained detectable even after 60 min. Averaged recovery curves are shown in Fig. 1 B. The same immobilization was observed when folded VSVG-YFP was measured in cells treated with brefeldin A, which prevents export of folded cargo from the ER. Thermally misfolded tyrosinase, a membrane protein that displays temperature-sensitive folding (Kamada et al., 2004), showed the same response to hypertonicity. When we tested the soluble, permanently misfolded cargo protein α1-antitrypsinnullHongKong (NHK), recovery was markedly suppressed, albeit to a lesser degree than the membrane proteins. In contrast, no such mobility changes

Figure 1. Diffusion of various cargo proteins in the ER was severely restricted by hypertonicity. (A) The indicated areas of a COS7 cell expressing VSVG-YFP at 40 °C were photobleached as described in Materials and methods. Images of a whole cell before and after photobleaching are shown. After the first series of FRAP (top), the osmolarity was adjusted to 930 mOsm, and images were recorded at 1, 30, and 60 min. The pink arrowhead indicates a spot photobleached at 1 min, and the yellow arrowhead indicates a spot photobleached at 30 min. (B) Quantitative FRAP of various cargo proteins when exposed to hypertonicity. Cells expressing misfolded VSVG-YFP (40 °C), folded VSVG-YFP (32 °C) in the presence of 10 μM brefeldin A, NHK, or tyrosinase (40 °C) were exposed to 930 mOsm. FRAP was recorded before (black) and 1 (red) or 60 min (green) after treatment. The normalized mean recovery rates are plotted. The number of experiments and mobile fractions are shown in Table I. Bars, 5 μm.
single oligosaccharide. To test for a link between glycosylation and immobility under hypertonic conditions, we designed YE mutants containing N-linked oligosaccharides. Because the C terminus of YFP protrudes from the fluorophore, we inserted a single N-linked glycosylation site (Mellquist et al., 1998) between YFP and the KDEL motif. The monoglycosylated YE, YE(gly)1, showed a slight decrease in the mobile fraction 1 min after the addition of sorbitol; however, by 60 min, the recovery had almost returned to the level observed under isotonicity (Fig. 2B). Marked immobilization was observed when a second N-glycosylation site was created in an external loop of YFP (see Materials and methods). The recovery rate of YE(gly)2 reached 32.8% at 1 min and 53.3% at 60 min (Table I). Because oligosaccharide chains are bulky (Petrescu et al., 1997), we tested whether the observed immobilization of the doubly glycosylated protein was caused by its increased hydrodynamic diameter by examining Y2E, a tandem YFP dimer (Fig. S5B, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). However, the mobile fractions were comparable with those of YE (Table I). These results suggest that it is the two N-glycans that are responsible for the hypertonicity-induced immobilization of VSVG-YFP. If so, the removal of a single N-glycan should rescue it unless the immobilization is the result of massive aggregation. To test this, we created two mutants: VSVG(N336S), which lacks the C-terminal glycosylation site, and VSVG(N336S/N179S), which contains no glycosylation site. The effects of the mutations were confirmed by immunoblotting (Fig. 2C). Neither mutant exited the ER at 37°C, and the kinetics of the recovery rates were especially slowed in the nonglycosylated mutant (Fig. 2C).

Next, we examined whether molecules other than secretory proteins might respond in a similar manner. We analyzed the mobility of the KDEL receptor, a multispansing membrane protein that recycles between the ER and Golgi apparatus. After hyperosmotic stress, FRAP analysis of KDEL receptor–YFP revealed a mobile fraction of 81.5% at 1 min and 82.8% at 60 min, although the recovery rates slowed (Fig. 2A and Table I). The maximum recovery rates of three other ER-resident proteins, esterase, Ero1L, and BiP, also returned to levels similar to those at isotonicity after 60 min (Table I). When we examined the effects on the simplest fluorescent protein, EYFP-ER (YE), the mobile fraction was also unchanged, although the recovery kinetics were reduced, presumably as a result of the cell shrinking (Fig. 2B, left; and Table I). Hyperosmotic stress had little effect on the motion of the lipid DiOC6 (Fig. 2A).

Multiple N-linked oligosaccharides strongly influence the mobility of cargo proteins

Next, we tried to identify the common element in cargo immobilized by hyperosmolality. Comparing the proteins listed in Table I revealed that all of the molecules showing potent immobilization were N-glycosylated on at least two sites; VSVG, tyrosinase, and NHK contain two, six or seven, and three oligosaccharides, respectively. In contrast, the KDEL receptor, BiP, and YE are not glycosylated, and both esterase and Ero1L contain only a single oligosaccharide. To test for a link between glycosylation and immobility under hypertonic conditions, we designed YE mutants containing N-linked oligosaccharides. Because the C terminus of YFP protrudes from the fluorophore, we inserted a single N-linked glycosylation site (Mellquist et al., 1998) between YFP and the KDEL motif. The monoglycosylated YE, YE(gly)1, showed a slight decrease in the mobile fraction 1 min after the addition of sorbitol; however, by 60 min, the recovery had almost returned to the level observed under isotonicity (Fig. 2B). Marked immobilization was observed when a second N-glycosylation site was created in an external loop of YFP (see Materials and methods). The recovery rate of YE(gly)2 reached 32.8% at 1 min and 53.3% at 60 min (Table I). Because oligosaccharide chains are bulky (Petrescu et al., 1997), we tested whether the observed immobilization of the doubly glycosylated protein was caused by its increased hydrodynamic diameter by examining Y2E, a tandem YFP dimer (Fig. S5B, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). However, the mobile fractions were comparable with those of YE (Table I).

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Nonetheless, in growth medium, the maximum recovery rates for both mutants were comparable with those for VSVG. However, hypertonicity-induced immobilization was not observed in these mutants. These data are consistent with the results in Fig. 2B and indicate that N-glycosylation but not folding or molecular crowding is essential for immobilization. Because most secretory proteins are N-glycosylated and the restriction of simple diffusion should be beneficial for avoiding nonproductive interactions, we hypothesized that the ER may be equipped with a mechanism that uses multiple N-glycans to limit the diffusion of cargo proteins. Indeed, examination using fluorescence loss induced by photobleaching (FLIP), in which an area of a narrow strip was repeatedly photobleached, also showed that the presence of double glycans markedly slowed long-range transport in the ER (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). If this hypothesis is correct, we should be able to detect the disturbance of the simple diffusion of cargo proteins in cells in growth medium. We used FCS to analyze cargo motion in detail. At a delay time of \( \tau \approx 1 \) ms, the decay profile of \( G(\tau) \) was clearly shifted to the right as compared with the nonglycosylated YE (Fig. 3A). Fitting each \( G(\tau) \) to a three-component model revealed that the slow components of YE(gly)\(_2\) were markedly increased compared with those of YE, and the population of freely diffusing molecules (the 0.207-ms component, which is equivalent to a diffusion coefficient of 27.2 \( \mu \text{m}^2/\text{s} \)) was decreased by nearly half. In contrast, YE and YE(gly)\(_2\) had only slightly different diffusion times in cell lysates (Fig. 3C). The decay curves fit well to a one-component model with diffusion times of 0.064 ms and 0.074 ms for YE and YE(gly)\(_2\), respectively. Because diffusion time is proportional to the cube of the molecule’s diameter, this result suggests that the hydrodynamic diameter of YE(gly)\(_2\) is only 55% larger than that of YE and, therefore, would not account for the presence of slow components in the cells. Thus, the slow component of YE(gly)\(_2\) observed under conditions of isotonicity most likely represents a large cellular component, such as membranes.

### Table 1. Summary of mobile fractions of various ER molecules after hypertonicity treatment

| Cargo and ER-resident proteins | +Sorbitol |
|--------------------------------|-----------|
|                               | 0 min     | 1 min | 60 min |
| VSVG (40°C)                    | 93.4 (12) | 10.8 (12) | 17.8 (12) |
| VSVG (32°C)                    | 97.5 (8)  | 15.7 (8)  | 9.5 (8)  |
| VSVG(N336S)                    | 86.9 (10) | 60.3 (5)  | 62.8 (8) |
| VSVG (null)                    | 87.6 (5)  | 69.7 (5)  | 71.1 (5) |
| Tyrosinase                     | 100.8 (8) | 7.2 (12)  | 19.4 (12) |
| \( \alpha_1 \text{AT}_{\text{NK}} \) | 89.3 (9)  | 50.1 (8)  | 27.1 (8) |
| KDELR                          | 91.8 (8)  | 81.5 (8)  | 82.8 (8) |
| Esterase                       | 72.9 (4)  | 53.8 (4)  | 70.2 (7) |
| Ero1L                          | 77.6 (5)  | 64.7 (3)  | 76.1 (4) |
| BiP                            | 81.5 (5)  | 52.0 (6)  | 81.7 (16) |
| Lectin-related proteins        |           |        |        |
| Calnexin                       | 89.6 (17) | 44.0 (18) | 86.6 (13) |
| Calnexin(Y163F)                | 82.4 (6)  | 66.3 (3)  | 79.7 (5) |
| ERGIC53                        | 77.7 (5)  | 49.3 (4)  | 68.7 (9) |
| ERGIC53(N516A)                 | 74.3 (4)  | 50.7 (3)  | 69.4 (6) |
| VIP36                          | 102.9 (3) | 88.9 (3)  | 84.6 (6) |
| VIP36(N166A)                   | 96.1 (5)  | 77.0 (3)  | 94.1 (6) |
| VIP                            | 95.2 (9)  | 18.1 (8)  | 18.1 (7) |
| VIP(N163A)                     | 82.5 (5)  | 44.8 (3)  | 78.5 (6) |
| UGOT                           | 88.8 (3)  | 31.2 (3)  | 69.2 (5) |
| Artificial cargo               |           |        |        |
| DiOC\(_6\)                     | 108.4 (12) | 94.3 (12) | 95.6 (12) |
| YE                             | 95.8 (7)  | 99.3 (7)  | 97.9 (7) |
| YE(gly)\(_1\)                  | 88.1 (4)  | 77.9 (4)  | 87.5 (4) |
| YE(gly)\(_2\)                  | 98.0 (9)  | 32.8 (9)  | 53.3 (9) |
| \( \text{Y}\_2E \)             | 81.8 (9)  | 71.3 (4)  | 87.7 (5) |

\( n \) is given in parentheses for the number of measurements. Mobile fractions of various molecules in the ER before and after osmotic stress were estimated. All molecules except DiOC\(_6\) were fused to YFP.
used exclusively to analyze molecular events on the cell surface (Sako, 2006; Jaiswal and Simon, 2007), part of the ER network resides in enough proximity to the cell surface that it should be possible to visualize a thin optical section of the ER. When the ER was illuminated by a laser at a shallow angle to the CCD camera, YE(gly)₂ was visible throughout the ER network (Fig. S2 A; available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). However, when illuminated at steeper angles, the image of the ER contained spots, but not the network. A projection of 1,000 images confirmed that the spots were confined within the reticular structure. To confirm that the individual spots represented images of single proteins, we examined the photobleaching properties of fixed proteins (Fig. S2 B). In 500 sequential images, the sudden disappearance of a few immobile fluorescent spots was observed. Because it was unlikely that photobleaching of two molecules occurred simultaneously, this observation suggested that individual fluorescent spots mostly represented single molecules.

To examine whether translational movements can be measured, we analyzed sequential images of YE(gly)₂ (Fig. 4 B and Videos 1 and 2; available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). We tracked the movement of a single molecule in consecutive images. Projection of streaming images showed that the spots were found throughout the reticular structure (Fig. 4 A), indicating that the fluorescent spots are not likely to represent colliding proteins at the three-way junction. Indeed, translational displacement of each spot was clearly detected when we compared consecutive images (Fig. 4 B). We quantitatively analyzed 557 molecules from four streaming images of YE(gly)₂ (Videos 1 and 2). When the mean square displacements (MSDs) of each spot were plotted against time, the increments were linear (Fig. 4 C), indicating that the motion is diffusive. The apparent diffusion coefficient was estimated to be 1.68 ± 0.045 μm²/s, which is one order of magnitude lower than the simple diffusion components of YE and YE(gly)₂ (27.2 μm²/s) obtained by FCS (Fig. 3 B) and most likely corresponds to the slow component.

However, the lifetime of each spot was very short (Fig. 4, D and E). It is conceivable that the short residence time indicated the rapid dissociation from the slowly diffusing components. On the other hand, it is also likely that the rapid disappearance of the spots was caused by the movement of the cargo from the evanescent layer as a result of the depth of the ER because a cell surface protein can be tracked over seconds (Fig. S2, C–F; and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). We reasoned that these possibilities could be distinguished if the lifetime distribution of the proteins was analyzed because they should follow the rule of probability measure, P(t), for freely moving particles in one dimension (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). Let A be a one-dimensional reflected

Figure 4. Analysis of the motion of a single YE(gly)₂ molecule. Images of YE(gly)₂ in the evanescent field were recorded at 32°C and a resolution of 15 ms (Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). (A) Four sequential images from Video 1. A projection of 1,000 images reveals traces of a reticular network (bottom). (B) The displacement of each spot in sequential images (Video 2). An image (green) was compared with the next consecutive image (red). (C) Translational diffusion of YE(gly)₂. Averaged MSD was plotted against time intervals (τ). Error bars represent 95% confidence intervals. (D) Appearance and disappearance of single molecules (numbered arrows in A) over nine frames. (E) A one-dimensional image of a single pixel width of the video was aligned with the progression of time. The kymograph is an image of 7 s in length and 19 μm in distance. (F) Lifetime distribution of each spot. Lifetime of the fluorescence spots was determined from four streaming images, each of which was obtained over 15 s, and the time distribution was counted. The lifetime dataset was normalized, and the best-fit value of d_A was calculated. Bar, 5 μm.
Brownian particle starting from \( x = a \) (0 < \( a \) < \( l/2 \)) at \( t = 0 \) with a reflecting barrier at \( x = l/2 \), and let \( T_0 \) be its first passage time to \( x = 0 \) (see Materials and methods). On the basis of the reflection principle at the barrier, the distribution of \( T_0 \) is equal to that of the first passage time of the free Brownian particle from \( x = a \) to \( x = 0 \) or \( l \). Therefore, if the diffusion coefficient of \( A \) is \( d_A \), the density of \( T_0 \) is equal to that of the first passage time of the one-dimensional diffusion motion \( M(t) = B/(2d_A t) \) (see Mobility measurements in living cells). Indeed, the simulated first passage time distribution is critically dependent on \( d_A \). One way to estimate \( d_A \) is to determine \( d_A \) from the lifetime distribution because \( d_A \) is theoretically identical to the mean translational diffusion coefficient. To obtain such a freely diffusing control molecule, we examined the model membrane protein inactive calnexin(Y163F), which lacks affinity for oligosaccharides (Kapoor et al., 2004).

Regardless of \( a \), the diffusion coefficient, \( d_A \), can be calculated from the thickness of the evanescent layer, \( l/2 \), or vice versa by using this equation if the normalized passage time (lifetime) distribution is given. Although the theoretical thickness of the evanescent layer in solution can be calculated from the optic parameters, the actual depth in cells cannot be predicted (Steyer and Almers, 1999). One way to estimate \( l \) in Eq. 1 is to determine \( d_A \) from lifetime distribution because \( d_A \) is theoretically identical to the mean translational diffusion coefficient.

We then applied the estimated value of \( l \), 0.201 \( \mu \)m, onto the lifetime distribution of YE(gly)\(_2\) and calculated the best-fit \( d_A \). The lifetime of YE(gly)\(_2\) (Fig. 4 F) appeared to be much shorter than that of calnexin(Y163F), and spots lasting >50 ms were rare. The best-fit \( d_A \) of YE(gly)\(_2\) was 5.37 ± 2.18 \( \mu \)m\(^2\)/s, which was significantly faster than the \( d_A \) of the inactive calnexin (1.64 \( \mu \)m\(^2\)/s), although their translational MSDs were nearly identical (compare Figs. 4 C with 5 C). Therefore, we reasoned from the FCS (Fig. 3, A and B) and MSD analysis (Fig. 4 C) that the slow component of YE(gly)\(_2\) was short lived and converted to the faster component, most likely by dissociating from membranes.
treated YE(gly)\textsubscript{2}. However, the lifetime of each spot was short (Fig. 6 B), which is similar to that observed for the freely diffusing calnexin mutant. These results indicate that YE(gly)\textsubscript{2} dissociates from the binding sites even under hyperosmolarity, and the disturbed diffusion observed with FRAP was caused by the immobilization of a binding partner.

The projection of multiple YE(gly)\textsubscript{2} images (Fig. 4 A) suggested that the observed spots do not represent cargo molecules trapped in ERESs because the cargo was distributed throughout the ER, whereas ERESs are immobile (Hammond and Glick, 2000). To confirm this and to examine the frequency of single cargo visits to ERESs, we expressed YE(gly)\textsubscript{2} by bead loading in cells stably expressing Sec13-mStrawberry. Simultaneous observation of the two-color imaging clearly showed that the majority of the observed spots of YE(gly)\textsubscript{2} was not in ERES (Fig. 7 A). This was most apparent in the kymograph showing ERES (Fig. 7, B and C) as a straight line in the time axis (Fig. 7 B). Quantifying the frequency of entry by counting the total intensity in the y axis indicated that YE(gly)\textsubscript{2} rarely visited ERESs (Fig. 7 B, right). Higher magnifications of the superimposed images also support this view (Fig. 7 C). At the very least, these results exclude the possibility that the fluorescent spots observed by TIRFM represent only YE(gly)\textsubscript{2} molecules trapped in ERESs.

Cytoplasmic actin regulates the motion of YE(gly)\textsubscript{2}

To understand how binding sites for glycoproteins are created, we studied the possibility that cytoskeletal elements such as microfilaments may act as transmembrane regulators of cargo diffusion.
Actin filaments form an extensive intracellular matrix that homogenously spreads throughout the cytoplasm and interacts with membranes (Stossel, 1984; McGrath et al., 1998). Further, it is known that actin is remodeled upon exposure to hypertonicity through the activation of various modulators (Di Ciano-Oliveira et al., 2006). Indeed, FRAP analysis of YFP-actin in cells after hyperosmolarity treatment showed that nearly one third of YFP-actin was immobilized (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1). In addition, when we estimated the change in the pseudoequilibrium constant of the actin monomer–polymer reaction, the equilibrium shifted toward polymer formation by approximately threefold. This estimation indicates that hyperosmolarity induces the rapid and sustained polymerization of actin.

To examine the role of actin polymerization in the immobilization of cargo proteins, we pretreated the cells with latrunculin B (LAT), which inhibits actin polymerization by sequestering the monomer (Spector et al., 1983), and examined the effect on VSVG-GFP immobilization. The treatment partially reversed the hyperosmolarity-induced immobilization (Fig. S4 B, left). In contrast, pretreatment with nocodazole, a drug that disrupts the microtubule cytoskeleton, had no effect (Fig. S4 B, right).

We next examined whether the same treatment might reduce the slow components of YE(gly)₂ at isotonicity. When we examined the mobility profile of YE(gly)₂ using FCS in cells treated with LAT, we found that the decay curve clearly shifted to a shorter delay time (Fig. 8 A, left; green line), whereas nocodazole caused no significant change (Fig. 8 A, dark yellow line). Assuming that the mobility components are identical, fitting to the three-component model showed that the LAT treatment reduced the slow fractions to 37.2%, but neither nocodazole (62.6%) nor vehicle alone (63.8%) had this effect (Fig. 8 A). Furthermore, the LAT treatment had little effect on G(0/H9270) of YE (Fig. 8 A, right; inset).

To confirm the dramatic effects of LAT on YE(gly)₂ diffusion, we measured the change in G(0/H9270) in a single cell before and after the treatment. G(0/H9270) was nearly identical at 0 and 5 min before the addition of LAT (Fig. 8 B). Immediately after the 5-min measurement, we placed medium containing LAT onto the cells and measured the same spot after a 4-min incubation (Fig. 8 B, 11 min). The curve was clearly shifted to the left, particularly around the delay time of 1 ms. The faster decay was still evident 8 min after LAT (Fig. 8 B, 15 min). Fitting showed that the fastest component increased from ~40 to ~70% (Fig. 8 B). Essentially the same effects were observed with cytochalasin B, which blocks monomer association/dissociation at the barbed end of actin polymers (Fig. 9 A; Cooper, 1987). To confirm that these reagents actually disturbed filamentous actin formation, we generated cells stably expressing YFP-actin and recorded images of the microfilaments in a single cell before and after treatment. The thick actin filaments largely disappeared after 15 min of incubation with LAT (Fig. 8 C). After the treatment with cytochalasin B, most of the actin became aggregated in a coarse networklike pattern, and actin asters formed.
effects of hypertonicity on the mobility of lectin-related membrane proteins (Table I). Calnexin is a well-characterized lectin-like chaperone with affinity toward monoglucosylated proteins (Schrag et al., 2001; Moremen and Molinari, 2006; Williams, 2006). Unlike our observations with VSVG, hypertonicity led to only a transient decrease in the mobile fraction for YFP-calnexin, and full mobility resumed at 60 min. Calnexin(Y163F) and UDP-glucose–glycoprotein glucosyltransferase (UGGT) showed similar responses. The intracellular C-type lectins ERGIC53 (Hauri et al., 2000a) and VIP36 (Kamiya et al., 2005) and their inactive mutants (N516A and N166A, respectively) also were not potently immobilized by hyperosmolarity. However, another lectin, VIPL (Nufer et al., 2003), showed potent and sustained immobilization. Although this immobilization was almost abolished when we used the inactive mutant N163A (Nufer et al., 2003), knockdown of VIPL slightly increased the faster component from 40.7 to 48.8% (Fig. S5 C).

Discussion

The endoplasmic reticulum is capable of exporting a large amount of properly folded secretory proteins. Because their quality should be affected by diffusional motion during folding, we examined the possibility that there is a general mechanism to limit simple diffusion in the ER. In this study, we have provided evidence that cargo diffusion in the ER lumen is controlled by the actin cytoskeleton through links to N-glycans.
function is less well documented, but a recent extensive proteomic study revealed that /H9252-actin and various actin-binding proteins are abundantly expressed in the ER (Gilchrist et al., 2006). Consistent with this, development in Dictyostelium discoideum lacking interaptin, an /H9251-actinin family protein localized in the ER, is delayed (Rivero et al., 1998). Requirement of the actin cytoskeleton for ER structure formation has also been reported in yeast (Voeltz et al., 2002) and in Caenorhabditis elegans (Poteryaev et al., 2005).

Both models assume the presence of lectinlike proteins and modulation of the affinity by oligomerization, a common feature of various lectins (Brinda et al., 2005). This evokes a well-characterized phenomenon in which some monomeric intracellular lectins have low or negligible affinity for ligands, but their oligomerization markedly increases the affinity (Hauri et al., 2000b; Kawasaki et al., 2006). For leguminous isolectins, forming trimers increases the ligand affinity >10-fold (Knibbs et al., 1998). Mannose-binding lectin, a serum protein that acts in innate immunity, forms covalently linked large oligomers; a reduction in its oligomerization correlates with reduced ligand binding (Larsen et al., 2004). ERGIC53 also forms covalently linked dimers and hexamers (Schweizer et al., 1988), which are required for sugar binding (Carriere et al., 1999). Monomeric subunits may exhibit significant sugar binding affinity only when clustered, as depicted in Fig. 10. To search for the key lectins involved in cargo function, we propose two models that could explain this mechanism (Fig. 10). According to the direct binding model (Fig. 10 A), affinity for N-glycans is induced by oligomerization through binding to actin microfilaments. However, it is also conceivable that the lectins become clustered if they are stalled in a microdomain created by microfilaments distributed on the membrane, as has been proposed for cell surface proteins (Morone et al., 2006). Therefore, we propose an alternative model in which glycoproteins are transiently trapped onto membranes by stochastically formed groups of high-affinity lectins (Fig. 10 B). In this model, we assume that the actin-based filament functions as a diffusion barrier for membrane proteins that can eventually cross the barrier, which should be continuously remodeled by actin turnover (Theriot and Mitchison, 1991; McGrath et al., 1998). In both models, immobilization of glycoproteins under hyperosmolarity can be explained by a solidified barrier that forms as a result of an actin equilibrium shift (Fig. S4 A).

The membrane skeleton, a dense meshwork of fibers that includes actin, directly attaches to the plasma membrane (Morone et al., 2006), and this undercoat structure is thought to be the basis of the aberrant diffusion of cell surface molecules (Kusumi et al., 2005). Consistent with this, it has been reported that the diffusion law of transmembrane proteins is actin dependent, and disruption of the actin-based cytoskeleton reverses restrictions in diffusion (Lenne et al., 2006). The role of actin filaments in ER function is less well documented, but a recent extensive proteomic study revealed that β-actin and various actin-binding proteins are abundantly expressed in the ER (Gilchrist et al., 2006). Consistent with this, development in Dictyostelium discoideum lacking interaptin, an α-actinin family protein localized in the ER, is delayed (Rivero et al., 1998). Requirement of the actin cytoskeleton for ER structure formation has also been reported in yeast (Voeltz et al., 2002) and in Caenorhabditis elegans (Poteryaev et al., 2005).
diffusion in the ER, we examined various lectinlike proteins and found that none of the major lectins satisfied model A. However, the response of VIPL to hypertonicity was distinct in that it was immobilized even after 60 min. Although an inactive form of VIPL, an N163A mutant, did not show the same extent of immobilization, this observation is still compatible with model B because substrate binding and microfilament-induced clustering can be cooperative. Consistent with this scenario, the presence of at least two oligosaccharides was necessary for immobilization. Thus, we think that multivalency may be the direct cause of hypertonicity-induced immobilization. This model does not exclude the involvement of other lectins. The weak effect of VIPL knockdown in reducing the slow population of YE(gly)₂ (Fig. S5 C) may support this interpretation.

Lifetime analysis of a single glycoprotein in an evanescent field indicated that the slow components of YE(gly)₂ are short lived (Fig. 3 B). This conclusion is based on the following: (1) the translational diffusion of YE(gly)₂ measured by TIRFM was nearly identical to that of an inert membrane protein (compare Figs. 4 C with 5 C); (2) the first passage time of YE(gly)₂ was shorter than that of calnexin(Y163F)-YFP; and (3) the lifetime of YE(gly)₂ was also short in a hypertonic condition despite its confined motion (Fig. 6 B). Lifetime frequency distribution is a useful tool to analyze the molecular dynamics of single intracellular proteins. It should be noted that there is a gap between the simulated curves based on Eq. 1 and the actual measurements. We think that this is caused by the increased frequency of photobleaching upon longer dwell time and that Eq. 1 assumes 100% reflection at the luminal surface of the ER. Nevertheless, we have demonstrated the transient binding of soluble proteins to membranes using various dynamic measurements, including FLIP. This transient binding should help in assuring the quality of cargo proteins by reducing the chances of aggregation and, at the same time, promoting access to the folding machineries on the membranes. Importantly, the binding reaction appears to be brief, so it should not inhibit luminal processes or export. This process resembles the calnexin cycle. We have previously shown that folding of nascent transferrin is transiently arrested in the calnexin complex while reglucosylation is allowed to proceed (Wada et al., 1997). Although this model has been questioned as a result of the subsequent elucidation of at least two oligosaccharides was necessary for immobilization. Consistent with this scenario, the presence of at least two oligosaccharides was necessary for immobilization. Thus, we think that multivalency may be the direct cause of hypertonicity-induced immobilization. This model does not exclude the involvement of other lectins. The weak effect of VIPL knockdown in reducing the slow population of YE(gly)₂ (Fig. S5 C) may support this interpretation.

Figure 10. Proposed mechanisms for the regulated diffusion of cargo glycoproteins. For either model, we assume that one or more lectins (shown as rods or bullets) with weak affinity for N-glycans transiently bind to N-glycans only when oligomerized. [A] Oligomerization is caused by direct tethering to polymerized actin. (B) A local concentration of the putative lectins occurs by confinement in a microdomain, which is transiently generated by an actin fiber fence, and the clustered lectins trap the glycoproteins. In either case, turnover of actin is greatly reduced under hyperosmolarity, and, thus, glycoproteins become nearly immobilized.

Materials and methods

Cells and expression of proteins

All proteins described in this study were genetically fused to enhanced YFP (EYFP, Clontech Laboratories, Inc.). We refer to EYFP as YFP. Unless otherwise stated, the fusion proteins used in this study were expressed in COS7 cells using the siliconized glass microbead-loading method (Nagaya et al., 2002) and were measured 30 min to 3 h after bead loading. VSVG-YFP, tyrosinase-YFP, and the KDEL receptor have been previously described imported through protein translocon channels, directional flow would be expected. Considering that the folding rates of proteins vary widely, the apparent lack of flow may be beneficial for the maturation in that it provides a delay time for export. On the other hand, acquisition of export competence may cause active motion. We believe that the simultaneous observation of cargo with ERESs (Fig. 7) has revealed a way to analyze cargo motion toward ERESs and to elucidate whether export-competent cargo proteins are stochastically trapped in ERESs or whether there is an active mechanism to ensure efficient export. These analyses will eventually allow a better understanding of how the ER compromises folding and export.
transfected with each vector using Lipofectamine 2000 and were directly analyzed. For enzymatic deglycosylation of cell lysates, cells in a 24-well dish were lysed with 100 μl of lysis buffer (0.5 M Tris, pH 7.5). After the addition of 5 μl of 10% Triton X-100, half of each sample was digested with 1,000 U PNGaseF (peptide-N-glycosidase F) for 1 h at 37°C. Mock and PNGaseF-digested lysates were then directly analyzed by immunoblotting using an anti-GFP antibody. The bands were visualized using ECL Western blotting detection reagents (GE Healthcare).

**Mobility measurements in living cells**

To reduce fluorescence background, protein expression and measurement were performed in COS7 cells cultured in 1% FBS containing phenol red-free MEM with Hank’s salts from which riboflavin and pyridoxal hydrochloride were omitted. The optical apparatus for FRAP and FCS were set up essentially as described previously (Kamada et al., 2004) except that the dishes were kept in a closed stage chamber (95% air and 5% CO2; Takaihit) and heated with an object heater (BiopTechs). Except for the experiments described in Figs. 2 C and S4 A, a circle of 4-μm radius was photo-bleached for five iterations, and pre- and postbleach images were recorded at the lowest laser intensity. For experiments shown in Figs. 2 C and S4 A, a radius of 2.34 μm was used. The osmolality of the medium was adjusted by adding sorbitol. FRAP data were normalized and fitted according to the method of Jacobson et al. (1976) with the exception of the data in Fig. S4 A, in which the protocol of Axelrod et al. (1976) was applied to determine the diffusion of rapidly diffusing molecules. To minimize the effects of macular crowding, FRAP was performed in two spots whose total fluorescence intensity was comparable with those used for FCS recordings.

FCS measurements were performed on a microscope (Confocor2; Carl Zeiss, Inc.) with a 40× NA 1.2 water immersion objective (C-Apochromat; Carl Zeiss, Inc.). The samples were excited at 514 nm with a 30-nW argon laser, and emissions were collected through a 530-600-nm bandpass filter. To avoid damage and photobleaching by continuous laser illumination during FCS, we chose a single spot in a cell and recorded the fluorescence intensity of each spot three times for 10 s, with 2-s intervals without recording at the same laser recording output to reduce the effect of the inhomogeneous population. Typically, laser power at the back aperture was ~5.0–6.5 μW. To avoid the crowding effects caused by overexpression, we measured spots whose initial count rates were <100 kHz (Kamada et al., 2004). From the 50–200 autocorrelation curves obtained, we excluded data whose initial count rates were >100 kHz and those whose mean intensities were not constant during a single recording period (Kamada et al., 2004). The autocorrelation function G(t) is defined as

$$G(t) = \frac{\delta f(t) + c f(t)}{c f(t)^2}$$

where $f(t)$ is the fluorescence at time t, $c f(t)$ is the mean fluorescence, and $\delta f(t) = f(t) - c f(t)$ is the fluctuation of the fluorescence at time t from its mean value. To obtain the diffusion time, we used an intensity-normalized three-dimensional diffusion model,

$$G(t) = 1 + \frac{1 - f_0 + f(e^{-t/T})}{1 - f_0} \left( \sum_{i=1}^{n} f_i \frac{T_{tr}(s - 2 f_0)^{1/2}}{1 + \tau_{tr}(s - 2 f_0)^{1/2}} \right)^n$$

in which $N$ is the mean number of fluorescent molecules in the confocal volume, $f_0$ and $T_{tr}$ are the fraction and decay time of the triplet state (Vidgren et al., 1995), respectively, $n$ is the number of the components subjected to the normalization constraint

$$\sum_{i=1}^{n} f_i = 1$$

and $s$ is the fraction of the distance from the laser beam focus in the radial direction to the axial direction. $T_{tr}$ is the diffusion time of the i th component. To estimate the number of diffusion components, we started from a one-component diffusion model and considered more components if this did not give a satisfactory fit. Diffusion coefficients were converted from the fitted diffusion time using a rhodamine green solution as a standard (280 μm/s). For estimation of hydrodynamic diameters, COS7 cells stably expressing YE or YE(gly)2 in a 35-mm dish were lysed with 100 μl of lysis buffer (0.5 M sodium cholate, 0.15 M NaCl, and 10 mM Tris, pH 7.5). The lysates were centrifuged at 10,000 g for 5 min at 4°C, and the supernatants were directly measured with FCS.

To perform quantitative FLIP, we photo-bleached a narrow strip (5 pixels wide) in the ER for 20 iterations. The fluorescence signal of the entire ER of each cell was measured before and immediately after the photo-bleaching and was normalized to the change in total fluorescence. Pixel time of bleaching and recording was 6.4 μs, and each image was composed of 1,024 × 1,024 pixels. The normalized data were fitted to a...
two-phase exponential decay form using Prism 4 (GraphPad Software) as described in Fig. S1 B because a single-phase exponential decay form failed to fit.

Objective-based TIRFM was obtained by using a 488-nm Sapphire laser (20 mW, Coherent) through a side port of an inverted microscope (TE2000, Nikon). The beam was focused on the periphery of the back focal plane of a 60×1.45 NA oil-immersion objective (Nikon). Streaming images were captured by a back-illuminated frame-transfer CCD digital imaging system with on-chip multipixel gain (Cascade 512B, Roper Scientific) at exposure times of ~5–30 ms through appropriate combinations of filters. The obtained image data were recorded and linearly adjusted with MetaMorph v6.2 (MDS Analytical Technologies). All images were processed using CANVAS version 9.05 (ACD Systems). Translational mobilities of fluorescent spots were measured using ImageJ version 1.36b (National Institutes of Health) with a SpotTracker2D plug-in (Sage et al., 2005). Kymographs, in which one-pixel lines were compiled sequentially along the length for each frame to create a composite image, were made with an Align Stacks plug-in. The diffusion coefficient, D, was determined from the relationship ωMSD = 4DT. Mathematical analysis or simulation was performed using OriginPro version 7.5 (OriginLab Corp.). To extract information on mobility from the lifetime distribution of observed single molecules, we used the fundamental solution of the diffusion equation

\[
\frac{\partial}{\partial t}u(t, x) = \frac{1}{2} \frac{\partial^2}{\partial x^2} u(t, x),
\]

with the initial condition u(0, x) = δ(a). The solution u(t, x) = P(t, x) is given by

\[
P(t, x) = \frac{1}{\sqrt{2\pi t}} \exp\left(-\frac{(a-x)^2}{2t}\right).
\]

In probabilistic interpretation, P(t, x) is the probability density of determining the Brownian particle at x = x₀ at time t, which starts from x = a at t = 0. For I and a (0 < a < l), let T_a,l be a first passage time of standard one-dimensional Brownian motion, B(t), from x = a to x = 0 or l. T_a,l is an epoch at which the particle reaches the positions x = 0 or l for the first time. In this sense, T_a,l represents the lifetime of the density of T_a,l is given by the equation

\[
p^A(t) = \frac{1}{\sqrt{4\pi d_A t^2}} \int_{-l}^{l} \left[ \sum_{n=-\infty}^{\infty} \left(2n + l + 1 - a\right) \exp\left(-\frac{(2n + l - a)^2}{4a d_A t}\right) \right] ds.
\]

Therefore, the density \( \tilde{P}_a(t) \) of \( T_{a,l} \) is given by Eq. 1.

**Online supplemental material**

Fig. S1 shows FLIP analysis of YE(gly)₂. Fig. S2 shows that when observed by TIRFM, a single spot of ER cargo represents a single molecule in the ER. Fig. S3 shows the simulation of lifetime frequency distribution according to Eq. 1. Fig. S4 shows that hypertonicity induces actin polymerization and that LAT treatment reduces hypertonicity-induced immobilization. Fig. S5 shows the effect of VPL knockdown on YE(gly)₂ diffusion. Table S1 provides a list of primers used to construct expression vectors. Videos show streaming images of YE(gly)₂ (Videos 1, 2, and 3), VSVG-YFP (Video 3), and calnexin(Y163F)-YFP (Video 4) in living cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704078/DC1.

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